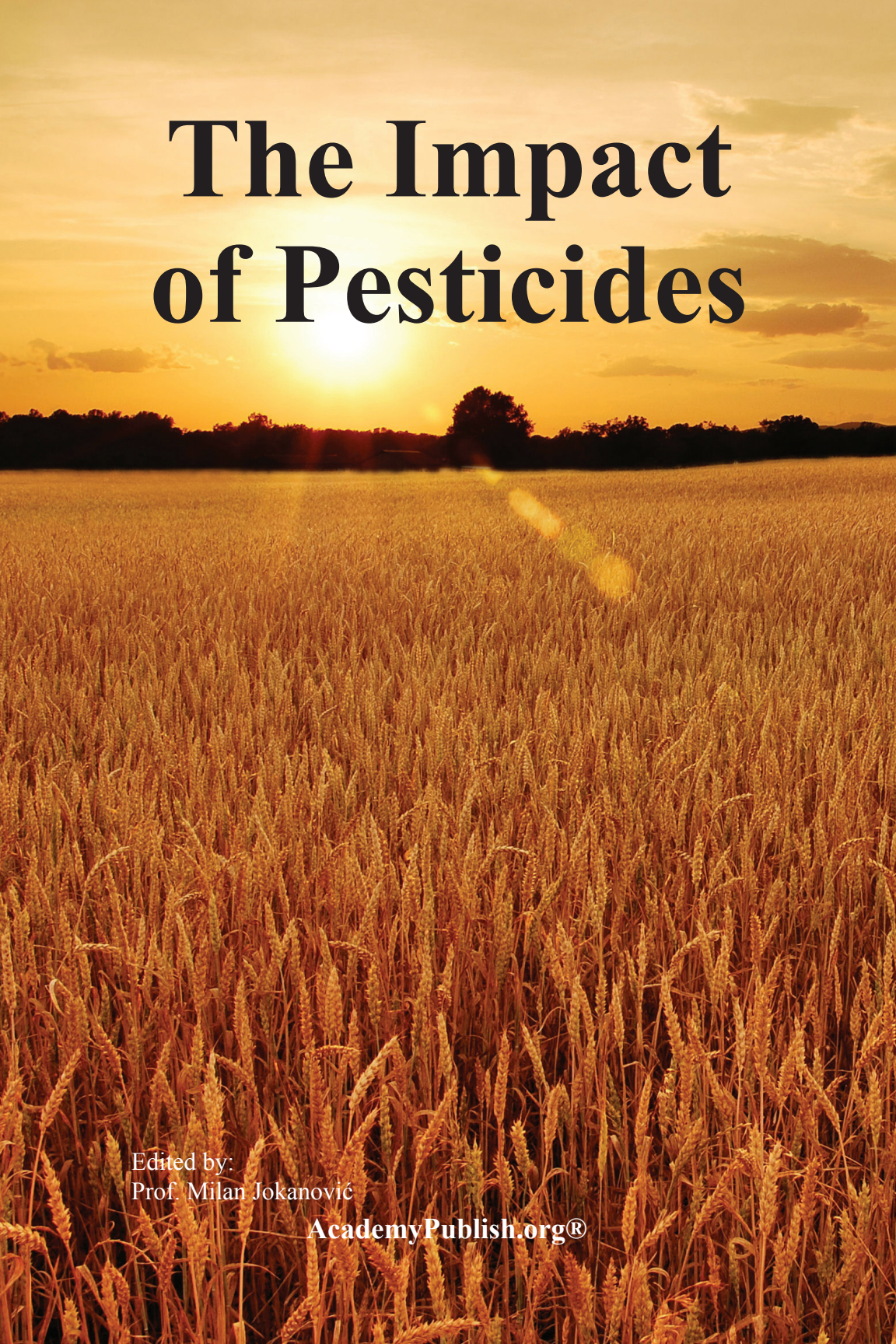
The Impact of Pesticides



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Section 1 HUMAN EXPOSURE TO PESTICIDES

Carbamates: Human Exposure and Health Effects, p.21-p.38
Simone Morais, Elsa Dias, Maria de Lourdes Pereira

Simone Morais is a Professor at the Department of Chemical Engineering at the Polytechnic Engineering Institute of Porto (Portugal) and permanent researcher at REQUIMTE, the largest network in Chemistry and Chemical Engineering established in Portugal. Her research interests include Analytical Chemistry and Electrochemistry, Food Safety and Air Pollution. S. Morais co-authored several papers in international refereed journals.
Simone Morais, Adjunct Professor
REQUIMTE, Instituto Superior de Engenharia do Porto, Rua Dr. António Bernardino de Almeida 431, 4200-072 Porto, Portugal.
sbm@isep.ipp.pt

Elsa Dias is Principal Technician of Clinical Analyses and Public Health at Laboratory of Clinical Pathology, Hospital D. Pedro (Aveiro, Portugal). In addition, she is a researcher at Aveiro University, where carbamate toxicological studies on laboratory animals have been undertaken.
Elsa Dias
Department of Biology, CICECO,
University of Aveiro, Aveiro, Portugal
elsadias@ua.pt

Maria de Lourdes Pereira is a professor of Cell Biology, Cytology and Histology, and Human Biology, and was Director of the Molecular and Cell Biology Master course. As a member of Research Centre on Ceramic and Composite Materials (CICECO) from Aveiro University, current research is directed to the adverse effects of some pollutants (heavy metals, pesticides) on different target organs of mice, combining histological, histochemistry, and ultrastructural approaches. M. L. Pereira co-authored several publications in the area of environment toxicology, and andrology, collaborating with some journals as referee.
Maria de Lourdes Pereira
Associate Professor with Aggregation
Department of Biology, CICECO,
University of Aveiro, Aveiro, Portugal
mlourdespereira@ua.pt

Neurotoxic Disorders and Medical Management of Patients Poisoned with Organophosphorus and Carbamate Pesticides , p.39-p.62

Professor Milan Jokanović is employed at Faculty of Medicine, University of Nish, Nish, Serbia. He is a member of Academy of Sciences and Arts of the Republic Srpska in Banja Luka. He is also a member of Editorial Board of "Toxicology"

published by Elsevier.
Professor Jokanović published more than 100 papers and book chapters on toxicology of organophosphorus compounds, antidotes and medical treatment of poisoning. He is also an expert for nonclinical and clinical drug development and regulatory affairs. More details about his work and expertise can be found at www.experta.co.rs.

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Betzabet Quintanilla-Vega, Norma Pérez-Herrera and Aurora Elizabeth Rojas-García

Betzabet Quintanilla-Vega, PhD
Associate Professor
Department of Toxicology
CINVESTAV-IPN
Mexico City, Mexico

Aurora Elizabeth Rojas-García, PhD
Associate Professor
Secretariat of Postgraduate and Research Autonomous University of Nayarit
Nayarit, Mexico

Norma Pérez-Herrera, PhD
Assistant Professor
Laboratory of Chronic and Degenerative Diseases Inter-Agency Unit of Clinical and Epidemiological Research Faculty of Medicine Autonomous University of Yucatan
Yucatan, Mexico

Occupational exposure to pesticides mixtures: oxidative balance, enzymatic biomarkers and genetic damage in an Argentinian population study, p.78-p.104
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Prof. Phd. Marta Ana Carballo
macarballo2003@yahoo.com.ar

Using a Toxicokinetic Modeling Approach to Determine Biological Reference Values (Bvrs) and to Assess Human Exposure to Pesticides, p.105-p.142
Aurélie Berthet, Michèle Bouchard, Mathieu Valcke, Roberto Heredia-Ortiz

Aurélie Berthet has a Bachelor's degree in biology and a Ph.D. in toxicology and public health. As part of her PhD project in human toxicology, she determined

the toxicokinetics of two fungicides widely used in agriculture, and for which toxicological data were very limited in humans. As postdoctoral researcher, she is currently carrying out toxicokinetics studies on dermal route using an in vitro system. The studied compounds are mainly pesticides and biocides. She is a SOT member and participates in an expert group on pesticide exposure.

Michele Bouchard is an associate professor in the Department of Environmental and Occupational Health of the University of Montreal. She is head of the Chair in Toxicological Risk Assessment and Management of the University and head of the Biomarker Unit of the Xenobiotics and Nanoparticles plateform funded by Canada Foundation for Innovation. Her researches aim at better assessing toxicological health risks in humans associated with environmental and occupational exposures to chemical compounds through the use of biomonitoring and toxicokinetic modeling. Studied contaminants include natural pyrethrins, pyrethroids, phthalimides, organophosphorus insecticides, carbamates, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, formaldehyde, selenium, mercury, arsenic, acrylamide and nanoparticles of titanium dioxide.

Mathieu Valcke has a Bachelor's degree in biochemistry and a Ph.D. in toxicology and risk assessment from the University of Montreal. He cumulates more than 10 years of experience in the field of public health, more specifically on topics related to the health risk assessment of environmental contaminants in soils, food and water. He also realized studies on the exposure and health risk related to the use of pesticides. He currently holds a position as the Chief scientific advisor of the Toxicological risk assessment Group at the National Institute of Public Health of the Province of Quebec, Canada, and has a clinical adjunct professor affiliation at the Department of Environmental and Occupational Health of the University of Montreal.

Roberto Heredia Ortiz is a research assistant in the Department of Environmental and Occupational Health of the University of Montreal. He worked on the development of models to reconstruct exposure to phthalimide fungicides from biomarker data, predict main exposure routes in workers and propose biological reference values. He is currently doing his Ph.D. in the Department on the comparison of toxicokinetic models and physiologically-based toxicokinetic models to describe the kinetics of benzo(a)pyrene biormarker, 3-hydroxybenzo(a)pyrene.

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About authors:
The authors belong to the Research Group in Environment and Health, University of Las Palmas de Gran Canaria (Spain). This is an interdisciplinary group composed

of medical researchers, veterinarians, biologists and pharmacists with extensive experience in studies involving monitoring of persistent organic pollutants (POPs) in human populations, and the toxic effects induced by environmental persistent organic pollutants, among them organochlorine pesticides, on human beings.

Contacts:
Luis D. Boada PhD, MD; Research Group in Environment and Health; Toxicology Unit. Dept. of Clinical Sciences. University of Las Palmas de Gran Canaria (Spain) and Instituto Canario de Investigación del Cáncer (ICIC). E-mail address: ldominguez@dcc.ulpgc.es. Phone: 34928453472.

Luis A. Henríquez-Hernández, PhD, VD; Research Group in Environment and Health; Toxicology Unit. Dept. of Clinical Sciences. University of Las Palmas de Gran Canaria (Spain) and Instituto Canario de Investigación del Cáncer (ICIC).). E-mail address: lhenriquez@dcc.ulpgc.es. Ph: 34928451461.

Manuel Zumbado, Research Group in Environment and Health; Toxicology Unit. Dept. of Clinical Sciences. University of Las Palmas de Gran Canaria (Spain) and Instituto Canario de Investigación del Cáncer (ICIC).). E-mail address: mzumbado@dcc.ulpgc.es. Ph: 34928453472.

Maira Almeida-González, PhD, VD; Research Group in Environment and Health; Toxicology Unit. Dept. of Clinical Sciences. University of Las Palmas de Gran Canaria (Spain) and Instituto Canario de Investigación del Cáncer (ICIC).). E-mail address: malmeida@dcc.ulpgc.es. Ph: 34928451461.

Pilar F. Valerón, PhD, BD; Research Group in Environment and Health; Toxicology Unit. Dept. of Clinical Sciences. University of Las Palmas de Gran Canaria (Spain) and Instituto Canario de Investigación del Cáncer (ICIC).). E-mail address: jfernandez@dbbf.ulpgc.es. Ph: 34928451421.

Octavio P. Luzardo, PhD, VD; Research Group in Environment and Health; Toxicology Unit. Dept. of Clinical Sciences. University of Las Palmas de Gran Canaria (Spain) and Instituto Canario de Investigación del Cáncer (ICIC).). E-mail address: operez@dcc.ulpgc.es. Ph: 34928451424.

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Prof. Dr. Krystyna A. Skibniewska, University of Warmia & Mazury in Olsztyn, Faculty of Technical Sciences, Head of the Chair of Foundations of Safety, Oczapowskiego 2, 10-719 Olsztyn, Poland. e-mail: kas@uwm.edu.pl
Nature of responsibilities at the university: safety of agricultural land, environment and food contamination, food safety. Author or co-author of 123 original papers, 4 monographs, 32 chapter in monographs, 152 conference papers, 4 books and lecture brochure.

Integrated Pest Management as a Tool to Mitigate Pesticide Negative Impact Into the Agro-Ecosystem: the Soybean Example p.165-p.190
Adeney de Freitas Bueno, Regiane Cristina Oliveira de Freitas Bueno

Adeney de Freitas Bueno graduated in Agronomy in 1998 and later mastered in Entomology in 2001 in the State University Júlio de Mesquita Filho (UNESP/ Jaboticabal, SP, Brazil). He was a Fulbright Exchange Student at 2003 at University of Nebraska Lincoln and finished his PhD at University of São Paulo (USP/ Ribeirão Preto) at 2004. He has been a soybean entomologist since 2006 working at the Embrapa Soybean in Londrina, Parana State, Brazil. He also is professor at the entomology graduated program of the Federal University of Parana and at the agronomy graduated program of Rio Verde University.

Regiane Cristina Oliveira de Freitas Bueno graduated in Agronomy in 2003 and later mastered in Entomology in 2005 in the State University Júlio de Mesquita Filho (UNESP/Jaboticabal, SP, Brazil). She finished his PhD at University of São Paulo (ESALQ/USP) at 2008. She was as post-doc student at Adeney de Freitas Bueno laboratory from 2009 to 2011. Since 2012 she is a professor of entomology at State University of Botucatu, São Paulo State, Brazil.

Overview of the State-of-Art of Dutch Surface Waters in the Netherlands Considering Pesticides p.191-p.202
M.G. Vijver, G.R. De Snoo

Dr. Martina G. Vijver is an assistant professor and researcher of the Department of Conservation Biology, of CML Leiden University. She works on the impact of toxicants, and focuses on mechanisms of effects as well as analysing results at

different spatial scales and at different ecological organizational levels. As a project leader of the Dutch pesticides atlas (www.bestrijdingsmiddelenatlas.nl) she works with many chemical surveillance data that are collected in different watersheds. These data were made accessible using a GIS framework. Many different projects have been developed; e.g. pesticides concentrations are statistically linked to land use, trend analysis are performed to observe water quality, and ecological impact of the pesticides are assessed. Martina G. Vijver has written approximately 65 publications.

Martina G. Vijver
Institute of Environmental Sciences (CML), Leiden University, The Netherlands
P.O. box 9518, 2300 RA Leiden.
Email: vijver@cml.leidenuniv.nl

Prof. dr. Geert de Snoo is director of the Institute of Environmental Sciences (CML) of Leiden University. He is full professor in Conservation Biology. His research is about the impact of chemicals on biodiversity and environmental quality, and about issues of sustainable land use, biodiversity and landscape quality. He is the initiator of the Dutch pesticides atlas (www.bestrijdingsmiddelenatlas.nl). He is also special professor at Wageningen University in the field of Nature Conservation on Farmland. Finally, he is a member of the Board for Authorisation of Plant Protection Products and Biocides (Ctgb), an Autonomous Administrative Body responsible for the admission of pesticides and biocides on the Dutch market. Geert R. de Snoo has written about 200 scientific and professional publications.

Geert R. de Snoo
Institute of Environmental Sciences (CML), Leiden University, The Netherlands
P.O. box 9518, 2300 RA Leiden.
Email: snoo@cml.leidenuniv.nl

Behavior of Pesticides and Their Transformation Products in River Water in Japan p.203-p.224
Kiniaki Kawata, Tomohiro Kose

Kuniaki Kawata is a Professor of Environmental Engineering at Niigata University of Pharmacy and Applied Life Sciences. He works on the environment behavior of chemicals including pesticides and the development of carbonaceous materials for the removal of chemicals. He received his PhD degree from Keio University, Japan, and has published more than 100 papers. E-mail address: kawata@nupals.ac.jp

Tomohiro Kose is an Assistant Professor of Environmental Engineering at Niigata University of Pharmacy and Applied Life Sciences. He works on environmental and indoor behavior of persistent organic pollutants including frame retardants and life cycle risk assessment of these chemicals used in electrical equipment. He received PhD degree from Hiroshima University, Japan, and published around 25 papers. E-mail address: tkose@nupals.ac.jp

About authors

An Overview of Organochlorinated Pesticide Residues in Albania. Case study: Porto Romano, Adriatic Sea p.225-p.239
Aurel Nuro, Elda Marku

Aurel Nuro is a chemist born in 1977 in Tirana, Albania where he graduated at the University of Tirana, Industrial Chemistry Branch in 1991. His PhD was obtained in 2001 at the Faculty if Natural Sciences at the same University. Dr. Nuro's main research fields include Organic Chemistry, Environment Pollution Control, Biomonitoring of organic pollutants and Ecotoxicology. His present employment started in 1993 as assistant lecturer and researcher in the Organic Chemistry Section at the Faculty of Natural Sciences, University of Tirana.
Department of Chemistry
Faculty of Natural Sciences,
Tirana University
ALBANIA
Tel/ Fax: +355 42 276 69
Mob: +355 68 40 56 176
E-mail: nuroaurel@yahoo.co.uk

Elda MARKU
1993 – to date Assistant Lecturer & Researcher in the Organic Chemistry Section, Department of General Chemistry, Faculty of Natural Sciences, University of Tirana.

Research Areas:
Organic Chemistry, Environment Pollution Control, Biomonitoring of organic pollutants, and Ecotoxicology

Department of Chemistry
Faculty of Natural Sciences,
Tirana University,
ALBANIA
Tel/ Fax: +355 42 276 69
Mob: +355 67 20 88 456
E-mail: eldamarku@hotmail.com

Pesticide Risk Index of Del Azul Water Creek (Argentina): Tool for Predicting its Overall Environmental Hazard p.240-p.263
Peluso Fabio, Grosman Fabián, González Castelain J., Othax N., Rodriguez L.

Authors are scientific researchers of the National University of the Centre of Buenos Aires province (UNCPBA). Dr. Fabio Peluso, Lic. José González Castelain, Mg. Sci. Natalia Othax and Mg. Sci. Lorena Rodriguez work at Flatlands Hydrology

Institute (UNCPBA, CIC, MA, located in AZul, Argentina) and Mg. Sci. Fabián Grosman at Multidisciplinary Institute of Ecosystems and Sustainable Development (UNCPBA, Faculty of Veterinary Sciences, located in Tandil, Argentina). The main research issues of the authors are the assessment of the local natural water quality (freshwaters and groundwaters) with relation to the human pollution and the development of tools for it management.

Dr. Fabio Peluso
Flatlands Hydrology Institute
Av. Italia 780
7300
Azul, Provincia de Buenos Aires
Argentina.
fpeluso@faa.unicen.edu.ar

Long-Term Monitoring of Pesticides in Water From Lake Biwa and Rivers Flowing into Lake Biwa p.264-p.285
Taizo Tsuda, Ph.D.

Taizo Tsuda, Ph.D.
Chemical Environment Section
Environmental Monitoring Division
Lake Biwa Environment Research Institute
5-34 Yanagasaki, Ohtsu, Shiga 520-0022, JAPAN
E-mail: tsuda-taizo@pref.shiga.lg.jp

Fields of Research:
Environmental chemistry, Monitoring of environmental chemical pollutants in lakes and rivers,
Bioconcentration of environmental chemical pollutants in fish

Time Trend Variation of Selected Pesticides Residues in Soil From Some Regions in Vietnam p.286-p.320
Toan Vu Duc

Toan Vu Duc, Vice Head of Division of Chemistry, Vietnam Water Resources University
Division of Chemistry
Department of Environment
Vietnam Water Resources University
175 Tay Son street, DongDa district, Hanoi, Vietnam
vuductoan@wru.edu.vn
vuductoan2001@yahoo.com

Education:
Bachelor's degree: Chemical Technology (Study at Department of Chemistry, University of Natural Science, Viet Nam).
Master's Degree: Master of Science in Environmental Technology (Study at Hanoi University of Technology, Viet Nam and Institute of Environmental Technology, University of Applied Science Basel, Switzerland).
Doctor's Degree: Environmental Technology (Study at Hanoi University of Technology, Viet Nam and University of Applied Science Basel, Switzerland).

Countries of work experience: Viet Nam, Switzerland, Thailand, United States of America.
Research Fields: Environmental Toxicology (Persistent Organic Pollutants (POPs)), Environmental Analysis, Environmental Impact Assessment, Wastewater treatment.

DDT Residues in Breeding Population of Booted Eagle (Aquila Pennata) Associated with Agricultural Land Practices p.321-p.338
Emma Martínez-López, Pedro María-Mojica, José F. Calvo, José E. Martínez, Antonio J. García-Fernández

Dr. Antonio Juan García-Fernández
ajgf@um.es
Murcia, Spain

Dynamic and Batch Adsorption Studies of Isoproturon on Activated Carbon p.339-p.353
Juan García, José Luis Sotelo, Araceli Rodríguez, Silvia Álvarez

José Luis Sotelo Sancho (Full Professor), Juan García Rodríguez (Associate Professor), Araceli Rodríguez Rodríguez (Associate Professor), Silvia Álvarez Torrellas (Ph. D. Student)

The authors have taught courses in chemical engineering, Biochemical Engineering, Food Sciences, Chemical Sciences, etc, at the undergraduate and postgraduate level. They have supervised numerous under- and postgraduate students in the fields of Chemical Engineering, Environmental Chemistry, etc. They are also currently supervising numerous students at the MA and PhD levels. They have also presented numerous papers at local and international conferences. They are also active in research publications having published research papers in several local and international journals of repute. They are participating as researchers in several projects supported by public and private funds which are focused on the development of integrated advanced oxidation processes for wastewater treatment coming from industry and sewage treatment plants.

Department: Chemical Engineering
Affiliated Organisation: Complutense University

Contact Details:
Grupo de Catálisis y Procesos de Separación (CyPS), Departamento de Ingeniería Química, Facultad de Ciencias Químicas, Universidad Complutense de Madrid, Avda. Complutense s/n, 28040 Madrid, Spain. E-mail: juangcia@quim.ucm.es

Section 3 PESTICIDE ANALYSIS

Modern Sample Preparation Methods for Pesticide Multiresidue Determination in Foods of Animal Origin by Chromatographic-mass Spectrometric Techniques p.355-p.379
Renato Zanella, Osmar Damian Prestes, Caroline Do Amaral Friggi, Manoel Leonardo Martins, Martha Bohrer Adaime

Authors are from the Laboratory of Pesticide Residues Analysis (LARP) from the Chemistry Department of the Federal University of Santa Maria (UFSM).

Contact: Prof. Dr. Renato Zanella, Depto. de Química, Campus da UFSM, 97105-900 Santa Maria - RS, Brazil. Phone/Fax: +5555 32208011, http://www.ufsm.br/larp

Analytical Methods to Assess the Impact of Pesticides on Human from Coffee p.380-p.390
Zenilda Cardeal

Zenilda de Lourdes Cardeal has a master's degree in analytical chemistry at the Universidade Estadual de Campinas, Brazil; PhD degree in Chimie from Université de Paris XI (Paris, France) and did a Post doctoral stage at the Royal Melbourne Institute of Technology (Australia, 2005). She is Lecture of analytical chemistry at the Universidade Federal de Minas Gerais, Brazil, since 1994. Works in the development of methods for the analysis of organic compounds at trace levels. His research work is focused on the following themes: environmental analysis, food analysis, toxicological analysis, gas chromatography, comprehensive two-dimensional gas chromatography, and solid phase microextraction.

About authors

Sensors - A Nanotechnological Approach for the Detection of Organophosphorous Compounds/Pesticides p.391-p.415
Sanjay Upadhyay, Mukesh K. Sharma, Mahabul Shaik, Ritu Das, V.K.Rao

Sanjay Upadhyay is working as a scientist in Defence Research & Development Establishment, Gwalior, India. He obtained his Ph. D. degree in chemistry from Banaras Hindu University, Varanasi, India. He finished his Postdoctoral work in 2007 from University of Tsukuba, Japan. His research interests include electroanalytical chemistry, bioelectrochemical sensor, DNA sensors, nanomaterials and quantum dots.

Mukesh Kumar Sharma is working as a scientist in Defence Research & Development Establishment, Gwalior, India. He received his Doctor Degree in Chemistry from Jiwaji University, India in 2010. The current research interests are nanomaterials, quantum dots, electroanalysis and electrochemical biosensor/ immunosensors.

Mahabul Shaik is working as a scientist in Defence Research & Development Establishment, Gwalior, India. He received his Post graduation degree in chemistry from Osmania University, Hyderabad, India in 2008. He is pursuing his Doctoral degree from Jiwaji University, .Gwalior, India. Currently he is working in the area of gas sensors, chemiresistors, nanomaterials and adsorption materials.

Ritu Das is a Doctoral student at Defence Research & Development Establishment, Gwalior, India. She received her post-graduation in 2010 from Jiwaji University, Gwalior, India The current research interest is electrochemical biosensor.

V.K.Rao is a scientist & Head, Biosensor Development Division in Defence Research & Development Establishment, Gwalior, India. He did Ph.D. in Chemistry from I.I.T., Chennai. Currently he is working in the area of gas sensors, biosensors, chemiresistors, nanomaterials and adsorption materials.

Editor's Preface

Pesticides are highly effective substances used in control of pests and vectors of human diseases. Their application in agriculture enabled increased crops yields and manufacturing of high quality products in order to satisfy the increasing food demands over the world. The increasing use of pesticides had caused concerns about their effects on human health and the environment.

The book is organized in three sections. The first section contains six chapters on human exposure to pesticides. Chapter 1 disusses human exposure and health effects of carbamate pesticides. Chapter 2 is related to neurotoxic disorders and medical treatment of patients poisoned with organophosphorus and carbamate pesticides. Chapter 3 comments on the role of human paraoxonase-1 (PON1) as a modulator of adverse effetcs of organophosphorus pesticides. Chapter 4 focuses on the occupational exposure to pesticides mixtures. Chapter 5 discusses a toxicokinetic modeling approach to determine human risks resulting from occupational and environmental exposure to pesticides. Chapter 6 discusses human exposure to organochlorine pesticides and the effects on ggrowth hormone/insulin-like growth factor system.
The second section of the book contains ten chapters related to pesticides in the environment. Chapter 7 discusses the effects of pesticides on the environment. Chapter 8 considers an integrated pest management system as a tool to mitigate pesticide negative impact on the ecosystem. Chapter 9 reports on Dutch surface water quality with respect to pesticides. Chapter 10 comments on behavior of pesticides and their transformation products in river water in Japan. Chapter 11 reports on the presence of organochlorine pesticide residues in mussels from the Albanian coast of the Adriatic Sea. Chapter 12 reports on pesticide residues in a creek in Argentina with a prediction of environmental hazard. Chapter 13 focuses on monitoring of pesticide residues in a Japan lake and river. Chapter 14 presents data on pesticide residues in soil in Vietnam. Chapter 15 focuses on DDT residues in an eagle population. Chapter 16 describes a method for removal of herbicide isoproturon from water sources using activated carbon.
The third section of the book contains three chapters related to pesticide analysis. Chapter 17 presents modern sample preparation methods for pesticide multiresidue determination in foods of animal origin by chromatographic-mass spectrometric techniques. Chapter 18 describes analytical methods used for detection and quantification of pesticides in coffee. Finally, Chapter 19 reports on a nanotechnological approach for the detection of organophosphorus pesticides.

The book is a compilation of works, addressing the effects of human exposure to pesticides, pesticides in the environment and pesticide analysis. It offers a large amount of information to the professionals interested in pesticides issues. The commitment of each of the contributing authors with the present project is gratefully acknowledged.

Professor Milan Jokanović
Belgrade, Serbia

Section 1

HUMAN EXPOSURE TO PESTICIDES

Carbamates: Human Exposure and Health Effects

Simone Morais, Elsa Dias and Maria de Lourdes Pereira

ABSTRACT

The extensive use of carbamate pesticides in modern agriculture has raised serious public concern regarding the environment and food safety. Due to their broad spectrum of biological activity, carbamates can be used as insecticides, fungicides, nematocides, acaracides, molluscicides sprout inhibitors or herbicides. Contamination of fruits and vegetables may result from treatment as well as from conditions such as improper use of pesticides, residues from preceding treatments in the soil and cross-contamination. Sources of residues in products of animal origin include contaminated water or feed, pesticide-treated housing, and contaminated milk. The presence of pesticide residues is a concern for consumers because carbamates are known to have potential harmful effects to other non-targeted organisms than pests and diseases. The major concerns are their toxic effects such as interfering with the reproductive systems and foetal development. In this chapter, the more relevant contributions, of the last ten years, to the current knowledge on several aspects regarding carbamate pesticides, such as mode of action, effects on human health, legislation, monitoring, human exposure to carbamate residues, risk and exposure assessment will be discussed.

INTRODUCTION

Pesticides are of vital importance in the fight against diseases, for the production and storage of food being widely used for pest control in agriculture, gardening, homes and soil treatment (Crespo-Corral et al., 2008, Janssen, 1997). In spite of their extensive use, an average of 35% of the produce is lost worldwide (Janssen, 1997). Carbamates represent one of the main category of synthetic organic pesticides since their introduction into the agrochemical market in the 1950s (Tomlin, 1997) and are used annually on a large scale worldwide (Paíga et al. 2009a). They constitute a versatile class of compounds used as insecticides, fungicides, nematocides, acaracides, molluscicides, sprout inhibitors or herbicides. Classification of pesticides to functionalities and chemical properties is available on several informative website (www.epa.gov/pesticides/about/types.htm and http://www.alanwood.net/pesticides). Although carbamates present low bioaccumulation potentials and short-term toxicity (relatively short biological half-lives and are fairly rapidly metabolized and excreted), they are considered hazardous to the environment and human health being included in the priority list released by the United States Environmental Protection Agency (US EPA, 1992).

Apart from their pesticide properties, the role of three carbamate insecticides (carbaryl, baygon and carbofuran), as possible cancer chemotherapeutic agents was

recently explored on preliminary studies *in vitro* using trypsinized squamous cell carcinoma, since they inhibit cellular metabolism including energy, protein, and nucleic acid metabolism, thereby, causing cell regression and death (Amanullah and Hari, 2011).

Three classes of carbamate compounds are known: i) the ester derivatives; ii) those with the general structure $R_1NHC(O)OR_2$, in which R1 and R2 are aromatic and/or aliphatic moieties, and iii) those containing a benzimidazole group (IPCS, 1986).

In this chapter, the more relevant contributions, of the last ten years, to the current knowledge on several aspects regarding carbamate pesticides, such as mode of action, effects on human health, legislation, monitoring, human exposure to carbamate residues, risk and exposure assessment will be discussed.

MODE OF ACTION AND TOXICOLOGY

The mechanism of carbamates poisoning, except herbicidal carbamates, involves carbamylating of the active site of acetylcholinesterase leading to the inactivation of this essential enzyme which has an important role in nervous system of humans, and other animal species (Ecobichon, 2001). Carbamate compounds are often called anticholinesterases. In the presence of inhibitors, acetylcholinesterase becomes progressively inhibited and is not further capable of hydrolyzing acetylcholine to choline and acetic acid (Jokanovic, 2009; Jokanovic and Maksimovic, 1997). Consequently, acetylcholine accumulates at cholinergic receptor sites and produces effects equivalent to excessive stimulation of cholinergic receptors throughout the central and peripheral nervous system. Inhibited enzyme can be spontaneously reactivated, with reversal of inhibition occurring typically with half-time of an hour or less after exposure (Jokanovic, 2009). This fact may reduce the possible period of intoxication in situations of accidental overexposure or suicide attempts. The inhibition of other esterases may also occur (Thompson, 1999).

Herbicidal carbamates (such as carbetamide) which are a limited number of compounds are not inhibitors of cholinesterase and are cell division inhibitors (Tomlin, 1997). Plants can metabolize carbamates in which arylhydroxylation and conjugation, or hydrolytic breakdown are the main routes of detoxification. The results of a number of studies suggest that carbamates are exclusively distributed *via* the apoplastic system in plants (IPCS, 1986).

The acute toxicity of the different carbamates ranges from highly toxic to only slightly toxic or practically non-toxic (IPCS, 1986). Concerning the main carbamate insecticides in use, their relative toxic potency estimated human values, (Erdman, 2003) vary from high toxicity (LD_{50} <50 mg/kg; for aldicarb, aldoxycarb, aminocarb, bendiocarb, carbofuran, dimetan, dimetilan, dioxacarb formetanate, methiocarb, methomyl, oxamyl and propoxur) to moderate toxicity (LD_{50} = 50-200 mg/kg; bufencarb, carbosulfan, pirimicarb, promecarb, thiodicarb, trimethacarb) and to low toxicity (LD_{50} > 200 mg/kg; fenocarb, carbaryl, isoprocarb, MPMC (Meobal), MTMC (Metacrate, Tsumacide), XMC (Cosban)).

The acute dermal toxicity of carbamates is generally low to moderate; an exception is aldicarb, which is highly toxic. Data are available for only a limited number of substances (IPCS, 1986).

Short- and long-term toxicity studies have been carried out. Some carbamates are very toxic and others are less toxic in long-term studies. For many years, long-term toxicity data on carbamates have been evaluated by the FAO/WHO Joint Meeting on Pesticide Residues (JMPR), and a number of ADI (the estimate of the amount of a substance in food, mg/kg body weight/day, that can be ingested daily over a lifetime without appreciable health risk to the consumer (WHO 1997)) for carbamates have been established (IPCS, 1986).

Among other pesticides, carbamates have been included in the list of known endocrine disruptor compounds (Schulte-Oehlmann et al., 2011). In this well-designed review authors provide a databanks of a wide range of pesticides with potentially endocrine-disrupting properties, discussion on some key questions regarding the multiplicity of variables involved.

The onset of clinical effects subsequent to carbamate exposure depends on the dose, route of exposure, type of carbamate involved, use of protective gear, and the premorbid state of the victim (Rosman et al., 2009). Ingestion or inhalation of carbamates results in a more rapid onset of clinical effects as compared with dermal exposure. Acute carbamate poisoning episodes were recently described among pesticide sprayers due to inadequate personal protection (Jensen et al., 2010). There is an increase of pesticide exposure and poisoning in children around the world with consequent morbidity and mortality (El-Naggar 2009; Balme et al., 2010; Jayashree et al., 2011) Clinical manifestations for carbamates (excluding herbicides) result from accumulation of ACh in the synapses and overstimulation of muscarinic and nicotinic receptors throughout target organs. The main clinical manifestations of the carbamate intoxication are muscarinic signs (miosis, salivation, sweating, lacrimation, rhinorrhea, abdominal cramping, vomiting, diarrhea, urinary incontinence, bronchospasm, dyspnea, hypoxemia, bradycardia, bronchial secretions, pulmonary edema and respiratory failure), nicotinic signs (less frequent; muscular twitching, fasciculations, muscle weakens including the respiratory muscles, paralysis, tachycardia, hypertension) and central nervous system signs (rare) (Rosman et al., 2009). The large demand for educational materials that summarize the acute toxicity of pesticides is illustrated by the publication of five editions of the Environmental Protection Agency's Recognition and Management of Pesticide Poisonings (US EPA, 1999; Frazier, 2007).

The medical management of carbamate poisoning consists of supportive measures and specific antidotal treatment, that is, the anticholinergic compound atropine (Rosman et al., 2009). Recovery without medical treatment of cases of accidental overexposure with various carbamate pesticides spontaneously occurred, generally, within 4 h of exposure that caused symptoms of headache, dizziness, weakness, excessive salivation, nausea, or vomiting. For accidental or intentional poisoning that produced symptoms such as visual disturbances, profuse sweating, abdominal pain, incoordination, fasciculations, breathing difficulties, or changes in pulse rate, treatment with atropine combined with general supportive treatment, such as artificial respiration and administration of fluids, has resulted in recovery of

individuals within 1 day (Jokanovic, 2009). Deaths occurred only in severe cases where treatment was delayed and/or insufficient atropine was administered. Furthermore, oximes have been tested in experimental studies and have been shown to be beneficial, alone and/or with atropine, in countering the toxicity of the carbamates isolan, thimetilan, pyramat, dimetilan, aldicarb, neostigmine, physostigmine, pyridostigmine and others (Dawson, 1995). Only carbaryl toxicity reacted inversely and was increased by oxime (Dawson, 1995; Jokanovic, 2009). Rosman et al. (2009) developed a simple decision-making algorithm for the medical first responders in a mass casualties event (such as the use of carbamates compounds by terrorists as a weapon), suspected to be caused by a cholinergic substance (organophosphate or carbamate). According to the proposed algorithm, treatment should consist of atropine and oxime regardless of the exact toxic compound involved. They speculated that in a mass casualties event, the benefits of using oximes outweigh the low level of potential risks (Rosman et al., 2009).

Health problems from pesticides in the absence of acute poisoning are also clinically important. Several carbamates are rated as probable or possible carcinogens in humans, according to the classification of USEPA (2004) and the IARC (IARC, 1991). Epidemiologic studies of pesticide exposure and cancer incidence was recently reviewed by Weichenthal and colleagues (2010). This survey illustrates that carbamates may induce different types of cancer at occupational levels. For example, applicators with the highest LDs of exposure to aldicarb demonstrated high incidence of colon cancer, when compared to nonexposed workers. However, melanoma was associated to carbaryl exposed applicators.

The evidence available today shows that both men and women can experience adverse reproductive effects from carbamate pesticides. When women are sufficiently exposed to certain pesticides, several types of adverse reproductive outcomes may occur, and developmental problems in their children may result. According to Frazier (2007), the main adverse reproductive effects associated to female exposures are neurodevelopmental or childhood behavioral problems (carbaryl), and possible childhood leukemia (propoxur). However, no significant effect on gestation, fertility and parturition indices, and average birth weight was demonstrated on experimental studies with rats exposed to propoxur (Ngoula et al., 2007). Regarding, male exposures associated with adverse reproductive effects, they are related with carbaryl, carbosulfan and carbofuran and consist in sperm aneuploidy and abnormal morphology in manufacturing workers (carbaryl); DNA fragmentation in men treated for infertility (carbaryl); chromosome aberrations and abnormal head morphology in mice spermatozoa (carbosulfan, carbofuran) (Giri et al., 2002; Meeker et al., 2004; Xia et al., 2005).

LEGISLATION

In recent decades, significant analytical developments have been achieved in pesticide residue analysis and, in many cases, focus has been put towards sample preparation and analytical detection (Soler et al., 2004). This has allowed maximum residue limits (MRLs) to become more and more stringent in food commodities and

in water (*Codex alimentarius.* 2009; Hamilton et al., 2003). Hamilton et al. (2003), in a IUPAC Technical Report, presented an interesting overview of regulatory limits for pesticides in water issued by WHO, Australia, the US, New Zealand, Japan, Canada, the European Union and Taiwan. The European Union has set new Directives for pesticides in fruits and vegetables in order to meet health concerns (Regulation EC no. 396/2005 that introduces changes to the European Directive 91/414/EEC) (European Comission, 2005). Typically, MRLs for carbamates range from 0.01– 0.05 mg/kg depending on the commodity and the pesticide (European Union, 2008). If a pesticide is not included in any of the above mentioned lists, the default MRL of 0.01 mg/kg applies (Art 18(1b) of Reg. (EC) No 396/2005). Recently, regulation (EC) No 901/2009 has been produced concerning a coordinated multiannual Community control programme for 2010 to 2012 to ensure compliance with MRLs and to assess the consumer exposure to pesticide residues in and on food of plant and animal origin (Morais et al., 2011). The Member States shall, during 2010 - 2012 analyze samples for the product/pesticide residue combinations. When pesticides are applied according to good agricultural practices, MRLs are not exceeded, but their incorrect application may leave harmful residues, which involve possible health risk and environmental pollution (Hassanzadeh et al., 2010)

ANALYTICAL METHODS

Pesticides comprise a large group of substances with the only common characteristic of being effective against a pest and constituting a challenge to the analyst (Soler et al., 2004; Paíga et al., 2009a, 2009b). Carbamates have been analyzed for various application areas such as environmental analysis, food safety, toxicology, and occupational health. Currently, most interest in the analysis of pesticides is in the field of food safety, thus especially in various types of vegetables and fruits. In addition, pesticides are analyzed in environmental samples, such as different water compartments, soil, sediments, sludge, and animal tissue like fish, and in human body fluids and tissues (Niessen, 2010). The presence of pesticide residues and/or their degradation products, which sometimes are more toxic than their precursors in the environment and in foodstuffs, calls for the use of very sensitive analytical methods, capable of determining these compounds at concentration levels equal or lower than the MRLs established by international organizations (Crespo-Corral et al., 2008). Different techniques have been employed for carbamates determination (Caetano et al., 2008; Wu et al., 2009; Somerset et al., 2011; Van Dyk and Pletschke, 2011; Llorent-Martínez et al., 2011) but the most efficient approaches involve the use of chromatographic methods. Liquid chromatography (LC) coupled with mass spectrometry (LC/MS) is one of the most powerful techniques for monitoring carbamates that are thermally unstable and hence do not perform well easily in a gas chromatography MS multimethod (Sagratini et al., 2007; Chung and Chan, 2010; Park et al., 2010). In particular, it has been shown that, in combination with tandem mass spectrometry (ion trap or triple quadrupole), LC is a very sensitive technique to reveal carbamates and other pesticides residues in fruits (Granby et al., 2004; Soler et al., 2005; Niessen, 2010). Instruments with either

atmospheric pressure chemical ionisation (APCI) or electrospray ionisation (ESI) are the most commonly used (Granby et al., 2004; Kruve et al., 2011).

Regarding sample preparation (Chen et al., 2008; Paíga et al., 2009a, 2009b), it traditionally involves an extraction with an organic solvent such as include matrix solid-phase dispersion, solid-phase extraction, supercritical fluid extraction, solid-phase microextraction, stir bar sorptive extraction and more recently the 'quick, easy, cheap, effective, rugged, and safe' (QuECheRS) method (Anastassiades et al., 2003; Wilkowska et al., 2011).

Nowadays, several rapid, relatively inexpensive, sensitive screening analytical biosensors that need little sample pre-treatment are constantly being developed for the identification and quantification of carbamate compounds (Caetano et al., 2008; Wu et al., 2009; Somerset et al., 2011; Van Dyk and Pletschke, 2011). Biosensors are based on the intimate contact between a bio-recognition element that interacts with the analyte of interest and a transducer element that converts the bio-recognition event into a measurable signal. Among the different types of biosensors, the electrochemical sensors are of special interest due to the high sensitivity inherent to the electrochemical detection and the possibility to miniaturize the required instrumentation, thereby making the construction of compact and portable analysis devices possible (Campàs et al., 2009; Somerset et al., 2011). Somerset et al. (2011) reported detection limit of 0.880 nmol/L for carbaryl, 0.249 nmol/L for carbofuran and 0.111 nmol/L for methomyl using a mercaptobenzothiazole-on-gold biosensor system. From the literature reviewed by Van Dyk and Pletschke (2011), the lower detection limits are also in the range of 0.1 nmol/L (for carbofuran, Ciucu et al., 2003) being clear that enzymatic methods, and biosensors, usually are not able to achieve the sensitivity of chromatographic methods. However, authors claimed that they can serve as a tool for screening of hundreds of samples in a short period of time complementing the existing methods and allowing for a more rapid assessment of problematic environments. Each method has unique advantages which can complement each other (Rodriguez-Mozaz et al., 2007; Van Dyk and Pletschke, 2011).

CARBAMATE BIOMARKERS

Biomonitoring is defined as the repeated, controlled measurement of chemical or biological markers in fluids, tissues or other accessible samples from subjects exposed or exposed in the past or to be exposed to chemical, physical or biological risk factors in the workplace and/or the general environment (Manno et al., 2010).

Some bioindicators are commonly used since they can reflect the effect of contaminants on cellular metabolism and global homeostasis (Tan et al., 2011). González-Fernández et al. (2008) focused on a wide range of conventional biomarkers and explored a preliminary working scheme for the integration of the triple -omic approach (transcriptomics, proteomics, and metallomics) in environmental monitoring. These authors found them useful and a comprehensive alternative in the study of environmental issues and the diagnosis of contamination threats. The expansion of omic technologies in recent years have contributed to

occupational environmental health research through the look at the complete complement of genes its expression and regulation, proteins and metabolites (Vlaanderen et al., 2010). These authors explored in a well design review the currently fields (genotyping, transcriptomics, epigenomics, proteomics and metabolomics) pointing for new insights in the near future. George and Shukla (2011) provided also a complete review of the research activities in the area of toxicoproteomics focusing on the effects of carcinogenic pesticides, namely carbamates. Although these authors considered proteomic technologies as relevant tools in this domain, other issues were also important to elucidate the sequential steps of pesticide-induced carcinogenesis (e.g. the analysis of metabolic activation of chemicals, genome analysis, mRNA measurements, and classical biochemical analysis). In their point of view a better dialogue among those areas and a transdiciplinary approach is needed to better understand the impact of these chemicals on oncology research.

The measurement of current perception threshold (CPT) using Neurometer CPT/Eagle, on the index finger and the great toe can be used as a biomarker to monitor the effects of carbamate exposure among exposed workers (Lubis et al., 2008). By using three neuroselective frequencies range (2000, 250, and 5 Hz) the CPT values were prominent among farmers on both the medial and peroneal nerves.

Sams and co-workers (2010) proposed the quantification of 5,6-dimethyl-2-(methylamino)pyrimidin-4-ol (MDHP) in urine, as a sensitive and specific biomarker of exposure to pirimicarb. Using liquid chromatography with mass spectrometry detection (LC–MS), these authors quantified this secondary metabolite in urine of exposed volunteers.

The osmotic fragility of erythrocytes as a potential biomarker of oxidative membrane damage in carbamate pesticide-induced damage to erythrocytes was recently described (Sharma et al., 2010).

As reported previously, several carbamates may reduce male fertility inducing alterations on testicular morphology. For this reason, the selection of histopathological markers is highly dependent upon the toxicant mechanism of action, dose, and duration of exposure (Moffit et al., 2007). These authors discussed the example of carbendanzim, a well known Sertoli cell toxicant, which was used to underline the role of cytoskeleton alterations as markers of testicular injury. These authors pointed also to multiple histopathological endpoints (e.g. seminiferous tubule diameter, sloughing and vacuolization of seminiferous epithelium, spermatid head retention, and germ cell apoptosis) for a better understanding of the the loss of testicular function.

HUMAN EXPOSURE TO CARBAMATE RESIDUES

The presence of pesticide residues is a concern for consumers because carbamates are known to have potential harmful effects to other non-targeted organisms than pests and diseases (Gilden et al., 2010). People have environmental exposures to pesticides mainly through diet. Human intake due to pesticide residues in food commodities is usually much higher than those related to water consumption and air

inhalation (except for occupational exposure or at home application, e.g. home gardens or the handling with domestic animals). The evaluation of pesticide residues in food is nowadays a priority objective to ensure food quality and to protect consumers against possible health risks (Sagratini et al., 2007).

Fruits and vegetables are important components of the human diet since they provide essential nutrients that are required for most of the reactions occurring in the body. A high intake of fruits and vegetables (five or more servings per day) has been encouraged not only to prevent consequences due to vitamin deficiency but also to reduce the incidence of major diseases such as cancer, cardiovascular diseases and obesity (Keikotlhaile et al., 2011). Contamination of fruits and vegetables may result from treatment as well as from conditions such as improper use of pesticides, residues from preceding treatments in the soil and cross-contamination (particularly during harvesting) (Janssen, 1997; Shi et al., 2011). Due to their high solubility in water, carbamates are distributed in aqueous food such as fruit and related derivatives. Sources of residues in products of animal origin include contaminated water or feed, pesticide-treated housing, and contaminated milk. Carbaryl is one of the residues most frequently reported in previous studies (Rawn et al., 2006; Paíga et al., 2009a, 2009b; Jensen et al., 2009; Hassanzadeh et al., 2010). Carbaryl controls a great number of species of insects on fruits, and other crops, forests, as well as on poultry, farm animals, and pets (US EPA, 2003). Hassanzadeh et al. (2010) studied the residue content of carbaryl applied on greenhouse cucumbers from Iran and its reduction by duration of a pre-harvest interval and post-harvest household processing. Carbaryl residues were detected in concentration ranges of 0.22–4.91 mg/kg indicating that residues in cucumber were higher than its European Union MRL (0.05 mg/kg) value 14 days after application. They suggested that a waiting period of more than 14 days should be observed before harvesting or consumption of cucumbers, in order to protect consumer health. They concluded that household processing, such as washing and peeling and refrigeration storage, was effective in reducing the carbaryl residue levels being peeling the most effective way to reduce the carbaryl residues of the cucumber samples.

Paíga et al. (2009a) analyzed a total number of 28 different fresh tomato samples and 6 processed tomato products from different locations in Northern Portugal. Although S-ethyl-N,N-dipropylthiocarbamate (EPTC) was not authorized for use in tomato cultures, it was detected in all fresh samples at the estimated level of about 4 μg/kg. Butylate was detected in tomato pulp and tinned tomato, at estimated levels of 4 μg/kg.

Caldas et al. (2011) characterized the dietary risks of organophosphorus and dithiocarbamate pesticides in a total diet study at a Brazilian university restaurant. They observed that dithiocarbamat reach a value of 0.08 mg/kg corresponding to an intake by fruit consumption of 0.112 μg/kg body weight per day.

Kobayashi et al. (2011) performed a survey of pesticide residues in 595 imported frozen products on the Tokyo market from April 1989 to March 2008. Carbaryl was detected in berries (blueberry, raspberry and strawberry) and methomyl in okra and spinach at the level of 0.005-0.01 mg/kg.

Jensen et al. (2009) assessed the cumulative acute exposure of the population of Denmark to 25 organophosphorus and carbamate pesticide residues from the consumption of fruit, vegetables and cereals. Residue data obtained from the Danish monitoring programme carried out in the period 2004–2007, which included 6704 samples of fruit, vegetables and cereals were used in the calculations. Exotic fruits, including passion fruit, mango, guava, carambola, kaki, rambutan and kumquat had the highest detection frequency (13.9%), followed by cherry (12.5%), mandarin (9.9%), lemon (8.6%), asparagus (7.7%), peach/nectarines (7.6%), rice (7.3%), grapefruit (6.9%), and pineapple (6.9%). Commodities with the highest mean concentrations were basil followed by lemon, grapefruit, dates, oranges and mandarin. Carbaryl was detected at concentrations ranging from 0.0018 mg/kg (in mushrooms) to 0.1369 mg/kg (in grape table) Jensen et al. (2009).

Rawn et al. (2006) determined seven parent N-methyl carbamate insecticides, in addition to two transformation products of aldicarb (aldicarb sulfoxide and aldicarb sulfone), and a single transformation product of carbofuran (3-hydroxycarbofuran) in infant and junior foods available on the Canadian retail market between 2001 and 2003. Carbaryl was the most frequently (7.6%) detected compound and concentrations ranged from 0.6 to 18 ng/g. Detectable levels of carbaryl were most frequently found in foods prepared with fruit. Methomyl was detected (0.8 ng/g) in one chicken with broth sample analyzed in the present study. In all cases, the concentrations observed were orders of magnitude below the maximum residue limits established for these compounds in the corresponding raw food commodities in Canada (Rawn et al., 2006). Dietary intakes of carbaryl and methomyl based on the consumption of infant foods tested ranged between 0.2–343 and 0.4–2.0 ng/kg body weight per day, respectively (Rawn et al., 2006). These authors (Rawn et al. 2004) also quantified the N-methyl carbamate concentrations for apple and grape juices available on the retail market in Canada. Carbaryl was the most frequently (58.6%) detected carbamate in juice samples studied. It was observed more frequently in grape juices than in apple or mixed juices. Oxamyl and methomyl were detected in apple juice samples, although they were below detection limits in all grape and mixed juice samples analysed. Maximum levels of carbaryl, methomyl and oxamyl were 93, 6.7 and 4.6 ng/L, respectively.

Kumari et al. (2004) reported the monitoring of pesticidal contamination of farmgate vegetables from Hisar, India. A total of 84 farm gate samples of seasonal vegetables was analyzed for organophosphates, synthetic pyrethroids, organochlorines and carbamates. Only aldicarb among the carbamates was detected in potato (at the range of 0.003-0.082 μg/g).

RISK ASSESSMENT

Common exposed professionals include pesticide applicators, and harvesting crops, and farmers. However, humans may be also exposed to carbamates through over-exposure at home application (e.g. home gardens, the handling with domestic animals). Biological and environmental monitoring are the main tools presently available for the “in-the-field” pesticide risk assessment, although some limitations

must be considered, since effective activities occurs in an open air (Colosio et al., 2011). For this reason, Colosio et al. (2011) developed an integrated user-friendly tool for risk assessment and management in agriculture, in which the computational modelling was added to biological, and environmental monitoring.

Most of the population may be exposed to carbamates through dietary (e.g. consumption of treated fruits, vegetables and contaminated water) (Lu et al., 2010). Hazard characterization involves comparing the pesticide exposure concentration with the ADI or the ARfD (the estimate of the amount of a substance in food that can be ingested over a short period of time, usually during one meal or one day, without appreciable health risk to the consumer) (WHO 1997; Keikotlhaile et al., 2011). In exposure assessment, the potential intake or consumption of pesticide residues is divided by the body weight and compared to ADI or ARfD (Exposure = (Concentration of pesticide residue x Food consumed)/ body weight) (Keikotlhaile et al., 2011).

Long-term averaged daily intake of contaminants such as pesticide residues in food may be estimated using NORMTOX (Ragas and Huijbregts, 1998; Ragas et al., 2009). Schulte-Oehlmann et al. (2011) reviewed the monitoring data over a 10-year period and revealed that although the percentage of foodstuff without detectable pesticide residues in Germany has continuously decreased from 64 to 51.5%, for carbaryl ARfDs were exceeded substantially. Among other pesticides, carbamates were also rated as endocrine-active factors (Schulte-Oehlmann et al., 2011). In view of the probable health risk of a wide range of pesticides, a new EU regulation is being prepared, including the test criteria for these chemicals to be finalized by 2013.

Humans are sequentially or simultaneously exposed to a wide range of pollutants, instead to only one. Due to the main limitations and complex approach to certainly evaluate the health effects of complex mixtures few studies were undertaken to explore this complexity. Recently, Ragas and co-workers (2011) have investigated the cumulative health risk effects of a wide range of stressors, including pesticides, on different target groups (young children, working adults and elderly). This survey was based on the time spent in different microenvironments, the outdoor movement, and food consumption patterns. Different scenarios for assessing mixture effects for a better understanding of mechanism of action, and interactions (antagonistic or synergistic) were considered. In conclusion, this review underlines the need for person-oriented exposure models that can simulate the cumulative exposure history of individuals based on realistic activity and consumption patterns; in addition, a better mechanistic understanding of the effects of cumulative stressors is relevant.

CONCLUSIONS

The increase of carbamate applications makes the implications for the environmental and biomedical research communities a significant endeavor. Diet, and principally fruit and fruit juices, is the major carbamates source for non-occupationally exposed population. The dietary acute intake of some carbamates (AChE inhibitors) as previously evaluated by the FAO/WHO Joint Meeting on

Pesticide Residues indicated a possible risk to health, which has restrained the *Codex Alimentarius* Committee from setting a maximum residue level for many of these compounds. As already stated by other authors (Van Dyk and Pletschke, 2011), an effective strategy for dealing with pesticide contamination in the environment has to commence with an assessment of the extent of the problem. However, monitoring programs for pesticides are scarce, particularly in developing countries. Furthermore, more research is needed in order to characterize the cumulative and synergistic effects of these pesticides, as well as, the interaction among those contaminants and other pollutants.

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Neurotoxic Disorders and Medical Management of Patients Poisoned with Organophosphorus and Carbamate Pesticides

Milan Jokanović

In this article the neurotoxic disorders appearing in patients poisoned with organophosphorus and carbamate pesticides are reviewed. Organophosphorus compounds cause four important neurotoxic effects in humans: the cholinergic syndrome, the intermediate syndrome, organophosphate-induced delayed polyneuropathy and chronic organophosphate-induced neuropsychiatric disorder. Compared to the cholinergic syndrome, that causes millions of cases of poisoning with fatality of more than 15% each year, other disorders involve much smaller number of patients. Carbamates have caused only cholinergic syndrome. This article is focused on neurotoxic disorders appearing after acute and chronic exposure to organophosphates or carbamates with emphasis on molecular mechanisms, clinical presentation, pathogenesis, and possibilities for prevention/medical treatment.

INTRODUCTION

Organophosphorus compounds (OPs) have been used as pesticides and developed as warfare nerve agents such as soman, sarin, tabun, VX and others. Pesticide poisoning results from occupational, accidental, and intentional exposure (Clark, 2002). The epidemiological pattern of poisoning shows significant variation in number of deaths and form of poisoning between developing and industrial countries (Baker et al., 1978; Jeyaratnam, 1990; Van der Hoek et al., 1998; Eddleston, 2000; Eddleston and Phillips, 2004). According to the World Health Organization, about 1 million accidental and 2 million suicidal poisonings with organophosphorus insecticides are reported per year, with more than 300000 fatalities (Jeyaratnam, 1990). Medical management is difficult, with case fatality generally more than 15% (Eddleston et al., 2008; Jokanović et al., 2010).

OP esters cause four neurotoxic disorders in humans: the cholinergic syndrome, the intermediate syndrome, organophosphate-induced delayed polyneuropathy (OPIDP) and chronic organophosphate-induced neuropsychiatric disorder (COPIND). These syndromes, arising from severe exposures, may be caused either by OP pesticides or warfare nerve agents. Most of the cases of poisoning can be prevented by better administrative control, restricted access to OP pesticides, effective measures of personal protection and education of OP pesticide applicators and medical personnel.

Carbamates (CBs) have caused only cholinergic syndrome. Serious poisonings with CBs are much less frequent when compared with OP intoxications and the chances for patient survival are much better. According to the 10 year experience (1997-2007) from the National Poison Control Center of the Military Medical Academy in Belgrade around 75% of carbamate poisonings were caused by a single compound carbaryl (Jokanović et al., 2010).

The cholinergic syndrome

Signs and symptoms of cholinergic syndrome occurring in acute poisoning with OP pesticides are predictable from their biochemical mechanism of action and are directly related to the levels of acetylcholinesterase (AChE) activity. In cases of

human poisoning, general acute symptoms of peripheral nicotinic and muscarinic intoxication are clearly apparent (World Health Organization, 1986). These symptoms include miosis (unreactive to light); sweating, rhinorrhea, lacrimation, and salivation; abdominal cramps and other gastrointestinal symptoms; respiratory difficulties and cough; dyspnea, constriction sensation in the chest, wheezing; twitching of facial muscles and tongue, tremors, and fasciculations; bradycardia and ECG changes, pallor, and cyanosis; anorexia, nausea, vomiting, diarrhea, and involuntary urination and defecation. These signs and symptoms are accompanied by central effects such as dizziness, tremulousness, and confusion; ataxia; headache, fatigability, and paresthesia. Finally, seizures, convulsions, twitching, coma, and respiratory failure may occur. If the poisoned patient survives the first day of poisoning, there are personality changes, mood swings, aggressive events and psychotic episodes including schizoid reactions, paranoid delusions, and exacerbations of preexisting psychiatric problems. Sleep is poor from nightmares and hallucinations; disturbances or deficits in memory and attention, and additional delayed effects also occur. Death usually occurs due to respiratory failure resulting from a combination of central and peripheral effects, paralysis of the respiratory muscles, and depression of the brain respiratory center (Karchmar, 2007; World Health Organization, 1986; IPCS, 1998; Marrs and Vale, 2006; Jokanović et al., 2011). The data presented in Table 1 summarize the muscarinic, nicotinic and CNS effects in patients poisoned with OP and CB pesticides observed at the National Poison Control Center in Belgrade during 1998-2007 period. These findings are consistent with the results of other studies on acute OP poisoning (Clark, 2002; Eyer et al., 2003).

The first four to six hours are the most critical in acute poisoning with OP pesticides. If there is improvement in symptoms after initial treatment then the patient is very likely to survive if adequate treatment is continued (IPCS, 1998).

Table 1. Muscarinic, nicotinic, and CNS effects in patients poisoned with OP and CB pesticides observed at the National Poison Control Center in Belgrade (1998-2007).

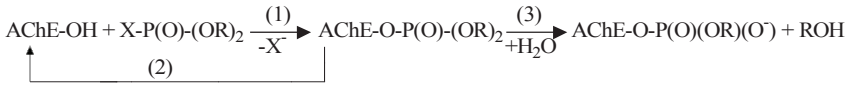
Muscarinic	No. (%) of Patients[1]	Nicotinic	No. (%) of Patients[1]	CNS	No. (%) of Patients[1]
Miosis	196 (61.8)	Fasciculations	46 (14.5)	Coma	80 (25.2)
Bronchorrhoea	164 (51.7)	Hypertension	35 (11.0)	Somnolence	23 (7.3)
GIT[2]	161 (50.8)	Fibrillation	32 (10.1)	Convulsions	14 (4.4)
Hypotension	88 (27.8)	Tachycardia	30 (9.5)	Sopor	13 (4.1)
ARI[2]	83 (26.2)	Tremor	4 (1.3)	Disorientation	4 (1.3)
Bradycardia	29 (9.1)	Arrhythmia	1 (0.3)	Agitation	1 (0.3)
ACF[2]	15 (4.7)				
Cardiac arrest	8 (2.5)				

[1]Compared to total number of patients poisoned with OPs and CBs during 1998-2007 (n=317).

[2]Abbreviations: GIT, gastrointestinal symptoms; ARI, acute respiratory insufficiency, ACF, acute circulatory failure.

Figure 1. Interaction of acetylcholinesterase (AChE-OH) with organophosphorus compounds. Reaction 1 shows interaction of organophosphate molecule with the serine hydroxyl group at the active site of AChE. Inhibited AChE cannot further serve its physiological function, which causes the accumulation of acetylcholine at the nerve endings. Reaction 2 is spontaneous reactivation of inhibited AChE which occurs relatively rapidly for dimethylphosphates and slowly for other OP

compounds. Reaction 3, called “aging”, represents non-enzymatic time-dependent loss of one alkyl group (R) bound to the phosphorus. X stands for acyl radical (i.e. Cl^-, F^-, CN^-, *p*-nitrophenol etc.).



The mechanism of OP poisoning involves inhibition of AChE at synapses and neuromuscular junctions in cholinergic pathways leading to accumulation of acetylcholine and overstimulation of postsynaptic muscarinic and nicotinic receptors (Figure 1). Inhibition of AChE occurs after phosphorylation of hydroxyl group at serine at the active site of the enzyme. Following inhibition, AChE can be spontaneously reactivated at the rate that depends on chemical structure of OP. For OP having dimethyl radicals the AChE reactivation is relatively rapid with a half-time of about 1-2 hours, while that for OP having diethyl functional groups is 31-57 hours. For OP belonging to the group of warfare nerve agents (soman, sarin, tabun, VX) having branched radicals spontaneous AChE reactivation does not occur at all. For certain OP, an additional step can occur on phosphorylated AChE known as aging reaction that represents non-enzymatic time-dependent loss of one alkyl group (R) bound to the phosphorus. The aging reaction depends on the chemical structure of the inhibitor and leads to a stable non-reactivatable form of phosphorylated AChE (Reiner and Pleština, 1979; Worek and Diepold, 1999; Jokanović and Stojiljković, 2006; Jokanović and Prostran, 2009).

The duration of effects is determined mainly by the properties of the compound: its liposolubility, the stability of the OP-AChE complex and whether it is reactivatable after the use of cholinesterase reactivators (such as oximes). It is important to note that only OP containing P=O bond (known as direct inhibitors) are potent AChE inhibitors; those having a P=S group (indirect inhibitors) must be metabolically activated to P=O group (Jokanović, 2001). The signs and symptoms of poisoning with direct inhibitors appear quickly during or after exposure, while those with indirect inhibitors appear slowly and last longer, even up to several days after cessation of exposure.

Clinical diagnosis of acute poisoning with OP compounds is relatively simple and is based on medical history, circumstances of exposure, clinical presentation, and laboratory tests. Confirmation of diagnosis can be made by measurement of erythrocyte AChE or plasma cholinesterase (ChE). Activities of these enzymes have been accepted as biomarkers of exposure and/or toxicity of OP. Erythrocyte AChE is identical to the enzyme present in the target synapses and its levels are assumed to reflect the effects in target organs. For that reason, erythrocyte AChE is regarded as biomarker of toxicity of these compounds. It is difficult to know, due to pharmacokinetic reasons, how closely AChE inhibition in erythrocytes reflects that in the nervous system since access to blood is easier than access to brain. Thus, the inhibition of AChE in erythrocytes may be overestimated relative to that in brain (Jokanović and Maksimović, 1997). In addition, AChE in brain is restored by *de novo* synthesis more rapidly than in erythrocytes where AChE activity is recovered via erythropoesis. Inhibition of ChE does not provide accurate information related to clinical severity of the poisoning. Many OP insecticides (e.g. chlorpyrifos, demethon, malathion) appear to be more potent inhibitors of ChE than they are of erythrocyte AChE and, as the consequence, ChE inhibition might occur to a greater extent than AChE inhibition.

Management of the cholinergic syndrome is described in the second part of this review.

Intermediate syndrome

The term Intermediate Syndrome (IMS) was first described by Senanayake and Karalliedde (1987) because it appeared in the interval between the end of the cholinergic crisis and the onset of OPIDP. Following exposure to various OP pesticides, clinical manifestations of IMS typically occur within 24 to 96 hours, and affect patients without fasciculation or other cholinergic signs. The reported incidence of IMS ranges from 7.7% to as high as 84% (Shailesh et al., 1994). Although IMS is well recognized as a disorder of neuromuscular junctions, its exact etiology, incidence, and risk factors are not clearly understood. IMS generally occurred among patients with prolonged and severe inhibition of AChE, however not every patient with severe AChE inhibition developes IMS. Other risk factors of IMS include delayed metabolism of OP pesticides due to toxicokinetic factors or impaired organ function, severity of poisoning, elevated muscle enzymes, and inadequate or late oxime therapy. IMS has been linked with exposure to specific OP pesticides having dimethyl phosphate structure (e.g. fenthion, dimethoate, monocrotophos, dichlorvos, methylparathion) but also developed after exposure to parathion (ethyl phosphate) and methamidophos (phosphoramidate) (De Bleecker et al., 1993; Yang and Deng, 2007). Two typical cases of IMS caused by fenthion and diazinon were recently described by Jokanović and coworkers (2010).

Marked weakness of neck flexion and varying degree of proximal limb muscle weakness, manifesting as weakness of shoulder abduction and hip flexion, are the regular clinical features. Respiratory insufficiency is also common and frequently draws medical attention to the onset of the syndrome. Other possible manifestations are involvement of muscles innervated by motor cranial nerves and decreased deep tendon reflexes. Studies conducted in nineties have shown that intermediate syndrome goes along with excretion of cholinesterase inhibitor metabolites in the urine and by severe depression in cholinesterase levels. It was suggested that the condition might reflect the recirculation of lipid soluble cholinesterase inhibitors from body fat compartments or gastric fluids (De Bleecker, 2006).

John and coworkers (2003) demonstrated an association between the development of IMS and increased activity of enzymes creatine phosphokinase and lactate dehydrogenase, the markers of muscle function, in 25 patients with acute organophosphate poisoning. Muscle injury was seen in all patients beginning at admission, peaking over the first 5 days and then declining over the next 5 days. Temporal profiles of blood muscle isoenzymes showed significantly greater muscle injury in those patients with greater severity of poisoning at admission, those who developed intermediate syndrome and in patients with longer duration intermediate syndrome. Khan et al. (2001) and Dhandapani et al. (2003) demonstrated that oxidative cellular damage of muscle membranes could be associated with muscle necrosis. In a prospective study conducted by De Bleecker (1995) muscle biopsy performed in 19 patients with OP poisoning showed only minimal number of necrotic fibers. IMS could be explained by the reduction in number of functioning cholinergic receptors at the postjunctional membrane, or a failure of acetylcholine release. All these abnormal findings on electromyography suggested a combined presynaptic and postsynaptic defect, without sensory impairment (Baker and Sedgwick, 1996).

With appropriate therapy, recovery from IMS occurs 5-18 days after the onset of weakness. The recovery among patients who survived IMS follows a distinct

pattern, starting first with muscle power recovery in cranial nerve-innervated muscles, followed by respiratory muscles, proximal muscles, and neck flexors. Since IMS carries a high risk of death among patients with respiratory failure, prompt recognition of the syndrome is the basis of IMS treatment. IMS management is mainly supportive since there are no specific antidotes available for this life threatening syndrome. As IMS generally takes place at the same time with severe OP toxicity and persistent inhibition of AChE, early gastrointestinal decontamination, followed by appropriate therapy involving atropine and oximes, and prompt institution of respiratory support, should be helpful in ameliorating the magnitude and/or the incidence of IMS. The prognosis of IMS appears to be favorable if respiratory failure can be promptly recognized and treated accordingly (De Bleecker et al., 1993; De Bleecker, 2006; Yang and Deng, 2007).

Organophosphate Induced Delayed Polyneuropathy

Organophosphate induced delayed polyneuropathy (OPIDP) is unique toxicological phenomenon in that it is caused by a single exposure to certain OP with effects usually appearing after 10 to 20 days or later. OPIDP is toxicologically different from OP poisoning in that it is based on different mechanisms which do not involve AChE and appear a few weeks after OP poisoning has been medically solved with standard therapeutic measures and patient dismissed from hospital. OPIDP is also a different syndrome from IMS.

Table 2. Organophosphorus pesticides reported to cause OPIDP in man (Jokanović et al., 2002; Lotti and Moretto, 2005; Jokanović and Kosanović, 2010).

OP insecticide	No of cases	Location	Year
Chlorpyrifos	2	Italy, India	1986
Dichlorvos	5	Romania, Turkey, Brazil, Korea, India	1980, 2002-2006
Ethyl parathion	1	Germany	1993
Fenthion	3	USA	1985
Isofenphos	1	Israel	1987
Isofenphos/phoxim	1	Italy	1995
Leptophos	80	USA	1974
Malathion	2	Japan, Turkey	1991, 2009
Merphos	1	USA	1977
Methamidophos	> 45	Sri Lanka, Italy, China, Turkey, USA	1981, 1998
Mevinphos	1	Serbia	2010
Mipafox	3	UK	1952
Omethoate	1	France	1972
Phosphamidon/ Mevinphos	1	China	2002
Trichlorfon	22	Romania, Iran, Japan, Hungary	1983 -1986
Trichloronat	1	Poland	1975

The interest in OPIDP appeared after thousands cases of poisoning with triorthocresyl phosphate (TOCP) that occurred mainly due to beverage and food contamination in USA in 1930 and Morocco in 1959 (Johnson, 1982; Morgan,

1982; Lotti, 1992; Jokanović et al., 2004). By the end of twentieth century, there were many cases of OPIDP due to TOCP poisoning in Romania, Sri Lanka, former Yugoslavia and China. In addition to TOCP, several other OP pesticides have been reported to cause OPIDP in man (Table 2) (Jokanović et al., 2002; Lotti and Moretto, 2005; Jokanović and Kosanović 2010). Cases of OPIDP caused by pesticides were discussed in more details by Lotti and Moretto (2005).

OPIDP is relatively rare neurodegenerative disorder in humans that is characterized by loss of function and ataxia of distal parts of sensory and motor axons in peripheral nerves and ascending and descending tracts of spinal cord. The early neurological symptoms usually are sharp, cramp-like pains in the calves, tingling in the feet and hands followed by distal numbness and paresthesia. Pain and weakness in muscles spread rapidly and patients become unsteady and unable to keep their balance. Progressive leg weakness occurs, together with depression of tendon reflexes. Symptoms may also appear in the arms and forearms. Sensory loss may be mild. Muscle tonus of the limbs gradually increase and spasticity appears in the lower limbs. Physical examination reveals distal symmetrical mainly motor polyneuropathy, with wasting and flaccid weakness of distal limb muscles, especially in the lower limbs. In severe OPIDP quadriplegia with foot and wrist drop were observed as well as mild pyramidal signs (Lotti, 1992). There may be some functional recovery in less severe cases with more distal involvement and sparing of spinal cord axons, but pyramidal and other signs of central neurological involvement may become more evident with time. The recovery affects only sensory nerves, while motor neurons may permanently lose its function as indicated by Morgan (1982) who described the lack of improvement during 47 years in 11 patients poisoned with TOCP. The prognosis for functional recovery depends on the degree of pyramidal involvement with ataxia and paralysis representing a permanent outcome of severe OPIDP. It appears that clinical signs of OPIDP in children are considerably milder than in adults (Jokanović et al., 2004; Lotti and Moretto, 2005; Jokanović, 2009b).

OPIDP is initiated by phosphorylation and subsequent aging of >70% neuropathy target esterase (NTE) in peripheral nerves. Carbamates, sulfonates and phosphinates also covalently bind to NTE but cannot undergo aging reaction, and as a result, these inhibitors do not cause OPIDP. When given to experimental animals before a neuropathic OP they protect from OPIDP by occupying NTE active site (Johnson, 1982; Lotti, 1992).

NTE was discovered by Martin Johnson, who described the most important toxicological and biochemical features of this esterase (Johnson, 1982). NTE is an integral membrane protein in vertebrate neurons present in all neurons, but not in glia. The active site of NTE contains Ser^{966} and two aspartates Asp^{960} and Asp^{1086} that appear essential for enzymatic activity. It was shown that human NTE catalyses hydrolysis of membrane-associated lipids with possible involvement in intracellular membrane trafficking. NTE may have important functions during brain development through involvement in a cell-signaling pathway between neurons and glial cells (Glynn, 1999; van Tienhoven et al., 2002; Glynn, 2006). Physiological role and importance of NTE were recently discussed by Jokanović et al. (2011).

Medical treatment of OPIDP in humans is symptomatic. Standard treatment of OP poisoned patients comprising atropine, oxime and diazepam was not effective in treatment of OPIDP. However, there were several reports in the literature describing attempts of treatment of OPIDP in animals and these studies were reviewed by Lotti (1992) and Jokanović et al. (2004; 2011), but none of these treatments have been tested in patients so far.

Chronic organophosphate-induced neuropsychiatric disorder

Chronic exposure to OP has been associated with impaired neurobehavioral performance in some, but not all, epidemiological studies (Ray and Richards, 2001). Chronic organophosphate-induced neuropsychiatric disorders (COPIND) occur without cholinergic symptoms and apparently are not dependent on AChE inhibition (Ray and Richards, 2001; Singh and Sharma, 2000). COPIND usually appears with a delay and persists for a long period possibly suggesting the permanent damage of the central nervous system (Savage et al., 1988; De Silva et al., 2006; Tan et al., 2009). The most common symptoms of COPIND include cognitive deficit (impairment in memory, concentration and learning, problems with attention, information processing, eye-hand coordination and reaction time), mood change (anxiety, depression, psychotic symptoms, emotional lability), chronic fatigue, autonomic dysfunction, peripheral neuropathy and extrapyramidal symptoms such as dystonia, resting tremor, bradikynesia, postural instability and rigidity of face muscles (Ahmed and Davies, 1997; Davies et al., 2000a; Singh et al., 2000; Ray and Richards, 2001; Salvi et al., 2003; Kamel et al., 2004; Roldan-Tapia et al., 2005; Tan et al., 2009; Jokanović et al., 2011). Suicidality and alcohol intolerance have also been reported (Davies et al., 2000a). Similar clinical features have also been reported by soldiers suffering from the Gulf-War Syndrome, which led to the, so far unproven, hypothesis that the illness was caused by chronic exposure to chemical agents with similar effects to OPs (Gronseth, 2005).

Diagnostic criteria for COPIND include (Davies et al., 2000b):

- Repeated exposure to organophosphates;
- At least four of the following: a) personality change and destabilization of mood, b) impairment of concentration, c) impaired exercise tolerance, d) reduced tolerance to alcohol, e) heightened sensitivity to organophosphates;
- At least three of the following: a) exacerbation of “dippers flu”, b) impulsive suicidal thinking, c) language disorder, d) heightened sense of smell, e) deterioration of handwriting.

With the aim to prove the harm due to chronic OP exposure, Jamal (1997) performed tests of peripheral and autonomic nerve functions in patients with COPIND. Results obtained in this study showed the absence of damage in some cases of COPIND, while others have shown only a few symptoms.

In several epidemiological studies conducted among farm workers and pesticide applicators, neuropsychological damage accompanied with damage of peripheral nervous system, anxiety and depression were predominant among the poisoned group (Steenland et al., 1994; London et al., 1998; Beseler and Stallones, 2008). Agricultural workers tested about 2 years after a pesticide poisoning episode showed significantly lower performance in verbal and visual attention, visual memory, sequencing and problem solving (Rosenstock et al., 1991). Levin et al. (1976) found a high level of anxiety in commercial sprayers of insecticides but not in farmers. Savage et al. (1988) showed abnormalities in psychometric testing and motor reflexes. Mild intoxication can also induce COPIND, farm workers with mild OP pesticides intoxication requiring no hospitalization performed worse on tests of cognitive and psychomotor function than nonpoisoned workers did tested 2 years later. During the process of dipping sheep into the basin with insecticide, workers exposed to OP compound developed greater vulnerability to psychiatric disorders such as significant anxiety and depression (Jamal et al., 2002; Kamel et al., 2005). Epidemiological study from Spain revealed a link between exposure to organophosphates and increased suicidal rate (Parron et al., 1996). A literature review of mortality and morbidity studies related to suicide among pesticide-

exposed populations, revealed high suicide rates in farming populations. Epidemiological studies conclude that acute and chronic OP exposure is associated with affective disorders (London et al., 2005; Jaga and Dharmani, 2007).
The underlying mechanism of COPIND has not been established. Tan et al. (2009) hypothesized that COPIND could be derived from withdrawal of OP pesticide after chronic low-level exposure or acute exposure. In addition, other scientists have suggested that mechanisms other than the inhibition of AChE might also be involved. Ray (1998) hypothesized that some protein targets present in brain, known to be sensitive to a number of OP compounds, may be changed even after low level exposure exerting both favorable and damaging manner. Several animal studies have shown that cognitive enhancing action of low doses of certain OPs, such as dichlorvos, in rats were unrelated to AChE inhibition. It was suggested that OP compounds may affect neuropeptide metabolism through the release of endogenous opiates and/or through interactions with yet unidentified receptors (Kohen et al., 1980; Kubek et al., 1997; Desi and Nagymajtényi, 1999). It has also been observed that administration of different OP compounds has different behavioral presentation, suggesting that observed effects are not entirely a result of AChE inhibition (Van Dongen and Wolthuis, 1989; Pope et al., 1992). Finally, London et al. (2005) reported that exposure to OP may cause serotonin disturbances in the central nervous system, which are implicated in depression and suicide in humans (London et al., 2005).

MEDICAL MANAGEMENT OF PATIENTS WITH CHOLINERGIC SYNDROME CAUSED BY ORGANOPHOSPHORUS PESTICIDES

General Measures

Treatment of OP pesticide poisoning should begin with decontamination and resuscitation if needed. Decontamination is vital in reducing the dose of the pesticide absorbed, but care must be taken not to contaminate others, such as medical and paramedical workers. In the case of ingestion, lavage can be performed, and activated charcoal administered. The patient should be observed carefully during the early stages of treatment because respiratory arrest may occur. Solvent vehicles and other components of the formulated OP pesticide may complicate the clinical picture and should be taken into consideration (IPCS, 1998).
Supportive measures should be directed towards the cardiorespiratory system with particular emphasis on maintenance of ventilation, cardiac rhythm and blood pressure; the removal by suction of respiratory and oral secretions which may cause respiratory distress; and the oxygenation of the patient. Severely poisoned patients disconnected from the ventilator when the general condition improves, must be carefully watched for rapid deterioration and development of the intermediate syndrome during the following few days in the Intensive Care Unit (IPCS, 1998). In addition, the patients should be warned to report to hospital if signs of organophosphate-induced delayed polyneuropathy appear 2-3 weeks after exposure.
Ingested organophosphates should be removed by early gastric aspiration and then lavage, with protection of the airway because they are mostly dissolved in aromatic hydrocarbons; this may be the best remedy in unconscious patients. Gastric lavage is most effective within 30 minutes of ingestion, but might be still effective up to 4 hours post ingestion, as organophosphates are rapidly absorbed from the gastrointestinal tract (WHO, 1986).
Administration of oral activated charcoal, in conventional doses, may be considered for reducing further absorption of some organophosphorus pesticides (WHO, 1986).

This recommendation was supported by Peng et al. (2004) who conducted a randomized controlled clinical trial involving 108 patients aimed to assess the efficacy of hemoperfusion with charcoal in treatment of acute severe dichlorvos poisoning. The authors concluded that the rapid fall in blood dichlorvos level and the dramatic clinical response suggest that hemoperfusion with charcoal is effective in the treatment of acute severe dichlorvos poisoning. However, two clinical trials designed to evaluate the effectiveness of activated charcoal in OP poisoned patients failed to confirm these results. A randomised controlled trial of single and multiple doses of activated charcoal in Sri Lanka failed to find a significant benefit of either regimen over placebo in more than 1000 patients poisoned with pesticides (Eddleston et al., 2005a). In addition, Eddleston et al. (2008) conducted an open-label, parallel group, randomised, controlled trial in three Sri Lankan hospitals aimed to assess whether routine treatment with multiple-dose activated charcoal offers benefit compared with no charcoal. Among 2338 patients who ingested pesticides (1310 cases of poisoning with OP and carbamate pesticides) there were no differences in mortality between patients treated with or no charcoal. The authors concluded that they cannot recommend the routine use of multiple dose activated charcoal in poisonings with OP and carbamate pesticides and suggest that further studies of early charcoal administration might be useful.

Specific Treatment
Atropine

Patients acutely poisoned with OP compounds should be treated with atropine, diazepam and an oxime. Atropine acts by blocking the effects of excess concentrations of acetylcholine at muscarinic cholinergic synapses following OP inhibition of AChE. Atropine is the initial drug of choice in acute OP poisoning. Atropine sulphate in combination with an oxime has been used in traditional therapy for OP intoxications including insecticides. Atropine can relieve the following symptoms of OP poisoning: sweating, salivation, rhinorrhoea, lacrimation, nausea, vomiting and diarrhea, and can help control of bradycardia and circulatory depressions, dilating the bronchi and abolishing bronchorrhoea. Atropine does not bind to nicotinic receptors and cannot relieve nicotinic effects in OP poisoning (World Health Organization, 1986).
According to IPCS (1998) an initial trial dose of atropine, 1 to 2 mg (0.05 mg/kg) intravenously, should be given slowly over three minutes, and then repeated every five to ten minutes if there is no observable adverse effect. In symptomatic children, intravenous dose of 0.015 to 0.05 mg/kg atropine should be administered every 15 minutes as needed. Atropine may then be repeated or increased in increments at 15 to 30 minute intervals until bronchosecretion is cleared and the patient is atropinized (dilated pupils, dry skin, skin flushing) which should be maintained during further treatment. Repeated evaluations of the secretions through regular auscultation of the lungs is the only adequate measure of atropinization in the severely poisoned patient. The dose may be increased as required. Patients poisoned with OP appear to be resistant to toxic effects of atropine and may require relatively large doses of atropine administered during prolonged periods. In severe OP poisoning total dose of atropine given during 5 weeks of treatment can be as high as 30000 mg (IPCS, 2002).

Diazepam

Benzodiazepines are CNS depressants, anxiolytics and muscle relaxants. Their main site of action is at the gamma-aminobutyric acid (GABA) receptor. The GABAA receptor is a ligand gated chloride ion channel and part of a superfamily of receptors which also includes the nicotinic acetylcholine receptor and the glycine receptor. GABA is the major inhibitory neurotransmitter in the mammalian central nervous system. Benzodiazepines, including diazepam, alter GABA binding at the GABAA receptor in an allosteric fashion but these drugs do not directly activate the receptors (Sellström, 1992; Marrs, 2004).

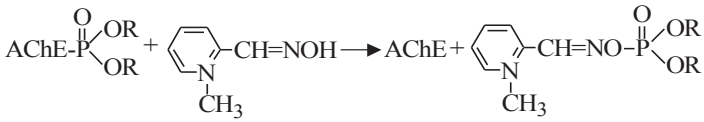
Currently, the most important anticonvulsant is diazepam. The combination of atropine and diazepam is more effective in reducing mortality than atropine or oxime alone. It was also shown that diazepam enhanced the efficacy of low doses of atropine. In the cholinergic nervous system, diazepam appears to decrease the synaptic release of ACh. The main consequence of the action of benzodiazepines in CNS is hyperpolarization of neurons making them significantly less susceptible to cholinergically-induced depolarization. The ultimate result is cessation of propagation of convulsions (Sellström, 1992; Marrs, 2004).

In patients poisoned with OP, benzodiazepines may have a beneficial effect in reducing anxiety and restlessness, reducing muscle fasciculation, arresting seizures, convulsions, controlling apprehension and agitation and possibly reducing morbidity and mortality when used in conjunction with atropine and an oxime. Diazepam should be given to patients poisoned with OP whenever convulsions or pronounced muscle fasciculation are present. In severe poisoning, diazepam administration should be considered even before these complications develop. The recommended dose of diazepam in cases of OP poisoning is 5-10 mg intravenously in the absence of convulsions and 10-20 mg intravenously in cases with convulsions, which may be repeated as required (Johnson and Vale, 1992; Jokanović, 2009a). WHO recommends the dose of diazepam of 5 to 10 mg intravenously slowly over three minutes which may be repeated every 10 to 15 minutes (maximum 30 mg) in adults and 0.2 to 0.3 mg/kg intravenously slowly over three minutes in children (maximum 5 mg in children up to 5 years old, and 10 mg in children older than 5 years) (IPCS, 1998).

Pyridinium Oximes

Extensive studies over the past few decades have investigated the mechanism of action of pyridinium oximes. The rate of spontaneous reactivation of phosphorylated AChE can be accelerated by pyridinium oximes that have a chemical structure which 'fits' the structure of the inhibited AChE. The oximes can only be of benefit as long as inhibited AChE is not completely converted to the aged form which is resistant to both spontaneous and oxime-induced reactivation. Oximes reactivate phosphorylated AChE by displacing the phosphoryl moiety from the enzyme by virtue of their high affinity for the enzyme and their powerful nucleophilicity. Phosphorylated oximes are formed during reactivation reaction (Figure 2) and some of them appear to be potent inhibitors of AChE (Luo et al., 1999; Ashani et al., 2003; Worek et al., 2007). It was shown that phosphorylated oximes of 2-substituted pyridinium compounds (e.g. PAM-2, HI-6) are rather unstable while those of 4-pyridinium aldoximes are markedly stable (Worek et al., 2000; Ashani et al., 2003; Kiderlen et al., 2005).

Figure 2. Reactivation of phosphorylated acetylcholinesterase with PAM-2 and formation of reactivated enzyme and phosphorylated oxime.

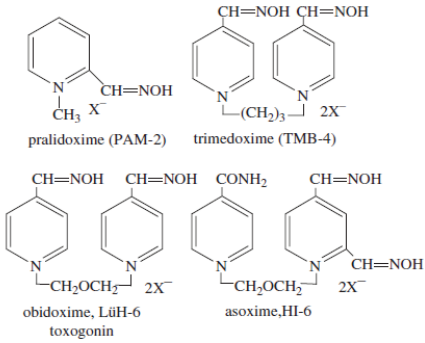


Pyridinium oximes are effective against OP-inhibited AChE in the peripheral nervous system, but have a limited penetration across the blood-brain barrier due to their pharmacokinetic profile and the presence of quaternary nitrogen atom(s) in their structure. However, it appears that oxime penetration through blood-brain barrier is underestimated since soman can cause seizure-related opening of the blood-brain barrier (Carpenter et al., 1990; Grange-Messent et al., 1999) and enable passage of higher oxime concentrations into the brain. It was shown that 0.5-1.0 LD50 of sarin caused a dose-dependent increase in permeability of blood-brain barrier in midbrain, brainstem, cerebrum and cerebellum in rats 24 hours after poisoning (Abdel-Rahman et al., 2002). Sakurada et al. (2003) have determined the amount of PAM-2 passing accross the blood-brain barrier at approximately 10% of the given dose which may be effective in reactivation of OP-inhibited AChE in brain. Additional data indicate indicate that in OP poisoning, when given with atropine, PAM-2 can pass bloodbrain barrier at higher concentrations.

In addition to performing AChE reactivation in OP poisoning, pyridinium oximes might also show direct pharmacological effects. This issue is discussed in detail by Jokanović and Stojiljković (2006) and Jokanović and Prostran (2009). In addition, the structure-activity relationships for pyridinium oximes developed as AChE reactivators were discussed by Jokanović and Prostran (2009) and Musilek and coworkers (2011).

Observational studies of pralidoxime and obidoxime suggest that the ability to reverse AChE inhibition with oximes varies with the pesticide ingested. AChE inhibited by diethyl OP pesticides, such as parathion and quinalphos, seems to be effectively reactivated by oximes, but AChE inhibited by dimethyl OP, such as dimethoate, monocrotophos and oxydemeton-methyl, apparently responds poorly. AChE inhibited by S-alkyl-linked OP, such as profenofos, is not reactivated by oximes at all. This difference is probably caused by variations in the rate of aging on inhibited AChE induced by different OP pesticides.

Figure 3. Chemical structure of pyridinium oximes used in the medical treatment of human OP poisoning. X indicates an anion.



Among the many classes of oximes investigated so far, those with clinical application can be divided in two groups - the monopyridinium and bispyridinium oximes. Currently, the only used monopyridinium oxime is pralidoxime (PAM-2), while the most significant bispyridinium oximes comprise: trimedoxime (TMB-4), obidoxime (LüH-6, Toxogonin) and HI-6, and their chemical structure is presented in Figure 3. There is still no international consensus on the choice of most effective oxime and on dosing regimen.

Pralidoxime

Pralidoxime administered to human volunteers at a dose of 10 mg/kg by intramuscular route, produced a plasma concentration of >4mg/L within 5-10 minutes and maintained levels above this threshold for an hour (Sidell and Groff, 1971). Adverse effects of PAM-2 iodide in volunteers include dizziness, blurred vision, occasional diplopia, impaired accomodation, nausea and headache (Jagger and Stagg, 1958; Sidell and Groff, 1971).
The clinical experience with the use of PAM-2 iodide, given with atropine and diazepam, in the treatment of the victims of Tokyo sarin attack victims in 1995 was extremely favourable (Stojiljković and Jokanović, 2005). However, PAM-2 should not be recommended as the drug of choice in poisonong with warfare nerve agents due to its lack of efficacy against tabun and soman (Kassa, 2005).
In poisoning with OP pesticides pralidoxime chloride should be administered to adults in a dose of 500 mg/h, continuously maintained until clinical improvement is obtained, or 30 mg/kg body weight bolus intravenously over 4 to 6 hours or 8 to 10 mg/kg/h intravenously until full recovery occurs. In children, pralidoxime chloride should be administered in a dose of 25 mg/kg intravenously for 15 to 30 minutes, followed by a continuous infusion of 10 to 20 mg/kg/h. The therapy can continue for 18 hours or longer, depending on the clinical status (IPCS, 1998).
It is essential to adjust the appropriate plasma concentration, i.e. for pralidoxime 20 to 40 mg/L and for obidoxime about 4 mg/L. This concentration is usually attained by a daily dose of 10 to 15 g PAM-2 Cl and 0.75 to 1.0 g obidoxime, respectively, either given divided in 4 to 6 single bolus doses or, preferably, by continuous intravenous infusion, following the first loading dose (2 g pralidoxime and 0.25 g obidoxime, respectively) (IPCS, 1998).

Obidoxime

When administered to human volunteers by intramuscular route obidoxime 5 mg/kg produced a plasma concentration > 4mg/L, from 5 minutes after injection to 3 hours (Sidell and Groff, 1970). Adverse effects of obidoxime in male volunteers were described as pallor, nausea, burning sensation, headache, generalized weakness, sore throat, and paresthesia of the face (Simon and Pickering, 1976; Eyer, 2003; Marrs and Vale, 2006). Following high doses of obidoxime (several grams per day) in severely OP-poisoned patients, hepatotoxic effects were occasionally observed including increased serum transaminases, jaundice and cholestasis (Eyer, 2003).
Obidoxime should be administered in adults at dose of 250 mg given by slow intravenous injection followed by continuous infusion of 750 mg/24h (0.4 mg/kg/h) to reach plasma concentrations of 10-20 μmol/l. Intramuscular dosing is possible when the intravenous route is inaccessible. In children, the dose of obidoxime is 3 to 6 mg/kg slowly administered intravenously over at least 5 minutes (IPCS, 1998).

Asoxime (HI-6)

Clinical studies showed that HI-6 dosed at either 250 or 500 mg by intramuscular route reached plasma concentrations > 4 mg/L in 4-6 minutes. This concentration was maintained for 125 minutes following the lower dose (250 mg) and 200 minutes following the higher dose (500 mg) (Kušić et al., 1985; Kušić et al., 1991). These authors have administered HI-6 four times a day as a single intramuscular injection of 500 mg with atropine and diazepam treatment. Oxime treatment was started on admission and continued for 2 to 7 days.
A clinical study performed on 22 healthy human volunteers did not reveal any adverse effects when HI-6 was given in doses up to 500 mg by oral route (Jovanović et al., 1990). HI-6 is considered to be a very promising bispyridinium oxime in medical treatment following exposure to most nerve agents. A disadvantage of HI-6 compared to other available oximes is its lack of stability in aqueous solutions. HI-6 was considered to be an effective antidote (in combination with atropine and diazepam) in treatment of patients poisoned with OP insecticides (Kušić et al., 1991).
It is important to note that oximes are not effective for improvement of outcomes if the patient develops severe complications such as aspiration pneumonia or hypoxic brain injury before treatment. Such complications take place with fast-acting pesticides such as parathion and dichlorvos (Eddleston et al., 2008).

Clinical experience with pyridinium oximes

A particular problem in interpreting the beneficial role and efficacy of oximes in clinical practice is a deficiency of published data, especially those evaluated in controlled clinical trials. Studies related to the efficacy of oximes in clinical setting showed the heterogeneity of therapeutic approaches (i.e., dose regimen, oxime choice and final outcome of the treatment). In most reports cited in this section chemical structure of OP pesticides was identified in blood/urine and there were adequate data on therapeutic measures taken.
Eddleston et al. (2005b) conducted a prospective study on 802 patients self-poisoned with chlorpyrifos, dimethoate, or fenthion. Compared with chlorpyrifos (8.0%), the proportion dying was significantly higher with dimethoate (23.1%) or fenthion (16.2%) as was the proportion requiring endotracheal intubation (chlorpyrifos, 15.0%; dimethoate, 35.2%, fenthion, 31.3%). Patients poisoned by diethyl OP pesticide (chlorpyrifos) responded well to pralidoxime, whereas those poisoned by two dimethyl OP pesticides (dimethoate, fenthion) responded poorly. Poor efficacy of pralidoxime in treatment of human dimethoate and fenthion poisonings was in agreement with experimental studies conducted by Jokanović and Maksimović (1995) who found that antidotal efficacy of obidoxime, trimedoxime, pralidoxime and HI-6 (given with atropine and diazepam) in rats dosed with 2 LD50 of the dimethoate, was low. However, there was a discrepancy between fenthion poisoned patients and animals in that pralidoxime was ineffective as an antidote in patients, while the four oximes showed considerable efficacy in rats.
Kušić et al. (1991) have tested the oxime HI-6 in OP pesticide poisoning in 60 patients. HI-6 was administered four times a day as a single intramuscular injection of 500 mg with atropine and diazepam treatment. Oxime therapy was started on admission and continued for 2 to 7 days. Most patients were treated with HI-6 and nine patients severely poisoned with quinalphos were treated with PAM-2 chloride (1000 mg four times per day). HI-6 rapidly reactivated human erythrocyte AChE inhibited by dietoxy OPs (phorate, pyridaphenthion, quinalphos) as well as that

inhibited by dichlorvos (a dimethoxy OP) with reactivation half-lives ranging from 0.5 to 3.5 h. AChE inhibited with other dimethoxy OPs (dimethoate, phosphamidon) was reported to be resistant to HI-6 treatment, whereas reactivation with malathion was slow (reactivation half-time 10 hours). Both HI-6 and PAM-2 successfully reactivated AChE in quinalphos-poisoned patients, with HI-6 acting as a faster AChE reactivator than PAM-2. No adverse effects were seen in patients treated with the oximes.

Nine patients intoxicated with OP pesticides were treated with PAM-2 methylsulphate (Contrathion) using a dose of 4.42 mg/kg as a bolus injection followed by continuous infusion 2.14 mg/kg/h. In patients with ethylparathion and methylparathion poisonings, enzyme reactivation could be obtained in some patients at oxime concentrations as low as 2.88 mg/L. In other patients, however, oxime concentration as high as 14.6 mg/L were ineffective. The therapeutic effect of the oxime seemed to depend on the plasma concentrations of ethylparathion and methylparathion. Due to AChE reinhibition, reactivation was absent as long as these concentrations remained above 30 μg/L (Aragao et al., 1996).

Willems et al. (1993) reported that ethyl parathion and methyl parathion could be effectively treated with PAM-2 methylsulfate (plasma concentrations 4 mg/L) and atropine when pesticide concentrations in plasma were relatively low. In severe poisoning with pesticide levels in plasma above 30 μg/L, high PAM-2 concetrations in plasma (14.6 mg/L) did not provide any improvement. In addition, PAM-2 at concentrations of 6.3 mg/L was not effective in AChE reactivation in dimethoate poisoning where AChE was inhibited with its active metabolite omethoate.

It was reported that in cases of life-threatening parathion poisoning obidoxime (Toxogonin) (250 mg administered intravenously as a bolus followed by infusion of 750 mg per day) was effective (Thiermann et al., 1997; Thiermann et al., 1999). However, AChE reactivation did not occur until the concentration of paraoxon in plasma was low. Oxydemeton methyl poisoning responded to obidoxime therapy only when the oxime was instituted shortly after poisoning. In cases when obidoxime treatment started too late there was no reactivation of erythrocyte AChE and one out of six treated patients died.

In a clinical study of 63 patients poisoned with OP pesticides, patients were divided into three groups: one was treated with atropine only, while the other two received atropine and either PAM-2 or obidoxime. Initial and maintenance intravenous doses for PAM-2 were 30 mg/kg and 8 mg/kg/h, respectively, and 8 mg/kg and 2 mg/kg/h, respectively, for obidoxime. The major clinical findings or AChE activities at the time of admission did not show statistically significant differences among the groups. Although the severity of intoxications (based on respiratory complications and duration of hospitalization) was higher in the atropine plus oxime groups, 12% and 50% of patients in the atropine and atropine plus obidoxime groups died, respectively. No mortality was found in the PAM-2 plus atropine group. Incidence of recurrent twitching and convulsions, repeated respiratory arrest, required mechanical respiration, required intensive care unit therapy and duration of hospitalization were lower in the atropine plus obidoxime group than in the atropine plus PAM-2 group. Three of the patients who received the obidoxime combination therapy developed hepatitis and two of them died due to hepatic failure, which may indicate overdosage of obidoxime (Balali-Mood and Shariat, 1998).

AChE inhibited by several OP pesticides, including dimethoate, demethon, triamiphos, ethoprophos, profenofos, fenamiphos and pyridafenthion, resists any attempt of reactivation with any oxime, probably due to variations in phosphoryl moiety and distribution of electronic charge (Jokanović and Maksimović, 1995; Bismuth et al., 1992).

In a randomised controlled trial, Pawar et al. (2006) studied the effect of very-high dose pralidoxime iodide (2 g loading dose, then 1 g either every hour or every 4 h for 48 h, then 1 g every 4 h until recovery) in 200 patients with moderate OP poisoning (excluding severely ill patients). Among OP pesticides involved there were chlorpyrifos (diethyl OP) and dimethoate (dimethyl OP). The high-dose regimen was associated with reduced case fatality, fewer cases of pneumonia, and reduced time on mechanical ventilation. This study suggests that large doses of PAM-2 could have benefit if patients are treated early and have good supportive care.

CLINICAL ASPECTS AND MEDICAL MANAGEMENT OF POISONING WITH CARBAMATES

Cases of accidental overexposure to or suicide attempts with various CB pesticides have followed similar clinical courses characteristic of cholinergic poisoning like that in poisoning with OP. Differences in severity, duration, and outcome have corresponded to differences in effective doses and in promptness and appropriateness of treatment. Spontaneous recovery without medical treatment has occurred generally within 4 hr of exposures producing symptoms of headache, dizziness, weakness, excessive salivation, nausea, or vomiting. More severe symptoms have generally prompted medical treatment. Following treatment with sufficient atropine, individuals have recovered from poisoning that produced such symptoms as visual disturbances, profuse sweating, abdominal pain, incoordination, fasciculations, breathing difficulties, or changes in pulse rate. Recovery has been complete in some cases within 2 hr and in all cases within one day. CB poorly pass the blood-brain barrier and effects on central nervous system seen in OP poisoning are absent or minimal. Deaths have resulted in severe cases where treatment was delayed and/or insufficient atropine was administered. It is important to note, however, that treatment with atropine combined with general supportive treatment, such as artificial respiration and administration of fluids, has resulted in recovery even in cases where symptoms progressed to pulmonary edema or coma (Baron, 1991; Rotenberg et al., 1995; Jokanović, 2009a).

Carbamylation of AChE is apparently a short-lived phenomenon, as CB are reversible AChE inhibitors that spontaneously reactivate with a half-life in the order of an hour or less. Although the immediate clinical picture of CB poisoning is similar to that of OP, reversible inhibition with spontaneous hydrolysis of the carbamylated AChE moiety results in less severe and less prolonged toxicity. Dimethyl compounds are a special case: they produce a carbamylated AChE, which may be reactivated with oximes. However, oximes are harmful when employed in animals poisoned with monomethyl carbamate, and a man who ingested carbaryl died 6 hours after a PAM-2 treatment (Karchmar, 2007). The use of oximes in the case of CB poisoning is controversial and considered contraindicated by some authors. Lieske et al. (1992) have found that pyridinium oximes (obidoxime, trimedoxime, pralidoxime, HI-6) enhance inhibition of both eel AChE and human serum ChE induced by carbaryl. The authors have proposed that oximes act as allosteric effectors of cholinesterases in carbaryl poisoning resulting in enhanced inhibition rates and potentiation of carbaryl toxicity. Inspite of this, some authors have reported beneficial effects of pralidoxime in aldicarb poisoning in humans (Garber, 1987; Burges et al., 1994). In experimental studies, oximes have been shown to be beneficial, alone and/or with atropine, in countering the toxicity of the carbamates isolan, thimetilan, pyramat, dimetilan, aldicarb, neostigmine, physostigmine, pyridostigmine and others (Bošković et al., 1976; Sterri et al., 1979;

Dawson, 1995). It appears that the only CB whose toxicity was increased by an oxime was carbaryl (Dawson, 1995).

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The Role of Human Paraoxonase-1 (Pon1) as a Modulator of Organophosphorous Pesticide Adverse Effects

Dr. Betzabet Quintanilla-Vega, Dr. Norma Pérez-Herrera and Dr. Aurora Elizabeth Rojas-García

Abbreviations: AChE, acethylcholinesterase; BER, base excision repair; CYP, cytochrome P450; CBT, childhood brain tumors; DAP, dialkylphosphates; DMP, dimethylphosphate; LBW, low birth weight; MN, micronucleus; OP, organophosphate; PON1, human paraoxonase 1; SB, spina bifida; SNP, single nucleotide polymorphism.

ABSTRACT

Gene-environment interactions have attracted attention in last decades, since they influence xenobiotic toxicokinetics, such as organophosphate (OP) pesticides, modifying their toxicity. Regarding OP, one of the genes is human serum paraoxonase-1 (PON1), an esterase predominantly synthesized in the liver and associated with high-density lipoproteins (HDL). PON1 is involved in deactivation of some OP and nerve agents. Plasma PON1 activity varies among individuals by a combination of genetic interactions with environmental-dietary factors, leading to a 40-fold variation. More than 200 SNPs have been identified in *PON1* gene, but genetic variations focus on a few in the promoter and coding regions affecting either the amount of the enzyme or the affinity for specific substrates. This chapter will discuss the contribution of PON1 polymorphisms, particularly those in the coding region, Q192R and L55M, which have shown more consistently to enhance the associations between OP exposure and adverse effects. OP exposure is of public health concern in developing countries, not only to workers (agriculture and manufacture) but to their families. Individuals carrying the risk alleles are more susceptible toward alterations on semen quality, neurological function, DNA integrity, among others, as well as brain tumors, neurodevelopment and cognitive alterations and malformations in children by paternal or prenatal OP exposures. Studies have shown that PON1 polymorphisms are relevant risk factors for having people affected by OP exposure but not consistently, therefore, the exact role of PON1 genetic variation on the susceptibility to OP toxicity is an important question that remains to be elucidated.

INTRODUCTION

Epidemiological studies have shown the global impact of anticholinesterase pesticides, after acute and chronic poisoning on public health worldwide, including developed and developing countries. The magnitude of acute poisoning is difficult to know, but it has been estimated on 250,000-500,000 events with 30,000 to 300,000 fatalities yearly and organophosphate (OP) pesticides are often the most involved. Registers show that OP and carbamates are involved in 40% of accidental pesticide poisonings in the developing world. A high proportion of poisonings in

developing countries is due to suicide attempts and by occupational exposure due to low economic status and poor educational levels. In addition to acute toxicity due to poisoning events that affects a high number of subjects, the delayed toxic effects of OP chronic poisoning have to be also considered (Colosio and Vellere, 2010).
Most OP pesticides are organothiophosphates that require metabolic activation through oxidative desulfuration to form the corresponding oxidative metabolites (oxons); this bioactivation is mediated by several cytochrome P450 (CYP) enzymes. While OP deactivation is mediated mainly by serum paraoxonase 1 (PON1) (Jokanovic, 2001).

Human paraoxonase 1 (PON1)

Human serum PON1 (EC 3.1.8.1) belongs to a multigene family comprised by *PON1*, *PON2* and *PON3* genes. PON1 is a 355-aminoacid calcium-dependent esterase, predominantly synthesized in the liver and closely associated with high-density lipoproteins. To date, there is no known physiological substrate and no clear described biological function. PON1 is involved in the deactivation of some active oxons of OP pesticides (Mackness et al., 1998), as well as of nerve gases like sarin and soman (Kanamori-Kataoka and Seto, 2009). Recently, other substrates have been described such as estrogen esters (Teiber et al., 2007) and its lactonase activity (Draganov et al., 2005). Different assays have been developed to explore PON1 activity in human populations. The arylesterase and oxonase activities are the most frequently used and are often determined using phenylacetate or any oxon (i.e paraoxon, diazoxon) as substrates in the presence of calcium; however, PON1 activity is currently performed using the non-toxic substrate 4-methyl-phenylacetate (4-CMPA). A two substrate assay protocol plotting PON1 activity toward 4-CMPA and phenylacetate has been developed, successfully resolving phenotypes of high and low activity (Richter et al., 2010).

PON1 genetic polymorphisms

A wide variability in human plasma PON1 activity has been reported and has been attributed to the presence of polymorphisms in *PON1* gene, among other factors. There are 200 single nucleotide polymorphisms (SNPs) in *PON1* described to date (Draganov and La Du, 2004). Four polymorphisms in the promoter region at positions -108 (T/C), -126 (G/C), -162 (A/G), and -909 (C/G) have been related with differences in PON1 activity and expression (Brophy et al., 2001; Leviev and James, 2000; Suehiro et al., 2000), and linkage disequilibrium has been reported among them (Blatter-Garin et al., 1997; Brophy et al., 2000; Rojas-García et al., 2005). On the other hand, two polymorphisms in the coding region at positions 55 and 192 have been reported; that at position 55 (Leu/Met) has been related with different PON1 activity, the 55M isoenzyme has lower enzymatic activity than the 55L isoenzyme (Blatter-Garin et al., 1997), due in part to linkage disequilibrium with the -108C allele (Brophy et al., 2001), and to an increase in the stability of the 55L isoenzyme (Leviev and James, 2000). Regarding *PON1* Q192R polymorphism, the two allozymes (R and Q) differ considerably in their affinity and catalytic activity toward a number of substrates (Draganov and La Du, 2004). Both isoforms hydrolyze phenylacetate at similar rates (La Du et al., 1986), but paraoxon and diazoxon hydrolysis rates are different (Li et al., 2000). Linkage disequilibrium between *PON1* 55 and 192 sites has also been reported (Blatter-Garin et al., 1997; Brophy et al., 2000; Rojas-García et al., 2005).
Several studies have reported the association between PON1 phenotype and genotypes, mainly those related to -108, 192, and 55 positions. In a study in a Hispanic-Caribbean population, polymorphisms at -108 and 55 sites showed a

contribution in PON1 activity of 17% and 12%, respectively (Chen et al., 2003), while other studies in Caucasian populations reported that *PON1* -T108C and M55L polymorphisms participated with 23% and 5%, respectively (Brophy et al., 2001). The contribution of polymorphisms in these sites in a Mexican population was 12% and 13%, respectively (Rojas-García et al., 2005). On the other hand, a high arylesterase activity was observed within the *PON1* 192RR genotype in Mexican individuals (Rojas-García et al., 2005), but not in African-American or Caribbean populations (Chen et al., 2003). Finally, in Caucasians populations, *PON1* 192QQ genotype was the one associated with increased arylesterase activity (Brophy et al., 2001).

The differences found in the phenotype-genotype relationship in human populations may be due to the modulating effect of a variety of other factors. PON1 activity increases over time from birth, reaching a plateau between 6 and 15 months of age in humans and at postnatal day 21 in rodents (Costa et al., 2005). No gender-related differences have been found in human populations (Geldmacher-von Mallinckrodt and Diepgen, 1988; Richter et al., 2009). Regarding external factors, cigarette smoke extract inhibited *in vitro* human plasma PON1 activity and this effect was antagonized by antioxidants (Costa et al., 2005), while heavy alcohol drinkers had 45% lower activity compared with non-drinkers (Rao et al., 2003). A study in 189 white men found a positive correlation between dietary and medicinal intakes of Vitamins C and E and serum PON1 activity; although other studies have shown no association (Costa et al., 2005). These data evidenced the importance of evaluating PON1 phenotype and genotype to provide better information about PON1 status.

The wide variability in human plasma PON1 activity has led to the hypothesis that individuals with high plasma PON1 activity would be more resistant to OP pesticide effects than individuals with low activity. The number of articles reporting the influence of PON1 polymorphisms on some adverse effects of OP exposure or degenerative diseases has increased in last decades, and several outstanding reviews have dealt with the evidence of the relationship between PON1 genotype or phenotype and diseases in several populations worldwide.

PON1 and male reproductive toxicity

The presence of PON1 activity in male reproductive system has been poorly explored and until recent years, the expression of *PON1* was reported in mouse testicular cells (Marsillach et al., 2008) and biopsies of humans (Marsillach et al., 2010). Some reports have related seminal PON1 activity and antioxidant status with semen quality, suggesting that the reduced PON1 activity may have a role in the pathogenesis of male subfertility (Verit et al., 2009). However, other studies have not shown such association (Marsillach et al., 2010). Regarding the most studied PON1 genetic polymorphisms, Q192R and L55M, the 55M allele showed a significant difference in the distribution between infertile (n=187) and fertile (n=194) men in a case-control study conducted in Slovenia and Q192R polymorphism did not. These results suggested that the 55M allele might represent a risk factor for infertility susceptibility in Slovenian men (Volk et al., 2011). Similarly, Lazaros et al. (2011) reported that both, 55M and 192R allele frequencies were significantly higher in oligospermic respect to normospermic men from Greece; in addition, men with 55LL/192RR genotype showed lower sperm concentration and motility.

Few studies have evaluated the role of *PON1* Q192R and L55M polymorphisms as modulators of the adverse effects of OP exposure on semen quality. Padungtod et al. (1999) showed that Chinese workers from a pesticide factory (n=60; parathion and methamidophos) carrying the 192QR/RR genotype had significantly lower sperm

count and morphology, as well as serum luteinizing hormone than wild-type individuals. In a cross-sectional study conducted by our group to evaluate the role of *PON1* Q192R polymorphism on the susceptibility to OP toxicity on semen quality in 54 agricultural workers in southern Mexico exposed mainly to OP (i.e. methamidophos), Pérez-Herrera et al. (2008) observed a dose-dependent relationship between OP exposure (as an exposure index) during 3 months before sampling (as a reflect of the exposure to sperm cells during one spermatogenic cycle) and sperm quality parameters in workers carrying the *PON1* 192RR genotype.

Modulation of OP neurological effects by PON1 polymorphisms

The inhibition of acetylcholinesterase (AChE) activity through the phosphorylation of a serine residue in the active site is the most studied adverse effect of OP exposure, which causes severe cholinergic symptoms that may lead to death (Gallo and Lawryk, 1991). Because of its function to deactivate the oxon of OP pesticides, PON1 has been associated with a protective role against OP neurotoxicity. Studies conducted in English farmers mainly exposed to diazinon with ill health reported a lower PON1 diazoxonase activity compared to a referent group, and the risk of developing health problems among exposed farmers was higher if carrying the *PON1* 192RR genotype (OR=3.7, 95%CI 1.2-11.5) compared to those who were 192QQ homozygous (OR=1.1, 95%CI 0.5-2.1) after controlling for potential bias. Additionally, there was an increase risk in farmers carrying the 55LL genotype (OR=4.3, 95%CI 1.1-17.3) (Cherry et al., 2002; Povey et al., 2007). On the other hand, studies were conducted in Gulf War veterans reporting symptoms of neurological damage to determine if PON1 activity and genotype (*PON1* L55M and Q192R polymorphisms) were related to these effects. Veterans were exposed to chemical agents including OP pesticides and nerve gases (Mackness et al., 2000; Hotopf et al., 2003). Significant lower PON1 activity toward paraoxon but not toward diazoxon was observed in veterans, and this effect was not explained by differences in *PON1* coding region polymorphisms. PON1 concentration was also lower in veterans, which was again not dependent of PON1 genotype. Authors conclude that a decreased capacity to detoxify OP pesticides may have contributed to the development of neurotoxicity in Gulf War veterans. Finally, Lee et al. (2003) performed a study in farmers from South Africa (n=100) and *PON1* Q192R polymorphism resulted an independent predictor of chronic toxicity, subjects carrying one or two 192Q alleles showed an increased risk (OR=2.9, 95%CI 1.7-6.9) of having anticholinesterase symptoms.

PON1 in Parkinson's and Alzheimer's diseases

Parkinson's disease is an idiopathic disorder of the dopaminergic nervous system characterized by progressive tremor, bradykinesia, rigidity, and postural instability (Ruiz et al., 2011). It has been postulated that exogenous compounds, including pesticides, might be involved in its etiology, and the relationship appears stronger for the exposure to herbicides and insecticides after long term exposures. However, the evidence is not sufficient to conclude that such a relationship exists for any particular pesticide or combined pesticide or other exogenous toxicant exposure (Brown et al., 2006). Additionally, several genetic factors have been proposed as risk factors for developing Parkinson's disease, and the association of PON1 polymorphisms is the most studied genetic predisposition factor.

A case-control study of Parkinson's disease conducted in a rural population of California's Central Valley (351 cases and 363 controls) living close to areas with extensive agricultural pesticide application, especially OP (diazinon, chlorpyrifos

and parathion) showed that carriers of the variant *PON1* 55MM genotype exposed to diazinon or chlorpyrifos exhibited a two- and three-fold increase, respectively in the risk of having Parkinson's disease compared to unexposed wild-type or heterozygous individuals; no increase in risk was noted for parathion. Authors conclude the importance of considering susceptibility factors when studying environmental exposures in Parkinson's disease (Manthripragada et al., 2010). Similar results reporting that the 55M allele was associated with Parkinson's disease have been reported by others (Carmine et al., 2002; Fong et al., 2005). By the contrary, there are some studies that were not able to show significant associations between *PON1* Q192R polymorphism and Parkinson's disease (Benmoyal-Segal et al., 2005; Dick et al., 2007; Taylor et al., 2002).

Another neurodegenerative disease that has been related to OP exposure is Alzheimer's disease, the most common cause of dementia in old-aged individuals, which is a complex disorder with a multi-factorial etiology (Cellini et al., 2006). It is characterized by: 1) deposition of oxidized low-density lipoproteins (LDL) forming the senile plaques, 2) structural changes in neurons, and 3) cell death in acetylcholine-producing neurons (Pola et al., 2003). Also, the implication of vascular factors in Alzheimer-type dementia is strongly suspected (Dantoine et al., 2002a). Studies in last decades have observed a significant association between Alzheimer's disease and pesticides exposure. Baldi et al. (2001) showed in a prospective cohort study of 1,507 French elderly a relative risk of Alzheimer's disease of 2.39 (95%CI 1.02 -5.63) for occupational exposure to pesticides (dithiocarbamates, carbamates and OP, among others) and 5.63 (95%CI 1.47 - 21.58) for Parkinson's disease after confounding factors were controled. It was not possible to identify the specific pesticides responsible for these observed effects. Similarly, Hayley et al. (2010) reported an association of Alzheimer's disease with OP pesticide exposure (hazard ratio (HR) of 1.53, 95%CI 1.05-2.23) in residents of an agricultural community, concluding that pesticide exposure may increase the risk of dementia and Alzheimer's disease later in life.

Serum PON1 activity decreases with aging and in disorders associated with a high risk of adverse cardiovascular events; therefore epidemiological studies have shown that PON1 activity is negatively correlated with vascular dementia. Additionally, there are studies showing the association between PON1 polymorphisms and the presence of Alzheimer's disease. In this regard, polymorphism *PON1* Q192R was reported as a reliable marker to distinguish patients with Alzheimer's disease from patients with vascular dementia or healthy subjects (Dantoine et al., 2002b; He et al., 2006; Scacchi et al., 2003). However, other studies have failed to found this association (Pola et al., 2003; Wingo et al., 2012) There have also been reported that the frequency of *PON1* 55MM genotype was significantly increased in Alzheimer's disease in patients from Canada (Leduc and Poirier, 2008). The role of PON1 polymorphisms in the promoter region is also controversial. Some results indicated that *PON1* C-161T polymorphism does not play a role in Alzheimer's disease (Cellini et al., 2006), while there is evidence of the association between *PON1* C-161T polymorphic site and Alzheimer's disease in Caucasians and African Americans (Erlich et al., 2006). A recent mini review concluded that the involvement of PON1 genotypes in the occurrence of neurodegenerative diseases remains unclear (Androustsopoulos et al., 2011).

Due to the complexity of the neurodegenerative diseases, and the inconsistency of published results, more studies are necessary to further clarify the effect of gene-environment interactions of PON1 in the development of diseases.

PON1 modulation of genetic damage

Few studies have explored the association between genetic polymorphisms on xenobiotic biotransformation enzymes and DNA damage caused by OP exposure. In this regard, in a study conducted in lymphocytes of *PON1* 192QQ and 192RR genotyped individuals and *in vitro* exposure to paraoxon, DNA damage measured by micronuclei (MN) frequency was different between genotypes. Paraoxon mainly caused DNA damage in lymphocytes from individuals with the 192QQ genotype compared to 192RR subjects (10.2 *vs*. 8.3 MN/1000 cells, $p<0.05$) (Rojas-García et al., 2009). Even though the evidence in human populations is still limited, most studies carried out in occupational workers seem to indicate the relevance of PON1 in the individual susceptibility to OP genotoxicity. A study conducted in vineyard workers exposed to pesticides in Brazil (108 agricultural workers and 65 controls) evaluated the DNA damage using the MN test in binucleated lymphocytes and the comet assay in peripheral leukocytes and subjects were genotyped for *PON1* Q192R polymorphism as well as for other genes: glutathione-S-transferases (*GSTT1, GSTM1* and *GSTP1), CYP1A1* and *CYP2E1*. The study showed a significant higher rate of MN and DNA damage in pesticide-exposed individuals and an effect of PON1 polymorphism, 192QQ individuals had significantly higher MN frequency (8.44 MN) than 192RR subjects (6.23 MN); an association between GSTM1, GSTT1 and CYP2E1 polymorphisms was also suggested. The conclusion of the study was a significant association of MN frequency in OP exposed workers with the *PON1* Q192R polymorphism (da Silva et al., 2008).

Similarly, a study conducted in 115 workers employed for spraying OP for community health programs and 115 controls from India, showed that PON1 activity toward paraoxon and phenylacetate was significantly lower in workers than in control subjects, but no significant differences were observed in the distribution of *PON1* Q192R and L55M genotypes and allelic frequencies between workers and controls. Further, the DNA damage was observed to be significantly higher in workers than in control subjects, individuals carrying the 192QQ or 55MM genotypes (with the least paraoxonase activity) showed significantly higher DNA damage compared to individuals with other genotypes and exposed to OP, suggesting that individuals with these genotypes are more susceptible toward OP genotoxicity (Singh et al., 2011a). In addition, in a study conducted in 150 workers occupationally exposed to OP and 134 normal healthy controls, the role of *PON1, CYP1A1, CYP3A5, CYP2C9* and *CYP2D6* genetic polymorphisms on the modulation of DNA, by means of the alkaline comet assay was explored. The results revealed that PON1 activity toward paraoxon and AChE activity were found significantly lower in workers as compared to control subjects. Workers with *PON1* (192QQ and 55MM) and *CYP2D6*3* (poor metabolizers) genotypes had significantly higher DNA damage when compared to other genotypes. Finally, a significant increase in DNA damage was observed in workers carries of *CYP2D6* and *PON1* (Q192R and L55M) polymorphisms (Singh et al., 2011b). These findings of gene-environment or gene-gene-environment interactions need further extensive studies.

There are also studies where PON1 and other gene polymorphisms did not influence the DNA damage. Liu et al. (2006) conducted a study in 91 fruit growers who experienced pesticide exposure and 106 unexposed controls. Several other polymorphisms on metabolic OP enzymes besides *PON1* Q192R were evaluated, such as CYP3A5, PON2 and some glutathione-S-transferasas (GSTM1, GSTT1, and GSTP1). The results showed that subjects exposed to pesticides had a significantly greater DNA damage than did controls. The multiple regression model revealed that

age, pesticide exposure, and *CYP3A5 A-44G* and *GSTP1* but not *PON1* Q192R polymorphisms were significantly associated with an increased DNA damage.
The interaction of PON1 genetic polymorphisms with other genes, such as those involved in the DNA repair has also been reported. The oxidative DNA damage caused by OP exposure (Piña-Guzmán et al., 2006) is repaired primarily via the base excision repair (BER) pathway, a multistep process that involves genes encoding for several proteins, which have been proposed as potential cancer susceptibility genes (Hung et al., 2005). In exposed individuals containing an increased body burden of reactive genotoxic agents, polymorphisms reducing repair capacity could also lead to enhanced genotoxic effects. A recent study carried out in 108 vineyard workers exposed to pesticides and 65 non-exposed subjects examined if two BER polymorphisms (*XRCC1Arg194Trp* and *OGG1Ser326Cys*) or the combined genotypes of these polymorphisms with *PON1* Q192R could modify individual susceptibility to DNA damage (Rohr et al., 2011). The results showed that individuals with the OGG1Cys variant allele showed higher DNA damage, detected by the comet assay in peripheral leukocytes, compared to wild-type individuals (OGG1Ser). They also found an association of *PON1* 192QQ genotype with higher MN frequency. Considering the combined influence of metabolizing *PON1* and the DNA repair *OGG1* genes, the authors observed significantly higher DNA damage in the exposed group with the less efficient *OGG1Cys* allele independently of the PON1 genotype. When evaluating the effect of XRCC1 and PON1 genotypes, the XRCC1Arg/Trp genotype had a genotoxic protective effect among *PON1* 192QQ individuals, suggesting that enhanced XRCC1 activity may provide some protection for the risk associated with the poor detoxification capacity due to *PON1* Q192R polymorphism. Considering that deficiencies in detoxication and DNA repair pathways of genotoxic contaminants may be linked to cancer, further studies will give information to establish a genetic association of *PON1* and other genes involved in OP metabolism with the risk of developing cancer.
Modulation of genetic damage in germ cells has been less explored. Pérez-Herrera et al. (2008) conducted a cross-sectional study in farmers with Mayan ascendancy from southeastern Mexico chronically exposed to pesticides, mainly OP. The role of *PON1* Q192R polymorphism on the susceptibility to DNA integrity (evaluated by in situ-nick translation; NT-positive cells) was evaluated. A significant association was found between OP exposure and NT-positive cells in homozygote 192RR subjects, and a dose-dependent relationship was observed between OP exposure during 3 months before sampling and NT-positive cells in homozygote 192RR farmers. The authors suggest that cells at all stages of spermatogenesis are targeted by OP, and that there exists an interaction between OP exposure and PON1 Q192R polymorphism on this effect.

PON1 effect on child development

The role of PON1 genotype-phenotype on modulating the effects of prenatal pesticide exposure on child development has been scarcely explored. Berkowitz et al. (2004) evaluated the effects of pesticide exposure (pentachlorophenol, chlorpyrifos and pyrethroids) on birth weight, length, head circumference and gestational age in 404 births and their relationship with PON1 activity (from maternal and cordon blood samples) and *PON1* C-108T polymorphism in New York State. A significant positive trend was found between maternal PON1 activity, but not PON1 genotype and head circumference among the offspring of mothers with chlorpyrifos exposure (urine levels of 3,5,6-trichloro-2-pyridinol) above the detection limit, concluding that chlorpyrifos may have a detrimental effect on fetal neurodevelopment among mothers who exhibit low PON1 activity. In addition, the

relationship between OP exposure and *PON1* C-108T/Q192R polymorphisms as modulators of birth outcomes was explored by Harley et al. (2011) in Mexican-American women enrolled in the CHAMACOS Study, which comprises a cohort of mothers and children from Latino farm workers families in the Salinas Valley, California (USA) where OP are applied. Infants with the *PON1* -108TT genotype had shorter gestational age and had smaller head circumference than those with the *PON* -108CC genotype, while maternal total dialkylphosphates (DAP) concentrations (OP metabolites) were associated with shorter gestational age only among infants carrying the susceptible *PON1* -108TT genotype (interaction p = 0.09). In addition, infants' arylesterase and paraoxonase activities were positively associated with gestational age.

The interaction between maternal exposure to pesticides and the risk for having a baby with low birth weight (LBW) was also evaluated by Moreno-Banda et al. (2009) in a cross-sectional study in 264 female floriculturists or partners of floriculture workers from Central Mexico. Floriculture mothers carrying the *PON1* 192RR genotype showed nearly six-fold higher risk for having a baby with LBW (OR=5.93. 95% IC 1.28-27.5) if working during pregnancy, concluding that PON1 genetic variability increases the probability of having children with LBW.

In the CHAMACOS Study, Eskenazi et al. (2010) also evaluated the relationship between PON1 and neurodevelopment in children exposed to OP *in utero*. Children with *PON1* -108CT and -108TT genotypes performed lower scores on the Bayley Scales of Infant Development than children with the *PON1* -108CC genotype. Furthermore, maternal DAP concentrations were negatively associated with Bayley' scores in children with *PON1* -108CC or -108CT genotypes. *PON* Q192R did not show a clear pattern of associations with this outcome. Engel et al. (2011) evaluated the prenatal exposure to OP, *PON1* Q192R polymorphism and congnitive development at 12 and 24 months of age in children of the Mount Sinai Children´s Environmental Health Cohort study (404 mother-infant pairs). A negative association between prenatal exposure to OP (by means of DAP and dimethylphosphate (DMP) urine concentrations) and poorer Bayley' scores at 12 months was observed among black and Hispanic participants. Children of mothers with the *PON1* 192QR/RR genotype had approximately a five-point decline on Bayley' score with each $\log_{10}$ unit increase in DAP or DMP levels. By the contrary, latter in childhood, children of mothers with the *PON1* 192QQ genotype had a decrement in perceptual reasoning score with each $\log_{10}$ increment in DAP and DMP metabolites, this effect was not observed in children with mothers carrying the *PON1* 192QR/RR genotypes. An explanation for this contradictory effect is not given yet.

The relationship among childhood brain tumors (CBT), residential insecticide exposure (mainly chlorpyrifos and diazinon) and *PON1* C-108T and Q192R polymorphisms was explored by Nielsen et al. (2005) in children enrolled in the U.S. West Coast CBT Study. The risk of CBT was significantly increased among children genotyped as -108T whose mothers referred using pesticides during pregnancy (the risk per -108T allele, OR=2.6, 95% CI, 1.2-5-5), suggesting that CBT risk may be inversely related to PON1 protein levels. *PON1* Q192R and L55M polymorphisms did not show significant interactions (Nielsen et al., 2005, 2010). Authors mentioned that these results must be interpreted with caution due to some limitations, such as the small sample size, no prior studies and technical problems with the DNA sampling.

Finally, González-Herrera et al. (2010) evaluated PON1 polymorphisms and haplotypes and the risk for having offspring affected with spina bifida (SB) in a total of 152 parents of children with open-dorsolumbar SB in Southeast Mexico. Sixty-

three percent of case parents reside in rural areas where pesticides (including OP) use is frequent in agriculture activities. Children with -108CT, 192RR and 55LM/MM genotypes of *PON1* and the haplotypes 221 and 222 (for *PON1* C-108T, L55M and Q192R, respectively) were significantly associated with the risk for having a child with SB. *PON1* -108CT, 55LM and 192RR genotypes were relevant in mothers, mothers/fathers and fathers, respectively. Albeit the associations of PON1 genotype or phenotype with altered child development, additional research is needed to further confirm the relationship with OP paternal exposures, highlighting the risk of OP susceptibility not only to parents but also to their offspring.

CONCLUDING REMARKS

Gene-environment interactions regarding pesticide toxicity is a multifactorial event involving genetic variability and complex pesticide exposure scenarios that make the positive or negative interactions between OP exposure and PON1 genetic polymorphisms with adverse toxic effects difficult to interpret. Results about the role of different PON1 genetic polymorphisms on modulating OP toxicity are not consistent, mainly due to factors such as relative small sample size, not accurate OP exposure characterization, particularly chronic exposures, complex pesticide exposures to many active ingredients, the inclusion of only one-two PON1 polymorphisms, and the lack of including other OP metabolizing enzyme polymorphisms. Additionally, one of the most studied polymorphisms, *PON1* Q192R, is substrate-dependent; thus the amino acid substitution (Arg/Gln) determines the rates of hydrolysis of different substrates, thereby the R and Q alleles may have a positive influence in the toxicity of some OP and a negative influence in the toxicity of others, this is, the effect of this PON1 polymorphism is likely to be exposure specific. Some of these limitations have not been resolved. Finally, since other environmental factors such as diet modify the enzymatic activity, PON1 phenotype has to be considered while evaluating OP risk assessment. Therefore, no conclusions can be given yet and further studies are necessary to establish PON1 role in OP toxicity.

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Occupational Exposure to Pesticides Mixtures: Oxidative Balance, Enzymatic Biomarkers and Genetic Damage in an Argentinian Population Study

Marta Ana Carballo , Elisa Carlotta Kleinsorge , María Fernanda Simoniello

INTRODUCTION

Modern agricultural practices have brought about green revolution in many countries and have impact provided global food security. Historically, Argentina has been among the world leaders in the production and/or export of agricultural products. The main reason for this is that it is a relatively sparsely populated country, but richly endowed with natural resources for production agriculture. According to data from the Food and Agriculture Organization (FAO) (FAOSTAT database), in 2006 Argentina accounted for only 0.59% of the world's population, but for a much higher 2.10% of the world's total land area. Furthermore, Argentina's shares of the world's arable land and the planet's area with permanent meadows and pastures were even higher, at 2.23% and 2.96%, respectively (Lence, 2010). At present, Argentina is the top exporter of soybean oil and soybean meal and the third-largest exporter of soybeans. In addition, is the world's second-largest exporter of corn, sunflower meal, and sunflower oil and must be considered that the relative incidence of crops in exports is even larger (80% of the total).

The most important development was the explosive growth of soybeans, which went from being essentially unknown in the early 1970s to becoming by far the most important crop. In 2005-07, more than half of the crop area and about 45% of the value of crops produced corresponded to soybeans. The evolving patterns in crop output were induced by changes in the relative profitability of the various crops, largely arising from shifts in world supply and demand, the introduction of new technologies, and domestic agricultural policies. Argentina is a large country with spanning regions of quite different suitability for agriculture. From a geographical point of view, its agricultural production can be classified into two main categories, namely, output from the Pampean region, and output from the non-Pampean region or "regional economies." The Pampean region comprises the center and East of the country and produces most of the grains, oilseeds, cattle, and milk. The non-Pampean region consists of the rest of the country, and it produces a relatively large range of agricultural goods. Primary agricultural exports from the Pampas and the non-Pampean regions increased by 46% and 29%, respectively, between 2000 and 2004 (World Bank, 2006).

A major common denominator of the agricultural products from the non-Pampean region is that they tend to be mostly consumed by domestic market. Many of the non-Pampean agricultural products come from perennial plants (e.g., fruits, grapes, tea, and mate), rendering them unresponsive to short run demand shifts (Reca, 2006). Other distinguishing characteristic of agriculture in the Pampas region as compared with the non-Pampean, is that the Pampas is generally more intensive in the use of machinery and management, and more extensive in the use of land and labor (Sturzenegger and Salazni 2008). Importantly, unlike most products from the

Pampas, large components of the non-Pampean output have traditionally received some form of government protection (Reca 2006; Lence, 2010).

SANTA FE PROVINCE

In Santa Fe province (Pampean region) there is 23 percent for cereals and oilseeds in the country, which guarantees a place of importance in the national agricultural scene. Several factors converge to it. On the one hand, the large area of the province from north to south, which gives it different climatic characteristics, is enabling a large range of crops. On the other hand, province has productive potential of soils, with virtually no limitations for agricultural activities. The main traditional crops include soybeans, wheat, corn, sorghum, sunflower, to which add so-called regional crops: rice, cotton, sugarcane and fruit and vegetable. The soybean crop is still the most relevant of oil seeds. Santa Fe holds first place among the producing provinces, with nearly 32 percent of the national total. Cereals and oil seeds are main crops par excellence, due to its mild climate and soils fittest. In the north, subtropical climate, high temperatures and little rainfall, the main industrial crops are like sugar cane and cotton.

Horticultural activity in Santa Fe Province, Argentina, has low participation respect other crops: 4.9 % to field and 1.2 % in crops under cover, being leafy vegetables, cruciferous crops and other crops such as tomato and pepper some of the most relevant (Giunta et al., 2004; 2005).

The so-called "Cinturón hortícola Santafesino" (horticultural belt of Santa Fe) contributes 1.2% to the regional and national market of products. This productive zone is in The Capital Department of Santa Fe Province (Argentina), an area constituted of approximately 3100 Hectares (ha), 1200 ha of which are cultivated in intensive form, and where 2000 people are employed as temporary workers, according to the season.

At present, the horticultural production is focused on leaf-vegetable crops, mainly lettuce, chicory and arugula (22%), spinach beet and spinach (13%), cabbage, broccoli and cauliflower (13%), tomato and pepper (15%), zucchini, gourd, and cucumber (10%) and other vegetables such as beet, radish, aubergine, leek and parsley in a much smaller percentage.

The horticultural productivity in the subtropical regions of the world is severely limited by the pests and diseases affecting crops. The losses in the field and the reduction of the commercial values of the products caused by pests and diseases make the horticultural business less profitable than expected. The fact that the quality of the products has become a priority worldwide has led to the generation of a group of quality standards in response to the demands of the consuming market. The main criterion used regarding this issue is related to the visual aspect related to the shape, the color and absence of damages. The use of agrochemicals is the most common method used for the control of pests and diseases but also one of the most important factors affecting natural resources as well as the health of the rural workers and potential consumers.

Occupational exposure to pesticides mixtures

The current social conditions of agrochemical use are far from laboratory conditions which determine their safety. In general, agrochemicals are rarely applied with suitable protection equipment. As a consequence, subjects who work and/or live near vegetable crops usually suffer from pesticide-induced illnesses, generally

considered as conditions typical of their daily lives, since they do not lead to a significant incapacity for work.

Toxicity of pesticides, expressed by the LD50, is reported only for individual products and not for mixtures. It is widely known that active ingredients, when combined, can increase their individual ability to cause damage or generate new kinds of damages.

The vast majority of toxicological studies of chemicals have focused on the evaluation of exposures to single compounds. Humans are exposed to complex and variable mixtures of chemicals, which may act independently as in a single exposure, but may also interact to modulate the effects of the mixture as a whole and the components therein. The risk assessment of real-life exposures is thus much more difficult than that of exposure to single agents. In assessing such risks from a public health perspective, it is necessary to assess whether the chemicals in a mixture interact to cause either an increased or a different overall response as compared with the sum of the responses of the individual chemicals present in the mixture, or whether the overall effect is simply a summation of the expected effect of each chemical (Hughes and Wood, 2002).

Two basic methodological strategies exist to study the toxicology of mixtures: component interaction analysis and whole mixture analysis. Component interaction analysis (bottom-up approach) can be applied to the analysis of simple mixtures with a small number of constituents and where the composition is clearly known. In the absence of specific knowledge of the composition of a mixture, or where there are numerous components (a complex mixture), whole mixture analysis may be more appropriate. However, such studies cannot define the extent of true interactions between components of the complex mixture without data on the fractions of the mixture (Carpenter et al., 2002).

When using a metabolite as a quantitative indicator of exposure it is important to be aware that various factors can affect the proportion of compound being metabolised by a particular route and therefore the amount of metabolite appearing in blood or urine.

Biological monitoring and biological effect monitoring have been little used to study combined effects of exposures to pesticide mixtures. Measurement of pesticides or their metabolites in asymptomatic populations provides no information on the combined effects of pesticides, even if parent compounds or specific metabolites are measured in biological fluids. A further limitation of biomonitoring is that strategies for biomonitoring the exposure are still strongly influenced by the availability of suitable biomarkers. In fact, for many pesticides, there are none. The alternative of biological effect monitoring may be more promising for the study of combined effects, when new techniques become more widely available. The present methods of biological effect monitoring are rather insensitive.

Humans are often exposed to different pesticides or pesticide mixtures, either simultaneously or in series, making it difficult to identify the effects of each one separately. Chronic exposure to pesticides involves exposure to complex mixtures of different types of chemicals, active ingredients and by-products, such as impurities, solvents and other compounds produced during the storage procedure, present in technical formulations. Moreover, although inert ingredients have no pesticide activity, they may be biologically active and sometimes the most toxic component of a pesticide formulation.

It is important to consider that each active ingredient has a specific mode of action for controlling a pest, and has its own possible side effects on the wild-life and humans exposed to it. Dangerous effects of pesticides in the environment have been documented in many investigations, on soil microorganisms and aquatic flora and

fauna. Occupational exposure to pesticides may increase the risk for adverse reproductive outcomes, brain and nervous system disturbances may cause immunodepression and lead to cancer in later life and can also induce heritable changes. Three million cases of pesticide poisoning, about 220,000 of which are fatal, occur world-wide every year (Raipulis et al., 2009). Of those interviewed in a survey argentina, 13% did not provide an answer in relation with this issue. With regards to the place where the accidents took place, they found that 50% were in the greenhouse, 25% in the open field, and 25% in the shed. As to the body areas affected, in 52% of the cases, intoxication with agrochemicals affected the body in general, whereas in 13% of the cases, lesions were produced in eye and 7% in other body areas (Paunero et al., 2009).
Genotoxicological biomonitoring of human populations is a useful tool to estimate the genetic risk posed by an integrated exposure to complex mixtures of chemicals. Cytogenetic studies refer to different typology of exposure and provide different information about the genetic risk associated with pesticide exposure. Few studies are available on acute pesticide exposure in poisoned subjects. The large majority of cytogenetic monitoring studies in human populations exposed to pesticides concern the genotoxic effects of chronic low doses of a single compound or of a complex mixture of chemicals (Bolognesi, 2003).

Biomonitoring and Biomarkers

Human biomonitoring depends on the use of biomarkers, defined as quantitative indicators of molecular and cellular events in biological systems, relevant to human health, development and aging. Biomarkers are measured in biological material (generally blood or urine) collected from patients or volunteer subjects in observational or intervention studies (Collins and Dusinska, 2009). The molecular epidemiological approach, which measures molecular or cellular biomarkers as indicators of disease risk or of exposure to causative or preventive factors, has applications in studies of environmental and occupational exposure, disease etiology, nutrition, lifestyle, and so on. It is a valuable adjunct to conventional epidemiology, and has the advantage that it requires far fewer subjects and much less time (and is therefore more economical) than the conventional approach. In addition, the biomarkers, if carefully chosen, can give useful information about the molecular mechanisms involved in disease etiology, for example if they reflect an early stage in the progression of the disease (Collins and Dusinska, 2009).
A working group formed by IARC (1997) has defined biomarkers as ‘‘any substance, structure or process that can be measured in the body or its products and may influence or predict the incidence or outcome of disease’’. This definition is further extended by the definition of WHO-ICPS (1993).
The primary purpose of using biomarkers of effect is surveillance, i.e., the identification of individuals or a population at risk of adverse health effects, so that preventive measures can be taken. Although a biomarker of effect is usually also related to exposure to a specific chemical, it is generally more closely related to the occurrence of an adverse health effect (De Zwart et al., 1999).
The selection of appropriate biomarkers is of critical importance because of the opportunity for greater precision in the assessment of risk in individuals or population sub-groups, with the consequent implications for mitigation and health protection. However, this selection will depend upon the state of scientific knowledge and be influenced by social, ethical and economic factors. The process of selection and validation requires careful consideration of the specificity and sensitivity of the biomarker as a measure of the contribution of the exposure to an

observed adverse health outcome. Subject to ethical considerations, the use of validated biomarkers to monitor exposed populations may provide the basis for early, health-protective intervention (Criteria EHC 155, 1993).
Sampling both exposed (treated) and control (reference) individuals on the same day reduce the likelihood of day-to-day experimental variation influencing results. This may not be feasible; but what should be definitely avoided is collecting samples from all exposed subjects and then from all controls (or vice versa), over different time frames (Dusinska and Collins, 2008).
A biomarker of effect can be objectively measured and evaluated as an indicator of normal biological or pathological processes, or toxicological responses to a chemical exposure. The most reliable biomarkers of effect are mechanistically based. The measurement of such biomarkers forms the basis of biological effect monitoring. Some biomarkers can be used as surrogate endpoints. These can substitute for a clinical endpoint, and should be able to predict clinical outcome.
Sensitive biomarkers of effect offer considerable potential for use in studies of individuals exposed to low levels of pesticides and may be invaluable as a bridge between studies in experimental animals and studies in humans and between those in cultured cells and in the intact organism. Much effort is now being devoted to the application of modern biological methods, including transcriptomics, proteomics, metabonomics and non- or minimally-invasive imaging, to identify and develop effective biomarkers of effect. Examples of the application of this approach have been published recently (Petricoin et al., 2002; Issaq et al., 2002). Any accessible biofluid or tissue can be used for biomarker assessment. Techniques now available offer high sensitivity and are applicable to a broad range of endpoints. However, for use in studies of pesticide interactions, it will be important to establish the mechanistic relationship between biomarkers of effect identified in this way and biological responses of concern. Adequate validation, demonstrating their reproducibility and reliability, will be necessary before adopting their widespread use in the study of the toxicology of mixtures.

Acetylcholinesterase and Butyrylcholinesterase

The existence of two types of cholinesterases has been proved: acetylcholinesterase (AChE), or 'true cholinesterase', which is found in erythrocytes and in cholinergic nerve terminals; and butyrylcholinesterase (BChE), or pseudocholinesterase, found in plasma, liver, smooth muscle and fat cells. It is well known that AChE can be an effect biomarker of organophosphorous (OP) and methyl-carbamic (MC) compounds. Also, there is evidence that AChE inhibition correlates with OP-induced symptoms of toxicity (Ranjbar et al., 2002).
The inhibition resulting in the accumulation of endogenous acetylcholine responsible for toxicity in the nervous system presents a dose–response pattern of relatively mild symptoms at a 50–60% inhibition of AChE, with weakness, headache, dizziness, nausea and salivation and a convalescence of 1–3 days. Moderate symptoms at 60–90% inhibition are reversed within periods of a few weeks and are characterized by sweating, vomiting, diarrhea, tremors, disturbed gait, pain in the chest and cyanosis of the mucous membranes. At 90–100% inhibition, the prognosis is death from respiratory or cardiac failure.
Biological effect monitoring could be an important component to study the interactive effects of pesticides and related compounds. To be effective, the biomarker should reflect a response (e.g. inhibition of AChE) that is common to several components of a mixture (e.g. OPs). This may be a more meaningful parameter than the measurement of a metabolite common to compounds of differing

potencies. Biomarkers of effect in current use lack sensitivity. For example, alkyl phosphates can be detected in the urine of individuals after exposure to amounts of OP well below those causing depression of AChE activity, although this may also reflect the concentration-effect relationship that exists for such compounds (Moretto and Lotti, 1998).

Measurements of AChE activity in red blood cells have routinely been performed to survey exposures to OPs in exposed environments. It has also been established that if AChE activity (based on individual pre-exposure level – baseline) decreases by 25%, a second measurement has to be carried out, and that if a decrease in AChE activity is confirmed, exposure has to be avoided for 14 days (Knudsen and Hansen 2007). Historically, the measurement of both cholinesterases in plasma and erythrocytes, which reflected the influence of absorbed OPs on the inhibition of these blood enzymes as surrogates of AChE in neural tissue and neuromuscular junctions, was carried out (Cocker et al., 2002). However, it is well recognized that this is a relatively insensitive indicator of an absorbed dose of OP (Reid and Watts, 1981; Drevenkar et al., 1991; Nutley and Cocker, 1993; Hardt and Angerer, 2000). Blood cholinesterase activity needs at least 15% depression from an individual's normal level of plasma or erythrocyte enzyme activity to be considered indicative of pesticide over-exposure.

Furthermore, due to the large inter-individual variability in cholinesterase activity, this approach requires the collection of both baseline and post-exposure samples from an individual and long-term precision of the methods as this directly influences the level of BChE or AChE depression that can be considered significant (Mason and Lewis, 1989).

In addition, the collection of blood samples is sometimes considered invasive and, in some occupational settings, logistically difficult. BChE and AChE measurements have been used for a number of years in cases of clinical poisoning and accidental OP exposure, and in monitoring workers with high risk of exposure. Depression of the plasma BChE enzyme activity is not necessarily associated with symptoms of anti-cholinergic toxicity and large depressions in BChE have been noted in the absence of any effect on erythrocyte AChE. Decreases in the red cell enzyme activity have been suggested to have closer relations to these symptoms. Therefore, in both clinical toxicology and monitoring high-risk occupational activities, the measurement of both enzymes has been recommended (HSE, 2000; Heath and Vale, 1992).

Oxidative Status

Oxidative stress is a mechanism that could link pesticide exposures to a number of health outcomes observed in epidemiological studies. In blood, normal erythrocyte function depends on the intactness of cell membrane, which is the target for many toxic factors including pesticides (Banerjee et al., 1999).

Free radicals are generally very reactive molecules possessing an unpaired electron. They are produced continuously in cells either as by-products of metabolism, or for example, by leakage from mitochondrial respiration. The most important reactions of free radicals in aerobic cells involve molecular oxygen and its radical derivatives (superoxide anion and hydroxyl radicals), peroxides and transition metals. Cells have developed a comprehensive set of antioxidant defense mechanisms to prevent free radical formation and to limit their damaging effects. These mechanisms include enzymes that inactivate peroxides, proteins that sequester transition metals and a range of compounds that scavenge free radicals. Reactive free radicals formed

within cells can oxidize biomolecules and this may lead to cell death and tissue injury (De Zwart et al., 1999).
Continuous exposure of aerobic organisms to prooxidant challenges has endowed living cells with efficient and sophisticated antioxidant systems. These can be divided into enzymatic antioxidant and non-enzymatic antioxidant systems. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHpx) have been distinguished as the most important members of the enzymatic defense systems against oxygen radicals. Obviously, assaying these enzymes can offer an indication of the antioxidant status of an individual. Besides measuring the enzymatic antioxidant systems in blood samples, non-enzymatic antioxidants, such as vitamin E and C, b-carotene, urate, retinyl esters and GSH, can be monitored as well (Jaeschke, 1995). The available data on experimental animals and humans, obtained both from in vitro and in vivo studies, indicate that the enzymes associated with antioxidant defense mechanisms are altered under the influence of pesticides (Barerjee et al., 1999, Ranjar et al., 2002, Gultekin et al., 2001).
Oxidative stress plays an important role in the toxicity of various xenobiotics, including pesticide mixtures. Lipid peroxidation is probably the most extensively investigated process induced by free radicals. The abundant presence of membrane phospholipids at sites where radicals in general and, more specifically, reactive oxygen species are formed, render them easily accessible endogenous targets rapidly affected by free radicals. The extent of lipid peroxidation in whole blood was evaluated by measuring the formation of thiobarbituric acid reactive substances (TBARS). The higher oxidative stress in pesticide sprayers is evidenced by an increased concentration of TBARS in plasma and red blood cells, changes in antioxidant status, and altered activities of cellular enzymes. The increased concentration of TBARS observed could be due to the increased peroxidation of membranes. However, oxidative stress is a balance between free radical production and antioxidant activity, and it is possible that the increased TBARS are due to a decreased antioxidant activity (Prakasam et al., 2001).

Comet Assay

The Single Cell Gel Electrophoresis (SCGE) or Comet assay is a very sensitive method for measuring DNA strand breaks in individual cells. The assay is now a well-established, simple, versatile, rapid, visual, sensitive, and extensively used tool to assess DNA damage and repair, both quantitatively and qualitatively in individual cell populations (Dusinska and Collins, 2008).
The version of the Comet assay developed by Singh et al. 1988, electrophoresis under highly alkaline conditions (pH>13), has been found to be up to two orders of magnitude more sensitive than neutral version (Ostling and Johanson 1984). This enables the DNA supercoils to relax and unwind and allows the detection of alkali-labile sites and single-strand breaks in DNA during electrophoresis. This method measures low levels of strand breaks with high sensitivity.
The simplest types of DNA damage detected by the Comet assay are double-strand breaks (DSBs). DSBs result in DNA fragments and can be detected by merely subjecting them to electrophoretic mobility at neutral pH. Single-strand breaks (SSBs) do not produce DNA fragments unless the two strands of the DNA are separated / denatured. This is accomplished by unwinding the DNA at pH 12.1. It is also possible that single-strand breaks can relax the DNA and hence can also be detected with the Comet assay at neutral pH. Other types of DNA damage broadly termed alkali-labile sites (ALS) are expressed when the DNA is treated with alkali at a pH greater than 13. Breaks can also be introduced at the sites of DNA base

modifications by treating the DNA with lesion-specific glycosylases / endonucleases and the fragments thus produced can also be detected by the Comet assay.
At the same time, by controlling the conditions that produce nicks at the sites of specific DNA lesions, the Comet assay can be used to detect various classes of DNA damage. While breaks increase DNA migration, DNA binding and crosslinks can retard DNA migration and can also be detected by the Comet assay. Therefore, increased migration in the Comet assay can be attributed to strand breaks, alkali-labile sites and incomplete excision repair sites, while decreased DNA migration could be attributed to crosslinks, DNA–DNA or DNA–protein interactions. Some other lesions of DNA damage such as DNA cross-linking (e.g. thymidine dimers) and oxidative DNA damage may also be assessed using lesion-specific antibodies or specific DNA repair enzymes in the Comet assay.
The assay can be performed both in vivo and in vitro in a variety of samples. Peripheral blood lymphocytes, nasal and buccal epithelial cells have extensively been used to assess human genotoxicity in clinically or occupationally exposed population (Valverde et al., 1997). Also, in vitro studies have been conducted in cell lines and primary cell cultures for environmental biomonitoring using fish, earthworms and molluscs (Akcha et al., 2003). The in vivo assay with different tissues and organs from mice has also been used (Sasaki et al., 2000) for both DNA damage and repair and widely used in genetic toxicology (Dhawan et al., 2002), human epidemiology (Dhawan et al., 2001; Bajpayee et al., 2002), monitoring of human genotoxicity (Kassie et al., 2000; Palus et al., 2003; Basaran et al., 2003; Piperakis et al., 2003), patients undergoing radio/chemotherapy (Vaghef et al., 1997), and aging (Piperakis et al., 1998; Singh et al., 2003). Also, the in vivo assay has been used to monitor the dietary factors in various diseases such as diabetes (Raslova et al., 2000; Pitozzi et al., 2003) and thalassemia (Anderson et al., 2001; Ruf et al., 2003).
Single Cell Gel Electrophoresis has gained wide acceptance as a valuable tool in fundamental DNA damage and repair studies, genotoxicity testing and human biomonitoring. Human blood cells are particularly useful for biomonitoring purposes as they are easily acquired (Dusinska and Collins, 2008).
The biochemical changes induced after exposure to pesticides or their active metabolites include target cell/receptor binding, protein and DNA adduct formation, and induction or inhibition of enzymes (Lopez et al., 2007). DNA damage and oxidative stress have been proposed as mechanisms that could mechanistically link pesticide exposures with a number of health outcomes observed in epidemiological studies (Muñiz et al., 2008).

BIOMONITORING OF PESTICIDE-EXPOSED WORKERS

The main of this chapter is to use a biomarker set for the evaluation of damage induced in humans exposed to different pesticide mixtures in Santa Fe province, Argentina, in order to estimate possible toxicity mechanisms and its relationship with labor feature. Two different populations were evaluated: a) farm workers and applicators from the horticultural productivity, b) pesticide applicators working in extensive crops. In both situations donors were used as control group. The assessment of damage generated by direct exposure to pesticide mixtures was carried out through oxidative stress, DNA damage and enzymatic biomarkers in peripheral blood.
Study population.
The Regional Ethical Committee established the regulations for the development of the study and informed consent was given by each individual prior to the beginning

of the study. A face-to-face questionnaire was completed to obtain information on a) standard demographic data (age, gender, etc), b) individual lifestyle (diet, smoking habit, alcohol and medicine consumption), c) occupational aspects (working hours/days, years of exposure to pesticides, use of protective measures, etc), and d) pesticides used.

The study involved 315 subjects divided into two groups. The first group: Intensive crop workers (horticultural crops) consisted of 53 pesticide sprayers or pesticide applicators and 59 agricultural workers or farmers, and the control group consisted of 112 donors, from the same area, with no history of occupational exposure to pesticides or any potential genotoxic agent. The second group: Extensive crop workers (cereals and oil seeds) consisted of 48 pesticide sprayers or pesticide applicators and a control group of 50 donors.

Peripheral blood heparinized samples were obtained from all the subjects involved in the study.

Biomarkers

The variable used to describe pesticide exposure were butyrylcholinesterase (BChE) and acetylcholinesterase (AChE). Other indirect measures of exposure included utilization of protective gear during mixing/loading or during application of pesticides. Farmers can be expected to accurately recall details of their use of pesticides because it is a significant part of their farming operations.

AChE activity in erythrocytes: An aliquot of washed erythrocytes were haemolyzed by adding demineralized water at a 1:10 dilution. The hydrolysis rate of acetylthiocholine iodide (substrate) in erythrocyte dilution was measured at 405 nm with spectrophotometer by the reaction with DTNB to give the yellow 5-thio-2-nitrobenzoate anion. Enzyme activity was expressed as U/L of Red Blood Cells (RBC) (Ellman et al., 1961).

BChE plasmatic activity: The hydrolysis rate of butyrilthiocholine (substrate) in plasma was measured at 405 nm with spectrophotometer by the reaction of thiocholine iodide with DTNB, to give the yellow 5-thio-2-nitrobenzoate anion. Enzyme activity was expressed as U/L. (Ellman et al., 1961.)

Biomarkers of effect concern an assessment of early or late adverse effects of a chemical or another factor on a physiological system, organ or organism. The primary purpose of using biomarkers of effect is surveillance, that is the identification of individuals or a population at risk to adverse health effects so that preventive measures can be taken. Although a biomarker of effect is usually also related to exposure to a specific chemical, it is generally more closely related to the occurrence of an adverse health effect (De Zwart, 1999). Pesticides are example of agents that act as pro-oxidants and elicit effects in multiple organs. In some cases, these prooxidant effects occur alongside pesticide-induced alterations in target enzymes (Limón-Pacheco and Gonsebatt, 2009). Besides, it was verified an increase of thiobarbituric acid reactive species (TBARS). To provide concise guidance for detecting DNA damage (strand breaks, alkali labile sites, crosslinking and incomplete excision repair sites) the single cell gel or Comet assay is used in cells sampled from individuals potentially exposed to genotoxic carcinogens (Albertini et al, 2000).

Catalase activity in erythrocytes (CAT): Erythrocytes were haemolyzed by adding ice-cold demineralized ultrapure water at a 1:100 dilution. CAT activity in haemolysate erythrocytes was measured by monitoring the decrease in H_2O_2 concentration spectrophotometrically over time (Aebi, 1984). The specific activity of each sample was calculated on the basis that one unit of enzyme activity was defined as the activity required to degrades 1 mole hydrogen peroxide during 60 s/g Hb.

Lipid peroxidation in erythrocytes (TBARS): MDA as a marker for lipid peroxidation in red blood cells (dilution 1:4 with ice-cold) was determined by measuring the formation of the colour produced during the reaction of thiobarbituric acid (TBA) with MDA (TBARS Assay) according to a modification of the method of Beuge and Aust (1978). The sample absorbance was determined at 535 nm and the TBARS concentration was calculated using the extinction coefficient 1.56 x 10^5 M^{-1} cm^{-1}. MDA concentration in erythrocytes was expressed as nmol/g Hb.
Alkaline Comet Assay: The standard procedure originally described by Singh et al. (1988) with modifications was used. Two slides were processed for each sample, including negative and positive (H_2O_2 50 μM) controls. DNA strand breaks were measured with the Comet assay. One hundred randomly selected Comet assays from each of two duplicate gels were analysed visually on a scale of 0–4 (categories depending on DNA damage level). The overall score, between 100 and 400 arbitrary units, is related to the DNA break frequency and a comet-like image indicates the presence of DNA breaks (Simoniello et al. 2008). The Damage Index Comet Assay (DICA) was calculated. Cell Viability using Fluorescent Dyes: The same cell suspension used in the comet assay was mixed with fluorescent DNA-binding dyes and examined by fluorescent microscopy to visualize and count cells with aberrant chromatin organization. The percentages of each of these cellular states in relation to the total cells were obtained (Simoniello et al., 2010).

RESULTS INTENSIVE CROP WORKERS

The demographic characteristics were similar in populations evaluated (workers and controls), except for occupational exposure. The average age group of workers was 35.37 ± 15.17 years and the control group was 37.70 ± 14.07 years, ranging between 18 and 65 years of age for all volunteers included. Comparing the two groups using t-test found no statistically significant differences in age ($p = 0.763$). The gender distribution for workers is 41% (n = 43) for females and 59% (n = 62) for males and in the case of the controls is 51% (n = 46) for females and 61% (n = 54) for males. No statistically significant differences were found regarding sex between exposed and controls ($p = 0.997$). The habits of individuals in both groups (exposed and control) were also evaluated founding no statistically significant differences in smoking ($p = 0.875$) or alcohol consumption ($p = 0.667$) between both groups.
A summary of the pesticides most commonly used in horticulture zone, the CAS number, the IARC classification and the US EPA and WHO hazard classification is presented in Table 1.

Table 1. List of pesticides most commonly used (questionnaire answers) by the exposed subjects, CAS number, IARC classification, US EPA classification, and WHO hazard classification. IARC Classification: 3: Not classifiable as to carcinogenicity to humans; NL: Not Listed. US EPA Classification: Group B: Probable human carcinogen; B2: Sufficient evidence of carcinogenicity from animal studies; Group C: Possible human carcinogen; Group D: Not classifiable as to human carcinogenicity; Group E: Evidence of non-carcinogenicity to humans. WHO hazard classification: Ia: Extremely hazardous; Ib: Highly hazardous; II: Moderately hazardous; III: Slightly hazardous; U: Unlikel to pose an acute hazard in normal use.

Pesticides	Compound	CAS Number	Chemical Class	IARC	US EPA	WHO
Fungicide	Captan	133-06-2	Thiophthalimide	3	NL	U
	Thiophanate methyl	23564-05-8	benzimidazole	NL	Suggestive	U
	Copper	7440-50-8	Inorganic-Copper	NL	D	NL
	Mancozeb	8018 01 7	Dithiocarbamate-Inorganic Zinc	NL	B2	U
Insecticide-Nematicide	Chlorpyrifos	2921-88-2	organophosphorus	NL	E	II
Insecticide	Cypermethrin	67375-30-8	pyrethroid	NL	NL	II
	Dimethoate	60-51-5	organophosphorus	NL	C	II
	Endosulfan	115-29-7	Organochlorine	NL	NL	II
	Imidacloprid	105827-78-9	Chloro-nicotinyl	NL	NL	II
	Malathion	121-75-5	organophosphorus	3	Suggestive	III
	Methamidophos	10265-92-6	organophosphorus	NL	E	Ib
	Chlorfenapyr	122453-73-0	pyrazole	NL	Suggestive	II
	Parathion	56-38-2	organophosphorus	3	C	Ia
	Permethrin	54774-45-7 51877-74-8	Pyrethroid	3	Suggestive	II
Herbicide	Glyphosate	1071-83-6	phosphonoglycine	NL	NL	III

In Table 2 farm workers show a significant decrease in the activity of both enzymes when compared with the control population ($p < 0.01$, Dunnett test). (AChE: 20% and BChE: 7.5%). Instead, a significant decrease in AChE (30%; $p < 0.01$, Dunnett test) was observed in pesticide applicators, only.

Table 2. Mean values and standard deviation of the cholinesterase activity in controls, farm workers and applicators intensive crops.

The analysis of oxidative status biomarkers is shown in Table 3 exhibiting a significant increase at the level of TBARS (33%) in pesticide applicators ($p < 0.01$, Mann-Whitney test) but no statistically significant differences were observed in the farm worker group ($p > 0.05$).

Cholinesterase activity (X±DE)	Controls (n=112)	Farm workers (n=59)	Pesticide applicators (n=53)
BChE (U l^{-1})	6875,60±1257	6372,05±1338*	6805,93 ± 1409
AChE (U l^{-1} RBC)	9303,98±1960	7526,02±1913*	6569,84±1341*

BChE and AChE: $P < 0.01$ ANOVA, * $p < 0.01$ Dunnet test

At the same time, CAT depletion was 48% in applicators ($p < 0.01$, Mann-Whitney test) and 15% in farm workers ($p > 0.05$, Mann-Whitney test) compared to controls. Prior to the implementation of comet assay, viability was assessed and expressed in terms of the percentage of viable cells, resulting above 95% of cell viability in all the samples. The analysis of the values obtained for genotoxicity biomarker (comet assay) showed a statistically significant increase ($p < 0.01$, Dunnet test) in both exposed populations (farm workers and applicators) when compared with the control group.

Table 3. Mean values and standard deviation of the oxidative and genotoxicity markers in controls, farm workers and applicators intensive crops.

Biomarkers of effect (X±DE)	**Controls (n=112)**	**Farm workers (n=59)**	**Pesticide applicators (n=53)**
CAT ($kU\ g^{-1}$ Hb)	138,95±34,37	116,10 ± 71,50*	71,37 ± 36,98*
TBARS ($nmol\ g^{-1}$Hb)	141,58±34,46	139,70 ± 32,03	188,98 ± 43,49**
Comet assay (DICA)	138,95±34,37	220,91±19,27*	214,65±14,80*

* $p < 0.05$; ** $p < 0.01$ Dunnet test.

No statistically significant differences with regard to confounding factors such as age, gender and smoking habits over biomarkers selected in pesticide applicators, were observed. Alcohol consumption has a statistically significant difference for the enzyme CAT ($p = 0.001$) in sprayers. When assessing the years of works as factor, statistically significant differences were found in BChE ($p = 0.024$) and DICA ($p = 0.001$). Taking into account that employee no uses appropriate personal protective equipment (PPE) and that 75% of workers do not use any protection (gloves, respirators, goggles, protective clothing, boots, etc.) it was considered a protective measure when at least one protection element was present. When used as a comparison factor (PPE) we observed statistically significant differences for DICA ($p = 0.022$) and TBARS ($p = 0.007$) using the U-Test.

When analyzing data related to farm workers no statistically significant differences ($p > 0.05$) were found considering gender, smoking and alcohol consumption, finding significant differences in age for DICA ($p = 0.013$) and CAT ($p = 0.025$) as well as in AChE ($p = 0.014$) and DICA ($p = 0.001$) in relation to years of works.

Correlations (Spearman rho) between all biomarkers were applied. The correlations were significant between DICA and AChE (-0.355) and also between BChE (-0.184) and CAT (0.560). AChE correlated with all variables (with DICA -0.355, with 0.192 BChE with CAT and TBARS -0.255). Furthermore, the oxidative status variables correlated with each other (-0.364).

In order to conduct a comprehensive analysis of pesticide mixture, which products were used during the seven days prior to sampling on each farm visited, was considered. Table 4 presents pesticide mixtures used to workers in the last week prior to obtain samples.

Table 4. Mixtures of pesticides used considering the location of the operation and the number of workers

Mix No.	Place Name	No. of workers exposed	Trade name of pesticide
0	Hospital Protomédico	10	No mention of the products used
1	Monte Vera	11	Methamidophos-Cypermethrin-Chlorpyrifos
2	Angel Gallardo	18	Cypermethrin, Chlorpyrifos-Sunfire (Chlorfenapyr)-Parathion-Chlorpyrifos
3	Hospital Protomédico	20	Terminator (Chlorpyrifos)-Kalibre (Cypermethrin)-Parathion, Chlorpyrifos
4	Chaco Chico	16	Attack (Cypermethrin)-Parathion-Point 70 (Imidacloprid)-Cercobin (benzimidazole) - Captan (flusilazole + carbendazim)-Dimethoate-Metafoz (Metamidofoz)-Manzate (Mancozeb)-Kalibre (Cypermethrin)
5	Recreo	18	Cypermethrin, Chlorpyrifos, Glyphosate
6	Candioti	12	Cypermethrin, Dimethoate, Parathion

Table 5. Evaluation of pesticide mixtures used and their influence on BChE, AChE, CAT, TBARS and DICA

	Mix 1	Mix 2	Mix 3	Mix 4	Mix 5	Mix 6	P*
BChE	6269,6± 1540	6642,5± 1373	7346,3± 955	5928,3± 941	5548,1± 1727	6368,9±1 418	0,003
AChE	6241,6± 193	6779,6± 806	6429,6± 978	9062,9± 1546	7173,5± 1748	6092,9±8 88	<0, 001
CAT	33,53±2 0,15	83,83±2 7,32	56,33±5 8,68	110,95± 101,0	88,76±3 8,31	108,07±1 01,08	0,159
TBARS	180,76± 22,28	198,09± 26,72	207,42± 35,63	123,76± 21,93	118,03± 21,13	165,76±2 6,21	<0, 001
DICA	200,00± 2,68	185,75± 7,28	218,15± 12,46	244,56± 12,70	214,50± 10,67	205,58±8 ,81	<0, 001

*p: Kruskal-Wallis test.

Table 5 shows that there are statistically significant differences when evaluating mixtures of pesticides used as a factor for all biomarkers except for CAT.

Results Extensive crop workers

Similar to previous intensive worker group, demographic characteristics were comparable in the both populations evaluated (workers and controls), except for the occupational exposure. The average age group of workers was 32,09 ± 12,18 years and the control group was 33,24 ± 13,05 years, ranging between 18 and 65 years of

age for all volunteers included. Comparing the two groups using T-test found no statistically significant differences in age (p = 0,653). The habits of individuals in both exposed and control groups were also evaluating no statistically significant differences in smoking habit (p = 0,667) or alcohol consumption (p = 0,875) between groups.
A summary of the pesticides most commonly used in horticulture zone, the CAS number, the IARC classification and the US EPA and WHO hazard classification is presented in Table 6.

Table 6. List of pesticides most commonly used (questionnaire answers) by the exposed subjects, CAS number, IARC classification, US EPA classification, and WHO hazard classification. IARC Classification: 3: Not classifiable as to carcinogenicity to humans; NL: Not Listed. US EPA Classification: Group B: Probable human carcinogen; B2: Sufficient evidence of carcinogenicity from animal studies; Group C: Possible human carcinogen; Group D: Not classifiable as to human carcinogenicity; Group E: Evidence of non-carcinogenicity to humans. WHO hazard classification: Ia: Extremely hazardous; Ib: Highly hazardous; II: Moderately hazardous; III: Slightly hazardous; U: Unlikel to pose an acute hazard in normal use.

Pesticides	Compound	CAS Number	Chemical Class	IARC	US EPA	WHO
Fungicide	Mancozeb	8018-01-7	Dithiocarbamate-Zinc	NL	B2	U
Insecticide - Nematicide	Chlorpyrifos	2921-88-2	Organophosphorus	NL	E	II
Insecticide	Cypermethrin	67375-30-8	Pyrethroid	NL	NL	II
	Dimethoate	60-51-5	Organophosphorus	NL	C	II
	Endosulfan	115-29-7	Organochlorine	NL	NL	II
	Pirimicarb	23103-98-2	Metil Carbamate	NL	Suggestive	II
	Alpha-cypermethrin	67375-30-8	Pyrethroid	NL	II	II
	Methamidophos	10265-92-6	Organophosphorus	NL	E	Ib
	Monocrotophos	6923-22-4	Organophosphorus	NL	NL	Ib
Herbicide	Glyphosphate	1071-83-6	Fosfonoglicine	NL	E	III
	Glyphosphate Trimesiun	81591-81-3	Fosfonoglicine	NL	E	III
	Flumetsulam	98967-40-9	Triazolo Pyrimidine	NL	E	U
	2,4 D	94-75-7	Clorofenoxid acid	2 B	D	II
	Paraquat	1910-42-5	Bipiridilium	NL	E	II
	Picloram	1918-02-1	Piridincarboxylic Acid	3	E	III

In pesticide applicators, Table 7 shows a significant decrease (21.8 % AChE and BChE by 5.12 %.) in the activity of both enzymes when compared with the control population ($p < 0.01$, T test).

Table 7. Mean values and standard deviation of the cholinesterase activity in controls and applicators extensive crops.

Cholinesterase activity (X±DE)	Controls (n=50)	Pesticide applicators (n=48)
BChE (U l^{-1})	6733,90 ± 1012	6388,52 ± 1442*
AChE (U l^{-1} RBC)	8870,62 ± 1376	6929,45 ± 1883*

* $p < 0.01$ T test.

Analyzing the biomarkers of oxidative status, Table 8 shows a statistically significant increase in TBARS levels (47%) in pesticide applicators ($p < 0.01$, T test). At the same time, CAT increase was 61% in applicators ($p < 0.01$, T test) compared to the control population.
Damage Index comet assay showed a statistically significant increase (43 %; $p < 0.01$, Mann-Whitney test) in applicators when compared with the control group.

Table 8. Mean values and standard deviation of the oxidative and genotoxicity markers in controls and applicators extensive crops.

Biomarkers of effect (X±DE)	Controls (n=50)	Pesticide applicators (n=48)
CAT (kU g^{-1} Hb)	169,09 ± 86,32	272,19 ± 126,71*
TBARS (nmol g^{-1} Hb)	191,33 ± 32,99	281,69 ± 66,09*
Comet assay (DICA)	148,92 ± 35,84	212,33 ± 33,70*

*$p < 0.01$, T test (CAT and TBARS); Mann-Whitney (Comet Assay)

When analyzing data related to extensive crops applicators, no statistically significant differences ($p > 0.05$) were found respect to age, smoking and alcohol consumption for all biomarkers selected.
Similar previous group, 56% of workers do not use any protection (gloves, respirators, goggles, protective clothing, boots, etc.) therefore was considered a protective measure used when at least one protection element was present. When PPE was used as a comparison factor we observed statistically significant differences in relation to AChE ($p < 0.01$, Mann-Whitney Test). Similar result was showed for AChE considering years of works ($p < 0.05$).
Correlations (Spearman rho) between all biomarkers were applied. The correlations between AChE and BChE (0.273) and between BChE and TBARS (0.265) were statistically significant. Also, AChE correlated statistically significant with DICA, CAT and TBARS. DICA significantly correlated with CAT (0.206) and TBARS (0.522).
In order to conduct a comprehensive analysis of pesticide mixtures, which products were used during the seven days prior to sampling on each farm visited, was taken into account
Table 9 presents pesticide mixtures used by workers in the last week prior to obtain samples. In Table 10 we can observe that statistically significant differences when

evaluating mixtures of pesticides used as a factor for biomarkers can be observed, except for BChE and CAT.

Table 9. Mixtures of pesticides used considering the location of the operation and the number of workers.

Mix No.	Place Name	No. of workers exposed	Trade name of pesticide
1	Monte Vera	7	Cypermethrin- Dimethoate- Glyphosphate
2	Progreso	3	Dimethoate-glyphosate-Preside (Flumetsulam)- 2.4 D
3	Santo Domingo	4	Methamidophos-2.4D-Glyphosphate
4	Santo Domingo	6	Cypermethrin- Endosulfan- Glyphosphate
5	Ceres	7	Dimethoate Methamidophos- Chlorpyrifos, cypermethrin- Glyphosphate- Gramoxone (paraquat) -2.4 D- Tordon (Picloran)-Paton 50 (Pirimicard)- sulfosate (Glyphosate Trimesiun) - Monocrotophos
6	San Joaquín	4	Glyphosphate
7	Cayastá	6	Fastac (Alfa-methrin)
8	Helvecia	11	Dimethoate-Manzate (Mancozeb)-Monocrotophos

Table 10. Evaluation of mixtures of pesticides used and their influence on BChE, AChE, CAT, TBARS and DICA.

	Mix 1	Mix 2	Mix 3	Mix 4	Mix 5	Mix 6	Mix 7	Mix 8	P*
BChE	6426,00 ±1786	4478,66 ±446	6618,33 ±634	5601,20 ±1221	6494,28 ±540	7379,75 ±2133	7097,00 ±1880	6380,43 ±1335	0.141
AChE	8149,25 ±1889	5633,33 ±225	4402,33 ±1114	6908,40 ±788	8803,71 ±1693	9389,25 ±1274	5411,00 ±1243	6482,43 ±1293	<0,001
CAT	269,98 ±25,02	283,54 ±146,71	198,89 ±31,22	222,88 ±72,75	206,75 ±118,83	186,76 ±35,17	281,76 ±98,01	346,10 ±157,96	0,167
TBARS	279,19 ±86,93	266,20 ±14,89	246,94 ±71,25	342,33 ±43,79	348,26 ±81,05	226,07 ±62,99	240,81 ±36,69	277,26 ±50,62	0,028
DICA	214,25 ±8,68	213,00 ±10,81	221,00 ±11,00	205,20 ±12,04	236,00 ±57,17	162,25 ±6,94	205,33 ±11,43	217,12 ±33,51	0,066

*P: Kruskal-Wallis test.

DISCUSSION

The agricultural workers included in this study were also exposed to a great number of pesticides (all of the subjects were exposed to more than two different pesticides), some of which are classified as being carcinogenic by the US Environmental Protection Agency (US-EPA) and hazardous by the World Health Organization (WHO), although not yet listed by the IARC (Table 1 and 6). Assessment of the associations with individual pesticide exposure is very difficult because most occupations involve the regular use of a large number of different pesticides, together with other chemicals such as co-formulants, which vary greatly in their potential toxicities and potencies. Furthermore, measurements of systemic exposure to pesticides were not taken and therefore correlations between increased genotoxicity biomarkers and the degree of exposure were not possible to obtain (Bull et al., 2006).

Different researches have recognized the invaluable role of AChE monitoring in rural workers at high risk of exposure to OPs and MC pesticides (Mc Cauley et al., 2006). In our work, in agreement with different reports (Ranjbar et al., 2002; Singh et al., 2007), AChE showed a significant decrease in intensive and extensive crop workers. Measuring BChE activities is a frequent marker of exposure in pesticide sprayers, is easier to assay and is more widely available; in our case, BChE activity inhibition was significant in intensive farm workers and extensive applicators but not in intensive applicators. This may be related to the differential profiles of cholinesterase inhibition that can be observed depending on the particular OP compound; for example, chlorpyrifos and malathion are preferential inhibitors of BChE whereas dimethoate is a preferential inhibitor of AChE. In the interpretation of cholinesterase monitoring results, we may consider that both groups had been exposed to different pesticide mixtures and take into account inter-individual variation and confounding factors in enzymatic activity.

Oxidative damage is thought to be an important mechanism of several pesticides (Banerjee et al., 1999, Prakasam et al., 2001). In blood, normal erythrocyte function depends on an intact cell membrane, which is the target for many toxics, including pesticides. The results of the present study indicate that CAT activity decreased significantly in both pesticide applicators and non-pesticide applicators of intensive crops ($p < 0.01$). On the contrary, CAT activity increased significantly in extensive pesticide applicators ($p > 0.05$). The available data on experimental animals (Seth et al., 2001), *in vitro* studies (Gultekin et al., 2000; Prasanthi et al., 2005) and *in vivo* studies (Ranjbar et al., 2002; Lopez et al., 2007) indicate that the enzymes associated with the antioxidant defense mechanism change under the influence of pesticides. These enzymes efficiently scavenge toxic free radicals and are partly responsible for protection against lipid peroxidation due to pesticide exposure (Banerjee et al., 1999). Hence, the increased level of TBARS observed in this work could be due to increased peroxidation of membranes and/or to decreased antioxidant activity, caused by exposure to pesticide mixtures.

Different OPs, such as phosalone, chlorpyrifos ethyl, and diazinon, have been reported to induce oxidative stress as shown by enhancement of MDA production (Gultekin et al., 2000; Prakasam et al., 2001; Altuntas et al., 2003; Catalgol et al., 2007). Carbamate pesticides may induce oxidative stress, which leads to the generation of free radicals and an alteration in antioxidant enzymes or OFR scavenging enzymes (Seth et al., 2001; Dettbarn et al., 2006). Some pyrethroids affect the flow of erythrocyte membrane due to increased lipid peroxidation (Kale et al., 1999, Gabbianelli et al., 2002; Nasuti et al, 2003). It is likely that the production of O^{2-} or the direct action of pyrethroid on the production of GPx could be the cause

of oxidative damage (Prasanthi et al., 2005; El Demerdash, 2007). Several different pathways by which oxidative DNA damage occurs have been proposed. These include chemical modification of nucleotides (Cicchetti and Argentin, 2003), direct action of ROS on DNA, or indirect lipid peroxidation degradation products (Collins, 1999). The Comet assay has been used to determine the extent of DNA damage in lymphocytes from rural workers occupationally exposed to a variety of pesticides (Garaj-Vrhovac and Zeljezic, 2001; Shadnia et al., 2005; Remor et al., 2008). Our results show that pesticide-spraying workers and farmers from intensive and extensive crops presented a significant increase in DICA when compared to controls ($p < 0.001$ in both cases).

Both applicator groups (from intensive and extensive crops) exhibited significant difference in DICA when years of exposure were considered ($p < 0.05$) and when we used the personal protective equipment (PPE) worn by individuals as a comparison factor($p < 0.05$). The positive genotoxicity observed in the exposed workers of this study may be due to the lack of protective measures or protective clothing, gloves or boots in a few cases. In other works carried out in Argentina, 86% of the workers interviewed declared to use PPE, but the authors commented that only 20% had the complete equipment, existing cases in which they wore gloves only. In the present work, 35% of the workers interviewed admitted not using PPE (Paunero et al., 2009); this finding agrees with the results indicated by Souza Casadinho (2003) with regard to the unawareness in relation to the danger of using agrochemicals, although it is widely admitted that horticultural activity is risky. In agreement with this, when asking about the aspects which they considered that should be improved, only 5 % of the workers interviewed mentioned aspects of hygiene and safety in the work.

An increase in micronuclei was seen in pesticide-exposed people who did not wear protective gloves (Bolognesi et al., 2002). At the same time, increases in the frequencies of chromosomal aberrations and micronuclei have been found in some studies where the population exposed to pesticides wore no protection during work activities (Costa et al., 2006) or little or no protective clothes (Dulout et al., 1985). Interestingly, several studies that reported a majority of workers using protective measures (>60%) concluded that the results were negative (Bolognesi et al., 2004; Pastor et al., 2001 and 2002; Piperakis et al., 2003 and 2006), suggesting the importance of PPE for preventing exposure. Therefore, field workers may be affected by a lack of available work-site laundering facilities, prolonging their exposure to pesticides and other farm chemicals.

The influence of confounding factors, such as age, gender, smoking and alcohol consumption, on the genotoxic effects of occupational exposure to pesticides was investigated and no significant differences were observed in relation to all biomarkers evaluated ($p > 0.05$). Other authors have reported similar results when evaluating micronucleus frequency in pesticide-exposed workers (Sailaja et al., 2006). Likewise, smoking failed to have a significant influence on the number of CA (Zeljezic & Garaj-Vrhovac, 2001), level of MN (Bolognesi et al., 2002) and increase in comet tail-length values (Garaj-Vrhovac & Zeljezic, 2001; Liu et al., 2006). However, the discrepancy in some reports is not surprising since the failure to show an effect of smoking could be due to the kind of exposure, target tissue, and individual susceptibility of subjects in the population. When individuals are exposed to mixtures, it is difficult to predict the final genotoxic effect because of the interaction that could occur between the agents involved, either maximizing or antagonizing the effect (Castillo-Cadena et al., 2006).

Correlation analysis in intensive crop workers revealed that the damage observed in lymphocytes using comet assay was positively associated with the presence of

changes in the antioxidant enzyme CAT, suggesting the influence of a possible mechanism of oxidative damage at this level. Furthermore, it was also significantly inversely associated with cholinesterase enzymes. The correlation between TBARS and AChE activity found in the present study is similar that obtained by other authors (Ranjbar et al., 2002; Akhgari et al., 2003; Singh et al., 2007). In extensive crop workers, correlations between AChE and BChE, and between BChE and TBARS were statistically significant. Also, DICA correlated with AChE, CAT and TBARS as well as parameter of oxidative stress each other.

Multiple chemical exposures are of great interest in Toxicology and Public Health (US-EPA, 1998). Most of the research has been performed on individual chemical agents (Groten, 2000) without considering that the effect of a chemical mixture could be either more or less powerful than the exposure to the individual compounds. Multiple exposures are a rule and not an exception in agricultural practice: pesticide applicators spray large amounts of agrochemical mixtures including a significant number of genotoxic compounds. The pesticides most often used are chlororganics and, more recently, carbamates, organophosphates and pyrethroids, which have been reported to be positive for genotoxic effects in experimental studies in bacterial and in mammalian systems (Bolognesi, 2003).

In this chapter, we considered pesticide mixtures used in the last week by applicators (Table 5 and 10). Although the number of people exposed to each mixture is small, its assessment may contribute to understanding their influence on the results. In Table 5 we reflect on the data obtained for biomarkers using pesticide mixture as factor analysis. For BChE were found statistically significant differences between the different mixtures ($p = 0.003$). Mix 5 (cipermetina, chlorpyrifos, glyphosate) had the highest enzyme inhibition. In AChE significant differences were also found ($p < 0.001$) and greater inhibition was in Mix 6 (dimethoate, cypermethrin, parathion). These data can be related to differential profiles of inhibition of cholinesterase enzymes that can be observed depending on each particular organophosphorus compound. Chlorpyrifos and malathion are preferential BChE inhibitors while dimethoate is a preferential inhibitor of AChE (Banerjee et al., 1999). When other biomarkers were analyzed, DICA and TBARS showed statistically significant differences ($p < 0.001$ in both), exhibiting its higher values for the Mix 4 (cypermethrin, parathion, imidacloprid, benzimidazole, flusilazole, carbendazim, dimethoate, metamidofoz, mancozeb). This mixture has different active ingredients, different biological targets and unknown proportions.

Table 10 showed significant differences for AChE ($p < 0.001$). Most enzyme inhibition was found for Mix 3 (methamidophos, 2.4 D, glyphosate). In this particular mixture, the only compound that has been described as having the ability to inhibit the enzyme is methamidophos. In several studies, has been evaluated as an important specific inhibitor of AChE (Jong et al., 1982, Singh et al., 1986). TBARS showed statistically significant variations ($p = 0.028$) using mixtures as a factor showing the highest values for the mixture 5 (methamidophos, chlorpyrifos, dimethoate, cypermethrin, glyphosate, paraquat, 2.4 D, picloran, pirimicard, glyphosate trimesiun, monocrotophos). Considering chemicals listed in this mix, it is important to note that some of them have been banned in several countries due to its high toxicity, such as monocrotophos and methamidophos (Finkelman, 1994; Arevila et al., 1997). is also very important to point out that in developing countries, the process of prohibition is limited (Repetto and Baliga, 1996).

In agreement with the results found for intensive crop workers, applicators exposed to more complex mixtures with different active ingredients, could be more injured than exposed to less complex mixtures.

In mixtures of pesticides is important to note that each active ingredient has a specific mode of action to control a pest, and has its own side effects on wildlife and humans exposed to it. Thus, occupational exposure to pesticides may increase the risk of adverse reproductive outcomes or disorders of the nervous system, causing immunosuppression and may even cause cancer in adulthood and also may induce heritable changes. The focus on chemical interactions, particularly in environmentally relevant concentrations, is an important step forward in our understanding of the impact of mixtures on human health and the environment.

CONCLUSION

Our study shows that, under the conditions of this experimental work, intensive and extensive crop workers exposed to pesticide mixtures have enzymatic alterations, modifications in oxidative balance and genotoxic damage when compared to controls. Further studies should be carried out enlarging the sample size and conducting a serial and routine monitoring of populations exposed to pesticide mixtures, using effect and exposure biomarkers.
In conclusion: a) farmers should be advised to use protective measures during all activities that imply pesticide handling, b) individuals under exposure risk must be monitored frequently to minimize potentially harmful effects of pesticides on the DNA, c) the evaluation of population risks associated with pesticide exposure in our country should proceed.

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Using a Toxicokinetic Modeling Approach to Determine Biological Reference Values (BRVs) and to Assess Human Exposure to Pesticides

Aurélie Berthet, Michèle Bouchard, Mathieu Valcke, Roberto Heredia-Ortiz

ABSTRACT

Exposure to various pesticides has been characterized in workers and the general population, but interpretation and assessment of biomonitoring data from a health risk perspective remains an issue. For workers, a Biological Exposure Index (BEI®) has been proposed for some substances, but most BEIs are based on urinary biomarker concentrations at Threshold Limit Value - Time Weighted Average (TLV-TWA) airborne exposure while occupational exposure can potentially occurs through multiple routes, particularly by skin contact (i.e.captan, chlorpyrifos, malathion). Similarly, several biomonitoring studies have been conducted to assess environmental exposure to pesticides in different populations, but dose estimates or health risks related to these environmental exposures (mainly through the diet), were rarely characterized. Recently, biological reference values (BRVs) in the form of urinary pesticide metabolites have been proposed for both occupationally exposed workers and children. These BRVs were established using toxicokinetic models developed for each substance, and correspond to safe levels of absorption in humans, regardless of the exposure scenario. The purpose of this chapter is to present a review of a toxicokinetic modeling approach used to determine biological reference values. These are then used to facilitate health risk assessments and decision-making on occupational and environmental pesticide exposures. Such models have the ability to link absorbed dose of the parent compound to exposure biomarkers and critical biological effects. To obtain the safest BRVs for the studied population, simulations of exposure scenarios were performed using a conservative reference dose such as a no-observed-effect level (NOEL). The various examples discussed in this chapter show the importance of knowledge on urine collections (i.e. spot samples and complete 8-h, 12-h or 24-h collections), sampling strategies, metabolism, relative proportions of the different metabolites in urine, absorption fraction, route of exposure and background contribution of prior exposures. They also show that relying on urinary measurements of specific metabolites appears more accurate when applying this approach to the case of occupational exposures. Conversely, relying on semi-specific metabolites (metabolites common to a category of pesticides) appears more accurate for the health risk assessment of environmental exposures given that the precise pesticides to which subjects are exposed are often unknown. In conclusion, the modeling approach to define BRVs for the relevant pesticides may be useful for public health authorities for managing issues related to health risks resulting from environmental and occupational exposures to pesticides.

INTRODUCTION

Populations may be occupationally or environmentally exposed to pesticides by different routes and different sources; the best approach to assess such exposure has been recognized to be through biological monitoring. It provides information on internal doses, and reflects actual rather than estimated exposure (He, 1993; Woollen, 1993; de Cock *et al.*, 1995; He, 1999; Jakubowski and Trzcinka-Ochocka, 2005). Furthermore, biomonitoring of exposure provides early warning signs before irreversible alterations occur and allows documenting inter- and intra-individual variability (Jakubowski and Trzcinka-Ochocka, 2005).

For many pesticides with short biological half-lives and largely eliminated in urine, urinary biomarkers are usually used as indicators of exposure (Barr and Needham, 2002). Nevertheless, for a proper interpretation of biomonitoring results, knowledge of the kinetics of the pesticide of interest and its metabolites as well as sample collection strategy is necessary (Aitio, 2006). Once the pesticide kinetics is documented in humans, biological guideline values aiming at preventing harmful effects may then be derived by linking safe exposure levels with corresponding urinary biomarker values (Hoet and Lison, 1996; Maroni *et al.*, 2000c). For the general population, such biological limit values have not yet been proposed by governmental organizations for pesticides (Valcke and Bouchard, 2009; Angerer *et al.*, 2011); biomonitoring values are rather compared to those obtained in large-scale longitudinal epidemiological studies and considered as a reference for baseline or control values (Barr, 2007; Angerer *et al.*, 2011; Göen *et al.*, 2011). For occupationally exposed individuals, the most often used biological reference values are the BEIs®, set by the American Conference of Governmental Industrial Hygienists (ACGIH), and BATs of the Deutsche Fortschungsgemeinschaft (DGF). The only differences between both values are that BATs are ceiling values rather than time-weighted averages and have been applied only to non-carcinogen chemicals (Drexler *et al.*, 2008; Ikeda, 2008; Angerer *et al.*, 2011). However, both correspond to compound levels in accessible biological matrices corresponding to inhalation threshold exposure limit values and no such values for pesticides have been proposed so far by these Agencies.

Recently, biological reference values (BRVs) have been proposed for specific urinary biomarkers of some organophosphorus insecticides (OPs), a carbamate insecticide and two phthalimide fungicides (Bouchard *et al.*, 2003, 2005, 2006, 2008; Gosselin *et al.*, 2004; Valcke and Bouchard, 2009; Heredia-Ortiz *et al.*, 2011; Heredia-Ortiz and Bouchard, 2011). These BRVs were established using toxicokinetic models developed for each substance, and relating the dose of the parent compound absorbed in the body to the time course of urinary metabolites under different exposure scenarios and to pesticide exposure doses causing early health effects in humans. Therefore, they correspond to safe biomarker levels in humans, regardless of the exposure scenario. The purpose of this chapter is thus to present a review of the toxicokinetic modeling approach used to determine these BRVs and to illustrate the convenience of this approach by presenting the example of malathion, chlorpyrifos, parathion, carbaryl, captan and folpet in the context of an occupational exposure and that of non-specific OP exposure in children.

DETERMINATION OF BIOLOGICAL REFERENCE VALUES USING A TOXICOKINETIC MODELING APPROACH

The overall approach involves three steps, namely developing the toxicokinetic model, performing model simulations and validation as well as sensitivity analysis, and deriving BRVs using modeling.

Toxicokinetic Model Development

The main challenge with biomathematical toxicokinetic modeling is the ability to provide a real representation of the kinetics of the studied substance and its metabolites in humans. Largely employed tools for such purpose are physiological-based pharmacokinetic (PBPK) models (Gerlowski and Jain, 1983; Balant and Gex-Fabry, 1990; Meibohm and Derendorf, 1997; Rescigno, 2003; Reddy, 2005). The core idea is to represent body organs, tissues or groups of tissues or organs as compartments and account for physiology, while independently computing the physicochemical and biochemical properties of the compound of interest from one body compartment to the other or within a given compartment. From a fundamental point of view, this type of modeling would be the best way to simulate the physiological path of any substance and/or its metabolites in the body. However, the construction of the model can mainly be hampered by the difficulty to estimate model parameters, which are generally extrapolated from *in vitro* to *in vivo* scenarios or from animals to humans. As a result, fine-tuning adjustments must be carried out after the model is completed and those corrections differ from one substance to the other. Explicit details on the kinetics (and often dynamics) of the pesticide are very important for this type of model to work properly.

Other toxicokinetic modeling approaches published in the literature to establish relationships between the exposure or absorbed doses of a compound in individuals and urinary biomarkers are in essence similar to PBPK modeling except that the emphasis is put on effective toxicokinetics of the chemical compounds of interest and representation of the body remains simple (Shimmins *et al.*, 1967; Laskarzewski *et al.*, 1982; Rowland and Tozer, 1995; Gosselin *et al.*, 2006). With this approach, only the physiological compartments relevant to the description of biomarker kinetics are represented and these may or may not include a single physiological stage or a set of various functions in a sole compartment. Therefore, in these simulations, only emerging phenomena in the course of a substance physiological kinetics are modeled. The advantage of this method is the simplicity of the model, which leads to metabolite dynamics that are analytically solvable equations. The same caveats present in the PBPK modeling are observed, namely, a particular model must be constructed for each metabolite since every molecule has a different physiological path and the particular kinetics lead to chemical-specific effective models. However, in the effective toxicokinetic models, a non-detailed knowledge of the kinetics is enough to accurately assess the relation between exposure doses and biological matrices of interest.

The key modeling stages of the latter modeling approach are mainly conceptualization of a kinetic model based on primary physiological determinants of the kinetics of the compound of interest and its metabolites, resolution of the corresponding linear system of first-order ordinary differential equations, determination of model parameters and validation of the toxicokinetic model. Subsequently, the mathematical models can be used 1) to estimate most probable

exposure scenarios on the basis of the time course of urinary biomarkers in individuals and 2) to reconstruct daily absorbed doses from biomarkers levels measured in accessible sampled matrices.

To describe the kinetics of a given pesticide and its metabolites in humans with such approach, the first step is the development of a mathematical model composed of various linked compartments. This model is specific to the substance of interest and links amounts absorbed and their rate of absorption to excretion rates. Its purpose is to simulate essential biological features of the dynamics with a minimum number of parameters to reproduce the measured urinary and blood time profiles of the molecule under study. The required degrees of freedom in the simulation are determined from direct best-fitting to experimental data on the time courses of the pesticide and its metabolites in accessible biological matrices (e.g. blood and urine).

The conceptual representation of the general modeling of the kinetics of non-persistent pesticides followed in past studies (Bouchard *et al.*, 2003, 2005, 2006, 2008; Gosselin *et al.*, 2004; Heredia-Ortiz *et al.*, 2011; Heredia-Ortiz and Bouchard, 2011) is depicted in Figure 1. Compartments represent burdens, on a mole basis, of the pesticide in the body or cumulative excretion of metabolites as a function of time. Variations in time in compartment burdens are described mathematically by systems of differential equations (See Table 1 for description of differential equations). Most effective toxicokinetic models are based on first-order transfer rates which lead to a linear set of first-order ordinary differential equations. By first-order processes, it implies that the rate of change in the amounts in a given compartment is proportional to the amounts in the compartment at all times. Therefore, the rates of change in the amounts of a substance in a given compartment are described mathematically as the difference between compartment rates of uptake and loss. Exchange rates between compartments, described by arrows, represent either the physical transfer of the same substance or the transfer (on a mole to mole basis) through the biotransformation of the studied toxic substance into its metabolites or primary metabolites into derivatives. This kind of models is not restrained to be linear. Any of the rates presented could be changed to take into account any non-lineal mechanism like saturation phenomena (not just an enzymatic-like one, *i.e.* Michaelis-Menten kinetics), storage or protein binding.

The kinetics of the studied pesticide and its experimentally relevant metabolites are modeled for different routes-of-exposure, for example oral, dermal and inhalation. The input doses per unit of time, bioavailable at each site of absorption, the skin, the respiratory tract (RT) and the gastrointestinal tract (GI), are described as $g_{dermal}(t)$, $g_{inh}(t)$ and $g_{oral}(t)$, respectively. They are linked to their specific input compartment, which represents the amount of pesticide available at each site of absorption and generally illustrated by the symbols D(t), RT(t) and GI(t). A blood compartment (B(t)) is then used to describe the body burden of the pesticide in blood and in tissues in dynamical equilibrium with blood, *i.e.* tissues that rapidly reach and maintain a fixed ratio with blood. Another compartment is sometimes added to regroup storage tissue burdens of pesticides that are slowly returned to blood. Metabolite specific compartments, that is a compartment for each metabolite or groups of metabolites (specific or not to the studied pesticide) are added to represent metabolite burdens in blood and in tissues in dynamic equilibrium with blood (*e.g.* $M_x(t)$, $M_y(t)$, …). Similarly, different excretion compartments are introduced to represent cumulative amounts of the urinary metabolites (*e.g.* $U_x(t)$, $U_y(t)$, …) or

faecal metabolites (*e.g.* $F_x(t)$, $F_y(t)$, …). Other excretion compartments may be also added to describe the excretion of the non-monitored metabolites (N(t)).

Once the model is functionally represented by compartments and systems of differential equations, equations are solved to yield the mathematical functions of the time courses of the pesticide and each of its relevant metabolites in the different compartments. Initial conditions for every compartment are set in the experiment to be zero at starting time of the kinetic modeling. The next step therefore consists in determining the analytical solutions of the various linear first-order differential equations for oral, respiratory, dermal exposure, and any other relevant route-of-entry in the body.

Subsequently, model parameters are determined and adjusted to available literature data on the human kinetic time course following controlled exposure doses, along with *in vitro* studies and *in vivo* animal kinetics to complete human data when necessary. More specifically, the model parameters are computed by best-fit adjustments of the explicit solutions of differential equations to these time course data using a mathematical software. To best-fit general analytical functions to data sets, several procedures exist, but the least-square algorithm is a method often used (Weisstein, 2010).

Model Simulations, Sensitivity Analysis and Validation

Once parameters are estimated, simulations of the time course of the pesticide and its metabolites in the body and in excreta are performed by solving numerically the differential equation system using a mathematical software (*e.g.* the Runge-Kutta method incorporated in MathCad). Hence, by introducing time varying inputs and using the same set of parameter values, the model predicts amounts absorbed under different exposure scenarios (single or repeated, intermittent or continuous inhalation, dermal or oral exposures) starting from measurements of urinary amounts of biomarkers accumulated over specific time periods. This approach has the advantage of estimating the body burden of a chemical and its internal evolution regardless the route-of-entry and without reference to ambient concentrations.

The sensitivity of each of the model parameter values is then tested to determine their capacity to influence the predicted urinary excretion at different cumulative times (*e.g.* 12-h or 24-h urinary excretion). This mathematical procedure allows determining how much the value of a dependent function is modified when a particular independent degree of freedom is changed, while keeping all the remaining parameters unaffected. Lastly, the developed model is validated using different sets of experimental data available in the literature and for different routes-of-exposure in humans.

Derivation of BRV Using Modeling

Once validated, toxicokinetic models may be used to assess health risks related to pesticide exposures. Indeed, as models can be used to reconstruct the absorbed doses from measurements of cumulative urinary biomarkers, it is thus possible to establish the average daily absorbed dose corresponding, for each chemical studied, to a safe exposure level preventing early biological effects. The selected safe exposure level is based on a conservative reference dose, such as a human no-observed-(adverse)-effect level (NO(A)EL) dose, below which individuals should

not present adverse health effects whatever their exposure conditions. As a first step, most sensitive route of exposure can be identified with the model. As a second step, BRVs in the form of amounts of urinary biomarkers, excreted over different time periods, can be established from model simulations of an exposure to the established reference (NO(A)EL) dose and considering the route of absorption providing the smallest BRVs, to protect individuals whatever their exposure conditions. This reference value may then be compared to urinary excretion values in workers or in the general population, including children (Figure 2).

APPLICATION TO OCCUPATIONAL EXPOSURES

The approach described above was applied in the context of occupational exposures to several pesticides, including organophosphate and carbamate insecticides as well as phthalimide-like fungicides.

Organophosphate and Carbamate Insecticides

Most organophosphate insecticides (OPs) exhibit similar kinetic patterns. They are metabolized to common alkyl phosphate (AP) derivatives and to residues specific to each compound, which are then largely eliminated in urine. These specific metabolites as well as APs are easily measured in urine within a few hours following exposure, whatever the route-of-entry, and even at absorbed doses lower than those inducing any sign of toxic effects (Morgan *et al.*, 1977; Richter *et al.*, 1992; Carrier and Brunet, 1999; Dennis and Lee, 1999; Griffin *et al.*, 1999; Bouchard *et al.*, 2003; Gosselin *et al.*, 2005). These similarities allow developing a general toxicokinetic model for OPs (Figure 1) and adjusting appropriate biological determinants of the kinetics of each compound, such as physicochemical properties, biotransformation, and excretion of specific metabolites.

Like OPs, carbamate insecticides induce an inhibition of cholinesterase (ChE) activity and are mainly eliminated in urine (over 80%) (Maroni *et al.*, 2000b). Therefore, the same general toxicokinetic model developed for OPs (Figure 1) may be applied to carbamates using the same compound specific adjustments, even if urinary metabolites are not common to those of OPs.

Once a chemical-specific model is developed and validated, it can be used to derive specific BRVs, that is urinary values of biomarkers of exposure to OPs or carbamates corresponding to available pesticide reference exposure doses (such as a NO(A)EL), which may be daily absorbed without causing inhibition of red blood cells – acetylcholinesterase (RBC-AChE) activity. Application of this toxicokinetic modeling approach to derive BRVs for different OPs (malathion, chlorpyrifos and parathion), and for the carbamate insecticide carbaryl in the context of occupational exposures is presented.

Malathion

Malathion (O,O-dimethyl S-1,2-di(ethoxycarbonyl)ethyl phosphorodithioate, CAS 121-75-5) is a non-systemic insecticide widely used in agriculture, in households and in public health programs to control insect pests. Like other OPs, this pesticide can exert neurotoxic effects in humans since it is a AChE inhibitor (Angerer *et al.*, 2007). Symptoms and light clinical signs were reported in individuals with ≥30% reduction of RBC-AChE activity (Sidell, 1994). Therefore, to prevent cholinergic health effects in workers, the ACGIH suggested a RBC-AChE activity of 70% of

the individual's baseline (*i.e.* 30% inhibition of the activity) as a BEI® (ACGIH, 2011). On the other hand, Bouchard *et al.* (2003) used a modeling approach to derive BRVs based on measurements of malathion urinary metabolites, which can be considered as more sensitive indicators of exposure.

The main urinary metabolites of malathion are the mono- and di-carboxylic acids (MCA and DCA), the phosphoric derivatives dimethyl dithiophosphate (DMDTP), dimethyl thiophosphate (DMTP) and dimethyl phosphate (DMP). In field studies, malathion exposure is assessed in using MCA and DCA as specific urinary biomarkers and DMDTP, DMTP and DMP as non-specific biomarkers (Bradway and Shafik, 1977; Coye *et al.*, 1986; Fenske, 1988; MacIntosh *et al.*, 1999; Adgate *et al.*, 2001; Márquez *et al.*, 2001; Cocker *et al.*, 2002).

Based on this knowledge of biotransformation pathways of malathion and of kinetic time courses in volunteers (Feldmann and Maibach, 1974; Jellinek *et al.*, 2000), Bouchard et al. (2003) developed a malathion-specific human toxicokinetic model (Figure 3). The model links absorbed doses of malathion following different exposure routes (oral, dermal, inhalation) and scenarios (single, repeated, intermittent or continuous exposure) to kinetics of the malathion metabolites excreted in urine. It includes inputs for different routes-of-entry (gastro-intestinal tract, skin or respiratory tract), a specific body compartment for the malathion burden in blood and tissues in dynamical equilibrium with blood, a storage compartment for malathion accumulated in lipids or bound to tissue proteins, a total metabolite compartment to describe the whole body burden of total metabolites, as well as specific compartments to describe unconjugated and conjugates MCA, DCA, DMDTP, DMTP and DMP metabolites generated in cascade in the body. Compartments were also introduced to represent cumulative amounts of each of these five malathion metabolites excreted in urine. All amounts are initially expressed on a mole basis and exchange rates between compartments represent either the physical transfer of malathion or its metabolites, or the transfer (on a mole to mole basis) through biotransformation of malathion to its metabolites or one metabolite to another. To validate the model, different sets of experimental data from studies of Maibach *et al.* (1971), Wester *et al.* (1983) and Dennis and Lee (1999) were used.

The model was then used to propose limit values of urinary MCA, DCA and phosphoric derivatives that may serve as guideline values not to exceed to prevent significant inhibition of AChE in workers. To define such BRVs, the repeated-exposure NOEL dose of malathion established at 0.2 mg/kg/day (0.61 µmol/kg/day) by Moeller and Rider (1962) to prevent inhibition of plasma and erythrocyte ChE activities was used and corresponding daily absorbed NOEL dose was derived. Simulated urinary amounts of the biomarkers in 24-h collections considering a typical 8-h dermal exposure in workers to a total absorbed dose corresponding to the daily absorbed NOEL dose were proposed as BRVs (see Bouchard *et al.*, 2003). To insure a margin of safety, simulations were conducted with the slowest absorption rate compatible with available experimental time-course data and without considering any baseline excretion.

With this approach, BRV values of 44, 13 and 62 nmol/kg were obtained for MCA, DCA and the sum of phosphoric acids, respectively, in 24-h urine collections, and values of 57 and 119 nmol/kg were respectively derived for the sum of acids or total metabolites (Table 2). These BRVs served in the context of a biomonitoring study in

greenhouse workers exposed to malathion following a spraying episode or work in a treated area (Bouchard *et al.*, 2003). As depicted in Table 3, urinary values of MCA and DCA in the workers under study ranged between 1.7 and 21% of the proposed BRV for MCA and 1.1 to 32% of the BRV for DCA. Similar results were obtained when comparing biomonitoring results of Márquez *et al.* (2001) on measured total amounts of MCA excreted over a 24-h period in three Spanish greenhouse workers following application of malathion (31.7, 8.6, and 6.3 nmol/kg, considering a body weight of 70 kg) with the proposed BRVs (Table 3).

Chlorpyrifos

Chlorpyrifos (O,O-diethyl-O-[3,5,6-trichloro-2-pyridyl]phosphorothioate, CAS 2921-88-2) is a non-systemic insecticide used to control a wide range of insect pests. Toxicity is induced by chlorpyrifos (CPF) bioactivation product, CPF-oxon, which is an AChE inhibitor (Namba *et al*., 1971; Huff *et al*., 1994; Sultatos, 1994).

According to studies in animals and humans, CPF is nearly completely metabolized to 3,5,6-trichloro-2-pyridinol (3,5,6-TCP) and AP metabolites, i.e. diethyl thiophosphate (DETP) and diethyl phosphate (DEP), whatever the route-of-entry. These metabolites are then essentially excreted in urine (Bakke *et al*., 1976; Nolan *et al*., 1984; Griffin *et al*., 1999). Several studies in field workers assessed CPF exposure by measuring urinary 3,5,6-TCP as a specific biomarker, and DETP and DEP as non specific biomarkers (Jitsunari *et al*., 1989; Fenske and Elkner, 1990; Cocker *et al*., 2002).

Considering this biotransformation data along with available kinetic time courses in volunteers, Bouchard et al. (2005) developed a chlorpyrifos-specific toxicokinetic model (Figure 4A). Similar to malathion model, this model describes the biodisposition kinetics of CPF and its metabolites in humans with a minimum number of compartments and parameters. It allows predicting the time courses of CPF and its metabolites under different exposure routes (oral, dermal and/or inhalation) and temporal scenarios (single or repeated intermittent or continuous exposures). The parameters were established using the data of Nolan et al. (1984) and Drevenkar *et al*. (1993) in individuals orally exposed to CPF. The model includes specific inputs to represent the amounts of CPF bioavailable at each site-of-entry (skin, gastro-intestinal tract, respiratory tract), a specific body compartment to describe the CPF blood burden (i.e. arterial and venous blood including tissue blood), a storage compartment to represent CPF in lipids or reversibly bound to tissue proteins, two metabolite compartments to represent distinctly the body burden of 3,5,6-TCP and AP metabolites, and two urinary compartments to represent separately the cumulative urinary excretions of 3,5,6-TCP and AP metabolites. Furthermore, based on the study of Smith *et al*. (1967), the model considers that each mole of CPF in the body is eventually broken down into one mole of 3,5,6-TCP and one mole of AP, and metabolite excretion occurs only through the renal route. The model was validated using the data of Nolan *et al*. (1984) in dermally exposed individuals (on the forearm), and the data of Brzak (2000) and Griffin *et al*. (1999) in orally exposed subjects.

As was done for malathion, the model then served to estimate BRVs for 3,5,6-TCP and AP metabolites in urine. The available repeated-exposure NOEL dose of 0.1 mg/kg/day of chlorpyrifos (20 µmol/day for a 70 kilogram individual), which did not induce significant inhibition of RBC-AChE activities, was used (Coulston *et al*., 1972; McCollister *et al*., 1974; Mattsson *et al*., 2001), and the corresponding daily

absorbed NOEL dose was estimated. Simulated urinary amounts of these metabolites in 24-h collections considering an 8-h dermal exposure to a total absorbed dose equivalent to the daily absorbed NOEL dose were proposed as BRVs. With these considerations, BRV values of 26 and 45 nmol/kg bw were obtained, respectively, for 3,5,6-TCP and APs in 24-h urine collections (Bouchard *et al*., 2005).

The BRVs were applied to practical situations, and hence were used to assess the importance of exposure and potential effects in the groups of workers assessed by Samuel *et al*. (2002) and Fenske and Elkner (1990). In both studies, total amounts of 3,5,6-TCP in 24-h urine collections were quantified in greenhouse workers following the onset of an application of CPF or manipulation of treated plants (Samuel *et al*., 2002), or in workers exposed to CPF following structural control treatment of houses (Fenske and Elkner, 1990). As displayed in Table 3, the workers of these studies presented 3,5,6-TCP values in 24-h urine collections lower than the proposed BRV.

Parathion

Parathion (O,O-diethyl O-*p*-nitrophenyl phosphorothioate, CAS 56-38-2) is a potent non-systemic insecticide and acaricide with some fumigant action. Like other OP insecticides, it exerts its neurotoxic action by inhibiting nervous system AChE activity, both in insects and humans (Sidell, 1994; Maroni *et al.*, 2000a; Gosselin *et al.*, 2004). Although there are now application restrictions due to its high toxicity, the use of parathion is still a concern in many <other> countries (Denga *et al.*, 1995; Akgur *et al.*, 1999).

The ACGIH® and the GCIHH® (German Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area) proposed a 30% inhibition of RBC-AChE activity compared to the individual's baseline as a biological reference value. A more specific biological reference value based on urinary concentration of *p*-nitrophenol (*p*-NP) parathion metabolite in a spot urine sample has also been proposed, given that it is detectable in urine far before any inhibition of enzymatic activity occurs (Arterberry *et al.*, 1961; Richter *et al.*, 1992). The ACGIH BEI® value was set to 0.5 mg of *p*-NP/g of creatinine (0.34 mmol *p*-NP/mol creatinine) while the GCIHH BAT® value was established at 0.5 mg of *p*-NP/L of urine. However, parathion is eliminated through the kidneys by glomerular filtration or active secretion (Michalke, 1984; Jokanovic, 2001), such that its urinary concentration decreases with increasing urinary flow rate (Boeniger *et al.*, 1993) and is thus subject to significant variations with time. A more reliable sampling strategy than spot urine samples is to quantify total amounts of metabolites excreted in urine over given time periods (Woollen, 1993; Ross *et al.*, 2001). In this perspective, Gosselin *et al.* (2004) developed a toxicokinetic model to absorbed doses of parathion to urinary biomarker measurements and to propose, using model predictions, BRVs for parathion metabolites, *p*-NP and APs, in the form of cumulative amounts of urinary metabolites following the onset of an occupational exposure (Figure 4B).

Again, the model is based on knowledge on biotransformation pathways of parathion. After systemic absorption, it is either rapidly hydrolyzed to *p*-NP and DETP or oxidized to paraoxon in the liver mainly, or stored in lipids. Paraoxon represents 20% of the biotransformation while the hydrolysis constitutes 80% of the biotransformed parathion (Poore and Neal, 1972; Hayes, 1982; Jokanovic, 2001).

Paraoxon is then either metabolized by an A-esterase into *p*-NP and DEP, or it binds to proteins such as AChE (less than 20%) (Hayes, 1982; Jokanovic, 2001). The metabolites *p*-NP and APs, *i.e.* DETP and DEP, are eliminated in urine and faeces.

The model and its parameter values is also based on available data on the kinetics of parathion and its metabolites in volunteers (Hartwell *et al.*, 1964; Hayes *et al.*, 1964; Maibach *et al.*, 1971; Feldmann and Maibach, 1974; Morgan *et al.*, 1977). The model consists of specific inputs to describe amounts of parathion bioavailable at the skin surface, GI tract or respiratory tract; a body compartment to represent tissue burdens of parathion and paraoxon that rapidly reach and maintain a fixed ratio with blood burden; a storage compartment to characterize storage tissue burdens of parathion and paraoxon that are slowly returned to blood; specific metabolite compartments for *p*-NP or AP metabolites (the sum of DETP and DEP) in blood and in tissues in dynamic equilibrium with blood; retention compartments which delays excretion of *p*-NP or APs in urine; urinary and faecal compartments to represent separately cumulative excretion of total *p*-NP or APs in urine and faeces, respectively.

Simulations were then performed to propose BRVs for *p*-NP and APs in urine using an 8-h dermal scenario to a total dose corresponding to an available repeated-exposure NOEL dose of 4.8 mg/day (*i.e.*, 58 µg/day/kg bw). The NOEL was determined from the volunteer studies of Edson *et al*., (1964) and Rider *et al.* (1969) based on the inhibition of plasma-ChE or RBC-AChE. BRVs for *p*-NP and APs of 6 and 2 nmol/kg bw, respectively, were derived in 12-h urine collections and 24 and 15 nmol/kg bw, respectively, in 24-h urine samples. These values are expressed as cumulative amounts in urine over given time periods following the onset of an exposure episode.

The suggested BRV value for *p*-NP may be considered protective on the basis of results obtained by Hayes *et al.* (1964) in volunteers exposed to parathion and showing no significant inhibition of RBC-AChE (0%) and plasma-ChE (6%) activities at *p*-NP value of 15.9 µmol in 24-h urine collections (*i.e.* 24 nmol/kg bw assuming a body weight of 70 kg). The current applicability of these proposed BRVs to practical biomonitoring situations is difficult due to limits of the usage of parathion in the USA since 1991 and increased parathion handling and postapplication restrictions (EPA, 1999a).

Carbaryl

Carbaryl (1-naphthyl methylcarbamate, CAS 63-25-2) is a contact insecticide of the carbamate family, which inhibits ChE activity in humans. Most studies in exposed workers measured ChE activity and the ACGIH recommends a RBC-AChE activity of 70% of the individual's baseline as a BEI® (ACGIH, 2011) while no biological reference value based on urinary biomarkers were proposed.

The main urinary carbaryl metabolite in humans is 1-naphthol (over 85%) while 4-(hydroxy)-1-naphthyl N-,ethyl carbamate (free or glucurono-conjugate), 1-naphthyl-glucuronide and 1-naphthyl-sulfate are minor urinary metabolites (Knaak *et al.*, 1965; Kozbelt, 1968; Chin *et al.*, 1974; Feldmann and Maibach, 1974). 1-Naphthol is rapidly excreted in urine mostly within 24-h after absorption and mainly as glucurono- and sulfo-conjugates.

Based on these available published human data along with data of May *et al.* (1992) on plasma concentration-time course of carbaryl in volunteers, Bouchard *et al.* (2008) developed a carbaryl-specific toxicokinetic model (Figure 5). This model relates the absorbed dose of carbaryl, the evolution of its body burden and that of its metabolites to the cumulative urinary amounts of biomarkers excreted over given time periods. Specific input compartments were introduced in the model to simulate absorption through the skin (slow release into the blood stream) and the GI tract; inhalation exposure was considered kinetically similar to a constant intravenous (iv) exposure, and was thus modeled by direct inputs to the blood compartment. The model is also comprised of a blood compartment to represent total carbaryl burden in blood and tissues in dynamical equilibrium with blood; separate metabolite compartments to describe independently the body burden of the relevant carbaryl metabolites, namely 1-naphthol, 4-(hydroxyl)-1-naphtyl-N-methylcarbamate and non-monitored carbaryl metabolites; specific excretion compartments to represent cumulative amounts of each urinary carbaryl metabolite, that is one compartment for the sum of free and conjugated 1-naphtol, one for 4-(hydroxyl)-1-naphtyl-N-methylcarbamate and another to describe excretion of non-monitored or non-observed metabolites in urine. Unlike OP models, as observed time courses of metabolites in urine decreased linearly, no storage compartment was added in this model (Bouchard *et al.*, 2008).

To determine a BRV for 1-naphthol in 24-h urine collections in order to prevent cholinergic effects in workers exposed to carbaryl, the available repeated-exposure NOEL dose of 0.06 mg/kg/day (0.3 µmol/kg/day) was used (Wills *et al.*, 1968) and corresponding daily absorbed NOEL dose was estimated. The latter then served to simulate a typical 8-h dermal exposure in workers to a total absorbed dose corresponding to this daily absorbed NOEL dose. Corresponding 1-naphthol excretion in 24-h collections following the onset of exposure was proposed as a BRV and was established at 32 nmol/kg bw/day (Bouchard *et al.*, 2008).

The proposed BRV was subsequently applied to occupational exposure situations, and total amounts of 1-naphthol excreted in the 24-h urine of greenhouse workers exposed to carbaryl reported in two studies (Samuel *et al.*, 2002; Bouchard *et al.*, 2008) was compared to the suggested reference value. Bouchard *et al.* (2008) quantified total amounts of 1-naphthol in 24-h urine collections following the onset of a carbaryl application or the beginning of work in treated area. Similarly, Samuel *et al.* (2002) determined 24-h urinary amounts of 1-naphthol in individuals who handled plants or flowers treated with carbaryl. In both studies, all workers except one exhibited 1-naphthol values below the proposed BRV (Table 3).

Overall, comparison of the different proposed BRVs for specific urinary biomarkers of chlorpyrifos, malathion, parathion and carbaryl showed that values are similar in all cases as presented in Table 2. Likewise, values for non-specific metabolites, such as APs, are also in the same range although toxicokinetic models are specific to each insecticide. Nonetheless, to suitably assess occupational exposure, BRVs for specific metabolites should be preferred to those of non-specific biomarkers, although the latter may provide valuable information on overall OP exposure.

Phthalimide-like fungicides

Fungicides constitute a large class of chemical substances frequently used in agriculture and horticulture, especially captan (*N*-(trichloromethylthio)-4-

cyclohexene-1,2-dicarboximide, CAS 133-06-2) and folpet (*N*-[Trichloromethylthio]phthalimide, CAS 133-07-3). In humans, both fungicides were initially classified as probable human carcinogens (B2) by the (EPA, 1975, 1999b) based on an increased incidence of duodenum tumors in rodents chronically exposed to high doses by gavage. However, in 2004 captan classification changed to "not likely" considering that the doses administered to mice were much higher than those encountered in occupational settings (EPA, 2004; Gordon, 2007). Both compounds are also considered as sensitizers and strong irritants of the eyes, skin and respiratory airways, but no systemic toxicity was reported (Hayes, 1982; Edwards *et al.*, 1991; Trochimowicz *et al.*, 1991; Tomlin, 1997; EPA, 1999b, 2004; Costa, 2008; Gordon, 2010; ACGIH, 2011). Some studies also reported skin and respiratory problems in workers exposed to captan or folpet (Burroughs and Hora, 1982; Lisi *et al.*, 1987; Guo *et al.*, 1996). As a result, occupational guidelines were derived for captan, namely a TLV®-TWA of 5 mg/m^3 (ACGIH, 2011), but none are available to date for folpet.

Recently, BRVs were also proposed for captan and folpet metabolites using a toxicokinetic modeling approach (Heredia-Ortiz *et al.*, 2011; Heredia-Ortiz and Bouchard, 2011). More specifically, based on available toxicokinetic data (Krieger and Thongsinthusak, 1993; Berthet *et al.*, 2011a, b), two toxicokinetic models were developed to allow a better assessment of the effect of the exposure route and temporal scenario on the biomarkers kinetics of captan and folpet (Heredia-Ortiz *et al.*, 2011; Heredia-Ortiz and Bouchard, 2011). These models were then used to reconstruct absorbed doses from biomarker data and define BRVs, or safe daily urinary biomarker levels corresponding of an exposure dose limit for workers. To illustrate the applicability of the general approach developed for OPs and carbamate insecticides to other compound families, the examples of captan and folpet are detailed below.

Captan

Toxicokinetic data on captan are well documented in *in vivo* studies in animals usually exposed to the radiolabelled compound as well as in *in vitro* studies. According to these studies, captan is an unstable molecule in aqueous medium and in biological fluids when in contact with thiols. Gordon *et al.* (2001) estimated a half-life in the order of a second (0.97 s) following addition of C^{14}-labelled captan (1 mg/L) to human blood. The first step in the metabolism of captan is the non-enzymatic breakdown of N-S link to form a tetrahydrophthalimide (4,5-cyclohexene-1,2-dicarboximide; THPI) and a derivative of the trichloromethylthio group, namely thiocarbonyl chloride. The latter in turn reacts with thiols to form thiophosgene, a transient metabolite, which readily reacts with cysteine or gluthathione to form thiazolidine-2-thione-4-carboxylic acid (TTCA). In biomonitoring studies (EPA, 1975), measurement of THPI is favored to TTCA since it is more stable in biological matrices and TTCA is less specific, being also a urinary metabolite of carbon disulfide (Amarnath *et al.*, 2001).

In order to determine exposure and health risks in workers from amounts of urinary biomarkers, Heredia-Ortiz and Bouchard (2011) developed a toxicokinetic model, which reproduces the time course of THPI biomarker in blood and urine for different temporal scenarios (single, repeated intermittent or continuous exposures) and exposure routes (oral, dermal, respiratory). The determination of model parameters was based on available published metabolism data (Gordon *et al.*, 2001) along with the human time-course data collected in volunteers orally and dermally

exposed to captan (Berthet *et al.*, 2011a, b). The model was also corroborated with the volunteer data of Krieger and Thongsinthusak (1993) as well as experimental *in vivo* kinetic data in animals (Seidler *et al.*, 1971; DeBaun *et al.*, 1974; Couch *et al.*, 1977; van Welie *et al.*, 1991; Fisher *et al.*, 1992) along with other non-public results summarized in reviews on captan (Larsen, 1996; Wilkinson, 2003; EPA, 2004; Food and Agriculture Organization of the United Nations (FAO), 2007; Gordon, 2010).

An approach similar to the one used for OP insecticides and carbaryl has been applied to establish the conceptual representation of the model (Figure 6A). The model consists of specific inputs to describe captan bioavailable at each absorption site (GI tract, skin and respiratory tract); blood compartments to represent captan and the experimentally relevant THPI metabolite in blood and in tissues in dynamical equilibrium with blood; excretion compartments to describe cumulative amounts of THPI in urine and faeces, respectively. In the model, inhalation was simulated by direct inputs to the blood compartment, given that captan is rapidly absorbed through the respiratory tract and this can be considered kinetically as an almost constant intravenous exposure. Furthermore, as a linear THPI elimination in blood of volunteers orally exposed to 1 mg/kg bw of captan was observed in the study of Berthet *et al.* (2011a), no storage compartment was added to represent an accumulation in lipids or a binding to tissue proteins.

Unlike OPs and carbamate insecticides, health effects induced by phthalimide fungicides in humans are not clearly characterized. Therefore, to determine a BRV for urinary THPI, Heredia-Ortiz and Bouchard (2011) selected an absorbed dose (RfD_{abs}) corresponding to the chronic oral reference dose (RfD) of 0.125 mg/kg bw/day proposed by the U.S. EPA (EPA, 2004) on the basis of a rat study. An oral absorption fraction of 1 was considered, such that the RfD_{abs} was set equal to the exposure RfD. The RfD was derived from a NOAEL of 12.5 mg/kg bw/day in a three-generation reproduction study in rats to which a safety factor of 100 was applied. Following model simulations of a dermal exposure to the value of the RfD_{abs}, the BRV obtained for workers in 24-h urine collections was 21.5 nmol THPI/kg bw/day.

Table 3 presents mean cumulative amounts of THPI in 24-h urine collections of workers exposed to captan following different activities in several studies (Winterlin *et al.*, 1986; Verberk *et al.*, 1990; de Cock *et al.*, 1995; Krieger and Dinoff, 2000; Hines *et al.*, 2008). These published values were found to be below the proposed BRV determined with the model and based on a conservative reference dose.

Folpet

As captan, folpet metabolism is well documented in animals and *in vitro* studies (Chasseaud *et al.*, 1974; Chasseaud *et al.*, 1991; Gordon *et al.*, 2001; Canal-Raffin *et al.*, 2008; Gordon, 2010). Folpet is rapidly metabolized to phthalimide metabolite (PI) and thiosphogene, an unstable metabolite which reacts with cysteine or glutathione to form TTCA. PI is also rapidly hydrolyzed to phthalamic acid (PAA), and in turn to phthalic acid (PA). According to studies in rats exposed to folpet following an oral, intratracheal, or intraperitoneal administration (Chasseaud *et al.*, 1974; Chasseaud, 1980; Chasseaud *et al.*, 1991; Canal-Raffin *et al.*, 2008), PAA is the main ring-metabolite (over 80%). However, this metabolite is a very unstable compound in human urine and it is more convenient to transform PI and PAA metabolites to PA in acid conditions, and to measure total PA equivalents (PA_{eq}) in urine as a biomarker of exposure (Berthet *et al.*, 2011c). Nevertheless, PA is also a

metabolite of phthalates, such that urinary background levels may be relatively high in some instances. Conversely, PI is a specific biomarker of exposure to folpet, stable in urine and easily measurable in blood and urine (Barr *et al.*, 2002; Canal-Raffin *et al.*, 2008; Berthet *et al.*, 2011d).

To describe and better understand the toxicokinetics of folpet and its ring metabolites in humans, Heredia-Ortiz *et al.* (2011) developed models to describe the kinetics of either PI or PA_{eq} (Figure 6B). Only the pathway leading to the formation of the PI and PA_{eq} (*i.e.* the sum of PI, PAA and PA metabolites) biomarkers of exposure was modeled. Conceptual representations and parameters values were based on the human time-course data in volunteers orally and dermally exposed to folpet (Berthet *et al.*, 2011a, b) and available published metabolism data on folpet (Gordon *et al.*, 2001; Gordon, 2010). Specific models were derived to represent the time courses of PI and PA_{eq}, given the differences in the kinetics of these two biomarkers. To simulate the kinetics of PI specifically, PAA and PA were considered as non-observed metabolites whereas to simulate the kinetics of PA_{eq}, PI, PAA and PA compartments were lumped.

In the models, input doses per unit of time, bioavailable at each site of absorption, the skin, the respiratory tract and the GI tract, were represented. Distinct GI compartments were used to simulate folpet in the GI after oral exposure as well as the almost instantaneously generated PI. Skin compartments were divided into epidermis and dermis compartments to simulate the kinetics of PI following dermal exposure while the dermis was considered in equilibrium with blood in the model for PA_{eq}. As for captan, inhalation exposures were modeled by direct inputs to the blood compartment and instant fragmentation at the N-S link, and accumulation in tissues was considered negligible (Berthet *et al.*, 2011a, b). Furthermore, compartments were considered to describe the body burden of PI, PAA, PA and excretion in urine.

Using these kinetic models, BRVs for PI and PA_{eq} in 24-h urine collections were derived using a RfD_{abs} established from the chronic oral RfD of 0.09 mg/kg bw/day suggested by the U.S. EPA (1999b). This RfD is based on observed skin and stomach damaged cells in rats exposed at 35 mg/kg and on a NOEL of 9 mg/kg per day to which a safety factor of 100 was applied (EPA, 1999b). As a conservative scenario, the oral absorption fraction was set to be 1, such that the RfD_{abs} was considered equal to the exposure RfD. Following model simulations of a dermal exposure to the value of the RfD_{abs}, the resulting BRVs obtained in 24-h urine collections were 36.6 pmol/kg bw for PI and 32.0 nmol/kg bw for PA_{eq}.

To obtain an indication of the risk associated with exposure to folpet in workers, observed maximum daily amounts of PI and PA_{eq} in the urine of workers following trimming and spraying activities over 3-7 days were compared to the proposed BRV in 24-h urine collections (Berthet *et al.*, 2011e). The results show that in most cases, studied workers had maximum daily excretions exceeding the proposed reference values for PI regardless of the activity, while urinary values were below the PA_{eq} BRV for all workers except one. To date, there is no other available study in workers exposed to folpet to compare these reference values.

Overall, as presented in Table 2, BRV values determined for THPI and PA_{eq} with the modeling approach are in the same range as those proposed for biomarkers of OPs and carbaryl exposure in 24-h urine collections. Conversely, PI value is lower,

but it is a minor metabolite of folpet and only a very small fraction reaches blood unchanged following an oral or dermal exposure.

APPLICATION TO A CHILDREN POPULATION

The toxicokinetic modeling approach previously described for occupational exposure to pesticides is also applicable to an environmental exposure of the general population. However, the approach requires some specific considerations. Contrary to occupational exposure, the exact route, source and onset of an environmental exposure to pesticides, leading to a given set of population biomonitoring data, are generally unknown. This is because there may be an exposure to multiple unidentified pesticides, that may or not share the same mechanism of action, and this may occur at varying moments of the day and for different combinations of exposure routes (*e.g.* oral, inhalation, dermal). To account for these uncertainties, assumptions need to be made in order to perform a health-based interpretation of biomonitoring data. This was recently done by Valcke and Bouchard (2009) with regard to OP exposure in a children population for which urinary concentrations of AP metabolites measured in first morning voids were available (Valcke *et al.*, 2006).

First, as the specific pesticides to which the children under study were exposed were unknown, these authors assumed that all the methylphosphate (MP) metabolites originated from an exposure to malathion only, whereas all the ethylphosphate (EP) metabolites stemmed from an exposure to CPF. These two assumptions were made based on the following elements (Valcke and Bouchard, 2009):

- CPF and malathion were among the OPs to which the studied population was most likely exposed at the time of sampling;
- the availability of appropriate chemical-specific toxicokinetic models for those OPs;
- the accessibility of human NOELs for these two pesticides (thus precluding the need for uncertain animal-to-human extrapolation), that are based on the inhibition of RBC-AChE activity, which is more sensitive to OP inhibition than the nervous system AChE, responsible for the toxic effect of OPs;
- the rather small NOEL values for malathion and CPF as compared to those of other OPs, providing greater safety given that at least part of the measured metabolites likely result from the biotransformation of other less toxic OPs.

Valcke and Bouchard (2009) then used the toxicokinetic models for malathion (Bouchard *et al.*, 2003) and CPF (Bouchard *et al.*, 2005) to generate "NOEL-biomarker equivalents" (NBEs) corresponding to amounts of MP/EP metabolites in an overnight urine collection, expressed in nmol/kg bw. Hence, these authors simulated the time course of the targeted urinary metabolites for various dermal or oral exposure scenarios to the relevant NOELs. They also compensated for the uncertainty associated with the elapsed time between the onset of the exposure and the morning collection of the urinary samples in the investigated children. Indeed, simulations performed with the models had previously shown that the lowest, thus most conservative, excretion values of AP urinary metabolites were obtained considering a dermal exposure scenario. On the other hand, urinary OP metabolites in children are generally attributed to dietary exposure (Fenske *et al.*, 2002; Curl *et al.*, 2003; Lu *et al.*, 2005; Valcke *et al.*, 2006; Lu *et al.*, 2008). Thus, to obtain a range of conservative NBEs that would encompass all the possible exposure regimens in children, three

scenarios were simulated, assuming that a urine sample was always collected in the morning (at 7:00 am) and that all the measured exposure had occurred the previous day:

- ingestion of a bolus NOEL at dinner (6:00 pm);
- ingestion of 1/3 NOEL at each daily meal, *i.e.* breakfast (7:00 am), lunch (noon) and dinner (6:00 pm);
- an 8-h dermal exposure to the NOEL, starting at 7:00 am.

The resulting NBEs were respectively 106, 127 and 40 nmol/kg bw for MP metabolites, whereas those for EP metabolites were 87, 52 and 32 nmol/kg bw. Accounting for the body weight of each child, AP measurements made in each urine void collected could thus be compared to these values to assess whether or not each child had likely been exposed to a dose exceeding the NOEL the day prior to urine sampling. As indicated in Figure 7, which computes distributions of ratios of the AP urinary measurements over the relevant NBEs, this occurred only for one of the 442 samples collected when looking at urinary concentrations of MP metabolites and assuming a more conservative (but less likely) dermal exposure scenario (Valcke and Bouchard 2009). The same observation could be made when the sum of ratios obtained for both MP and EP metabolites were calculated in order to consider the possible co-exposure to multiple OPs, given that they all share a common mechanism of toxicity (AChE inhibition of activity).

Several authors have used non-toxicokinetic approaches to pesticide dose estimates (and resulting risks) from urinary biomonitoring data in spot urine samples typical of non-occupational settings. Such approaches rely on the underlying assumptions that pesticide exposure follows steady-state conditions and that daily creatinine excretion is constant, two assumptions that allow estimating pesticide exposure dose from the urinary measurements of parent compounds (Acquavella *et al.*, 2004; Alexander *et al.*, 2007) or the metabolites (Fenske *et al.*, 2000; Mage *et al.*, 2004; Curwin *et al.*, 2007; Mage *et al.*, 2008). Furthermore, biomonitoring equivalents (BEs), which correspond to a metabolite concentration in different matrices associated to steady-state exposure to a reference dose, have also been determined for several pesticides i.e. DDT/DDE, deltamethrin, hexachlorobenzene, triclosan, cyfluthrin and 2,4-D (Angerer *et al.*, 2011). BEs were actually suggested as a prioritization tool for risk management of population exposure to chemicals, including pesticides (Hays *et al.*, 2007).

A particularly interesting feature of the toxicokinetic approach described in this chapter is that by relying on amounts of metabolites excreted in urine rather than their concentrations, the toxicokinetics of the substance can be accounted for adequately, leading to robust internal dose estimates. This is true as long as a sufficient volume of urine is collected. Indeed, less uncertainty is associated with greater volumes collected; thus collection of first morning voids is suggested as they better reflect 24-h excretion volumes (Scher *et al.*, 2007). This sampling strategy also reduces the uncertainty associated with the daily excretion rates of creatinine, which are known to exhibit significant intra- and inter-individual variability (Mage *et al.*, 2004; Fortin *et al.*, 2008; Mage *et al.*, 2008). As a result, biomonitoring data can be interpreted both individually and at a population scale.

CONCLUDING REMARKS

In summary, this chapter showed the applicability of a toxicokinetic approach for the derivation of convenient BRVs for various types of pesticides. The various presented

examples highlight the importance of knowledge on urine collections (*i.e.* spot samples or timed-collections), sampling strategies, metabolism, relative proportion of different metabolites in the sampled matrix, route of exposure and background contribution of prior exposures. It also shows that focusing the approach on specific urinary biomarkers appears more accurate in the case of occupational exposure. Conversely, applying the approach to non-specific metabolites, but common to a category of pesticides, appears more accurate for environmental risk assessment where the pesticides to which subjects are exposed are unknown. The BRVs derived with the modeling approach may be readily used to provide a health-based interpretation of exposure in workers and the general population as well as facilitate health risk management of pesticides by public health authorities.

Table 1. First-order linear differential equations describing the kinetics of a generic model.

Compartment	Differential equations
Gastrointestinal tract	$\frac{\partial GI(t)}{\partial t} = D_{oral}(t) - k_{GIB}GI(t)$
Skin	$\frac{\partial S(t)}{\partial t} = D_{dermal}(t) - k_{SB}S(t)$
Pesticide in blood and tissues in equilibrium with blood	$\frac{\partial B(t)}{\partial t} = D_{inh}(t) + k_{GIB}GI(t) + k_{SB}S(t) - (k_{met} + k_{other} + k_{BU} + k_{BNO})B(t)$
Metabolite in blood and tissues in equilibrium with blood	$\frac{\partial B_{met}(t)}{\partial t} = k_{met}B(t) - (k_{metBU} + k_{metBNO})B_{met}(t)$
Pesticide in urine	$\frac{\partial U(t)}{\partial t} = k_{BU}B(t)$
Metabolite in urine	$\frac{\partial U_{met}(t)}{\partial t} = k_{metBU}B_{met}(t)$

Table 2. Biological reference values (BRVs) for specific metabolites of six different pesticides in 24-h urine collection of workers

Pesticides	Urinary biomarker	BRV[a] (nmol/kg bw/day)	Urine collection (following the onset exposure)	Reference dose used to derive the BRV
OP insecticides				
Chlorpyrifos				
	3,5,6-TCP	26	24-h urine	0.23 µmol/kg/day
		76	48-h urine	(NOEL based on an
	Total APs	45	24-h urine	oral exposure in
		99	48-h urine	humans)
Malathion				
	MCA	44	24-h urine	0.61 µmol/kg/day
	DCA	13		(NOEL based on an
	MCA + DCA	62		oral exposure in humans)
	Total APs	57		
	Total metabolites	119		
Parathion				
	p-NP	6	12-h urine	18.2 µmol/kg/day
		24	24-h urine	(NOEL based on a
	Total APs	2	12-h urine	dermal exposure in
		15	24-h urine	humans)
Carbamate insecticides				
Carbaryl				
	1-naphthol	32	24-h urine	0.3 µmol/kg/day (NOAEL based on ab oral exposure in humans)
Phthalimide fungicides				
Captan				chronic oral RfD of
	THPI	21.5	24-h urine	0.125 mg/kg bw/day
Folpet				chronic oral RfD of
	PI	0.04	24-h urine	0.09 mg/kg bw/day
	PA_{eq}	32	24-h urine	

pesticide and adjusted for the body weight.

[a] BRV corresponds to mean cumulative amounts of the studied metabolite measured in 12- or 24-h urine of workers exposed to the specific

Table 3. Mean cumulative amounts of metabolites observed in 24-h urine collections of workers assessed in different studies and comparison with proposed biological reference values below which health risks should be negligible.

References	N[a]	Studied urinary biomarker	Mean cumulative amounts of studied metabolite in 24-h urine (range) (nmol/kg bw)[b]	Average fraction of the proposed BRV (range)	Activity[c]	Crops[d]
Malathion						
Márquez *et al*. (2001)						
	3	MCA	15.5 (6.3 – 31.7)	0.35 (0.14 – 0.72)	Applicators	Greenhouse
Bouchard *et al.* (2003)						
	11	MCA	2.54 (0.73 – 9.25)	0.06 (0.02 – 0.21))	Applicators	Greenhouse
		DCA	0.99 (0.14 – 2.21)	0.08 (0.01 – 0.32)		
Chlorpyrifos						
Fenske and Elkner (1990)						
	8	3,5,6-TCP	2.01 (0.52 -3.12)	0.08 (0.02 – 0.12)	Structural control treatment	Greenhouse
Samuel *et al.* (2002)						
	1	3,5,6-TCP	0.10	0.004	Applicator	Greenhouse
	3	3,5,6-TCP	0.38 (0.3 – 0.47)	0.01 (0.01 – 0.02)	Handling	Greenhouse
Carbaryl						
Samuel *et al.* (2002)						
	2	1-naphtol	1.03 (0.62 – 1.44)	0.03 (0.02 – 0.05)	Applicators	Greenhouse
	12	1-naphtol	16.2 (0.71 – 103)	0.51 (0.02 – 3.23)	Handling	Greenhouse
Bouchard *et al.* (2008)						
	2	1-naphtol	2.21 (1.06 – 3.36)	0.08 (0.04 – 0.11)	Applicators	Greenhouse
	5	1-naphtol	8.47 (0.85 – 20.6)	0.29 (0.06 – 0.65)	Handling	Greenhouse
Captan						
Winterlin *et al.* (1986)						

References	N^a	Studied urinary biomarker	Mean cumulative amounts of studied metabolite in 24-h urine (range) (nmol/kg bw)[b]	Average fraction of the proposed BRV (range)	Activity[c]	Crops[d]
	3	THPI	2.71[e]	0.13	Loader/mixer/applicators	Grapes
	16	THPI	2.29[e]	0.11	Harvesters	Grapes
Verberk *et al.* (1990)						
	6	THPI	3.79[f]	0.18	Dipping bulbs in captan solution	Flower-bulbs
De Cock *et al.* (1995)						
	14	THPI	0.45	0.02	Applicators	Fruit
Krieger and Dinoff (2000)						
	41	THPI	0.13 – 0.35	0.01 – 0.02	Harvesters	Strawberries
Hines *et al.* (2008)						
	14	THPI	0.13 (0.09 – 0.18)	0.01 (0.004 – 0.01)	Applicators	Strawberries
Berthet *et al.* (2011)						
	2	THPI	0.16 (0.07 – 0.24)	0.01 (0.004 – 0.01)	Loader/mixer/applicators	Apple trees
	2	THPI	0.06 (0.005 – 0.11)	0.002 (0.0002 – 0.005)	Pruning, thinning	Apple trees
Folpet						
Berthet *et al.* (2011)						
	3	PI	0.043 (0.019 – 0.071)	1.17 (0.52 – 1.94)	Loader/mixer/applicators	Grapes
		PA_{eq}	21.5 (9.01 – 45.2)	0.67 (0.28 – 1.41)		
	3	PI	0.052 (0.011 – 0.074)	1.42 (0.30 – 2.02)	Pruning, thinning	Grapes
		PA_{eq}	17.4 (7.36 – 28.2)	0.54 (0.23 – 0.88)		

[a] Number of workers participating in the study.
[b] Mean cumulative amounts of the studied metabolite (range) measured in 24-h urine of workers exposed to the specific pesticide and adjusted for the body weight (50 kg and 70 kg for women and men, respectively, were taken when not indicated in the study).
[c] Activities performed by workers during the studied period of pesticide exposure.
[d] Studied crop fields.
[e] Mean 24-h urinary excretion was assumed to be 1 liter.
[f] Mean 24-h creatinine was assumed to be 1.5 g/l (or 0.013 mol/l).

Figure 1. Representation of a generic toxicokinetic model.

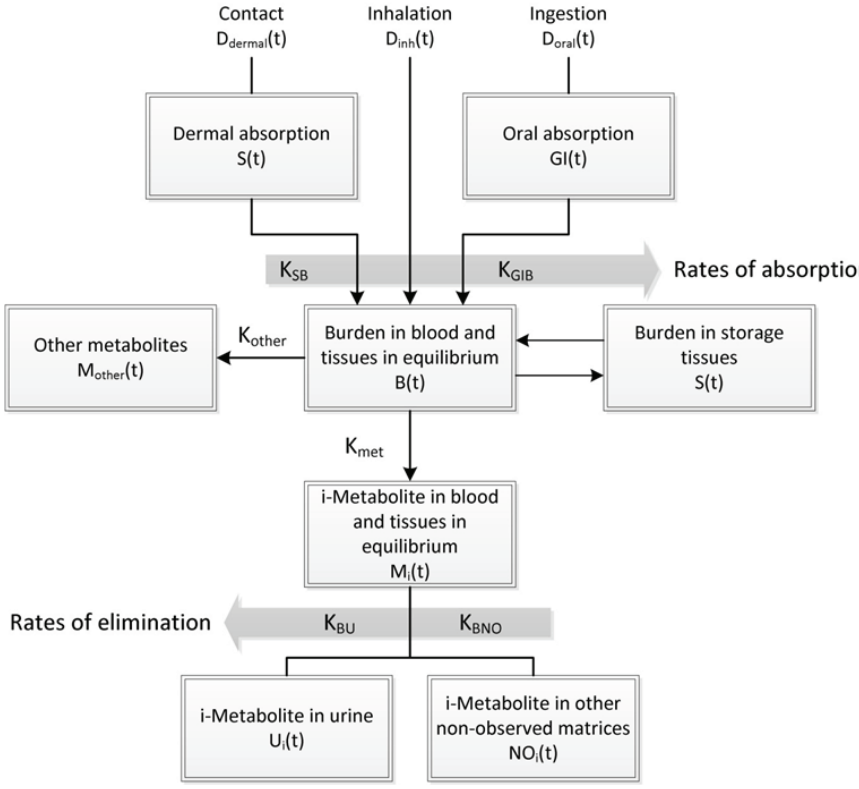


Figure 2. Representation of a BRV determination using a toxicokinetic approach in workers or in the general population

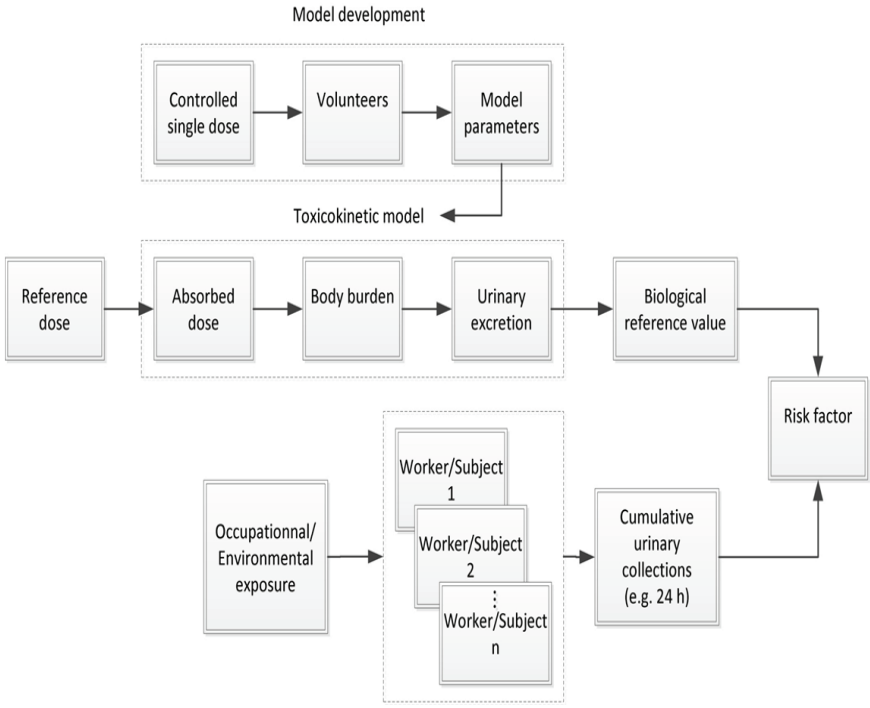


Figure 3. Conceptual representation of the kinetics of malathion and its metabolites (adapted from Bouchard *et al.*, 2003)

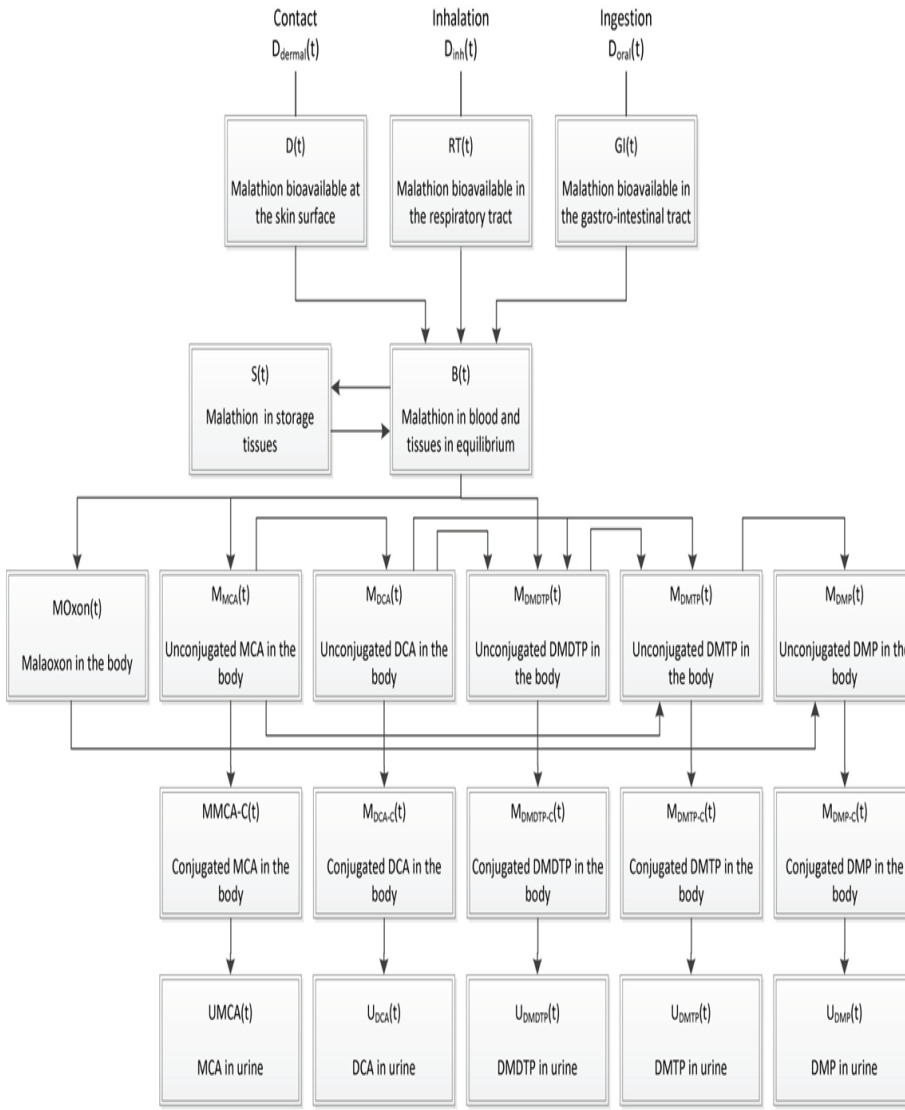


Figure 4. Conceptual representation of the kinetics of chlorpyrifos (A) and parathion (B), and their metabolites (adapted from Bouchard *et al.* (2005) and Gosselin *et al*. (2005))

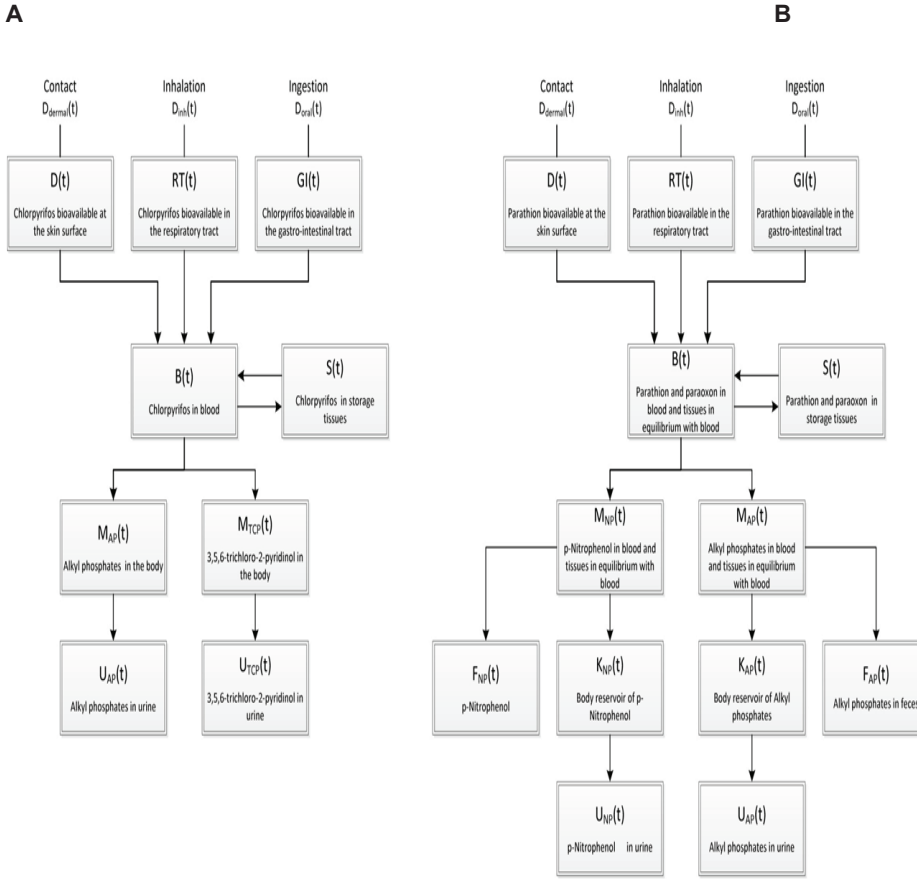


Figure 5. Conceptual representation of the kinetics of carbaryl and its metabolites (adapted from Bouchard *et al.*, 2008)

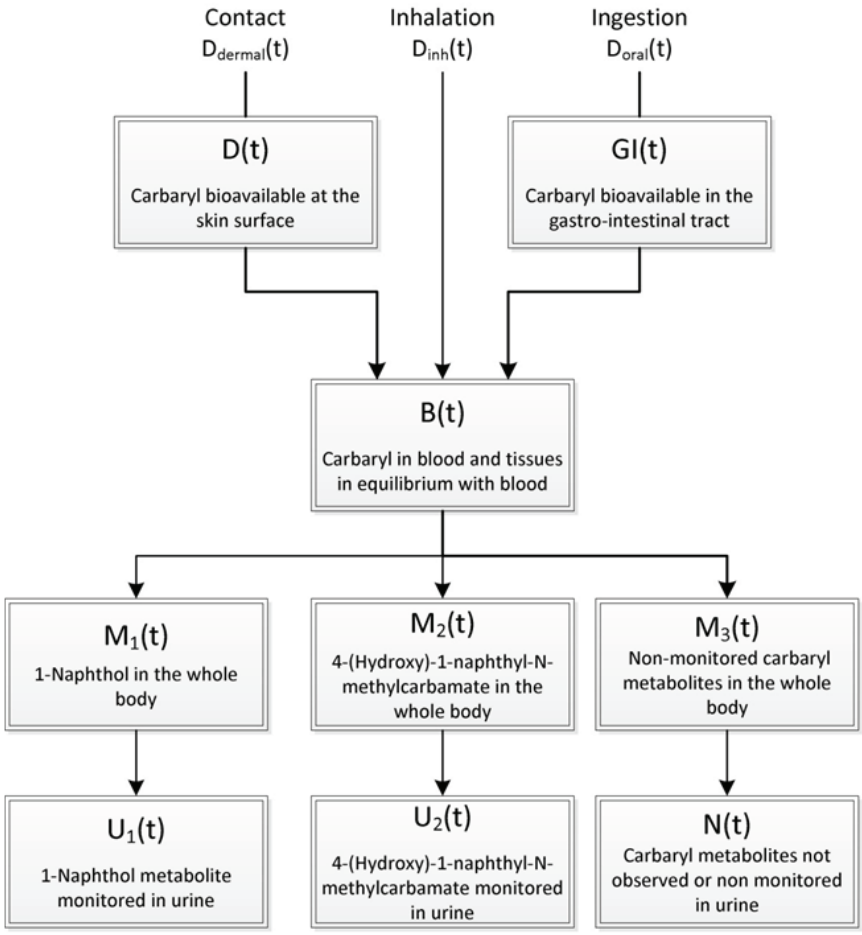


Figure 6 Conceptual representation of the kinetics of captan (A) and folpet (B), and their metabolites (adapted from Heredia and Bouchard (2011) and Heredia *et al.* (2011))

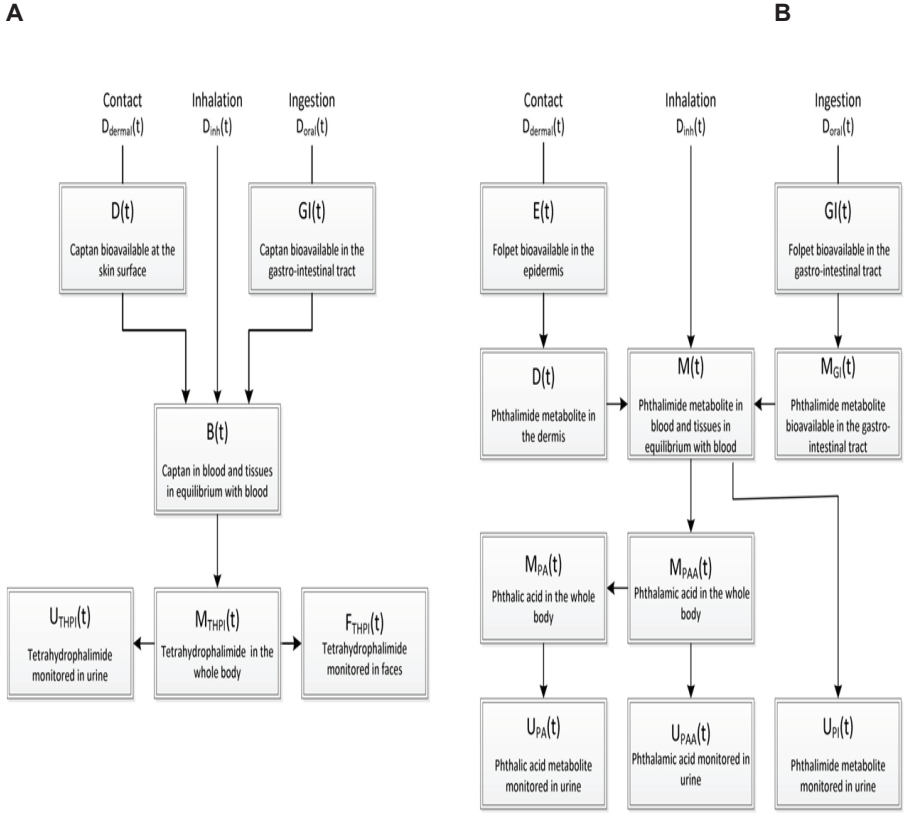
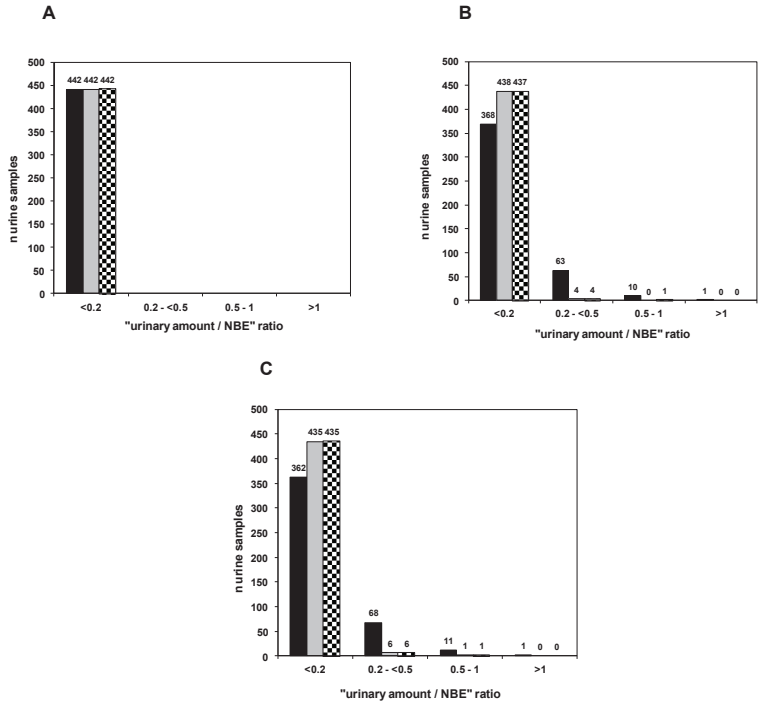


Figure 7. Distribution of ratios obtained between the metabolite measurements in the 442 available urine samples collected in children and the relevant NBEs determined using a toxicokinetic approach by Valcke and Bouchard (2009). Distributions are shown considering EP metabolites (A), MP metabolites (B) and both EP and MP metabolites (C) measured in first morning urine samples. NBEs were modeled for an 8-h dermal exposure to the NOEL (black), the ingestion of a bolus NOEL at dinner (grey), and the ingestion of 1/3 NOEL at each meal (black squares).



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Influence of Organochlorine Pesticides on GH-IGF System

Luis D. Boada MD, Luis A. Henríquez-Hernández, Manuel Zumbado, Maira Almeida-González, Pilar F. Valerón, and Octavio P. Luzardo

INTRODUCTION

Growth hormone (GH) is a glycoprotein that promotes anabolism and regulates metabolism in a wide range of organs (Maura and Haymond, 2005). In adult humans, GH is secreted into the bloodstream by the anterior pituitary gland and acts directly on some tissues, but also indirectly through the production of insulin-like growth factors (IGFs). These IGFs are so called because they bear protein sequence similarities with insulin and they have insulin synergizing activity. The liver is the main source of IGFs (Scarth, 2006). In fact, many of GH's actions are mediated indirectly through the up-regulation of systemic IGF release by the liver (Scarth 2006). Two distinct IGFs are important for human development and maintenance. IGF-1 is considered to be the more important of the two during adult life, whereas IGF-2 seems to be more relevant during foetal development than adult life (Gohlke et al., 2004). In any case, correct functioning of the GH–IGF axis is required for successful human development as well as for maintenance of healthy adults (Rosenfeld et al., 1999). IGF-1 has been established as a useful marker for monitoring the status of the GH-IGF axis (Schneider et al., 2006).

GH and IGF-1 have anabolic effects and they act through its binding to membrane-bound receptors (GHRs and IGF-1Rs, respectively). Activation of these receptors induce a second messenger cascade within the target cell leading to altered gene transcription and ultimately the anabolic effects (Scarth, 2006). Although GH and IGF-1 have net anabolizing effects leading to tissue hypertrophy/hyperplasia, some of these anabolic effects are thought to play a key role in the GH/IGFs potential to increase benign and malignant neoplasms (Foulstone et al. 2005). Most of IGFs actions are mediated in a traditional endocrine fashion through stimulation of their production in the liver by GH. However, IGFs can also act in an autocrine/paracrine manner (Cheema et al. 2005). Plasma and tissue GH and IGFs exist as both free and protein bound entities. It is generally considered that only the free hormone potentiates the mitogenic activity of these hormones, so an increase in the levels of their binding proteins reduces their activity in most cases. This is not always the case, and in some circumstances protein bound IGFs could increase activity relative to the free hormone (Duan and Xu, 2005). IGF binding proteins (IGFBPs) are a family of six proteins consisting of between 216 and 289 amino acids. The different IGFBPs have varying affinities for IGFs, have different effects on their activity and are expressed at varying levels in different tissues (Bach et al., 2005). IGFBP-3 is quantitatively the most

important IGFBP for binding IGF-1 in plasma, complexed with a third protein called acid-labile subunit (ALS). Higher plasma levels of this protein have been implicated in reduced incidence of cancer by virtue of its lowering free IGF-1 levels (Morimoto et al. 2005). It is important to point out that although the majority of research has focused on the role of IGFBPs in modulating IGF activity, IGFBPs have recently been shown to possess IGF-independent actions of their own (Bach et al. 2005).

Physiologically, IGF-I serum values are low at birth, rise during childhood with peak levels during puberty, and decline gradually with age thereafter, reaching a plateau in early adulthood (Hoppe et al., 2004; Löfqvist et al., 2005; Rogers et al., 2005; Chellakooty et al., 2006). Puberty has been suggested as a sensitive period for the programming of adult IGF-1 levels. Indeed, IGF-1 peak levels at puberty seem to determine IGF-1 levels in adulthood (Sandhu et al., 2006). An inverse relationship between age and IGF-I has been described (Baibas et al., 2003; Lukanova et al., 2001; Holmes et al., 2002), the same as the existence of a sexual dimorphism with respect to IGF-I levels, with men having higher levels than women, especially in older people (Landin-Wilhemsen et al., 1994; Kaklamani et al.,1999). In this sense, estrogens seem to exert an evident effect on GH/IGF system (Scarth 2006). Estrogens induce GH secretion, and, as a consequence, levels of GH vary with the menstrual cycle and the increased estradiol levels are correlated with higher GH, but not IGF-1, concentrations (Lissett and Shalet, 2003). In fact, estrogens seem to exert an inhibitory effect on IGF-1 serum levels (Scarth, 2006). As a consequence, there is a negative association between estrogens and IGF-I and a positive association between androgens and IGF-I (Scarth, 2006). Thus, adult women show, physiologically, lower levels of IGF-I than adult men.

As a consequence of the previously reported data, elevated GH/IGF activity could induce deleterious effects for human beings. A relevant side effect of elevated GH/IGF activity also seems to be an increased incidence of various cancers such as those of the prostate, endometrium, breast, lung, prostate, cervical and colorectal cancer (Scarth, 2006). Furthermore, IGF-1 has been involved in the development of other pathologies including diabetes, osteoporosis, dementia and cognitive disease, and renal and cardiovascular diseases (Vaessen et al., 2001; Renehan et al., 2004; Sunyer et al., 2005; Renehan et al., 2006; Landi et al., 2007). In children alterations in the levels of IGF-I have also been related to asthma and alterations in growth.

There is a close relationship between diet, and GH/IGF system. Thus, many studies seem to indicate that IGF-1 may mediate the association between protein intake and growth in children, and evidence suggest that protein restriction results in low IGF-1 concentrations in healthy children (Smith et al., 1995). Therefore, dietary restriction decreases the serum concentration of IGF in both human and animals (Fontana et al., 2009). Whereas it is well known that serum levels of IGF-1 are clearly influenced by dietary and lifestyle factors (Kaklamani et al., 1999; Baibas et al., 2003), the effects of xenobiotic exposure on the GH–IGF axis has not been enough explored.

Currently, human beings are potentially exposed to a large range of structurally diverse compounds. Long-term exposure to these pollutants, mainly through environmental and dietetic means, potentially could modulate the GH/IGF axis. In this

picture, chemical contamination of environment and food by organochlorine pesticides, should also be taken into account in any study regarding exogenous determinants of GH/IGF system.

HUMAN EXPOSURE TO ENVIRONMENTAL CONTAMINANTS: THE CASE OF ORGANOCHLORINE PESTICIDES

Human body burden of organochlorine pesticides (OCs) resulting from the universal presence of these contaminants in the environment is an issue of public health concern because they have been linked with the pathogenesis of cancer, asthma, diabetes, and growth disorders in children (Snedeker et al., 2001; López-Cervantes et al., 2004; Sunyer et al., 2005; McGlynn et al., 2006; Lee et al., 2006). OCs include insecticides DDT-derivatives (OC-DDTs), such as DDT and its metabolites (DDE, DDD), other cyclodiene-derivative pesticides (OC-cyclodienes), such as aldrin, dieldrin, and endrin, and hexachlorocyclohexanes, such as lindane. Although in western countries most OCs were banned in the late 1970's, persistent OC residues can be measured in environmental and biological samples as a result of their bioaccumulation (Snedeker, 2001; Jaga and Dharmani, 2003). Most OCs are considered endocrine disrupters and carcinogens (Soto et al., 1995; Snedeker, 2001).

Exposure of human beings to OCs largely derives from ingestion of contaminated food (Snedeker, 2001; Jaga and Dharmani, 2003) although environmental, occupational and other domiciliary exposures should not be excluded. In any case, it is well known that foods are the main source of human exposure to environmentally persistent pesticides, and that the group of DDT derivatives (OCs-DDTs) is one of the groups of pollutants that are usually detected in foods all over the world (Jaga and Dharmani, 2003; Zumbado et al., 2005). Dietary exposure to OCs results in the bioaccumulation of these chemicals in the human body (Jaga and Dharmani, 2003), and this circumstance should be borne in mind in any study regarding exogenous and dietary factors related to the IGF system.

POTENTIAL CONSEQUENCES OF ORGANOCHLORINE PESTICIDES EXPOSURE ON GH-IGF SYSTEM

It has been suggested by others the possibility that exposure to environmental pollutants, such as organochlorine compounds, could be an exogenous factor capable of modulating the IGF system (Tomei et al., 2004; Ceccatelli et al., 2006; Boada et al., 2007; Davis et al., 2009).

There are relatively few reports on disruption of the GH–IGF axis by pesticides and other environmental pollutants, compared with the number of studies looking at their effects on reproductive endocrinology. Although most papers explore the effects of pesticides on GH/IGF system as a consequence of their estrogenic/antiandrogenic action, the possibility exists that pesticides could also influence GH-IGF system directly. Thus, environmental pollutants could alter the normal synthesis and/or

secretion of IGF-1 (Tannheimer et al., 1998; Randi et al., 2005; Wang et al., 2005), or could act altering the IGF signalling pathway in both, in vitro and in animal models. These effects have been described for some OCs, i.e. o,p'-DDT and p,p'-DDE (Holloway et al., 2007); and for a number of aryl hydrocarbon receptor (AhR) ligands, such as dioxins, dibenzofurans, and polychlorobiphenyls. Similar results have been described for organophosphates (such as aldicarb, methomyl, metribuzin, linuron, and diisopropylfluorophosphate); for herbicides, i.e. atrazine; for detergent-derived products, such as 4-nonylphenol; for other environmental organohalogenated contaminants, i.e. hexachlorobenzene; and for policyclic aromatic hydrocarbons, such as benzo[a]pyrene (Scarth, 2006).

However, studies involving human populations are scarce. In human beings, occupational exposure to urban pollutants has been also described to cause an alteration on IGF-I levels in adults (Tomei et al., 2004), and recently it has been suggested that OCs could modulate the IGF-system in a way that is highly influenced by gender, age and by chemical or combination of chemicals implicated (Boada et al, 2007; Zumbado et al., 2010). Our recently published studies point to the possibility that DDT-metabolites and non-DDT-derivative-OCs (aldrin) could to exert a negative influence on IGF-I levels (in fact, a negative modulation of the GH axis).Thus, IGF-1 levels were significantly lower in women who showed detectable levels of DDD than in women who presented non-detectable levels of this metabolite, specially in 36-50 years old women. Also in women, a similar negative relationship was also found between IGF-1 serum levels and the levels of the cyclodiene pesticide aldrin. Furthermore, in oldest men aldrin seemed to exert a similar negative effect on IGF-1 serum levels (Boada et al., 2007). Additionally, and similarly to adults, IGF-1 serum levels were lower in pre-pubertal male children who showed detectable values of DDT-metabolites DDE, and DDD than in pre-pubertal male children with undetectable levels of these metabolites (Zumbado et al., 2010). This negative influence of OCs on GH/IGF system agree with those studies suggesting that prenatal exposure to DDT-metabolites may induce decreased height and weight in children (Ribas-Fito et al., 2006). It is possible that these OCs exert such inhibitory effects on the IGF-system indirectly, due to their estrogenic and antiandrogenic actions (Soto et al., 1995; Sohoni and Sumpter, 1998). Even more so, the fact that the negative association between IGF-I and DDE and DDD, was observed in the group of pre-pubertal male children suggest an anti-androgenic effect of these DDT-metabolites . These results seem to indicate that people with physiologically low levels of IGF-1 seem to be the most sensitive group to suffer the negative effect potentially exerted by OCs on GH/GF system (prepubertal male children, women and older men).

Because the liver is the main source of IGF-I in the circulation (Scarth, 2006) the possibility exists that the inverse relationship found between some OCs and IGF could be an indirect effect of these environmental pollutants on liver tissue. In fact, DDT and DDE have been related to increased risk of primary liver cancer (McGlynn et al., 2006). In any case, other unknown hepatic or non-hepatic

mechanisms may be involved (such as genetic polymorphisms related to xenobiotic-metabolizing enzymes or to IGF-receptors) (Scarth, 2006). Due to the fact that it has been suggested that IGF-1 peak levels at puberty could determine IGF-I levels in adulthood, the negative effect exerted by DDT-metabolites on GH/IGF system could be, therefore, a determinant factor in the modulation of GH-IGF axis with potential consequences in chronic IGF-related disorders later in life (Sandhu et al., 2006).

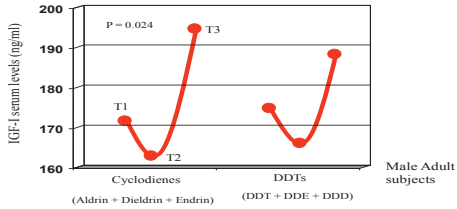
It has to be highlighted, that a non-linear dose-response curve has been observed between Total cyclodienes Body Burden (sum of serum levels of aldrin, dieldrin and endrin) and IGF-1 in men (Fig. 1) and between Total DDT Body Burden (sum of the serum levels of the most environmentally relevant DDT-derivatives, p,p'-DDT, p,p'-DDE, and p,p'-DDD) and IGF-I in pre-pubertal male children (Boada et al., 2007; Zumbado et al., 2010). The term "non-linear" is used to describe dose-response relationships in which the direction of a response changes with increasing or decreasing dose. This non-linear dose-response curve has been described previously in human beings and in animal models for a mixture of organochlorines (Weltje et al. 2005; Kortenkamp, 2006).

CONCLUSIONS

As stated previously by others, the mixture of environmental contaminants is the most frequent circumstance found in human populations and the real problem is the biological effect exerted by the simultaneous exposure to many environmental chemicals because the combination of pollutants in human tissues seems to have a very different biological action to that of chemicals taken individually (Ibarluzea et al., 2004; Weltje et al. 2005; Kortenkamp, 2006). It should be highlighted that the possible existence of hormetic responses could indicate that low-dose exposure could be as harmful as high doses for human beings. Nevertheless, investigating the possible biological effects of mixtures is a complex issue because the mechanisms of action for individual compounds are often poorly understood, and some chemicals may act through different routes depending on their concentration. For the majority of xenobiotics, among them pesticides, the small number of human in vivo studies performed and the lack of standardization in the methods used to assess changes in GH/IGF levels complicate quantitative comparisons of the different outcomes. Consequently, more studies are required in order to establish the effects of long-term exposure to low doses of pesticides on GH-IGF system.

FIGURES

Fig. 1. An "hormetic" effect("U" shaped dose-response curve)was observed between Total cyclodienes Body Burden (sum of serum levels of aldrin, dieldrin and endrin) and IGF-1 in men.



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Section 2

PESTICIDES IN THE ENVIRONMENT

Modifying Influence of a Pesticide Dump on Plants and Animals of Wood, Meadow, Pond and Lake Ecosystems

Krystyna A. Skibniewska, Jozef Szarek, Janusz Guziur, Miroslaw Grzybowski, Katarzyna Sawicka-Kapusta, Marta Zakrzewska

ABSTRACT

A team of specialists from various specializations studied in 2001-2009 influence of a pesticide dump on plants and animals living on a territory of a buried organochlorine pesticide wastes and its neighborhood. The dump in a form of 32 concrete wells and two unsecured pits was located in a sandy hill. Due to low light soils potential of accumulation, low levels of the contaminants (dichlorodiphenyltrichloroethane, lead and cadmium) were determined in soils, sediments and plants. There were well-marked differences of contaminants concentrations in tissues of animals living close to the dump, in comparison to those living at greater distances, anyway, the levels did not exceeded the Polish maximum limits for food of animal origin. Influence of the hazardous substances was clearly revealed in the change of phytosociological relations of the wood and the lake near the source of contamination. Pathomorphological analysis of organs and cells of mice and carps confirmed statistically more morphological lesions in rodents living in the area of the pesticide dump and in carps bred in the pond nearby the tomb. The results of the research can serve as a methodology for searching for lost hazardous substances dumps in the environment.

INTRODUCTION

All over the world, the 20th century left behind numerous improperly-secured dumping sites of plant protection chemicals past their expiry date and other hazardous substances, which are slowly being released to the environment, posing a threat to human health. A significant part of the deposits contained organochlorine compounds (OCls). Even 50 years ago, DDT (dichlorodiphenyltrichloroethane) was commonly applied throughout the world. Apart from helping farmers, it prevented millions of deaths by mass control of insects transmitting dangerous diseases such as malaria, plague, typhus fever or dysentery. Initially, it seemed safe, since it did not cause any visible symptoms of poisoning. Over time, it was realized that this compound decomposed in the environment only to a low degree and it accumulated in the fat of living organisms and when their weight declined rapidly it caused poisoning (Jandacek and Tso, 2001). Even at low concentrations in living organisms, it induces carcinogenic processes (Ennaceur et al., 2008), acts as an endocrine disrupter, causes disorders of the reproductive process (Ben Rhouma et al., 2001) and leads to diseases, e.g. osteoporosis or nervous system disorders (van Wendel de Joode et al., 2001).

In the 1960s and 1970s, many countries introduced a ban on the application of DDT-based preparations. In Poland, they were allowed to be used until 1974. The remaining, unused reserves were to be deposited in a manner specified for hazardous waste. The most reasonable and safest way, so it seemed, was to deposit those substances underground. Thus, many so-called pesticide dumps were created; concrete bunkers or wells made of rings, often leaky, sometimes located in the

vicinity of subsoil waters and therefore at risk of being washed off. Under favourable hydrogeological conditions, the contamination could spread significant distances.

It has become one of the most serious ecological problems in Poland. It concerns not only the environment and people living near pesticide dumps, but also tourists who stay in their vicinity, occasionally drinking water or eating agricultural produce grown near such disposal sites. Pesticide dumps can release toxic substances to the environment for many years, posing a threat to underground waters (including drinking water) and local crops. Naturally, the greatest threat is posed by objects unidentified both in geological and construction terms, particularly those whose existence has been forgotten.

The problem of pesticide dumps is still waiting to be completely solved. Numerous marked or unknown dumps, disposal sites and landfills containing pesticides withdrawn from use have been created all over Europe, as a consequence of insufficient knowledge concerning unfavourable effects of crop protection chemicals on the environment. The advances in knowledge of the harmful effects of OCls on the environment have resulted in a Europe-wide campaign to liquidate old, poorly-protected pesticide dumping sites. In Poland, an inventory of pesticide disposal sites was initiated at the end of the 20th century and led to the creation of a database of about 60,000 tonnes of hazardous substances located in 340 pesticide dumps in various parts of the country (Amador, 1992; Zaleska and Hupka, 1999) and the realization that not all deposits had been found.

There is no doubt that hazardous substances dumps should be detected and liquidated. Since 2002 a team of scientists from many specializations: ecologists, hydrologists, chemists, microbiologists, botanists, human and veterinary pathologists, have worked on a territory of a pesticide dump and its surroundings. The basic aim of the studies was to develop a methodology for searching for lost hazardous substances dumps. At the same time influence of the hazardous substances stored at the dump on the living organisms has been specified.

STUDY AREA

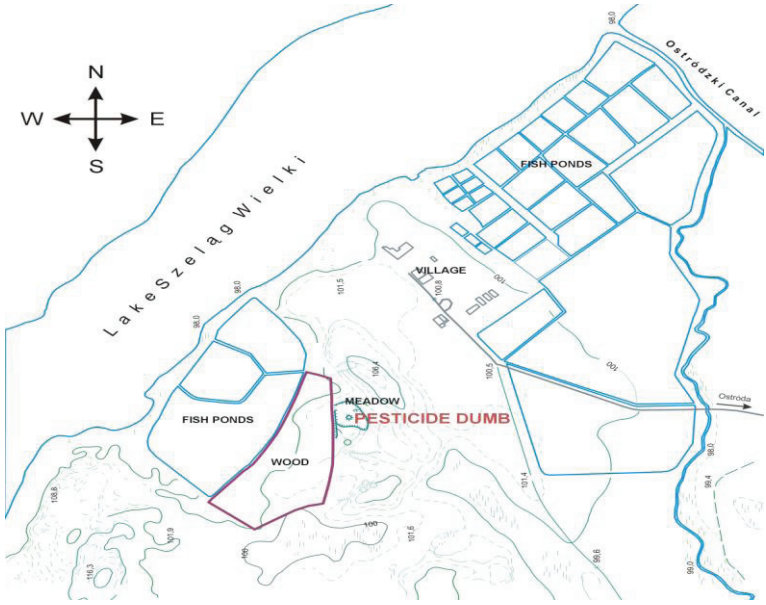
A pesticide dump was created in 1974 in the village of Warlity Wielkie near Ostróda in the province of Warmia and Mazury (Figure 1). The pesticide dump (PD) was located on a sandy hill ranging from 100.0 to 104.0 m above sea level (zone I of the study) with a bog at a foot of the hill (zone II). PD was situated on the boundary of the mixed forest (zone III), near a village (about 500 m), fishponds (about 100 m from the nearest one), drinking water intakes (about 600 m) and a lake (about 750 m). In the documents of the local authorities, this object was recorded as a pesticide dump in the form of 9 wells containing about 8.5 tonnes of mainly DDT preparations which had been withdrawn from use, with the addition of medicines and chemical reagents past their expiry date. In November 2004, this dump was liquidated as a part of the national campaign co-financed by the European Union (the campaign was completed in 2007 with liquidation of all recorded PDs); there were 32 wells and two unsecured pits found in the pesticide dump area, of which 53.78 tonnes of hazardous substances were taken away.

SEARCHING FOR EVIDENCE OF POLLUTION

In 2002-2004 and 2006-2008, the area of the pesticide dump and surrounding ecosystems were the subject of research studies. Samples for analysis were collected from the area of the dump itself and from the meadow, wood, bog, fishponds and

neighbouring lake. The research included analyses of soil, water, plant and animals living in the area, namely, fish from the ponds as well as black striped-field mice and yellow-necked mice.
First, an assessment was made to establish to what extent the contaminants had penetrated through soils and bottom deposits to organisms of animals and plants in various ecosystems located in the vicinity of the pesticide dump. The analysis included residues of organochlorines withdrawn from use in Poland: DDT and its metabolites and lindane, as well as lead and cadmium. It was assumed that products of chemical

Fig. 1. The study area



synthesis, which are pesticides, are usually contaminated with heavy metals. Additionally, the research analysed the degree and the character of morphological lesions in internal organs of various animals living near the pesticide dump and assessed their correlation with the level of contamination of those animals with the examined xenobiotics, in order to determine indicative morphological lesions in animals (mice and fish) evoked by the contamination of their body with xenobiotics, as well as selecting and classifying the bacteria species from soil and bottom sediments of surface waters which could be used as organisms indicating environmental contamination.
The analysis of soil and bottom sediments confirmed the washing away of hazardous substances from the PD to the environment (Skibniewska et al. 2003). However, although the leaking dump had been affecting the surrounding environment for a few dozen years, it did not exert a strong pressure on the nearby plants. It was observed that specific species subject to the analyses revealed a similar content of DDT and heavy metals (apart from samples collected directly from the dump) in various conditions of the habitat and at various distances from the pesticide dump. In spite of the fact that plants classified as marker plants had been selected for the research (moss - *Ceratodon purpurens*, mouse-ear hawkweed -

Hieracium pilosella, southernwood - *Artemisia abrotanum*, common reed - *Phragmites australis*) and although they were collected from sites situated along the runoff of the ground water towards the lake, increased levels of the examined compounds were not found in the examined material, which disqualifies plants as a tool for searching for forgotten dumping sites of hazardous waste located in the area of light soils that surrounded the pesticide dump. Due to a low level of accumulation, studies on singling out a species that could indicate the examined compounds were not possible.

On the other hand, the harmful impact of the PD was very clearly revealed in the change of phytosociological relations of the wood and the lake near the source of contamination (Grzybowski et al. 2004, 2005, 2010a-d). The harmful effect of the pesticide dump was demonstrated very clearly in the change of phytosociological relations of the lake, whose phytocenotic diversity index (H = 1.66) and colonisation index (Z = 0.78) classified the examined lake as an aging reservoir. An increased acreage of pollution-tolerant plant communities was observed along the border directly neighbouring the potential outlet of the contamination route from the pesticide dump. Collected floristic and phytosociological data concerning the research areas proved helpful in assessing the ecological condition of the ecosystems under the influence of the pesticide dump, the rate of spontaneous disappearance of pesticide ground contamination, as well as in the assessment of the risk to surface waters for an area contaminated with pesticides. It was confirmed that studies on phytosociological relations could be useful in revealing the sources of hazardous substances penetrating the environment.

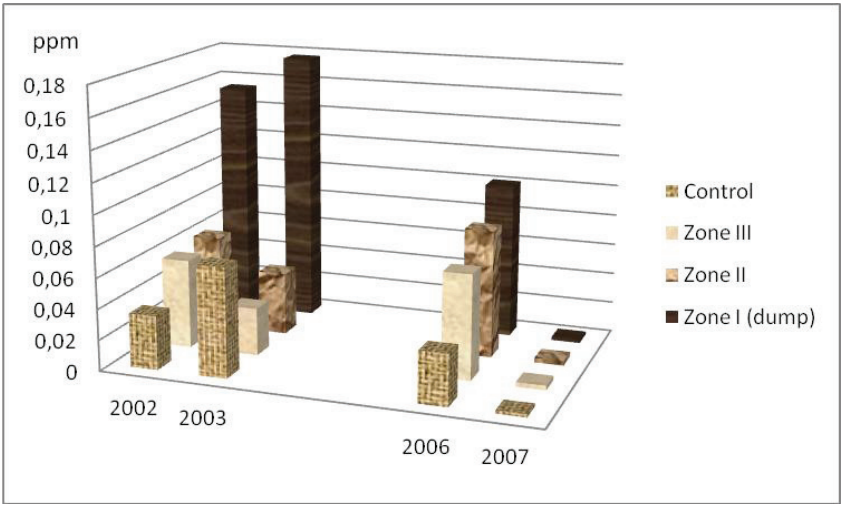
It was demonstrated that the long-term effect of pesticides on the lake water from the reclaimed area did not affect the general seasonal dynamics of the microorganism community. However, it is significant that in the quality and quantity relations between the sample collection sites (in the bottom sediments) an increased amount of fungi in relation to the control sample was found.

Increased levels of the residues of xenobiotics, both OCls, and heavy metals, were determined in the fat of black striped-field mice (*Apodemus agrarius* Pallas 1771) and yellow-necked mice (*Apodemus flavicolis* Melchior 1834), caught directly in the area of the pesticide dump and surrounding ecosystems (Figure 2). Before the liquidation of the dump, the surface of the pesticide dump was the most contaminated area and the rest of the land was to a small degree contaminated with residues of the compounds under examination. The process of liquidation (in 2004) caused serious secondary, although short-lived, contamination of this area. In this case, mice, as representatives of small animals living over a limited area, proved to be good indicators of environmental contamination. It should be mentioned that before dump liquidation quite high concentrations of DDT and its metabolites were determined in control material, though control mice were collected about 4 km far from the experimental area.

The chemical analysis of DDT residues and its metabolites in the fat of carp bred in the fish farm situated in the examined area did not provide any clear evidence of environmental pollution (Skibniewska et al. 2004). The concentration of the contaminants was well below the Polish maximum limits for food of animal origin (1.0 mg kg^{-1} of fat). Since the fish were bred in a 2-year cycle, the period of accumulation was not long. However, the contamination levels in fish bred in the pond situated in the closest vicinity to the pesticide dump were generally higher in comparison to the level determined in fish from the pond situated 2 km away. Additionally, the fat of breams netted at the outlet of the ditch piping water away from the dump area contained more ΣDDT in comparison to the fat of fish sampled

in distant parts of the lake. It is also worth noting that the share of DDT in the composition of metabolites was much higher in fish from the pond close to the PD.

Fig. 2. Content of ΣDDT in muscle fat of wild mice



Content of lead and cadmium in muscle tissue of carp brewed in the fish ponds was also low (Skibniewska et al., 2008). Other fish (roach and bream) netted in the lake situated in the basin of the PD contained the heavy metals in concentrations not exceeding the Polish maximum admissible levels

PATHOMORPHOLOGICAL ANALYSIS

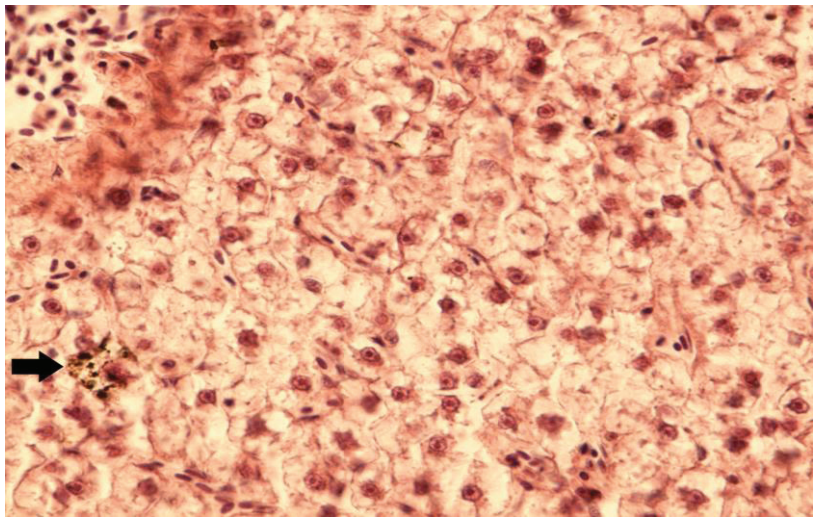
The best tool for searching for forgotten dumping sites of hazardous substances was found in a pathomorphological analysis. A higher intensity of pathomorphological lesions was observed in rodents, particularly in black striped-field mice, living within the area of the pesticide dump or in its direct vicinity(Szarek et al, 2007, 2009). Those lesions were statistically significantly more intense in consecutive years of research. The number and degree of intensity of morphological lesions were higher in carp living in a pond situated near the pesticide tomb in comparison with carp from the pond which was far from the source of DDT (Szarek at al., 2006, 2010). Two years after the liquidation of the pesticide dump, it was still affecting the fish bred in a nearby pond.

The distribution of morphological lesions in the organs of carp (*Cyprinus carpio* L.), black striped-field mice and yellow-necked mice, as well as the character of those deviations from the norm demonstrate that the cause of their origin is attributable to the PD in the vicinity of the animals. Carp hepatopancreas (particularly hepatocytes) and livers of black striped-field and yellow-necked mice can be considered as biomarkers and morphological analysis can be a key tool in this assessment.

Regressive metamorphoses near blood vessels were observed in the animals mentioned above. Foci of parenchymal degeneration and necrosis, covering a relatively low number of cells, usually adjoined the walls of venous and arterial vessels, which demonstrated a tendency to plethora. Melanomacrophages were present among hepatocytes in the liver stroma. They occurred individually or

formed clusters (Figure 3). Ultrastructural patterns revealed a proliferation of mitochondria, their polymorphism, swelling and dilution of the matrix (Figure 4, 5). Rough endoplasmatic reticulum was subject to defragmentation and vesicular transformation. Those lesions were relatively often accompanied by the presence of myelin-like structures in necrotic microfoci hepatocytes. In own research, the animals that were most susceptible to environmental contamination proved to be black striped-field mice and, to a slightly lower degree, yellow-necked mice and carps. The described lesions were found only sporadically in animals feeding in the area far from PD.

Fig. 3. Hepatopancreas of the carp (*Cyprinus carpio* L.) living in a pond situated close to pesticide dump - cluster of melanomacrophages (arrow) located among hepatocytes. HE stain., magn. x 520.



CONCLUSIONS

The serious problem posed by hazardous substance dumping sites, both for humans and for the natural environment, with particular focus on species biodiversity, requires finding appropriate analytical methods, research tools and biomarkers or bioindicators. The problem is all the more serious as this field of knowledge has significant gaps, not only as regards the Polish literature but also on a global scale. Long-term, multi-dimensional, and therefore innovative, environmental studies were required to achieve this aim. The research on the impact of the hazardous waste disposal site on species biodiversity and environmental safety was aimed at creating a certain model of analyses, which could be used as a scheme for monitoring potentially threatened places for rapid intervention and for preventing ecological disasters, not only in Poland but also all over the world.

The results of the research can be a significant point of reference, making it possible to observe and to assess changes occurring in the natural environment under the influence of a hazardous waste dumping site. Additionally, the information collected will facilitate the monitoring of such environments for their effective reclamation or

early detection of the contamination inflow from the so-called wild or not-localized sites. This is important in view of the safety of the natural environment, its broadly understood protection and ensuring the preservation of species biodiversity.

Fig. 4. Fragment of binuclear hepatocyte of the black striped-field mice (*Apodemus agrarius* Pallas 1771) - proliferation of mitochondria. Magn. x 4400.

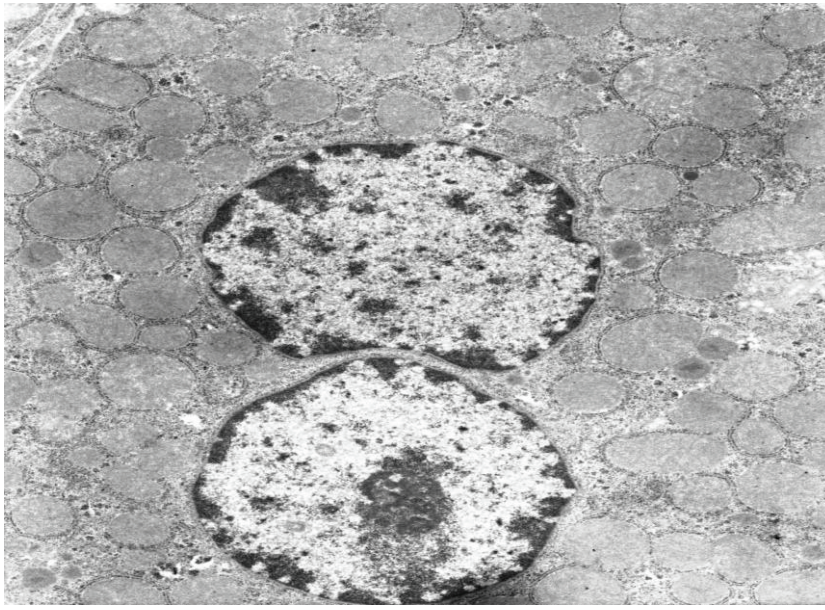
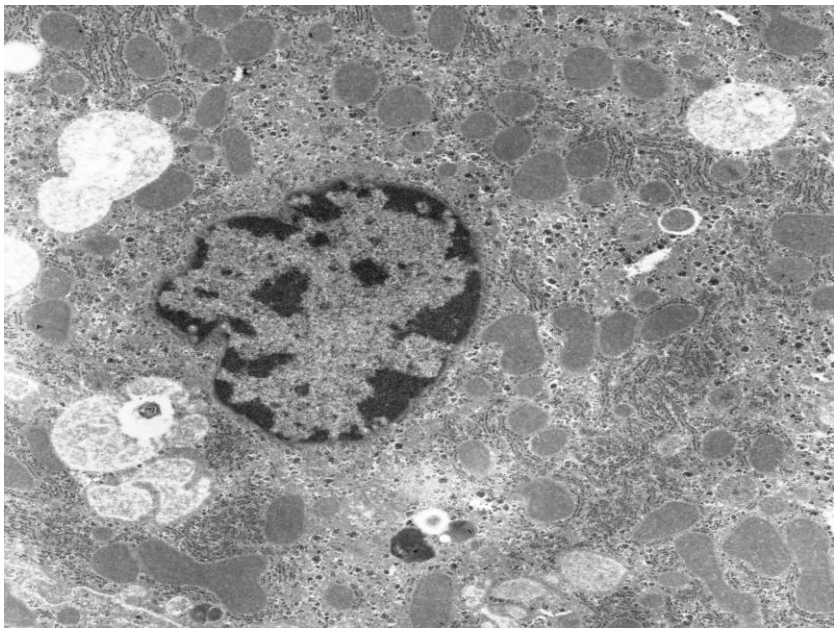


Fig. 5. Fragment of hepatocyte of the black striped-field mice (*Apodemus agrarius* Pallas 1771) - swelling of mitochondria with damage of matrix. Magn. x 4400.



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Integrated Pest Management as a Tool to Mitigate the Pesticide Negative Impact Into the Agroecosystem: The Soybean Example

Adeney de Freitas Bueno & Regiane Cristina Oliveira de Freitas Bueno

ABSTRACT

The increasing global food demands have lead to a constant battle for higher crop yields. Combined with the relative low costs of some insecticides such event has triggered an excessive use of pesticides in the agriculture. Among the side effects of pesticide abuse, especially the non-selective ones are: the reduction of efficacy of the natural biological controls; fast pest resurgence; and resistance selection to the insecticides used, besides the danger to the environment and human health. To address these problems and still maximize agricultural production, pest control programs must be guided by a proper Integrated Pest Management (IPM) approach. The IPM is based on the premise that plants can tolerate certain levels of injury with no economically significant reduction of yield; and insecticides should be used only as a complementary method, since the natural biological control is responsible to keep pests under control. Therefore, the most appropriate timing to initiate insecticide sprays on soybean fields is thoroughly discussed in this chapter. The safety of waiting for 30% defoliation at the soybean vegetative developmental stage or 15% defoliation, when at the reproductive stage, has been proved through experiments performed in different areas and with distinct soybean cultivars. Similarly, the economic threshold for stink bugs has already been evaluated through experimental field results comparing the use of insecticide when the mean stink bug population of 0.5 insects per sample is found, or when two stink bugs per sample are found. It can be concluded that anticipating stink bug control on soybean field is an expensive and excessive practice that only increases the negative impact of the chemicals used in the agroecosystem. Respect for the economic threshold and the option for pesticides more selective to beneficial arthropods is the clue to the possibility in reducing the use of chemicals in the agriculture, thus improving its sustainability.

THE SOYBEAN CROP SCENARIO

The soybean [*Glycine max* (L.) Merrill] crop is extensively cultivated in a large amount of field areas throughout different countries. It supplies half of the global demand for vegetable oil and protein (OERKE & DEHNE, 2004) with a worldwide production estimated at 256 million metric tons, which illustrates its significant economic importance all over the world. Among the largest world soybean producers are the United States of America, which produced around 91.4 million metric tons during the 2010 crop season, and Brazil, the second largest producer, which yielded approximately 69 million metric tons of soybean during the 2009/2010 crop season (USDA, 2010).

The global soybean production, however, still could be increased if problems with pests were avoided (OERKE, 2006). Therefore, in order to mitigate the negative consequences of pest outbreaks and improve profits, the soybean growers try to control pests with the use of chemicals, an attempt that can rather bring lots of

negative effects (ZALUCKI et al., 2009). Within this context, the main method of pest control adopted by the majority of the soybean growers is often the erroneous and abusive application of pesticides, without considering the economic threshold levels recommended (which is defined as the insect population level that justifies the control) (SONG & SWINTON, 2009) besides using dangerous and harmful pesticides (CARMO et al., 2010). Those issues should be emphasized among the reasons by which Brazil is nowadays ranked as the largest world consumer of pesticides (CORRÊA-FERREIRA et al., 2010). An excessive use of agrochemicals is also performed in other countries, endangering, without any doubt, the sustainability of this crop on a global perspective.

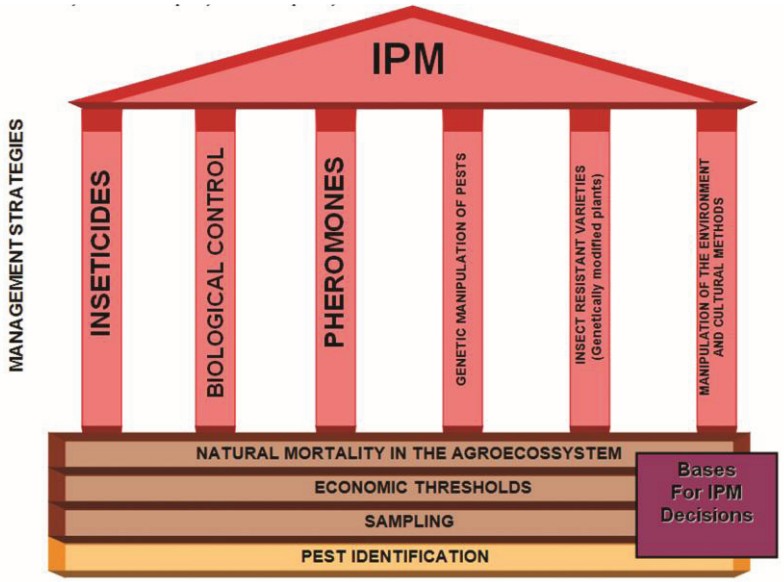
THE INTEGRATED PEST MANAGEMENT APPROACH

Integrated pest management of soybean (Soybean-IPM) is an approach (grouping different technologies) used on the management of this crop aiming at maintaining the sustainability of the agro-ecosystem by keeping it as close as possible to a biological equilibrium. This concept for soybean was established at the end of the 50's decade and searches primarily the consonance of the control method based on the ecological, economical and social principles.

The definition of the IPM, however, has been damaged along the years and, many times, even misinterpreted by some people that simplify excessively its complexity. The assertions that the IPM should only consider the lower utilization of insecticides or its rational use are, many times, very common. It is true that the adoption of the Soybean-IPM leads to a more rational use of insecticides, but the lessening, however, is a consequence of its adoption and cannot be misunderstood as its definition. This misunderstanding about the IPM complexity does not occur only with the soybean crop, but also on various other economically important crops.

Because of this simplistic vision of the IPM, other denominations for this "approach" has also been created as, for example, "Ecological Management of Pests", which in its essence aims at mainly inserting the complexity that is inherent to this theme and that, unfortunately, was been lost along the years within the IPM concept. Surely, it is not easy to define all the complexity of the IPM in a summarized form and for that reason its graphical representation, as shown on **Figure 1**, might more easily illustrate the complexity that the concept really has.

Figure 1. Illustration of an Integrated Pest Management program in analogy to the construction of a house (adapted from GALLO et al., 2000).

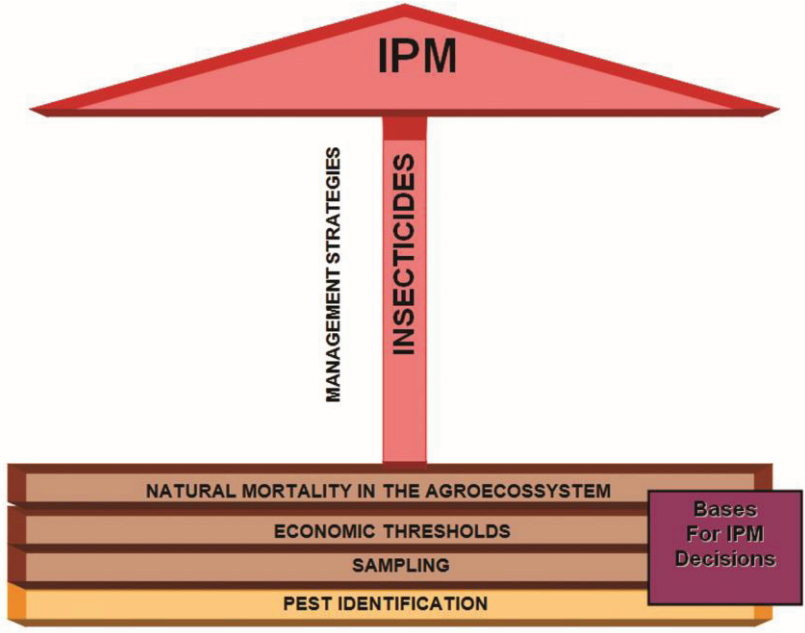


As shown in **Figure 1**, which resembles a "house", the IPM needs a solid base to sustain itself. Moreover, as a house, it also requires walls (management strategies) for maintaining standing such extent of complexity. It is difficult to achieve success in the Soybean-IPM when essential steps of its bases are not performed or when it is inadequately done. As an example, the sampling procedures have to be thoroughly performed since they will indicate the real amount of insects present within a given area of the crop field and, therefore, will provide adequate parameters allowing the obedience to the accurateness of the economic threshold levels for the control of the target-pests. Unfortunately, in recent years the sampling performed with the aid of a sample-cloth, an indispensable tool for the precision of this procedure, have been abandoned by several soybean growers. Without a precise sampling (**Figure 2**), the farmer will certainly be using insecticides wrongly (without a technical criteria), many times disrespecting the economic threshold levels (ETs) recommended by the research. In that case, wrong decisions and unnecessary insecticide applications, which might be used preventively, may be carried out, thus aggravating yet more the negative impact of the pesticides on the agro-ecosystem.

Figure 2. Appropriated sampling procedure with the aid of a sample-cloth. First, you position yourself in the area to be sampled (**A**). Second, with the sample-cloth placed in the middle of two soybean rows in a way to cover the soil and on the rows, sampler must agitate the uncovered rows to move all the insects to the sample cloth (**B**). Finally, you scout the insects sampled over the cloth (**C**).

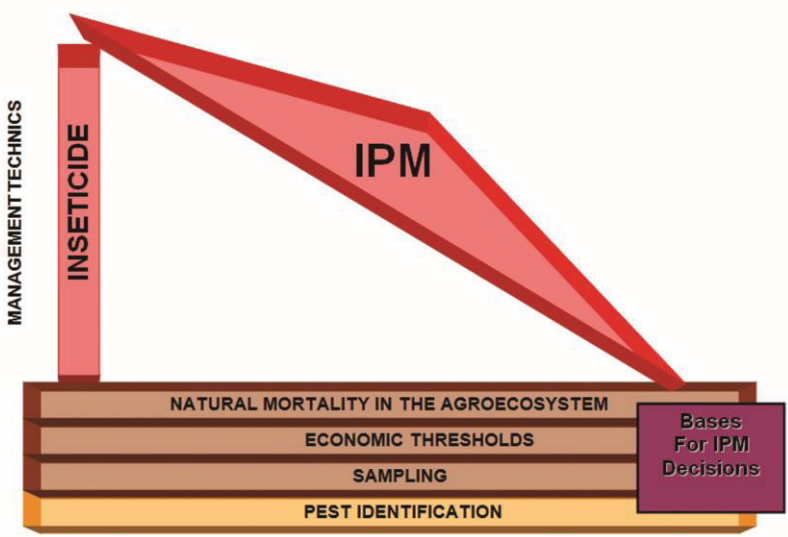
In this context, it is important to emphasize that among the pest control mostly used on soybean crops, many times used exclusively, is indeed the spray of chemicals. The use of a single practice contradicts the IPM recommendations that indicate, whenever possible, the use of different management tactics in a harmonious manner. Hence, without a solid base and attributing the sustainment of the Soybean-IPM, only and exclusively to chemical control, the Soybean Integrated Pest Management becomes extremely fragile (**Figure 3**).

Figure 3. Illustration of the fragility of the Integrated Pest Management when it is based on a single control method in analogy to the sustainment of a house roof (adapted from GALLO et al. 2002).



Especially when only pesticides are used to control pest outbreaks, without carrying out the proper sampling and/or not using the economic threshold levels, the overuse of chemicals is very common, what might impair all the Soybean-IPM technology (**Figure 4**), mainly when non-selective pesticides to beneficial arthropods are sprayed.

Figure 4. Illustration of the consequences from the wrong insecticide use (non-selective product or the abandon of Economic Thresholds = spraying at the wrong timing) endangering Soybean-IPM sustainability in analogy to a house roof (adapted from GALLO et al. 2002).

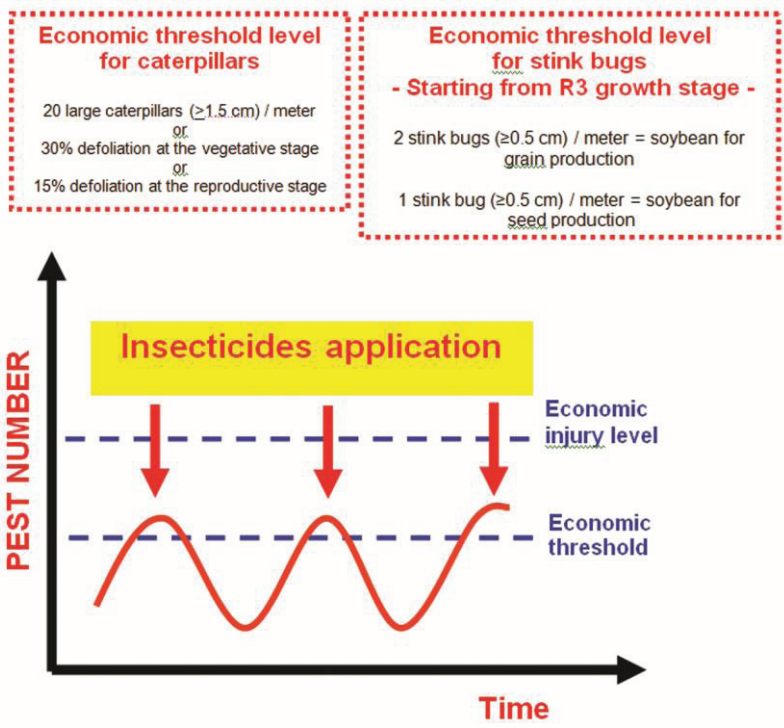


Therefore, aiming at mitigating the harmful effects that can be caused by pesticides, the adoption of the Soybean-IPM with the use of selective pesticides to beneficial insects (and less harmful to the environment) sprayed only when necessary (at levels equal or above the recommended economic thresholds) is of crucial importance.

Economic Thresholds

The IPM is based on the premise that crop plants can tolerate certain levels of injury with no economically significant yield reduction (HIGLEY & PETERSON, 1996). In this context, STERN et al. (1959) defined the lowest pest population that is able to cause economic damage to plants as the Economic Injury Level (EIL). However, to avoid reaching the EIL and the consequent losses on productivity, several factors should be taken into consideration, such as the time needed for the control measures to become efficient against the pests, or climate factors that can delay the implementation of a control measure, among others. Therefore, the decision of whether or not to control a pest population should always be made before the EIL is reached. The appropriate time to start the control measure in order to prevent the pest population from reaching the EIL is termed the Economic Threshold (ET) (PEDIGO et al., 1986). Therefore, insecticides should not be preventively applied on the soybean crop and their use is only justifiable when the pest population is equal or higher than the recommended ETs (**Figure 5**).

Figure 5. Graphical representation of the moment in which the pest control measures have to be adopted on the soybean field according to the Integrated Pest Management (IPM) recommendations.



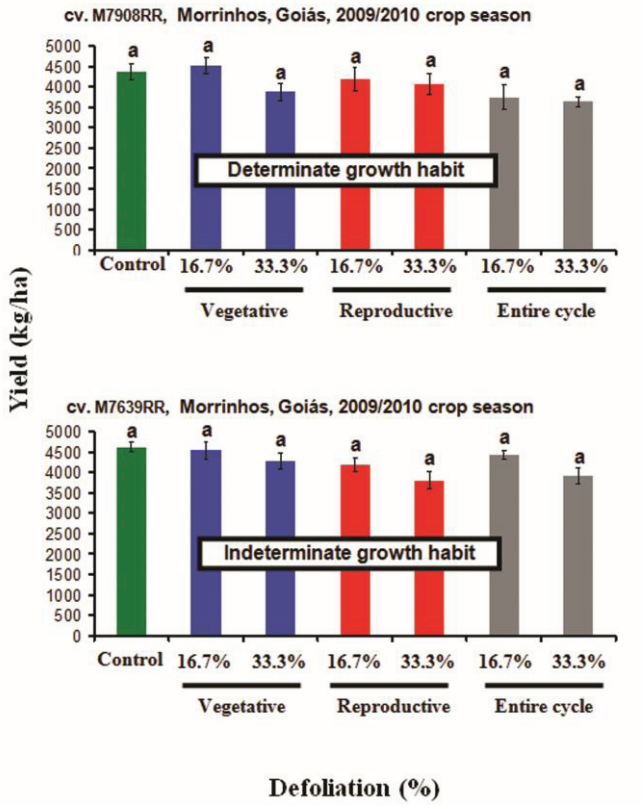
It is exactly regarding to the Economic Thresholds in which some questions on the viability of this technology relies. The economic threshold levels for soybean pest were determined years ago (1980's). Thus, it is the frequently questioned details about its nowadays validity, chiefly considering the newer and more productive soybean cultivars, sometimes of different cycle and growth habit (early cycle and indeterminate growth habit). Aiming at eliminating this kind of doubt, some research results will be following discussed, focusing specially on the more recent data, to validate these threshold levels and offer the necessary confidence to growers necessary to the use of this technology.

Economic Threshold for Defoliator Insects

The level of defoliation tolerated for the soybean crop before starting any control measure may slightly vary in different countries of the world. For example, in the US the defoliation level tolerated before start spraying insecticide is 35% at the vegetative stage of the crop and 20% at the reproductive stage (ANDREWS et al., 2009). In Brazil, the economic threshold recommended to initiate the control of defoliator insects is 30% defoliation at the vegetative stage or 15% when the crop is at the reproductive stage (TECNOLOGIAS, 2010). It is important to emphasize that the soybean plant tolerates some defoliation without significant decrease on yield

(HAILE et al., 1998). Earlier results report defoliation levels of until 50% without yield reduction (PICKLE & CAVINESS, 1984). Many of these studies used to determine the economic threshold currently recommended for controlling the major defoliator pests, however, were carried out in the 70's or 80's, although some recently published research papers have shown that these levels are still reliable (COSTA et al., 2003, REICHERT; COSTA, 2003). Among the recent research works, BUENO et al. (2010) showed that even the newer cultivars, regardless of the type of growth habit (determinate or indeterminate), tolerate the defoliation levels advocated by the economic threshold without a significant reduction in productivity (**Figure 6**). So far, there is no scientific evidence showing that more recent cultivars (early maturity group and indeterminate growth habit, for example) are more sensitive to leaf area losses.

Figure 6. Mean soybean production (±SE) with seeds at 13% moisture after different defoliation intensities (%) at different developmental stages of two soybean cultivars (M7908RR and M7637RR) grown in the municipality of Morrinhos, State of Goiás, Central Brazil, during the 2009/2010 crop season. Means followed by the same letter are not statistically different between each other by the Tukey test at 5% probability in each experiment (adapted from BUENO et al. 2010).



It is important to emphasize that the soybean plant has the characteristic of producing leaf area in excess. This characteristic, which is also present in other plant species, allows that even with some defoliation, these plants still achieve maximum interception of solar radiation for photosynthesis (BROUGHAM, 1956, 1958; & DONALD DAVIDSON, 1958, and Watson, 1958, MURATA, 1961, STERN & DONALD, 1962). This happens because a small loss of leaf area can be compensated by the greater light penetration until the lower leaves, which were once shaded, leading to an increased total production of photosynthesized products by the plant and making them producing a grain yield similar to the plants without defoliation or even inducing a slightly higher yield than the non-defoliated ones (TURNIPSEED, 1972).

In addition to the defoliator insects of the soybean crop, there are the stink bugs, which usually are a complex of different species that attack the pods sucking the grain contents. These pests are gaining importance in Brazil and some other countries. Questions about the viability of the ET recommended to initiate the control for this pest are also raised, particularly with respect to the early soybean cultivars with indeterminate growth habits.

Economic Thresholds for Stink Bugs

A study in this subject was carried out in the field, in the municipality of Arapongas, State of Paraná, South Brazil, during the 2010/2011 crop season. This study aimed at comparing the efficiency of the management used for different intensities of stink bugs infestations [ET (2 stinkbugs ≥ 0.5 cm / meter); ¼ ET (0.5 stinkbugs ≥ 0.5 cm / meter)] and the application of insecticides mixed with herbicides and fungicides (an increasingly common practice adopted by some Brazilian soybean growers in an attempt to reduce control costs) in the management of pests of the soybean crop. The treatments evaluated were applications of insecticides (or mixtures of them) at different crop developmental stages (**Table 1**). The experimental area was sown with the soybean cultivar 'BMX Potência RR' (maturity group 6.7 and indeterminate growth habit). The applications of herbicides and fungicides were equally carried out for all treatments, including the control plot. The pest population was weekly assessed with the aid of a sample-cloth on four sites per plot.

The results of this study indicated that, in general, even with a smaller population of stink bugs in the treatment with ¼ of the ET (0.50 stink bugs ≥ 0.5 cm/meter - treatment 2) as compared to the other tested treatments (**Figure 7**) this treatment did not have any significant gain in productivity (**Table 2**). In contrast, this treatment had higher number of insecticide applications and, consequently, higher environmental costs, since six applications of insecticides were performed, while the treatment 1, which followed the ET recommended by research for soybean destined to the grain production (2 stink bugs ≥ 0.5 cm/meter), only two insecticides applications during the crop cycle was needed (**Table 1**).

Table 1. Distribution of the applications of the different treatments [grams of active ingredient (a.i)/ha] evaluated in the control of stink bugs on the soybean crop. Municipality of Arapongas, State of Paraná, South Brazil, 2010/11 crop season.

Treatments	Dates of insecticide applications (Developmental stages of the crop)								
	12.08.2010 (V7)	12.22.2010 (V11)	01.05.2011 (R2)	01.11.2011 (R3)	01.24.2011 (R4)	01.29.2011 (R5.2)	02.05.2011 (R5.4)	02.11.2011 (R5.5)	02.18.2011 (R6)
1	-	-	-	-	Lambdacialotrin 26.5 + thiamethoxam 35.25	Lambdacialotrin 26.5 + thiamethoxam thiamethoxam 35.25	-	-	-
2	-	-	Lambdacialotrin 26.5 + thiamethoxam 35.25	Lambdacialotrin 26.5 + thiamethoxam 35.25	Lambdacialotrin 26.5 + thiamethoxam 35.25	-	Lambdacialotrin 26.5 + thiamethoxam 35.25	Lambdacialotrin 26.5 + thiamethoxam 35.25	Lambdacialotrin 26.5 + thiamethoxam 35.25
3	Lambdacialotrin 3.75	Lambdacialotrin 3.75	-	-	Lambdacialotrin 26.5 + thiamethoxam 35.25	-	-	Lambdacialotrin 26.5 + thiamethoxam 35.25	-
4	-	-	-	-	-	-	-	-	-

Figure 7. Mean population (±SE) of stink bugs along the soybean crop developmental stages after different treatments (indicated by the arrows) for pest control. Municipality of Arapongas, Sate of Paraná, South Brazil, 2010/2011 crop season.

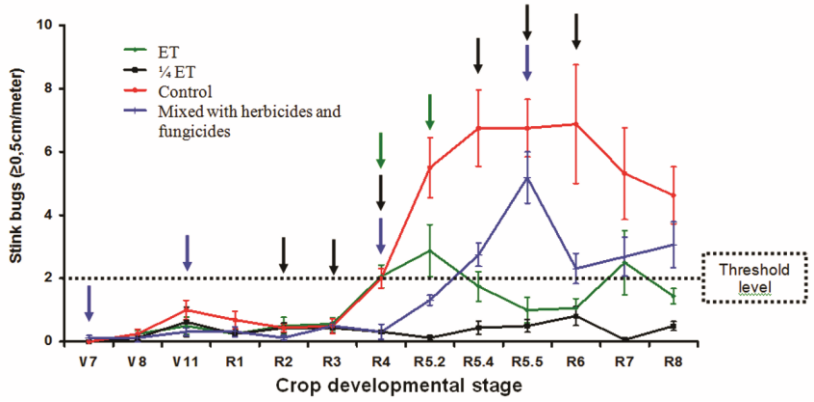


Table 2. Soybean yield and seed quality after the adoption of different management practices for the control of stink bugs. Municipality of Arapongas, State PF Paraná, South Brazil, 2010/2011 crop season.

	Treatment	Production (kg/ha)	Tetrazolium Test (%)[2] Stink Bugs Injury (scale 6 to 8)
1	Economic threshold (ET) for stink bugs	3812.5 ± 96.5 a[1]	4.5 ± 2.6 b[1]
2	¼ of the ET for stink bugs	3992.9 ± 116.5 a	1.0 ± 0.4 b
3	Insecticide mixed with herbicide and fungicides	3678.9 ± 76.6 a	4.8 ± 2.3 b
4	Control	3267.2 ± 39,9 b	13.7 ± 2.2 a
	CV (%)	**4.78**	**30.00**

[1]Means followed by the same letter in the column are not statistically different between each other by the Tukey test (P>0.05); [2] Original results followed by the statistics performed on data transformed by $\sqrt{X}$ (this data indicated the percentage of seeds with dead embryo due to stink bug injury).

Currently in Brazil, the infestations with stink bugs have increased significantly on the soybean crop and this is especially true with the species *Euschistus heros* (Hemiptera: Pentatomidae) (BUENO et al., 2007). This has occurred due to a combination of factors such as: 1) selection of resistant populations of stink bugs to the main insecticides used; 2) Low number of insecticides with different mechanisms of action; 3) deficiencies in the application technology of these products; and 4) ecological imbalance caused by the abusive and disordered use of broad spectrum insecticides, early in the development of the culture (BUENO et al., 2011; CORRÊA-FERREIRA et al., 2010). Therefore, the increased use of insecticides on the soybean fields will only contribute to aggravate the problems cited. Thus, similarly to the previously mentioned for defoliators, also for the stink bugs complex the overuse of insecticides brings more harms than benefits, especially when considering that there is no indication that the recommended economic threshold of 2 stink bugs ≥ 0 5 cm/meter is not safe to ensure the yield associated to the sustainability of the culture.

In the results obtained in that experiment, the observed productivity differed only in relation to the control treatment with no difference in productivity among the other treatments (**Table 2**). By analyzing the quality of the grains it can be observed that the damage from stink bugs (scale of 6 to 8, which means the % of seeds with embryos killed by the stink bugs) in the tetrazolium test, the result was statistically different only for the control treatment that had 13.7% of the grains with dead embryos. The treatment 1 (economic threshold recommend for stink bugs); 2 (1/4 of the economic threshold for stink bugs); and 3 (use of insecticides in combination with herbicide and fungicide) were statistically similar and showed percentages of seeds with dead embryos lower than 6% (**Table 2**). Such intensity of damage (6%) is still allowed, even in the category of soybean for seed production, which is more rigorous than the experiment performed, which was carried out in a soybean field aimed to grain production.

Thus, it is safe to affirm that controlling preventively the stink bugs [in the example used: treatment 3 (with four insecticide applications); and treatment 2 (¼ of the economic threshold recommended for stink bugs, with six insecticide applications)] is not feasible, mainly for not providing significant benefits on productivity or quality of the output attained, besides greatly increasing the number of chemical applications and consequently the production costs and the negative impact of it on the environment. On the contrary, the treatment which waited until the economic threshold level recommended for stink bugs had been reached, in addition to the reduction of environmental risks showed a practical and easily measurable advantage, which is the lower production cost due to the lower use of chemicals. Therefore, the chemical application at the right moment can be considered the most sustainable treatment among all the different management practices evaluated and must always be adopted by growers to reduce the negative impacts caused by agricultural chemicals in the environment since it rationalizes the use of pesticides.

Selectivity of pesticides to beneficial insects

Among the beneficial insects of agronomic importance in the soybean crop, the natural biological control agents are noteworthy for their contribution in maintaining the pest populations below the economic threshold levels. However, although many examples of successful biological control indeed exist, the chemical control is still essential to ensure good yield. The pesticides such as insecticides, fungicides, herbicides, and acaricides, represent important tools for crop management and play a significant role in the success of agricultural production. The most suitable products for use in the Soybean-IPM, however, are those that combine a good control of target pests with a minimal impact on the activity of natural enemies (beneficial arthropods). The integration of chemical products with biological control, in most cases, is crucial for the success of agriculture and reduction of negative impacts of their use. Hence, the selectivity of the insecticides to natural enemies is of great importance and should always be considered when choosing the best product to be used.

It is important to emphasize that in stressing the importance of the use of selective pesticides in agriculture one should not considered only the insecticides and acaricides, but also the herbicides, fungicides, plant growth regulators, foliar fertilizers and other chemicals that might be applied over the top of plant canopy. These other products are most often neglected in this matter of selectivity to biological control agents and may also have negative effects on beneficial arthropods.

It is important to point out that the ways in which an agrochemical may or may not be selective, is not a unique one and all of them must be considered when classifying a product as selective or harmful to a specific natural enemy. The selectivity, in general, may be physiological and/or ecological; and both are complementary and very important for the balance of the agro-ecosystem.

Physiological selectivity

The physiological selectivity is inherent to the chemical compound and is related to the tolerance of a natural enemy when it is subjected to direct contact with that specific product, with its residues, or by its ingestion. This type of selectivity is due to physiological differences among the species involved regardless if they are pests, predators, parasitoids, pathogens or pollinators, and can be determined by the concentration of a product that results in good control of the pest, without affecting the beneficial arthropods. This selectivity is, for example, what occurs with some phosphatic insecticides when there is a reduction in the penetration of the tegument, or an increase in the degradation of toxic molecule through the enzymatic system of a beneficial insect.

In this context, it is important to emphasize that there are different intensities of selectivity, which means that the pesticides are not only classified as selective or harmful. The degree of physiological selectivity of a product is usually expressed by the ratio of the median lethal dose (LD_{50}) to the pest and the natural enemy or the relationship between the dosages recommended for the control of the pest and the LD_{50} to the natural enemy. Therefore, if a curve of mortality of the pest and of the natural enemy is drawn for a given product, the dose to obtain the best selectivity can be defined as the point on the curve where the difference between the mortality of the pest and of the natural enemy is the maximum.

It is often difficult to achieve the optimal level of physiological selectivity. Many of the insecticides act on the central nervous system and there is a great similarity in the transmission of nerve impulses, not only among the different insect orders

(involving pests and natural enemies), but also from other animal phyla, which leads to the similarity of response of the pest and of the natural enemy to a given dose of an insecticide. However, it is at least possible to increase the degree of physiological selectivity, for example, by reducing the doses, provided it is done responsibly and supported by research data that assure the maintenance of an acceptable level of pest control.

It is important to stress that in a balanced agricultural system, the expected result of the use of an insecticide does not necessarily need to be 100% mortality of the pest, as it is generally regarded by many growers, but surely to reduce the population of this pest to a level below the economic injury level. Contrary to what one might think, 100% control of a given pest, may be not a satisfactory result of a control tactic, due to the indirect harmful effects caused to natural enemies as a result of the unavailability of preys or hosts, which among a number of side effects, can result in the rapid resurgence of the pest.

The new advances in pest control, especially with the development of insecticides with juvenile hormones, growth inhibitors and some insecticides of biological origin and, more recently, the biotechnology with the insecticide plants, have broadened the possibilities of the use of the physiological selectivity. The insecticides chitin synthesis inhibitors and accelerators of ecdysis are characterized by presenting low toxicity to vertebrates and to many beneficial arthropods, essentially by its action of ingestion, which gives them a high degree of selectivity in relation to other insects and arthropods that do not eat the treated foliage. The use of these products is feasible in pest management programs and should be the choice whenever possible instead of the broad-spectrum options.

Another example of selective pesticides is the ones usually called biopesticides. Biopesticides are derived from animals, plants or microorganisms such as bacteria and fungi. Bacillus thuringiensis (Bt) is one of the most widely used biopesticides and its varieties of strains are pathogenic to a wide range of pests, including Lepidoptera and Diptera. When Bt is ingested by the insect, the protein crystal is solubilized in their intestines and the formation of proteins called delta endotoxins, that are toxic to a large number of insects, occurs. Another example is the insecticide derived from a fermentation process using the bacterium *Saccharopolyspora spinosa*. This biopesticide has shown high specificity in the pest control, being very selective to predators in general but usually not selective to parasitoids (Hymenoptera).

Ecological selectivity

Ecological selectivity is another type of selectivity that always will complement the physiological selectivity and must also be taken into consideration. It is nothing more than the use of agrochemicals in an ecologically selective manner that minimizes the exposure of the natural enemies to these products and, at the same time, controls the pest-insects. The ecological selectivity is still divided according to the way in which exposure to the agrochemicals is differentiated between pests and the beneficial arthropods and may be temporal or spatial. An example of ecological spatial selectivity is the favorable and appropriate manner of a systemic insecticide application in the soil, which is then absorbed by the roots and circulates in the plant sap, reaching only the sucking pests, preserving their natural enemies that do not come in contact with the chemical product. Another example is the application of the insecticide only in restricted areas, especially within patches of pests that have little mobility in the field, such as mites and mealybugs.

Another important example in the soybean crop is the application of insecticides on the border of the field mixed with sodium chloride (common table salt) to control stink bugs. This practice is effective because the stink bugs are concentrated on the edges of the crops at the beginning of the infestation and the salt acts leading the insect to a longer feeding period within those areas and, consequently, increasing the contact with the product. These techniques allow the spatial separation of the insecticide and beneficial arthropod, thus preserving the latter of poisoning that would occur if it came into contact with the chemical.
An example of temporal ecological selectivity is the not applying insecticides in the middle of the day, avoiding the hours in which pollinators most visit the flowers, thus obtaining a separation in time between the product application moment and the period in which the beneficial arthropods that, otherwise, would be exposed to contamination. These practices allow that a chemical product, even when non-selective physiologically, has an ecological selectivity, preserving this beneficial arthropods in the area and, consequently, minimizing the negative impact of the chemical control on pollinators.
As we can see, the ecological selectivity is only possible due to differences in behavior or habitats between species, enabling the product to be in contact with a given species and not with another. This strategy is based on the ecological differences between pests, natural enemies and pollinators and requires a thorough understanding of the bio-ecological aspects of pest and beneficial arthropods which sometimes might not be available for some important species.

How the studies on selectivity of agrochemicals to natural enemies can aid on the reduction of the negative impact of pesticides of the soybean agroecosystem

In classifying a chemical as selective or harmful to beneficial arthropods is of great importance to consider all possible aspects. Worldwide, studies of selectivity have been the subject of many discussions, many times without reaching a common sense concerning methodologies or the standardization of the procedures used to evaluate the side effects of pesticides on beneficial organisms. This lack of consensus creates some contradictions in the results and conclusions found.
Some of the causes of these contradictions are the many tests carried out under field conditions, where the direct effects of the toxicity of the insecticide were not clearly defined. Many times, the elimination of the predator is due to the suppression of their food supply (insect pest). Different species may also respond differently to pesticides, in addition to the differences in the dosages of the products and of the insect developmental stages. As earlier mentioned, an evaluation methodology that considers all these bio-ecological differences is extremely necessary. In this context, in 1974, a group to work for the international scientific cooperation in the study of selectivity of pesticides to beneficial organisms, the International Organization of Biological Control (IOBC) was formed. One of its main goals is the global fomentation of selectivity tests based on a standard methodology. Since then the IOBC has promoted studies to standardize selectivity tests. According to this organization, the insect must be first submitted to an extreme contamination condition and if a high percentage of the population does not die, then the product can be considered harmless. Otherwise, it should go through complementary tests under greenhouse conditions and then in the field.
Among the species of natural enemies studied some, in addition to their potential as control agents, present a high degree of compatibility with the combined use of chemical compounds as, as for example, the egg parasitoids. These natural enemies are the insects that have attracted more interest worldwide, for killing their hosts

before the pest emergence and their attack to the plants. In addition, species of the genus *Trichogramma*, for example, have been successfully marketed and released for the control of a diversity of pest lepidopterans in various parts of the world. This continuing success, however, depends on the use of chemicals that do not interfere with the performance and development of their populations through the physiological selectivity.

Although many products still need to be tested for most biological control agents of economical importance for the soybean crop, some results have already been obtained and are available in the literature. Therefore, the use of more selective products, according to the results available to date should be encouraged and can be used as one of the selection criteria for choosing the pesticides that will be applied in the field. Some of the pesticides, at the doses used in the soybean crop, have already been evaluated for their selectivity to the egg parasitoids *Trichogramma pretiosum* and *Telenomus remus*. Some of these results are shown on **Tables 3, 4 and 5**.

Table 3. Effect of different insecticides application (E %) at four different developmental stages of the egg parasitoids Trichogramma pretiosum and Telenomus remus under laboratory conditions, followed by the classification according to the IOBC (C) protocol.

Treatment (grams of active ingrediente ha^{-1})	*Trichogramma pretiosum*										*Telenomus remus*							
	egg		larva		pupa		Adult				larva		pupa		Adult			
							24h		48h						24h		48h	
	E%	C	E%	C	E%	C	E%	C	E%	C	E%	C	E%	C	E%	C	E%	C
Acephate 375	-	-	-	-	-	-	60.6	2	100	4	-	-	24.9	1	-	-	-	-
Acephate 525	-	-	-	-	-	-	100	4	100	4	5.6	1	10.1	1	100	4	100	4
Acetamiprid 50	-	-	-	-	-	-	55.6	3	98.1	3	-	-	8.9	1	0.3	1	6.7	1
Alpha-cypermethrin 10	60.1	2	47.7	2	27.8	1	-	-	-	-	-	-	-	-	-	-	-	-
Azaractina 100 mL ha-1	-	-	-	-	11.2*	1	-	-	-	-	-	-	-	-	-	-	-	-
PsinSNPV10x1011 cpi/ha					6.3*	1	-	-	-	-	-	-	-	-	-	-	-	-
AgMNPV165x109 cpi/ha	-	-	-	-	2.6*	1	-	-	-	-	-	-	-	-	-	-	-	-
Beta- cyfluthrin 9.375 + Imidacloprid 75	-	-	-	-	-	-	78.8	2	94.0	3	-	-	44.1	2	88.9	3	98.3	3
Beta- cyfluthrin 9.375 + Imidacloprid 75 + spiromesifen 60	-	-	-	-	-	-	30.5	2	79.7	3	-	-	34.3	2	92.4	3	96.4	3
Beta- cyfluthrin 12.5 + Imidacloprid 100	-	-	-	-	42.7 / 88.5*	2/3	98.3	3	100	4	3.41	1	1.42	1	98.2	3	100	4
Bifenthrin 5	-	-	-	-	25.1	1	100	4	100	4	52.1	2	36.4	2	100	4	100	4
Buprofezin 150 + vegetal oil 0,25%	-	-	-	-	-	-	0	1	25.4	1	-	-	55.8	2	0.9	1	4.6	1
Clorfluazurom 10	1.5	1	0	1	28.5	1	-	-	-	-	-	-	-	-	-	-	-	-
Clorfluazurom 35	6.7	1	4.5	1	44.3	2	-	-	-	-	-	-	-	-	-	-	-	-
Chlorpyrifos 240	25.9	1	43.7	2	57.5	2	-	-	-	-	-	-	-	-	-	-	-	-
Chlorpyrifos 384	-	-	-	-	98.9	3	100	4	100	4	100	4	100	4	100	4	100	4
Chlorpyrifos 480	-	-	-	-	-	-	99.0	4	100	4	-	-	99.7	4	100	4	100	4
Diflubenzurom 20	-	-	-	-	0 / 6*	1	6.7	1	14.8	1	4.1	1	1,7	1	7.1	1	76.9	2
Endosulfan 525	38.2	2	87.3	3	22.9	1	-	-	-	-	-	-	-	-	-	-	-	-
Endosulfan 875	83.3	3	88.8	3	83.2	3	-	-	-	-	-	-	-	-	-	-	-	-
Esfenvalerate 7.5	99,5	4	99.7	4	100	4	-	-	-	-	-	-	-	-	-	-	-	-
Spinosad 24	100	4	100	4	100 / 89.3*	4/3	94.5	3	98.7	3	15	1	29.6	1	70.6	2	100	4

Table 3 (continuation)…

Treatment (grams of active ingrediente ha^{-1})	*Trichogramma pretiosum*										*Telenomus remus*							
	egg		larva		pupa		Adult				larva		pupa		Adult			
							24h		48h						24h		48h	
	E%	C	E%	C	E%	C	E%	C	E%	C	E%	C	E%	C	E%	C	E%	C
Spiromesifen 60	-	-	-	-	-	-	2.5	1	6.8	1	-	-	-	-	-	-	-	-
Flufenoxurom 10	-	-	-	-	4.8 / 9.1*	1	2.9	1	0	1	2.0	1	0	1	27.9	1	69.3	2
Gama- cyhalothrin 3.75	-	-	-	-	40,8	2	99,4	4	100	4	60,6	2	29,6	1	100	4	100	4
Lambda- cyhalothrin 3.75	100	4	33.6	2	31.9	2	-	-	-	-	-	-	-	-	-	-	-	-
Lambda- cyhalothrin 21.2 + Thiamethoxam 28.2	-	-	-	-	64.3	2	-	-	-	-	-	-	-	-	-	-	-	-
Lambda- cyhalothrin 26.5 + Thiamethoxam 35.25	-	-	-	-	-	-	88.3	3	100	4	-	-	51.7	2	-	-	-	-
Lufenurom 7.5	58,8	2	15,2	1	37.7 / 34.7*	2	0	1	21.6	1	-	-	-	-	-	-	-	-
Methamidophos 480	-	-	-	-	-	-	-	-	-	-	-	-	58.9	2	-	-	-	-
Methamidophos 480 + thiamethoxam 25	-	-	-	-	-	-	94.9	3	99.8	4	-	-	71.7	2	95.0	3	99.8	4
Methomyl 86	44.9	2	50.6	2	52.3	2	-	-	-	-	-	-	-	-	-	-	-	-
Methomyl 215	-	-	-	-	92.8*	3	-	-	-	-	-	-	-	-	-	-	-	-
Methomyl 322.5	95.7	3	90.8	3	69.0	2	-	-	-	-	-	-	-	-	-	-	-	-
Methoxyfenozide 19.2	4.5	1	5.3	1	7.0	1	-	-	-	-	-	-	-	-	-	-	-	-
Methoxyfenozide 21.6	-	-	-	-	9.4	1	1.1	1	0	1	0	1	0	1	0	1	100	4
Methoxyfenozide 36.0	-	-	-	-	4,1	1					10.6	1	7.4	1	9.9	1	0	1
Permethrin 49.92	-	-	-	-	61.6	2	99.3	4	100	4	-	-	-	-	-	-	-	-
Teflubenzurom 7.5	22.3	1	31.5	2	0	1	-	-	-	-	-	-	-	-	-	-	-	-
Teflubenzurom 12	47.0	2	38.7	2	20.3	1	-	-	-	-	-	-	-	-	-	-	-	-
Thiamethoxam 25	-	-	-	-	-	-	72.5	2	97.2	3	-	-	-	-	-	-	-	-
Thiamethoxam 50	-	-	-	-	-	-	87.7	3	95.2	3	-	-	-	-	-	-	-	-
Thiodicarb 56	100	4	70,0	2	69,1	2	-	-	-	-	-	-	-	-	-	-	-	-
Triflumurom 14.4	93.4	3	100	4	3.8	1	-	-	-	-	-	-	-	-	-	-	-	-
Triflumurom 24	96.4	3	100	4	54.6	2	-	-	-	-	-	-	-	-	-	-	-	-
Piriproxifem 100	-	-	-	-	-	-	2.6	1	40.8	2	-	-	37.3	2	-	-	-	-
Zeta- cypermethrin 10	45.0	2	13.5	1	11.3	1	-	-	-	-	-	-	-	-	-	-	-	-

*Experiment performed with the application of the insecticide on the eggs of the parasitized host; (**C**) IOBC classification = Class 1- harmless (E<30%), Class 2 - slightly harmful (30≤ E≤79%), Class 3 - moderately harmful (80 ≤ E ≤99%), and Class 4 – harmful (E>99%).

Table 4. Effect of different herbicides application (E %) on four different developmental stages of the egg parasitoids Trichogramma pretiosum and Telenomus remus under laboratory conditions, followed by the classification according to the IOBC (C) protocol.

Treatment (grams of active ingrediente ha^{-1})	*Trichogramma pretiosum*										*Telenomus remus*							
	egg		larva		pupa		Adult				larva		pupa		Adult			
							24h		48h						24h		48h	
	E%	C	E%	C	E%	C	E%	C	E%	C	E%	C	E%	C	E%	C	E%	C
2,4-D 1209	-	-	-	-	37.3	2	5.2	1	0	1	17.8	1	15.5	1	0	1	0	1
Clomazone 1000	-	-	-	-	17.9	1	-	-	-	-	0	1	35.9	2	0	1	86.3	3
Clorimuron 20	100	4	18.5	1	18.5	1	-	-	-	-	-	-	-	-	-	-	-	-
Paraquat dichloride 600	-	-	-	-	41.3	2	96.2	3	100	4	0	1	7.3	1	93.6	3	100	4
Paraquat dichloride 600 + diurom 300	-	-	-	-	26.9	1	-	-	-	-	0	1	12.8	1	1.1	1	92.3	3
Fluazifop 125	3.1	1	19,5	1	19.5	1	-	-	-	-	-	-	-	-	-	-	-	-
Flumioxazine 60	-	-	-	-	16.8	1	31.6	2	44.6	2	1.8	1	0.2	1	0	1	59.6	2
Fomesafen 250	8.3	1	13.9	1	13.9	1	-	-	-	-	-	-	-	-	-	-	-	-
Glyphosate 960 (GlizC)	18.5	1	16.7	1	16.7	1	-	-	-	-	-	-	-	-	-	-	-	-
Glyphosate 960 (Roundup Transorb®)	15.2	1	12.6	1	12.6	1	-	-	-	-	-	-	-	-	-	-	-	-
Glyphosate 960 (Roundup Original®)	37.2	2	31.7	2	11.3	1	-	-	-	-	-	-	-	-	-	-	-	-
Glyphosate 972 (Roundup ReadyC)	100	4	23.5	1	23.5	1	-	-	-	-	-	-	-	-	-	-	-	-
Glyphosate 1200 (Roundup ReadyC)	-	-	-	-	17.5	1	-	-	-	-	0.5	1	36.5	2	0	1	91.2	3
Glyphosate 1981.25 (Roundup WGC)	-	-	-	-	-	-	75.0	2	91.6	3	-	-	-	-	-	-	-	-
Glyphosate 2592 (Roundup Transorb®)	-	-	-	-	32.5	2	-	-	-	-	27.0	1	23.7	1	0	1	100	4
Glifosato 2880g (Gliz®)	-	-	-	-	16.1	1	63.1	2	91.0	3	0	1	36.6	2	2.1	1	100	4
Glyphosate 720 + imazetapir 90	-	-	-	-	27.1	1	33.8	2	374	2	0	1	53.7	2	9.8	1	0	1
Lactofen 165	12.6	1	8.3	1	8.3	1	-	-	-	-	-	-	-	-	-	-	-	-
S-metolachlor 1920	-	-	-	-	4.4	1	-	-	-	-	19.3	1	21.1	1	0	1	37.8	2

(C) IOBC Classification = Class 1 – harmless (E<30%), class 2 – slightly harmful (30 ≤ E ≤79%), class 3 - moderately harmful (80 ≤ E ≤99%), class 4 – harmful (E>99%).

Table 5. Effect of different fungicides application (E %) on four different developmental stages of the egg parasitoid Trichogramma pretiosum and Telenomus remus under laboratory conditions, followed by the classification according to the IOBC (C) protocol.

Treatment (grams of active ingrediente ha^{-1})	*Trichogramma pretiosum*										*Telenomus remus*							
	egg		larva		pupa		Adult				larva		pupa		Adult			
							24h		48h						24h		48h	
	E%	C	E%	C	E%	C	E%	C	E%	C	E%	C	E%	C	E%	C	E%	C
Azoxystrobin 50	6.2	1	13.1	1	13.1	1	17.3	1	0	1	29.5	1	38.2	2	14.8	1	23.4	1
Azoxystrobin 60 + cyproconazole 24	13.9	1	4.9	1	4.9	1	22.4	1	0	1	16.1	1	5.8	1	8.8	1	63.2	2
Carbenzadim 250	-	-	-	-	10.1	1	15.8	1	0	1	21.4	1	27.7	1	13.3	1	10.7	1
Epoxiconazole 12.5	-	-	-	-	0	1	31.1	2	76.8	2	22.7	1	34.3	2	0	1	0	1
Epoxiconazole 30 + pyraclostrobin 79.8	-	-	-	-	0	1	53.6	2	4.5	1	35.9	2	29.1	1	10.2	1	29.1	1
Flutriafol 125	-	-	-	-	0	1	64.8	2	89.2	3	35.0	2	37.2	2	6.5	1	8.5	1
Flutriafol 60 + thiophanate-methyl 300	-	-	-	-	12.5	1	36.2	2	0	1	26.4	1	12.4	1	0	1	5.7	1
Thiophanate-methyl 125	25.7	1	11.5	1	11.5	1	-	-	-	-	-	-	-	-	-	-	-	-
Tebuconazol 120 + trifloxystrobin 60	-	-	-	-	0	1	16.1	1	0	1	24.3	1	25.9	1	0	1	9.1	1
Tebuconazol 200 + trifloxystrobin 100	100	4	43.0	2	25.6	1	-	-	-	-	-	-	-	-	-	-	-	-
Tebuconazol 150	-	-	-	-	13.0	1	30.2	2	64.6	2	41.9	2	21.8	1	0	1	17.7	1
Thiophanate-methyl 400	-	-	-	-	2.4	1	20.0	1	0	1	50.6	2	8.8	1	0	1	48.2	2

(C) IOBC Classification = Class 1 – harmless (E<30%), class 2 – slightly harmful (30 ≤ E ≤79%), class 3 - moderately harmful (80 ≤ E ≤99%), class 4 – harmful (E>99%).

THE SAFETY OF THE SOYBEAN-IPM ASSOCIATED TO YIELD AND SUSTAINABILITY

The guarantee of positive results obtained with the adoption of Soybean-IPM, which combines high yield and lower environmental impact caused by pesticides, is a pre-requisite to a massive adoption of this technology. Therefore, in order to evaluate the influence of different pest control practices on its population as well as on the soybean yield, BUENO et al. (2011) carried out some experiments at different locations in important producing regions of Brazil. In these experiments, the authors analyzed the feasibility and safety of the Soybean-IPM more accurately using newer cultivars with different characteristics. All experiments were conducted in a randomized block experimental design with four treatments (**Table 6**) and four replications, each with an area of 25 m x 25 m. The most important results obtained (**Table 7**) ratify what had been previously published in the literature showing that the soybean-IPM is crucial to combine high yield with a more friendly agriculture (lower use of chemicals). The yield, where the insecticides were overused mixed with herbicides and fungicides [conventional management usually used by some soybean producers (CM)], without considering the economic threshold level was not higher than that obtained in the treatment where the IPM was adopted (**Table 7**). In this context, it should also be emphasized that the treatment CM had a greater number of pesticide applications, which besides not providing better control and having higher costs, can contaminate humans and the environment. In addition it can also aggravate the problems of pest resurgence and secondary pest outbreaks, among other problems previously discussed in this chapter (MEISSLE et al., 2010, TANG et al., 2010).

Table 6. Magament used including dosages per hectare of the products used and crop developmental stage in which the treatment applications were performed on five soybean experiments submitted to different pest management systems, installed at distinct municipalities of two soybean producing Brazilian states [Goiás (GO) and Paraná (PR)], in the 2008/2009 and 2009/2010 crop seasons. Adapted from BUENO et al. (2011).

Treatment[1]	2008/2009 crop season			2009/2010 crop season	
	Castelândia, GO	Santa Helena de Goiás, GO	Senador Canedo, GO	Morrinhos, GO	Arapongas, PR
IPM	**Methoxyfenozide 36 g:** R2; **Methamidophos 480 g**: R5.4 and R7.	**Methoxyfenozide 36g:** (R1) 43 and (R3) 57 DAE.	**Endosulfan 350g:** R4; **Methamidophos 600g:** R5; R5.3; R7.1 and R7.3.	**Methoxyfenozide 36g:** R4 and R5.2.	**Methoxyfenozide 36g:** R3; **Acephate 300g:** R5.1; **Methamidophos 480g:** R5.2.
Conventional Management (CM) (use of insecticides mixed with herbicides and fungicides)	**Alpha-cypermethrin 10g:** V2 and V6; **Beta-cyfluthrin 12,5g + Imidacloprid 100g:** R2 and R5.1; **Lambda-cyhalothrin 21,2g + thiamethoxam 28,2g:** R7.	**Alpha-cypermethrin 10g:** V6 and V8; **Beta-cyfluthrin 12,5g + Imidacloprid 100g:** R2 and R5.2.	**Alpha-cypermethrin 10g:** V7; R2; R5 and R7.1; **Beta-cyfluthrin 12,5g + Imidacloprid 100g:** R1; R5.3 and R7.3.	**Permethrin 20g:** V3; V6 and R4; **Beta-cyfluthrin 12,5g + Imidacloprid 100g:** R2.	**Lambda-cyhalothrin 18,75g:** V7; **Beta-cyfluthrin 12,5g + Imidacloprid 100g:** R1; R5.1 and R5.2.
Control (C)	**Without pest control**	**Without pest control**	**Without pest control**	**Without pest control**	**Without pest control**

[1]**IPM** = Integrated Pest Management; **CM** = conventional management usually adopted by Brazilian soybean growers wit overuse of insecticide **C** = Control treatment: withou est control.

Table 7. Soybean yield (Mean±SE) kg/ha obtained in experiments carried out under different pest management systems, at five different municipalities of two soybean producing Brazilian states [Goiás (GO) and Paraná (PR)], in the 2008/2009 and 2009/2010 crop seasons. Adapted from BUENO et al., 2011).

Treatment[1]	2008/2009 crop season[2]			2009/2010 crop season[2]	
	Castelândia, GO	Santa Helena de Goiás, GO	Senador Canedo, GO	Morrinhos, PR	Arapongas, PR
IPM	3,180.40 ± 185.43 a	2,447.01 ± 178.60[ns]	2,913.56 ± 200.37[ns]	4,179.25 ± 128.64 ns	2,992.57 ± 65.86 a
Conventional Management (CM) (use of insecticides mixed with herbicides and fungicides)	2,981.49 ± 178.97 a	2,441.33 ± 208.19	2,832.85 ± 277.65	3,902.50 ± 84.18	3,175.72 ± 51.49 a
Control (C)	2,555.12 ± 73.14 b	2,228.62 ± 166.52	2,487.32 ± 71.71	3,797.50 ± 96.81	2,667.83 ± 89.42 b
CV (%)	5.54	4.54	13.86	5.79	3.84

Treatment[1]	2008/2009 crop season[2]			2009/2010 crop season[2]	
	Castelândia, GO	Santa Helena de Goiás, GO	Senador Canedo, GO	Morrinhos, PR	Arapongas, PR
IPM	3,180.40 ± 185.43 a	2,447.01 ± 178.60[ns]	2,913.56 ± 200.37[ns]	4,179.25 ± 128.64 ns	2,992.57 ± 65.86 a
Conventional Management (CM) (use of insecticides mixed with herbicides and fungicides)	2.981,49 ± 178,97 a	2.441,33 ± 208,19	2,832.85 ± 277.65	3,902.50 ± 84.18	3,175.72 ± 51.49 a
Control (C)	2.555,12 ± 73,14 b	2.228,62 ± 166,52	2.487.32 ± 71.71	3,797.50 ± 96.81	2,667.83 ± 89.42 b
CV (%)	5.54	4.54	13.86	5.79	3.84

[1]**IPM** = Integrated Pest Management; **CM** = conventional management usually adopted by Brazilian soybean growers wit veruse of insecticides; **C** = Control treatment: without pest control. [2] Means followed by the same letter in the column are no tatistical different between each other the Tuk tes P>0.0 . [ns] Non s nificant

FINAL REMARKS

In order to maintain the sustainability of agriculture at median and long terms, a better alternative to the overuse of pesticides is the integrated pest management (IPM), which aims towards the rational use of insecticides as well as the harmonious integration of different control strategies (ZALUCKI et al., 2009). In the IPM approach, the natural biological control of pests is always prioritized according to which other auxiliary tactics, including the use of selective pesticides, are only used as complementary resources, and harmoniously applied in order not to impact the biological control agents in a correct practice of IPM, whose concept contains economic, ecological, and toxicological principles. In this context, the selectivity either physiological or ecological is obligatory for the biological control to have full success and thus mitigate the risks and negative impacts of chemical control.

A great example of how the IPM can be a tool to mitigate the negative impact of pesticides on agriculture can be observed in the soybean crop in Brazil. Prior to the adoption of the Soybean-IPM in Brazil, at the beginning of the 1970s, an average of six insecticide applications were made per crop season, using broad-spectrum insecticides. After Brazil has adopted the Soybean-IPM, in addition to the use of more selective products to protect natural enemies and beneficial insects, insecticides began to be used more appropriately, with growers considering the economic thresholds for the pest control. As a result, the use of pesticides was reduced to approximately two applications per crop season (BUENO et al., 2010). The Soybean-IPM technology has unfortunately been abandon by some Brazilian soybean growers making the number of insecticide application increase again nowadays in this country. Comparing these situations, the advantages of using the IPM methods cannot be ignored, because they are economically and environmentally feasible (KOGAN, 1998).

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Current Pesticide Loads in Dutch Surface Waters

Martina G. Vijver, Geert R. De Snoo

Key words: measurements, pesticides, surface water, MPC exceedance

INTRODUCTION

The Netherlands is one of the world's foremost agricultural producers, with an estimated 22,900 km^2 of the total land mass of around 33,700 km^2 devoted to agriculture or horticulture. In international comparisons of the economic significance (export value) of the agricultural sector the Netherlands inevitably ranks in the top three (with the USA heading the list). Land use is consequently highly intensive: in terms of output per hectare or head of livestock the Netherlands ranks among Europe's leaders. To achieve such high outputs a vast range of agricultural chemicals are used, including fertilisers, veterinary drugs, crop protection agents and other biocides.

At the same time the Netherlands is a country with a multitude of watercourses, characterised as it is as by its location in the delta of several major rivers like the Rhine and Meuse that flow out to sea via numerous channels. Equally characteristic are the countless man-made ditches, canals and lakes created for the purposes of water management, quite a challenge as around half the country is below sea level. It is no wonder, then, that the Netherlands has a long tradition of managing water quantity and water quality.

The widespread and heavy use of agricultural chemicals begs many questions, one of which is: to what extent do these practices pose a potential threat to the environment? Unsurprisingly, then, within both Europe and the Netherlands in recent decades policies have been introduced that seek to reduce dependency on pesticides, reduce pesticide usage and reduce emissions of these chemicals to the environment. Market approval of pesticides is tightly regulated and application of such compounds is also subject to stringent regulation, in agricultural settings as well as elsewhere. Farmers must have a spraying diploma, equipment must be approved, and agricultural plots must be separated from their surroundings by a (narrow) buffer zone.

Thanks to these policies there have been major improvements in surface water quality in Europe and specifically the Netherlands. The question, though, is whether these efforts have been sufficient. Pesticides are still frequently encountered in high concentrations in surface waters (Vijver et al. 2008), at levels often exceeding water quality standards, with potential damage to the aquatic ecosystem as a result. This implies a substantial challenge for policymakers, and it is therefore of key importance to know exactly where and when what pesticides occur in Dutch surface waters and to what extent this leads to statutory limits being exceeded.

This chapter reports on Dutch surface water quality with respect to pesticides by considering the following questions:

- To what extent do pesticide levels in Dutch surface waters exceed environmental standards?
- In which regions and during which periods of the year do the worst problems occur?
- Which pesticides exceed the standards most frequently?
- What can be said about general water quality with regard to the overall pesticide load and is there geographical differentiation?

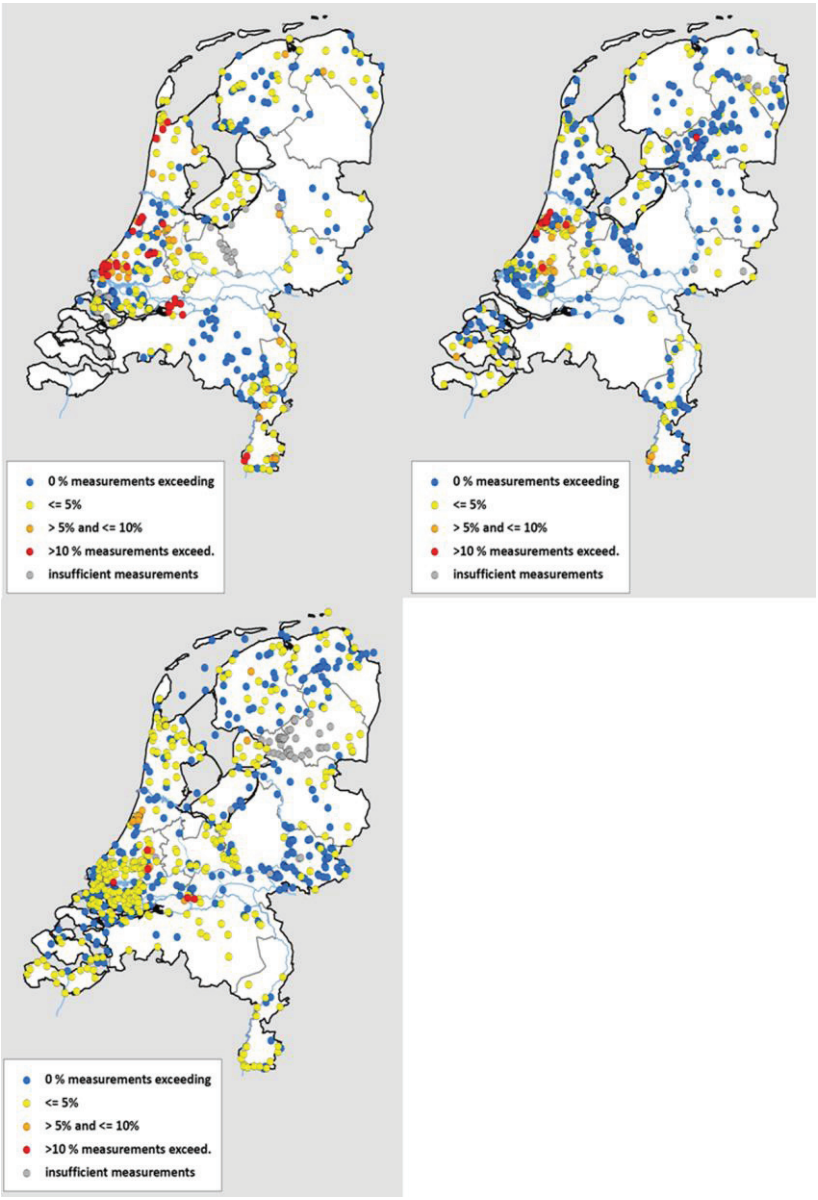
The surface water environmental standard: MPC

In the Netherlands and many other European countries the Maximum Permissible Concentration (MPC) has long been in use as the key environmental standard for safeguarding surface water quality. The MPC of a substance is the environmental concentration of the substance at which 95% of ecosystem species are protected against the substance's effects. The MPC is based on the intrinsic properties of the substance, i.e. its potential effects on living organisms. The MTR is derived using all the toxicity data available on the substance in question (provided they are sufficiently reliable), for as many different groups of organisms as possible. Collecting toxicity data and assessing their reliability is therefore a key part of the procedure for deriving an MTR. If toxicity data are available for only a limited number of species, but an estimate needs to be made of potential damage to the overall ecosystem, a safety factor is adopted that ranges from 10 to 1000. The methodology and safety factors to be used are standardised and grounded in a Technical Guidance Document (TDG, 2003) drawn up by the European Commission.

MPC exceedance by pesticides: spatial pattern

To obtain a nationwide picture of the spatial pattern of pesticide levels in Dutch surface waters, data were taken from the Dutch Pesticides Atlas (www.bestrijdingsmiddelenatlas.nl), an open-access internet resource developed by Leiden University that comprises over 3000 maps showing measured concentrations of pesticides in surface waters during the period 1997-2009. The data derive from the monitoring programmes of all the Netherlands' water boards and similar 'competent authorities'. At each monitoring site the individual measurements have been aggregated on an annual basis, as measurements may have been made on

Figure 1: Percentage of measurements exceeding the MPC in 1998, 2004 and 2009. Blue dots: no exceedances; yellow: less than 5%; orange: 5-10%, red: > 10% exceedances. Grey dots: sites with less than 10 measurements, for which no values were calculated.

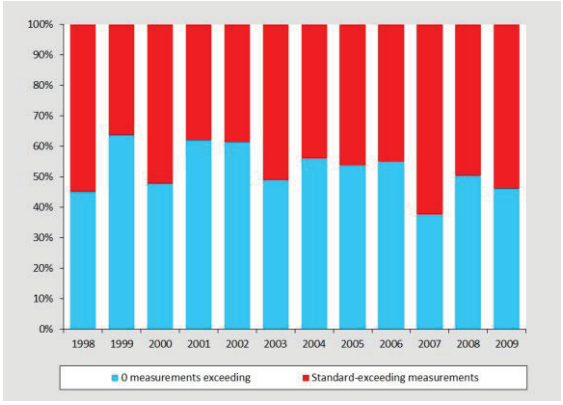


several different occasions at any given site. To compare the measured concentration with the MPC the designated method for this standard was followed, with the measurements being averaged over each month and then aggregated to the annual level by taking the 90-percentile value (i.e. the value below which 90% of the measurements lie). The results for three years in the period 1997-2009 are portrayed in Figure 1, which shows the percentage of measurements exceeding the MPC of the compound in question.
The first thing to note in this figure is the increase in national coverage from 1998 to 2009. This holds not only for the total number of monitoring sites included under the monitoring programmes, but also for the more balanced distribution of sites across the country.
The locations marked blue are 'clean': here no pesticides were found in concentrations exceeding the MPC (only sites where at least 10 measurements were made). At the locations marked yellow, less than 5% of the measurements exceeded the statutory limit. There are even regions in the Netherlands where over 5% (marked orange) or over 10% (red) of the measurements exceed the MPC level.
If we consider the percentage of pesticides exceeding the standards (not shown here) we see the same picture as for the measurements as such, that is to say, at most Dutch monitoring sites standards exceedance is due to a small number of compounds and to no more than several measurements a year. This holds for the entire country and is not specific to any individual region.

MPC exceedance by pesticides: temporal pattern

Year-on-year variation per monitoring site
Pesticide concentrations in Dutch surface waters vary from year to year. To gain an impression of how 'clean' the Netherlands is over the years, pesticide concentrations have been compared with the MPC. Because the MPC of a compound may change in the course of time, for this purpose the most recent MPC values were used (2009 database). Based on the monitoring results Figure 2 compares, for each of the years examined, the relative shares of 'clean' monitoring sites and sites with one or more MPC exceedances.

Figure 2: Share of monitoring sites with MPC exceedance. Blue: sites with no exceedance, red: sites with one or more compounds observed at a level exceeding the MPC.

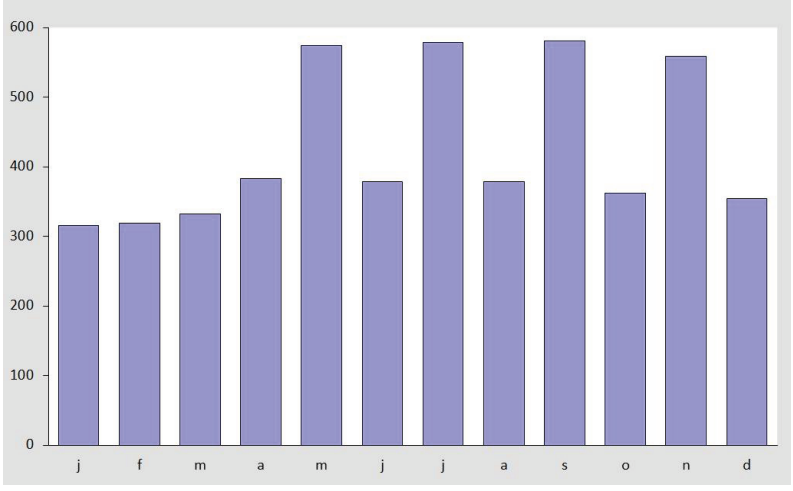


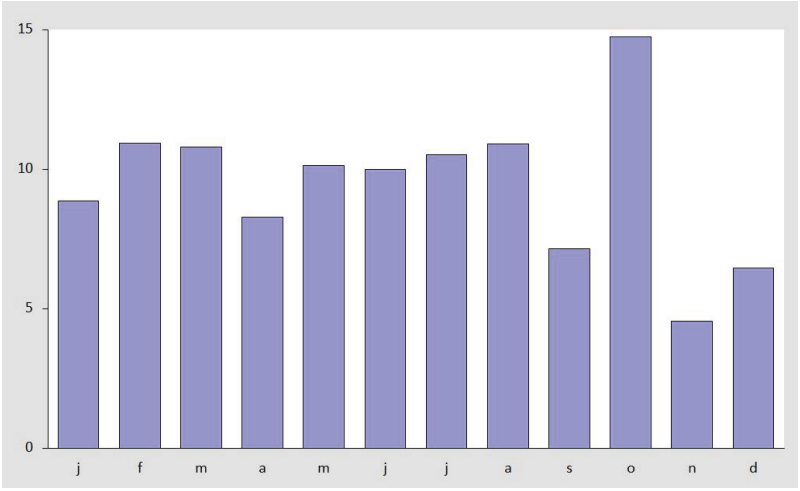
In 1998 around 45% of the 389 sites at which monitoring took place showed no MPC exceedance. In 2009 that percentage was approximately the same: 46%. This conclusion holds broadly for all the intervening years: over the years the percentage of 'clean' locations where there was absolutely no exceedance of the MTR standard has remained roughly the same. Similarly, the percentage of sites with measurements exceeding the MPC has also remained essentially unchanged over the years. At the same time, though, between 1998 and 2009 the number of monitoring sites rose by around 60%: from 447 in 1998 to 713 in 2009.

Seasonal variation of compounds

Water boards and other 'competent authorities' monitor the chemical quality of watercourses all the year round. Crops have a sow-grow-harvest cycle, during the various phases of which pesticide use is bound to vary, and the pesticide levels monitored at any given site correlate very much with the particular activities taking place on adjacent plots. Over the years approximately the same number of compounds are monitored each month. Nationwide, in 2009 the number varied from just over 300 to around 600.

Figure 3: Number of pesticides recorded monthly in 2009 (above) and percentage of compounds exceeding the MTR in each month of that year (below). J = January, F = February, M = March, etc.





The greatest number of pesticides are recorded in the months of May, July, September and November (see Figure 3). These figures derive from measurements at around 400 monitoring sites (www.bestrijdingsmiddelenatlas.nl). In the winter monitoring takes place at considerably fewer sites. This varied monitoring intensity is geared to the months in which most crops have their growing season and are thus still in the field. And this has a direct relation with the months in which pesticide use is highest.

In the months of February to August the percentage of compounds exceeding the standards is often around 10%. In October this percentage peaks further, with around 15% of the pesticides monitored exceeding the MPC. In this particular month about 300 compounds are included in the monitoring programme. The lowest percentage of exceedances occurs in the winter months of November and December. These colder and often wetter months are obviously outside the growing season for many crops, implying less pesticide use, with the possible exception of soil fumigants used to prepare the soil for the next early growing season. During this period monitoring is often focused more on the larger watercourses into which multiple streams flow. It should be noted, though, that there is little variation in the percentage of compounds exceeding the standards in the course of the year, varying essentially between 5 and 10% of the compounds. All in all, then, year-round monitoring of water quality, even outside the growing season of many crops, is by no means superfluous.

Which compounds cause most breaches of MPC standards?

Table 1 shows the compounds that exceeded the MPC most frequently in 1998, 2004 and 2009, ranked according to degree of exceedance using the following procedure. a) Compounds are ranked on the basis of the weighted number of monitoring sites at which the MPC for the compound was exceeded, i.e. correcting for the number of monitoring sites by taking the *percentage* exceedance of the standards. b) Compounds monitored at fewer than ten sites have been ignored. c) Allowance has been made for the degree of standards exceedance, weighting the results according to the following classes: 0 (<=MPC); 1 (> MPC and <= 2x MPC), 2 (>2x MPC and <5x MPC) and 5 (> 5x MPC exceedance). The compounds are

ranked on the basis of the degree of exceedance per monitoring site and the number of sites where the compound in question was monitored.

Table 1 Pesticides most frequently exceeding the MPC in 1998, 2004 and 2009, ranked according to the degree of exceedance, as explained in the text.

year	compound	no. of monit. sites	% exceedance	no. of water boards	no. of measurements	% exceedance
2009	Captan	38	47	4	194	13
2009	Desethyl-terbuthylazin	63	37	4	299	10
2009	Imidacloprid	452	44	26	2133	28
2009	Triflumuron	24	21	2	142	4
2009	Dicofol	24	17	2	142	3
2009	Omethoaat	31	16	3	169	3
2009	Foraat	51	14	3	313	2
2009	Captafol	15	27	1	29	14
2009	Fipronil	69	12	6	230	7
2009	Pyraclostrobin	66	17	5	341	7

year	compound	no. of monit. sites	% exceedance	no. of water boards	no. of measurements	% exceedance
2004	Imidacloprid	171	44	14	819	29
2004	Fenamifos	21	33	4	70	17
2004	Aldicarbsulfoxide	47	34	6	246	20
2004	ETU	19	26	2	118	6
2004	Pirimifos-methyl	161	20	15	899	7
2004	Chloorpyrifos	153	16	15	847	3
2004	Abamectine	63	13	9	292	9
2004	Carbendazim	211	17	19	1040	12

year	compound	no. of monit. sites	% exceedance	no. of water boards	no. of measurements	% exceedance
1998	Tetrachloorvinfos	24	71	2	288	14
1998	Pirimifos-methyl	43	67	4	361	34
1998	Bromofos-methyl	29	48	3	308	7
1998	Difenoconazool	20	35	2	189	5
1998	Aldicarbsulfoxide	38	61	4	246	26
1998	Propoxur	80	33	10	457	8
1998	Fenthion	46	43	6	426	26
1998	DDT, 24 en 44	46	22	7	263	44
1998	Telodrin	117	21	12	567	22

Accumulated Exceedance

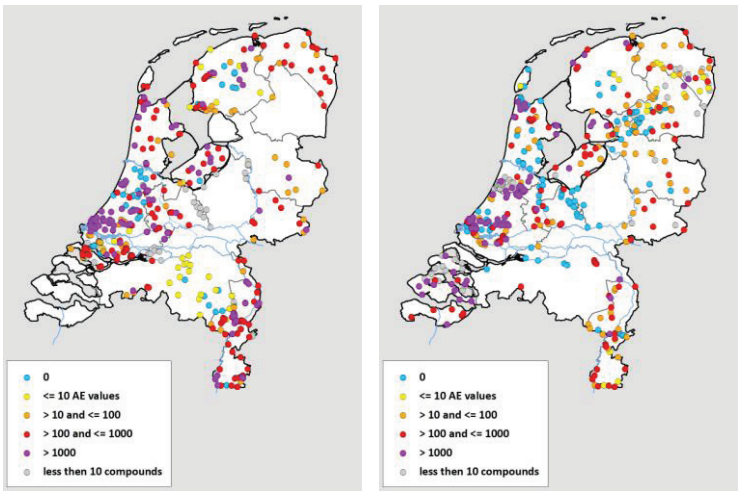
It is useful to express surface water pesticide loads in a single environmental quality indicator that takes in all the compounds detected at a given location. For this purpose the indicator Accumulated Exceedance (AE) is used, defined as follows:

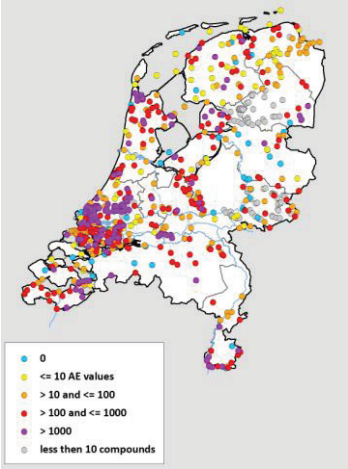
$$AE_{NRC} = \sum_{x=1}^{n} \left[\frac{conc_x - NRC_x}{NRC_x} \right]$$

where $conc_x$ is the measured concentration of compound x, n the total number of compounds and NRC_x the negligible risk level of compound x (= MPC/100).

For each monitoring site and each monitoring day the AE is calculated as indicated, summing the results for all the compounds for which monitoring data are available and then aggregating the results over a whole year. If fewer than ten compounds are measured in a particular sample, however, that sample is not included in the analysis. If the compound is present at a concentration below or equal to the NRC, it follows from the formula that the compound's contribution to the sum is zero. For compounds with a measured value below the limit of report (LOR) the concentration is taken to be zero, even though the actual concentration may have exceeded the NRC, and in some cases even the MPC. This procedure means that measurements reported as <LOR in fact lead to an underestimate of the AE. The results of the AE calculations are reported in map form in Figure 4 for the years 1998, 2004 and 2009.

Figure 4: Spatial mapping of Accumulated Exceedances (AE) in 1998, 2004 and 2009.

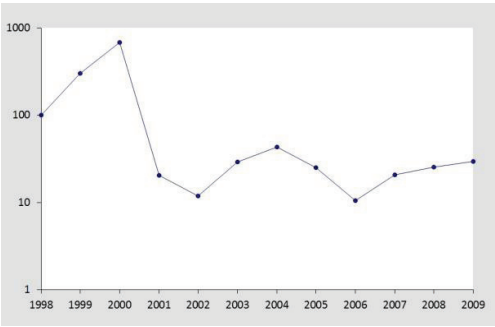




Over the years, the distribution of results over the various AE classes has remained largely unchanged. Areas with high AE values (in purple and red) are to be found in the south-west of the Netherlands, particularly in lower-lying polders and bulb-growing districts. Each year these areas are confronted with high pesticide loads. Noteworthy changes in pesticide loading can be seen along the Meuse and in the central part of the country. In the latter case the deterioration of water quality between 1998 and 2009 can be ascribed to the fact that in 1998 fewer than 10 compounds were monitored in this region, while in 2009 this figure was over 100. In the region along the Meuse the number of compounds monitored between 1998 and 2009 changed less dramatically: from around 30 to the range 31-100 compounds.

AE_{NRC} was also calculated for all the years in the form of a spatial analysis (not reproduced here). For the purpose of year-on-year comparison, the values for all the monitoring sites were summed, yielding a single value for each year for the Netherlands as a whole. The absolute value of this indicator can only be interpreted in combination with trends in the number of monitoring sites and the number of compounds monitored each year. The year-on-year environmental burden expressed as AE_{NRC} is shown in Figure 5.

Figure 5: Annual change in calculated Accumulated Exceedance, based on the results of the AE_{NRC} calculation, indexed to the year 1998 (=100). Note that the y-axis has a logarithmic scale.



For the years 2003-2009 the calculated AE values remain fairly stable and are around 30% of the value for 1998. This means that if the year 1998 is to be compared with the year 2009 based on these measurements there has been a 70% improvement in Dutch water quality, i.e. a 70% reduction in surface water pesticide loads.

INFLUENCES ON TRENDS

Reference year

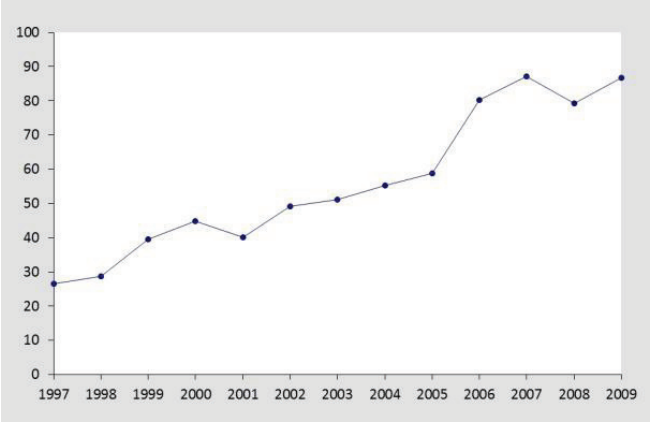
The question, obviously, is whether the observed changes in environmental burden are not due simply to the (rather arbitrarily chosen) initial and final years of the time series. The fact that 1998 has been taken as the reference year gives rise to the calculated difference between 2009 and that year. If 1997 (not included in the graph) or 1999 or 2000 had been taken as the reference year, the reduction would have been substantially greater. However, the difference between 1997 and 1998 is not due to: 1) changes in the number of compounds monitored, which remained virtually unchanged in these two years, 2) the number of monitoring sites, which in 1998 was in fact higher than in 1997, or 3) the compounds monitored, which also remained virtually unchanged. The year 1998, it appears, just happens to have a relatively low AE in the time series 1997-2000.

The overall trend of the last recent years is obviously not influenced by the choice of reference year, though. For a better understanding of AE_{NRC} Figure 6 shows the average number of compounds monitored annually per site as the basis for calculating Accumulated Exceedance.

Number of compounds

In the initial years the environmental burden was calculated on the basis of around 25-30 compounds per monitoring site on average, while in the final years of the time series this figure had risen to just over 80-90 compounds. Between 2003 and 2009 the total AE remained more or less stable, however. The number of compounds monitored, on which this calculation is based, rose by a factor of more than three between 2003 and 2009, however.

Figure 6: Average number of compounds monitored annually per site, as the basis for calculating Accumulated Exceedance.



When this result (Figure 6) is combined with the results on pollution load (Figure 5) it can be concluded that although substantially more compounds are now monitored, this has not led to an increase in AE. A second point to be noted is that compounds for which the measured value does not exceed the Limit of Report do not contribute to AE either (for these were assigned a zero value), but obviously do contribute to the total number of compounds over which the calculation is performed.

CONCLUSIONS

In the Netherlands surface water pesticide levels still frequently exceed statutory limits. Over the years, the relative area (amount of monitoring locations) of the country where there are no MPC exceedances has remained fairly constant. However, the absolute number of monitoring sites has increased, and the same trend can be observed with respect to the number of sites where limits were exceeded. Certain compounds that were no longer on the market were nonetheless still found in surface waters.

The percentage of monitored compounds found in concentrations exceeding the MPC decreased from 4% in 1997 to 1% in 2009. The remaining problems are thus due to only a small number of pesticides.

The months in which the greatest number of compounds as well as monitoring sites exceed the standards are May and July. At the same time exceedances are also observed in periods in which fewer crops are grown, including the winter months. Although the regional spread of MPC exceedance varies, the highest environmental loads are often to be found in the south-west of the country. As explained above, this load is calculated as the so-called Accumulated Exceedance (AE), covering a major portion of the pesticides monitored.

It can be concluded that pesticide loads in Dutch surface waters have declined, but not everywhere. From 1998 to 2009 it can be said that in this respect there has been a 70% improvement in general water quality.

ACKNOWLEDGEMENTS

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Behavior of Pesticides and Their Transformation Products in River Water in Japan

Kuniaki Kawata and Tomohiro Kose

INTRODUCTION

Pesticides serve an important role in increasing agricultural productivity. Several hundred different pesticides are used worldwide for agricultural production. In Japan, more than five hundred compounds are registered as pesticides for agricultural use. Manufactured pesticides exceeded 200 varieties and 250 metric kilotons in FY2009. Paddy rice farming has been playing an important role in crop production throughout the world. In Japan, paddy fields covered a total area of 2.496 million ha, accounting for 54.3% of all land cultivated in FY2010. Approximately 170 pesticides designated for rice farming are applied to paddy fields in Japan.

Pesticides applied to paddy fields have continually caused great concern because of pesticide runoffs into rivers: some fraction of the applied pesticides flow from the paddy fields into the rivers through drainage channels. Numerous reports have described pesticide behaviors in paddy fields and rivers in Japan. In this section, we summarize the behaviors of applied pesticides on paddy fields and the runoff ratios of pesticides from paddy fields to rivers. Second, we describe the daily variation of pesticides based on hourly and bihourly variations. Third, the concentration levels of pesticides in river waters and the typical weekly variations of pesticide concentrations are summarized. Fourth, we describe concentration levels of some pesticides and their transformation products in river water. Finally, from the standpoint of the human health protection, we discuss the impact of some pesticides regulated by the Environmental Quality Standard in Japan.

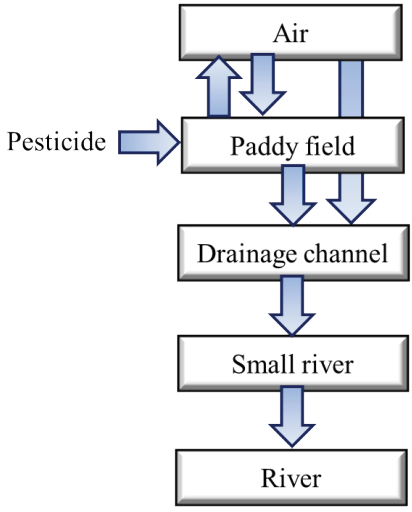
FATES OF APPLIED PESTICIDES IN PADDY FIELDS

Insecticides and fungicides are sprayed to paddy fields by aerial and ground applications. A diagram of pesticides applied to a paddy field is presented in Fig. 1. Parts of the aerially applied pesticides exist in the air around the applied paddy areas (Kawata 2009). Pesticides in the air drift to leeward sites. They can be a major source of that detected in the drainage channels and rivers immediately after aerial applications (Yoshida et al. 2007). Most of the applied pesticides became distributed between the soil and water in the paddy fields.

Regarding herbicides, most are applied by ground application (Amano et al. 2001, Maeda et al. 2008). The applied herbicides are dissolved or suspended in paddy water. Some pesticides in the paddy water are drained with drainage and leakage of the paddy water (Amano et al. 2001, Maeda et al. 2008, Morohashi et al. 2012). The

ratios of the pesticide amounts in the paddy soils increase according to the decrease of paddy water volumes. The half-lives of herbicides mefenacet, dymron, and bromobutide in the water were estimated as 1.8–7.7 days, 2.1–9.6 days, and 2.4–3.1 days (Ishii et al. 2004, Morohashi et al. 2012). The decrease of residue pesticides in the paddy soils can be interpreted using first-order reaction kinetics. In addition, the respective half-lives of phthalide, mefenacet, dymron and bromobutide in the soil were estimated as 20-31 days, 12-21 days, 30 days and 5.2-10 days, respectively (Iwashita et al. 2008, Ishii et al. 2004, Morohashi et al. 2012).

Fig. 1. Diagram of an applied pesticide moving from a paddy field to a river.



RUNOFF OF PESTICIDES FROM PADDY FIELDS TO RIVERS

Some pesticides applied to paddy fields flowed into rivers via drainage channels. The runoff events of the aerially applied pesticides occurred in the nearest drainage channels immediately after the application. The runoff events were also caused by rainfall (Hasukawa et al. 2009; Maeda et al. 2008). It is reported that transport of pesticides by surface runoff during rainfall events is a major process contributing to pesticide contamination of rivers (Fox et al. 2007; Guo et al. 2004). However, the runoff immediately after the application was the major process for the aerial applied pesticides to paddy fields. In cases of ground applications, the runoff of pesticides from paddy fields was caused mainly by the drainage of paddy waters at several days after application, and by rainfall (Morohashi et al. 2012).

The runoff ratios of pesticides from paddy fields to drainage channels or rivers are presented in Table 1. The reported values depended on the paddy fields themselves and water control as well as the application methods and weather conditions as described above. Among the pesticides, the runoff ratios of fungicides and

insecticides were 0.06–9.4% and 0.05–3.8%. However, those of herbicides were 0.1–43%; more than 10% of some herbicides, such as bromobutide, cafenstrol, dymron and simetryn, drained off of paddy fields. Therefore, herbicides have the potential outflows from the paddy fields with drained water.

Table 1. Runoff ratios of pesticides from paddy fields in Japan.

Pesticide	CAS No.	Use*	Runoff ratio (%)	Prefecture	Year	Reference
Bromobutide	74712-19-9	H	1.60, 25.5	Shiga	2002	Sudo and Kawachi 2006
			29.6	Shiga	2005	Hasukawa et al. 2009
			12– 43	Niigata	2009	Morohashi et al. 2012
			24.5, 30.8	Shiga	2005, 2006	Kawasaki et al. 2008
			0.8	Aomori	2006	Ministry of Environment 2007
			0.8	Saitama	2006	Ministry of Environment 2007
			16, 17. 9	Hokkaido	2008, 2010	Ministry of Environment 2009, 2011
Buprofezin	69327-76-0	I	0.3, 1.3	Niigata	1995, 1996	Mitobe et al. 1999
Cafenstrole	125306-83-4	H	8.9–26.7	Shiga	2002	Sudo and Kawachi 2006
			4.74	Shiga	2005	Kawasaki et al. 2008
Clomeprop	84496-56-0	H	0.0004	Niigata	2003	Kawata et al. 2005
			0.9	Shiga	2005	Hasukawa et al. 2009
Dinotefuran	248583-16-1	I	0.1–11.4	Akita	2009, 2010	Ministry of Environment 2010, 2011
Dymron	42609-52-9	H	22.5	Ibaraki	1998	Nakano et al. 2004
			26.3, 36.5	Shiga	2005, 2006	Kawasaki et al. 2008
			16-17	Ibaraki	2007, 2008	Ministry of Environment 2008, 2009
			0.1–6.4	Shimane	2009	Ministry of Environment 2010
Esprocarb	85785-20-2	H	8.2	Ibaraki	1998	Nakano et al. 2004
			0.32, 0.84	Shiga	2005, 2006	Kawasaki et al. 2008
Etofenprox	80844-07-1	I	0.05–0.2	Niigata	1995-1997	Mitobe et al. 1999
Fenitrothion	122-14-5	I	0.6–1.8	Niigata	1995-1997	Mitobe et al. 1999
Fenobucarb	3766-81-2	I	3.2	Niigata	1997	Mitobe et al. 1999
Flutolanil	66332-96-5	F	0.8–7.0	Niigata	1995-1997	Mitobe et al. 1999
Isoprothiolane	50512-35-1	F	5.0, 8.1, 9.2	Niigata	1995-1997	Mitobe et al. 1999
			1.8–6.6	Shiga	1996-2001	Sudo et al. 2002
Mefenacet	73250-68-7	H	14.5	Ibaraki	1998	Nakano et al. 2004
			1.1–2.0	Shiga	2002	Kawasaki et al. 2008
			5.33, 5.49	Shiga	2005, 2006	
			5.2–7.6	Shimane	2006-2008	Ministry of Environment 2007-2009

Table 1. (Cont.) Runoff ratios of pesticides from paddy fields in Japan.

Pesticide	CAS No.	Use*	Runoff ratio (%)	Site	Year	Reference
Mepronil	55814-41-0	F	6.1	Niigata	1995	Mitobe et al. 1999
Oxaziclomefone	153197-14-9	H	0.037	Niigata	2003	Kawata et al. 2005
			7.7	Saitama	2006	Ministry of Environment 2007
Pencycuron	66063-05-6	F	0.7–4.0	Niigata	1995-1997	Mitobe et al. 1999
Phthalide	87-41-2	F	0.3, 0.4	Shiga	2005	Hasukawa et al. 2009
			1.6, 1.7	Niigata	2005	Shiota et al. 2006
			1.7, 2.4	Niigata	2006	Maeda et al. 2008
			1.6–3.1	Niigata	1995-1997	Mitobe et al. 1999
Pencycuron	66063-05-6	F	0.7, 1.9, 4.0	Niigata	1995-1997	Mitobe et al. 1999
Pretilachlor	51218-49-6	H	14.1	Ibaraki	1998	Nakano et al. 2004
			9.14, 11.3	Shiga	2005, 2006	Kawasaki et al. 2008
			2.2 –26. 53	Akita	2006-2008	Ministry of Environment 2007-2010
Pyributicarb	88678-67-5	H	9.6	Ibaraki	1998	Nakano et al. 2004
Pyridaphenthion	119-12-0	I	1.3– 3.8	Niigata	1995-1997	Mitobe et al. 1999
Simetryn	1014-70-6	H	10.2–25.1	Shiga	2002	Sudo and Kawachi 2006
			18.6, 21.5	Shiga	2005, 2006	Kawasaki et al. 2008
			22.9	Saitama	2006	Ministry of Environment 2007
Thenylchlor	96491-05-3	H	0.9, 13.9	Shiga	2002	Sudo and Kawachi 2006
			5.52	Shiga	2005	Kawasaki et al. 2008
Thiobencarb	28249-77-6	H	0.1–1.6	Shiga	2002	Sudo and Kawachi 2006
			0.32, 0.84	Shiga	2005, 2006	Kawasaki et al. 2008
			15.9	Saitama	2006	Ministry of Environment 2007
Tricyclazole	41814-78-2	F	0.06– 9.4	Niigata	1995-1997	Mitobe et al. 1999

* F, Fungicide; H, herbicide; I, insecticide.

VARIATION OF PESTICIDES IN RIVER WATER

Daily Variation

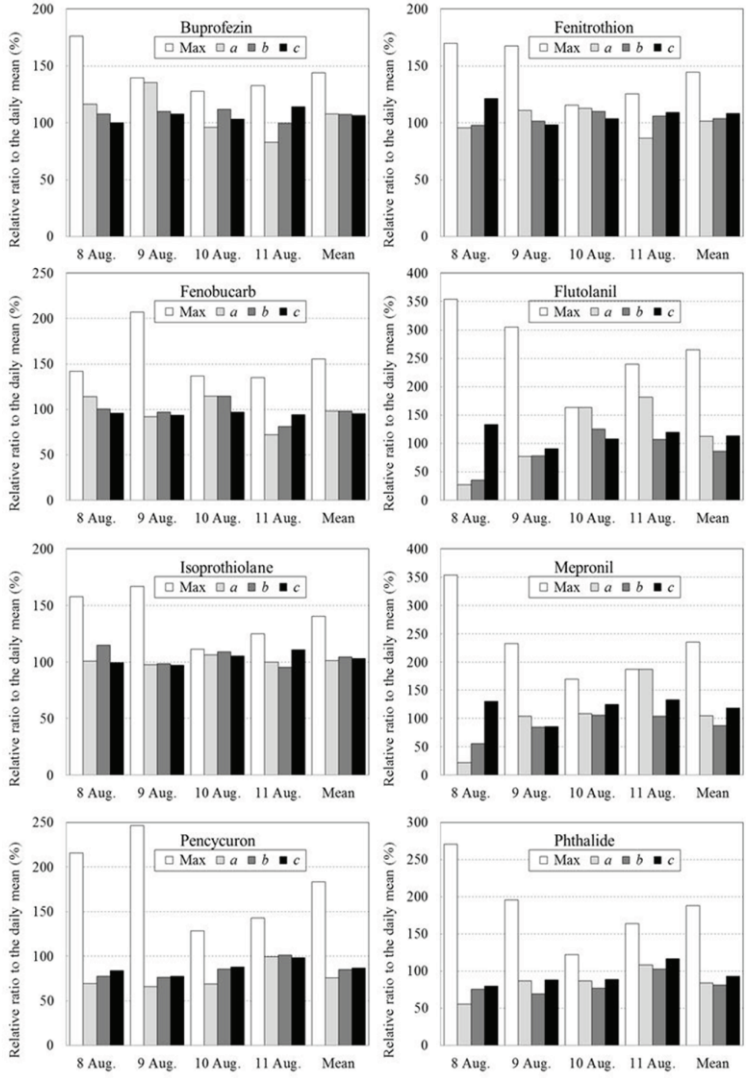
Rivers with cultivated areas in their basins are affected directly by these applied pesticides in the areas. Therefore, these rivers have been used to evaluate the pesticide runoff events from the cultivated areas. The pesticide concentrations in the waters from these rivers varied markedly during short periods. Hourly and bihourly variations in 11 pesticide concentrations in river waters after aerial applications during August 7 to August 12 were reported (Tanabe and Kawata, 2009). The targets were mainly applied aerially to paddy fields in the contributory area during the investigated period. The pesticides were detected at high concentrations after their applications. Differences in the times between the applications and high concentrations depended on the distances of the applied area and the sampling site as well as their properties and applied amounts (Tanabe and Kawata, 2009).

Figure 2 presents the relative concentration ratios of four kinds of concentrations of the eight pesticides out of the 11 compounds for the daily representative mean values from August 8 to 11. Buprofezin, fenitrothion and fenobucarb are insecticides; flutolanil, isoprothiolane, mepronil, pencycuron and phthalide are fungicides. The pesticide concentrations at 10 a.m. and the mean values at 10 a.m. and 4 p.m. were within 70–150 % of the daily mean concentrations, except for the values (less than 70%) of flutolanil, mepronil and phthalide on August 8. Data of that day showed increases of pesticide concentrations during 6 to 10 p.m. The concentrations at 10 a.m. for flutolanil on August 10-11 and mepronil on August 11 were greater than 150% of the corresponding daily means, because the concentrations increased at around 10 a.m. In these cases, the pesticide applications during the day or the previous day caused significant variations in their concentrations in the river waters. However the mean values at 10 a.m., 2 p.m. and 6 p.m. were within 70–150% of the daily mean concentrations. Therefore, the concentration at 10:00 a.m. is regard as the representative daily value in many cases in the river. However, the concentration might be different from the daily mean value during the day or on the day following the pesticide application. Therefore, the mean concentrations at 10 a.m., 2 p.m. and 6 p.m. of a day were recommended as the best representative value of the day in this case (Tanabe and Kawata, 2009).

Long Term Variation

It is difficult to evaluate the appropriate variations and the maximum concentrations in river waters by daily or weekly samplings immediately after the applications. However, long- term variations of pesticide concentrations in river waters were commonly evaluated based on weekly or biweekly samplings. The concentration levels of some pesticides in river waters are presented in Table 2. The concentration level of a pesticide in a river was affected mainly by the amount of a pesticide applied in the basin, the distance from paddy fields, and the flow rate of the river water.

Typical weekly variations of pesticide concentrations in the Shin River, Niigata Prefecture, are depicted in Fig. 3. The maximum concentrations of herbicides



mefenacet and simetrin were observed in May and July, respectively; those of fungicides (phthalide and isoprothiolane) and insecticides (fenitrothion and febobucarb) were observed in July–August (Mitobe et al. 1999). The herbicide concentrations were generally higher in May
and June than in July and August, which reflects that the herbicides are applied in the paddy fields of the investigated river basin mostly during May through June. On the other hand, insecticides and fungicides were detected at the highest concentrations in June–July and July–August, respectively. The difference depended on their application periods, i.e., the insecticides were applied mainly in June–July, and the fungicides in July –August.

Table 2. Pesticide concentrations in river waters in Japan.

Pesticide	CAS No.	Use*	Concentration (ng/L)		Site	Prefecture	Month	Year	Reference
			Max	Mean					
Atrazine	1912-24-9	H	150-170	60-140	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
			180-210	5-160**	Shin R.	Niigata	Apr.-Sep.	1996-1997	Mitobe et al. 1999
			190-430	–	40 rivers	Mie	May-Feb.	1999, 2000	Hayakawa et al. 2000
Bromobutide	74712-19-9	H	3440-9760	–	Drainage channels	Shiga	May-Aug.	2002	Sudo and Kawachi 2006
			1400-4900	–	Tadagawa R.	Hyogo	Apr.-Feb.	2004-2005	Yoshida and Fujimori 2005
			49400, 78500	–	Shiratori R.	Shiga	Apr.-Oct.	2005, 2006	Kawasaki et al. 2008
			6200	720	Shinano R.	Niigata	May-Jul.	2007, 2008	Goto et al. 2009
			4400	430	Agano R.	Niigata	May-Jul.	2007, 2008	Goto et al. 2009
			3300-15000	–	Kakogawa R.	Hyogo	May-Nov.	2008	Suzuki et al. 2010
			3700	–	Tsurumi R.	Kanagawa	Apr.-Mar.	2008-2009	Sakai 2010
Buprofezin	69327-76-0	I	12-28	12-20	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
			20-820	20-140**	Shin R.	Niigata	Apr.-Sep.	1997	Mitobe et al. 1999
Cafenstrole	125306-83-4	H	5700-10200	–	Drainage channels	Shiga	May-Aug.	2002	Sudo and Kawachi 2006
			480-1600	–	Tadagawa R.	Hyogo	Apr.-Feb.	2004-2005	Yoshida and Fujimori 2005
			4020	–	Shiratori R.	Shiga	Apr.-Oct.	2005, 2006	Kawasaki et al. 2008
			140	47	Shinano R.	Niigata	May-Jul.	2007, 2008	Goto et al. 2009
			79	29	Agano R.	Niigata	May-Jul.	2007, 2008	Goto et al. 2009
			23-400	–	Kakogawa R.	Hyogo	May-Nov.	2008	Suzuki et al. 2010
			80	–	Tsurumi R.	Kanagawa	Apr.-Mar.	2008-2009	Sakai 2010
Diazinon	333-41-5	I	50-320	24-57	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
			13	–	Sakura R.	Ibaraki	Apr.-Aug.	2008	Iwafune et al. 2010
			2800	–	Tsurumi R.	Kanagawa	Apr.-Mar.	2008-2009	Sakai 2010
Dymron	42609-52-9	H	13500, 38000	–	Shiratori R.	Shiga	Apr.-Oct.	2005, 2006	Kawasaki et al. 2008
			790	–	Miya R.	Nagano	Apr.-Oct.	2003-2007	Ishimota et al. 2010
			1000-5400	–	Tadagawa R.	Hyogo	Apr.-Feb.	2004-2005	Yoshida and Fujimori 2005
			4760, 6320	–	Sakura R.	Ibaraki	Apr.-Aug.	2007, 2008	Iwafune et al. 2010
				23.3	Tsurumi R.**	Kanagawa	June-Aug.	2008	Ninomiya et al. 2010

Table 2.（Cont.）Pesticide concentrations in river waters in Japan.

Pesticide	CAS No.	Use*	Concentration (ng/L)		Site	Prefecture	Month	Year	Reference
			Max	Mean					
Esprocarb	85785-20-2	H	150-5000	30-110*	Shin R.	Niigata	Apr.-Sep.	1995-1997	Mitobe et al. 1999
			100-470	60-140	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
			680-2380	–	Shiratori R.	Shiga	Apr.-Oct.	2005, 2006	Kawasaki et al. 2008
			100	32	Shinano R.	Niigata	May-Jul.	2007, 2008	Goto et al. 2009
			495, 1070	–	Sakura R.	Ibaraki	Apr.-Aug.	2007, 2008	Iwafune et al. 2010
			30	–	Tsurumi R.	Kanagawa	Apr.-Mar.	2008-2009	Sakai 2010
Etofenprox	80844-07-1	I	50-150	10-100*	Shin R.	Niigata	Apr.-Sep.	1995-1997	Mitobe et al. 1999
			37-200	8-56	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
			200	–	Tadagawa R.	Hyogo	Apr.-Feb.	2004-2005	Yoshida and Fujimori 2005
Fenitrothion	122-14-5	I	820-4200	50-420*	Shin R.	Niigata	Apr.-Sep.	1995-1997	Mitobe et al. 1999
			84-1700	36-210	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
			200	–	Tadagawa R.	Hyogo	Apr.-Feb.	2004-2005	Yoshida and Fujimori 2005
			990	–	Tsurumi R.	Kanagawa	Apr.-Mar.	2008-2009	Sakai 2010
Fenobucarb	3766-81-2	I	160-1900	20-190*	Shin R.	Niigata	Apr.-Sep.	1995-1997	Mitobe et al. 1999
			56-1300	29-150	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
			43	11	Shinano R.	Niigata	May-Jul.	2007, 2008	Goto et al. 2009
			15	7.1	Agano R.	Niigata	May-Jul.	2007, 2008	Goto et al. 2009
			93, 135	–	Sakura R.	Ibaraki	Apr.-Aug.	2007, 2008	Iwafune et al. 2010
				1.6	Tsurumi R.**	Kanagawa	June-Aug.	2008	Ninomiya et al. 2010
			60	–	Tsurumi R.	Kanagawa	Apr.-Mar.	2008-2009	Sakai 2010
Fenthion	94734-40-4	I	10-30	10-20*	Shin R.	Niigata	Apr.-Sep.	1996, 1997	Mitobe et al. 1999
			230	–	Tsurumi R.	Kanagawa	Apr.-Mar.	2008-2009	Sakai 2010
Flutolanil	66332-96-5	F	120-3900	50-400*	Shin R.	Niigata	Apr.-Sep.	1995-1997	Mitobe et al. 1999
			28-200	21-58	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
			810	–	Tadagawa R.	Hyogo	Apr.-Feb.	2004-2005	Yoshida and Fujimori 2005
Iprobenfos	26087-47-8	F	720-3600	30-220*	Shin R.	Niigata	Apr.-Sep.	1996, 1997	Mitobe et al. 1999
			210-870	79-180	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001

Table 2.（Cont.）Pesticide concentrations in river waters in Japan.

Pesticide	CAS No.	Use*	Concentration (ng/L)		Site	Prefecture	Month	Year	Reference
			Max	Mean					
Isoprothiolane	50512-35-1	F	3400-5300	180-540*	Shin R.	Niigata	Apr.-Sep.	1995-1997	Mitobe et al. 1999
			1300-8200	290-810	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
			130-270	–	Tadagawa R.	Hyogo	Apr.-Feb.	2004-2005	Yoshida and Fujimori 2005
			7.5	5.5	Shinano R.	Niigata	May-Jul.	2007, 2008	Goto et al. 2009
Mefenacet	73250-68-7	H	410-6100	40-100*	Shin R.	Niigata	Apr.-Sep.	1995-1997	Mitobe et al. 1999
			1000-3200	280-830	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
			1370-4900	–	Drainage channels	Shiga	May-Aug.	2002	Sudo and Kawachi 2006
			930	–	Miya R.	Nagano	Apr.-Oct.	2003-2007	Ishimota et al. 2010
			430-1300	–	Tadagawa R.	Hyogo	Apr.-Feb.	2004-2005	Yoshida and Fujimori 2005
			3890-22600	–	Shiratori R.	Shiga	Apr.-Oct.	2005, 2006	Kawasaki et al. 2008
			220	69	Shinano R.	Niigata	May-Jul.	2007, 2008	Goto et al. 2009
			57	32	Agano R.	Niigata	May-Jul.	2007, 2008	Goto et al. 2009
			1160, 1170	–	Sakura R.	Ibaraki	Apr.-Aug.	2007, 2008	Iwafune et al. 2010
			49, 72	–	Kakogawa R.	Hyogo	May-Nov.	2008	Suzuki et al. 2010
			1100	–	Tsurumi R.	Kanagawa	Apr.-Mar.	2008-2009	Sakai 2010
Mepronil	55814-41-0	F	780-6600	60-100*	Shin R.	Niigata	Apr.-Sep.	1995-1997	Mitobe et al. 1999
			190-900	63-310	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
			320	–	Shinano R.	Niigata	May-Jul.	2007, 2008	Goto et al. 2009
Pencycuron	66063-05-6	F	590-2300	130-380*	Shin R.	Niigata	Apr.-Sep.	1995-1997	Mitobe et al. 1999
			160-300	28-300	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
Phthalide	87-41-2	F	1800-3900	110-650*	Shin R.	Niigata	Apr.-Sep.	1995-1997	Mitobe et al. 1999
			130-990	38-150	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
			150-170	–	Tadagawa R.	Hyogo	Apr.-Feb.	2004-2005	Yoshida and Fujimori 2005
			680	260	Anno R.	Niigata	Aug.	2005	Shiota et al. 2006
			860	170	Anno R.	Niigata	Aug.-Dec.	2006	Maeda et al. 2008
			62	16	Shinano R.	Niigata	May-Jul.	2007, 2008	Goto et al. 2009
			16	9.3	Agano R.	Niigata	May-Jul.	2007, 2008	Goto et al. 2009

Table 2.（Cont.）Pesticide concentrations in river waters in Japan.

Pesticide	CAS No.	Use *	Concentration (ng/L)		Site	Prefecture	Month	Year	Reference
			Max	Mean					
Pretilachlor	51218-49-6	H	80-5100	40-270**	Shin R.	Niigata	Apr.-Sep.	1995-1997	Mitobe et al. 1999
			490-5200	160-710	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
			2160-7270	–	Drainage channels	Shiga	May-Aug.	2002	Sudo and Kawachi 2006
			470	–	Miya R.	Nagano	Apr.-Oct.	2003-2007	Ishimota et al. 2010
			310-740	–	Tadagawa R.	Hyogo	Apr.-Feb.	2004-2005	Yoshida and Fujimori 2005
			13600-15600	–	Shiratori R.	Shiga	Apr.-Oct.	2005, 2006	Kawasaki et al. 2008
			1550, 2020	–	Sakura R.	Ibaraki	Apr.-Aug.	2007, 2008	Iwafune et al. 2010
			160-420	–	Kakogawa R.	Hyogo	May-Nov.	2008	Suzuki et al. 2010
			90	–	Tsurumi R.	Kanagawa	Apr.-Mar.	2008-2009	Sakai 2010
Propyzamide	23950-58-5	H	440-2600	400-440	Shin R.	Niigata	Apr.-Sep.	1996-1997	Mitobe et al. 1999
				10	Yodo R.	Osaka		2008	BYQ 2011
Pyributicarb	88678-67-5	H	40-1600	30-240*	Shin R.	Niigata	Apr.-Sep.	1995-1997	Mitobe et al. 1999
			82-700	29-160	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
			78-90	–	Tadagawa R.	Hyogo	Apr.-Feb.	2004-2005	Yoshida and Fujimori 2005
			7.5	7.1	Shinano R.	Niigata	May-Jul.	2007, 2008	Goto et al. 2009
Pyridaphenthion	119-12-0	I	1900-10000	160-920*	Shin R.	Niigata	Apr.-Sep.	1995-1997	Mitobe et al. 1999
			22-140	22-59	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
Simazine	122-34-9	H	40	40	Shin R.	Niigata	Apr.-Sep.	1996	Mitobe et al. 1999
			16-180	16-99	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
			40-180	–	40 rivers	Mie	May-Feb.	1999, 2000	Hayakawa et al. 2000

Table 2.（Cont.）Pesticide concentrations in river waters in Japan.

Pesticide	CAS No.	Use*	Concentration (ng/L)		Site	Prefecture	Month	Year	Reference
			Max	Mean					
Simetryn	1014-70-6	H	910-2100	–	Shin R.	Niigata	Apr.-Sep.	1995-1997	Mitobe et al. 1999
			230-1600	57-170	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
			10200-25100	70-80**	Drainage channels	Shiga	May-Aug.	2002	Sudo and Kawachi 2006
			80	–	Miya R.	Nagano	Apr.-Oct.	2003-2007	Ishimota et al. 2010
			160	–	Tadagawa R.	Hyogo	Apr.-Feb.	2004-2005	Yoshida and Fujimori 2005
			12700, 49800	–	Shiratori R.	Shiga	Apr.-Oct.	2005, 2006	Kawasaki et al. 2008
			2310, 2340	–	Sakura R.	Ibaraki	Apr.-Aug.	2007, 2008	Iwafune et al. 2010
			42-510	–	Kakogawa R.	Hyogo	May-Nov.	2008	Suzuki et al. 2010
Thenylchlor	96491-05-3	H	50-320	76-700	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
			2200, 3500	10, 160**	Shin R.	Niigata	Apr.-Sep.	1996, 1997	Mitobe et al. 1999
			900-13900	–	Drainage channels	Shiga	May-Aug.	2002	Sudo and Kawachi 2006
			880	–	Shiratori R.	Shiga	Apr.-Oct.	2005	Kawasaki et al. 2008
			17	8.2	Shinano R.	Niigata	May-Jul.	2007, 2008	Goto et al. 2009
			126, 169	–	Sakura R.	Ibaraki	Apr.-Aug.	2007, 2008	Iwafune et al. 2010
Thiobencarb	28249-77-6	H	300-4300	50-83	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
			440	40, 70**	Shin R.	Niigata	Apr.-Sep.	1996, 1997	Mitobe et al. 1999
			800-1600	–	Drainage channels	Shiga	May-Aug.	2002	Sudo and Kawachi 2006
			35	11	Shinano R.	Niigata	May-Jul.	2007, 2008	Goto et al. 2009
			2.6	–	Agano R.	Niigata	May-Jul.	2007, 2008	Goto et al. 2009
			16, 20	–	Kakogawa R.	Hyogo	May-Nov.	2008	Suzuki et al. 2010
Thiuram	1634-02-2	F		100	Yodo R.	Osaka		2008	BYQ 2011
Tricyclazole	41814-78-2	F	30-5100	30-4800**	Shin R.	Niigata	Apr.-Sep.	1995-1997	Mitobe et al. 1999
			36-7700	35-7700	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001

* F, Fungicide; H, herbicide; I, insecticide. ** Median. ** Tsurumi R,.Sakai R. and Kashiwao R.

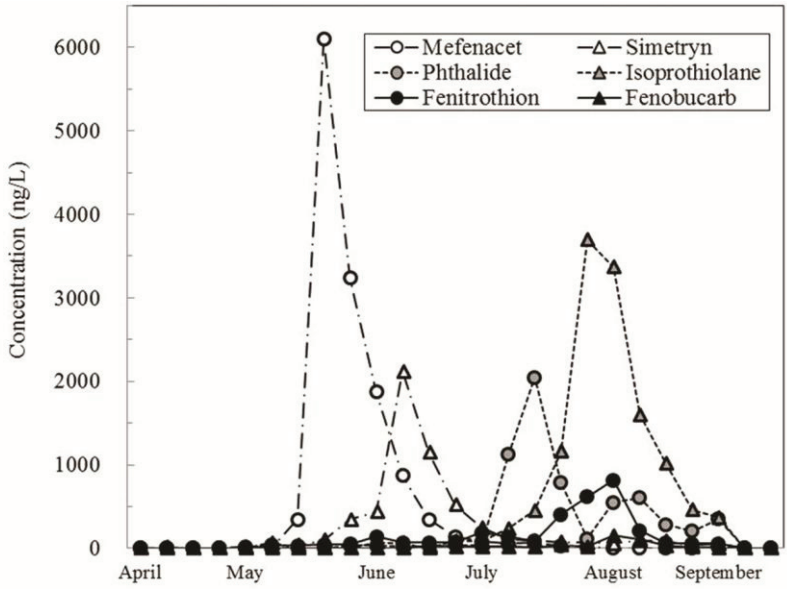


Fig. 3. Typical weekly variation of pesticide concentrations in Shin River, Niigata Prefecture (constructed based on Mitobe et al. 1999).

PESTICIDES AND THEIR TRANSFORMATION PRODUCTS

Organophosphates and Their Sulfoxide Oxons

Sulfoxide oxons are typical transformation products of organophosphorus pesticides. The thiono (P=S) type organophosphates are easily oxidized to their oxono (P=O) type oxons by chlorination (Kamoshita et al. 2007, Onodera et al. 1976). The oxons derived from the organophosphates have become a human health concern, because some oxons are reported to inhibit the acetylcholinesterase activity, and because this inhibition was stronger than that caused by the parent organophosphate. Moreover, some oxons were degraded only slightly compared to the parent organophosphates. Therefore, they can remain in water for longer times than the organophosphates can (Arai et al. 2005).

The concentrations of some oxons as well as the original organophosphates in river waters are presented in Table 3. Butamifos oxon, fenitrothion oxon, fenthion oxon, isoprothiolane oxon and Isoxathion oxon were detected in river waters together with their parent pesticides. In contrast, isoprothiolane oxon and tolchlofos methyl oxon were only detected without their parent pesticides.

Iwafune et al. (2010) reported that the ratios of fenthion oxon to fenthion were 0.048-0.062 in Sakura River. The ratios of butamifos oxon to butamifos were 0.30-0.61 (mean 0.42) in the Shin River (Mitobe et al. 1999). Regarding fenthion, two additional transformation products, fenthion sulfone and fenthion sulfoxide, were

detected at 16 and 158 ng/L in maximum, respectively (Iwafune et al. 2010). Their ratios to fenthion were 0.375–6.28 for the sulfone and 3.70–51.1 for the sulfoxide. An oxon of pyributicarb, a carbamate herbicide, was detected in the Chitose River, Hokaido (Kanetoshi et al. 1999). The maximum concentrations of pyributicarb and its oxon were 690 ng/L and 500 ng/L, respectively.

Bromobutide and Bromobutide-debromo

Bromobutide, a commonly used amide herbicide in Japan, is well known to degrade to bromobutide-debromo, N-(α,α-dimethylbenzyl)-3,3-dimethylbutyramide via metabolism (Isobe et al. 1984) and a photochemical reaction (Takahashi et al. 1985). Bromobutide-debromo was detected in river water to the degree presented in Table 4. The molar concentration ratios, R (%), of bromobutide-debromo to the sum of bromobutide and bromobutide-debromo in the river water were 100% at maximum in Sakura River (Iwafune et al. 2010) and 5.3–100 (56 in mean) in Shin River (Mitobe et al. 1999). The R values in paddy waters increased as time elapsed to 31–34% at 18 days after application (Morohashi et al. 2012), which implies that bromobutide degradation to bromobutide-debromo was promoted in the river after runoff from paddy fields.

Triazine Herbicides, Their Dealkylated Metabolites and Hydroxylated Metabolites

Atrazine and simazine are typical s-triazine herbicides. They degrade to dealkyl compounds and hydroxyl compounds. The reported concentrations of atrazine and simazine are presented in Table 2. Moreover, atrazine was detected at 144 ng/L at maximum from the Urikai River, Hokkaido, in 2006 (Ministry of Environment 2007). Simazine (1800 ng/L) and its transformation product hydroxysimazine (94 ng/L) were detected in groundwater from the lower reaches of the Shinano River. Transformation products of two kinds, deisopropylyatrazine and didealkylatrazine, were also detected at 110 ng/L and 2000 ng/L, respectively (Tanabe and Kawata 2004).

Other Pesticides and Their Hydrolysates

Benfuracarb (CAS No. 82560-54-1) and carbosulfan (CAS No. 39995-74-9) are carbamate insecticides. Their hydrolysates, carbofuran and carbofuran-3-hydroxy, were detected with benfuracarb (Table 5) from the Sakura River (Iwafune et al. 2010). A triazole herbicide, cafenstrol (CAS No. 125306-83-4), is degraded gradually to its main metabolite, cafenstrol-descarbamoyl, by photolysis in water. The transformation product was detected in the Sakura River as shown in Table 5. Clomeprop (CAS No. 84496-56-0) is an anilide herbicide. Although clomeprop itself shows no herbicidal activity against plants, its hydrolyzed phenoxyacetic acid, 2-(2,4-dichloro-3-methylphenoxy) propanoic acid (DMPA), has an auxinic herbicidal activity. DMPA and clomeprop were detected (Table 5) from the Sakura River.

Dichlobenil, 2,6-dichlorobenzonitrile (CAS No. 1194-65-6), is an aromatic nitrile herbicide. that is degraded to the persistent metabolite 2,6-dichlorobenzamide

(BAM) in water (Pukkila et al. 2009) and soil (Holtzeet al. 2006). The concentration levels of dichlobenil and BAM in river waters are presented in Table 5. BAM was detected in the Shin River and the Shinano River together with its parent pesticide. The ratios of 2,6

Table 3. Concentrations of oxons and their parent pesticides in river waters.

Pesticide	CAS No.	Use *	Concentration (ng/L)		Site	Prefecture	Month	Year	Reference
			Max	Mean					
Butamifos oxon			20	–	Shin R.	Niigata	Apr.-Sep.	1996	Mitobe et al. 1999
Butamifos	36335-67-8	H	50-210	40-50**					
Butamifos oxon			14-18	–	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
Butamifos			28-34	–					
Fenitrothion oxon			4	–	Sakura R.	Ibaraki	Apr.-Aug.	2007, 2008	Iwafune et al. 2010
Fenitrothion	122-14-5	I	63, 387	–					
Fenthion oxon			3	–	Sakura R.	Ibaraki	Apr.-Aug.	2007, 2008	Iwafune et al. 2010
Fenthion	94734-40-4	I	3, 43	–					
Isofenphos oxon			45-120	25-79	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
Isofenphos	25311-71-1	I	<20	–					
Isoprothiolane oxon			10	–	Shin R.	Niigata	Apr.-Sep.	1996	Mitobe et al. 1999
Isoprothiolane	50512-35-1	F	3700	180**					
Isoxathion oxon			3300	70**	Shin R.	Niigata	Apr.-Sep.	1997	Mitobe et al. 1999
Isoxathion	18854-01-8	I	290	–					
Tolchlofos methyl oxon			16	–	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
Tolchlofos methyl	57018-04-9	F	<10	–					

* F, Fungicide; H, herbicide; I, insecticide. ** Median.

Table 4. Concentrations of bromobutide and its debromo metabolite in river waters.

Pesticide	Concentration (ng/L)		Site	Prefecture	Month	Year	Reference
	Max	Mean					
Bromobutide-debromo	50-190	20-50*	Shin R.	Niigata	Apr.-Sep.	1995-1997	Mitobe et al. 1999
Bromobutide	930-1900	30-110*					
Bromobutide-debromo	27-56	14-23	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
Bromobutide	240-610	67-150					
Bromobutide-debromo	228, 296	–	Sakura R.	Ibaraki	Apr.-Aug.	2007, 2008	Iwafune et al. 2010
Bromobutide	4870, 11100	–					
Bromobutide-debromo	223, 318	–	Sakura R.	Ibaraki	Apr.-Aug.	2009, 2010	Iwafune et al. 2011
Bromobutide	11900, 13700	–					

* Median.

Table 5. Concentrations of pesticides and their typical transformation products in river waters.

Transformation product (Pesticide)	CAS No.	Use*	Concentration (ng/L)		Site	Prefecture	Month	Year	Reference
			Max	Mean					
Carbofuran	1563-66-2		257, 153	–	Sakura R.	Ibaraki	Apr.-Aug.	2007, 2008	Iwafune et al. 2010
Carbofuran-3-hydroxy	16655-82-6		1	–				2008	
(Benfuracarb)	82560-54-1	I	4, 2	–				2007, 2008	
(Carbosulfan)	55285-14-8	I	<1	–				2007, 2008	
Cafenstrol-descarbamoyl			330–, 859	–	Sakura R.	Ibaraki	Apr.–Aug.	2007-2010	Iwafune et al. 2010, 2011
(Cafenstrol)	125306-83-4	H	441–815	–					
DMPA			829–1540	–	Sakura R.	Ibaraki	Apr.-Aug.	2008	Iwafune et al. 2010, 2011
(Clomeprop)	84496-56-0	H	39–100	–					
BAM			160, 200	40-80**	Shin R.	Niigata	Apr.-Sep.	1996, 1997	Mitobe et al. 1999
(Dichlobenil)	1194-65-6	H	30, 90	20, 30**					
BAM			62-240	31-88	Shinano R.	Niigata	Apr.-Aug.	1996	Tanabe et al. 2001
(Dichlobenil)			18-76	15-29					
Pyrazolynate-destosyl			73–629	–	Sakura R.	Ibaraki	Apr.-Aug.	2007, 2008	Iwafune et al. 2010, 2011
(Pyrazolynate)	58011-68-0	H	7–66	–					

* F, Fungicide; H, herbicide; I, insecticide. ** Median.

dichlorobenzamide to dichlobenil were 0.88-–11 (mean 3.3) in the Shin River (Mitobe et al. 1999). A pyrazol herbicide, pyrazolynate(CAS No. 58011-68-0), is degraded to its destosyl compounds. Both compounds were detected in the Sakura River.

Acephate, O,S-dimethyl acetylphosphoramidothioate (CAS No. 30560-19-1) is an organophosphorus insecticide that is well known to degrade to methamidophos, O,S-dimethylphosphoramidothioate (CAS No., 10265-92-6), via hydrolysis. Methamidophos is also an organophosphate, but it has been banned for use as an insecticide in Japan. Although acephate and methamidophos were detected in agricultural soil at 0.01 mg/kg and 0.02 mg/kg (Ministry of the Environment 2009), respectively, at maximum, acephate was not detected (<8 μg/L) in river waters in Japan (BYQ 2011).

IMPACT OF PESTICIDES ON HUMAN HEALTH

In Japan, four pesticides are regulated with environmental quality standards (EQSs) for water pollution by the basic Environment Low. In addition, 12 pesticides are designated as monitored substances. The monitored substances are identified as needing further observation, and the guideline values (MGVs) are given for the 11 pesticides except for one herbicide, chlornitrofen. These standards and guidelines particularly apply to annual mean value.

The impact of some pesticides with EQSs and MGVs is discussed from the standpoint of the human health protection by comparing their maximum concentrations in river water with their EQSs and MGVs. Table 6 shows the target regulated pesticides except for 1,3- dichloropropene, dichlorvos and EPN, which have not been detected in the past 15 years. Among the pesticides, maximum values of fenitrothion exceeded the MGV value twice out of 55 times of sampling in the Shin River in 1997. The exceeded values were 3.1 μg/L (3100 ng/L) in April and 4.2 μg/L in August (Mitobe et al. 1999). Although the annual mean of the site was not reported, the median, 0.42 μg/L, was lower than the MGV. Therefore, the levels of pesticides found in the river waters were less than their respective regulation-mandated limits.

Table 6. Maximum concentrations of regulated pesticides in river waters

Pesticide	CAS No.	Use*	EQS or MGV（μg/L）	Max concentration in river water（μg/L）	Reference
Chlorothalonil	1897-45-6	F	50	<0.5***	BYQ 2011
Copper 8-quinolate	10380-28-6	F	40	<0.4***	BYQ 2011
Diazinon	333-41-5	I	5	2.8	Sakai 2010
Fenitrothion	122-14-5	I	3	4.2	Mitobe et al. 1999
Fenobucarb	3766-81-2	I	30	1.9	Mitobe et al. 1999
Iprobenfos	26087-47-8	F	8	3.6	Mitobe et al. 1999
Isoprothiolane	50512-35-1	I	40	8.2	Tanabe et al. 2001
Isoxathion	18854-01-8	I	8	<0.08**	BYQ 2011
Propyzamide	23950-58-5	H	8	2.6	Mitobe et al. 1999
Simazine	122-34-9	H	3**	0.18	Hayakawa et al. 2000
Thiobencarb	8249-77-6	H	20**	0.44	Mitobe et al. 1999
Thiuram	1634-02-2	F	6**	0.1**	BYQ 2011

* F, Fungicide; H, herbicide; I, insecticide. ** EQS. *** Annual mean.

CONCLUSION

In this section, we particularly examined the behavior and distribution of pesticides in rivers in Japan. We summarized the behaviors of pesticides that are typically applied on paddy fields and the runoff of pesticides from paddy fields to rivers. The runoff of pesticides from paddy fields was caused mainly by paddy water drainage and rainfall. The maximum runoff ratio was greater than 30%. Second, we described the daily variation of pesticides in a river based on hourly and bihourly variations. The mean concentrations at 10 a.m., 2 p.m., and 6 p.m. of each day were recommended as the best representative values of the day in investigated cases. Third, the concentration levels of pesticides in river waters and the typical weekly variations of pesticide concentrations were summarized. The typical seasonal variations of pesticides reflect the pesticide application periods. Fourth, we described concentration levels of some pesticides and their transformation products in river water. The transformation product ratio to the parent pesticide in water increased as time elapsed after application. Finally, we discussed the impacts of some pesticides. The levels of pesticides found in the river waters were less than their regulated levels.

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An Overview of Organochlorinated Pesticide Residues in Albania.
Case study: Porto Romano, Adriatic Sea

Aurel Nuro, Elda Marku

ABSTRACT

The findings reported here are parts of a biomonitoring study to determine the concentration and distribution of organochlorinated pesticide residues in mussel's *Mytilus Galloprovincialus* in a station considered hot-spot of Albania Coast, Porto-Romano located in Adriatic Sea. Organochlorinated pesticides such as DDT, Lindane, Hexachlorbenzene, Heptachlor and Aldrine were used widely before 90' in Albania for agricultural purposes. The former Chemical Enterprise located in Porto-Romano produced pesticides for almost two decades, mostly Lindane. In working period Lindane Plant has generated pollution because of discharging industrial wastes directly to the sea. As a result of recent development in agriculture, after 90' the use of pesticides in general has decreased. Tones of pesticides due to the inappropriate conditions of conservation and storage have been damaged in this area but not only. Considerable part of stock pesticides finished also to the sea because the mismanagement and atmospheric factors.

Study of pesticide residues in marine waters located in this area was carrying out using sentinel organisms. Mussels have been shown to concentrate many organic contaminants by a factor of 10 above ambient sea water levels, providing a direct representation of pollutant bioavailability. The mussel *Mytilus Galloprovincialis* were used because of their physiology and accumulation processes are well known, have a large geographic distribution in the Adriatic Sea and easy to find. Mussel samples were collected in November for years 2002-2008. The direct measurements of chlorinated contaminants need sophisticated and costly methods, with some difficulties for their application to the monitoring of Water Sea. Biomonitoring is based on the ability of mussels to concentrate chemical contaminants in tissues in relation to their biodisponibility.

The mussel samples firstly were homogenized with anhydrous sodium sulfate after that extracted by water bath assisted extraction. Clean-up procedure was performed using sulfuric acid for lipids hydrolyze followed by a second purification on florisil column. HP 6890 Series II gas chromatograph equipped with a ^{63}Ni micro-electron capture detector was used for determination and quantification of organochlorinated pesticides. The column used for separation of pesticides was a HP-5 (25 m x 0.33 mm I.D., 0.25µm film).

The presence of organochlorinated pesticide residues in mussel samples indicates the presence of these contaminants in Porto-Romano station. The old industries represent the major polluting factor, followed by agricultural contribution and other possible sources of contamination such as the water-based contribution, atmospheric

factor, etc. Levels of pesticides in analyzed mussel samples were between 98.2-611.3 ng/g fresh weights. There is a comparability between our data obtain for other areas of Adriatic Sea. Note that the schedule in years was not a regress linear function as expected. These could be affected by other possible sources of contamination such as mismanagement of oddments pesticides, irrigation of surfaces where these pesticides were used by rainwater, drift water-based contribution and atmospheric component. The most frequently detected pesticides were *p,p*-DDE followed by HCHs and HCB. Impact of organochlorinated pesticides in this area will be evident for years to come because of their stability and slow degradation in marine waters, adding the shift of them between water column and respective sediment. Trend of pesticides in this area must continue because of pesticide presence in elevated concentrations.

Keywords: Biomonitoring; organochlorinated pesticides; water analysis; gas chromatography

INTRODUCTION

Persistent organochlorines such as organochlorinated pesticides (OCPs), polychlorinated pesticides (PCBs) and Dioxins are a group of compounds of great chemical stability and persistence whose presence in the environment is a clear indication of anthropogenic pollution. The massive use of pesticides for agricultural purposes caused their widespread diffusion to all environmental compartments including a wide range of organisms up to the humans. The term pesticide is used to indicate any substance, preparation or organism used for destroying pests. Synthetic pesticides have been used since in the early to mid twentieth century. The modern history of pesticides dates back to World War II when for the first time the insecticidal properties of DDT were recognized. DDT was first introduced on a large scale to fight fleas, lice, flies and mosquitoes and reduce the spread of insect borne diseases such as malaria and yellow fever. Many public health benefits have been realized by the use of pesticides, but their potential impact on the environment is substantial too (Di Muccio, 1996). OCP are used extensively as insecticides sterilizes, and herbicides. In particular, those used as insecticides are extremely toxic to living bodies. Most OCPs have been progressively restricted and then banned in the 1970s in most industrialized countries a widespread environmental pollution has resulted from their use in agriculture and civil uses. Organoclorine pesticides (OCP) in general are lipophilic compounds with noticeable chemical and environmental stability. The accumulation of organochlorine compounds is connected with their chemical structure and their physical properties such as polarity and solubility. Bioacumulation of these compounds from organisms depend on feeding behaviours, habitat, age, sex, state of health as well as the lipid composition of the animal's tissue or organ.

Before 90' organochlorinated pesticides were used widly in Albania for agricultural purposes. The main agricultural areas were in the western of the country (Shkodra,

Durresi, Tirana, Fieri, Lushnja, Vlora) but almost every were in the country had been developed different directions of agricultural (fruits, corns, vegetables, etc.). The most used organochlorinated pesticides were DDT, Lindane, HCB, Aldrins and Heptachlors. For some decades until 1990, the former chemical plant in Porto-Romano, Durres produced sodium dichromate, for leather tanning, and pesticides, such as lindane (gamma-HCH) and thiram. Both productions processes have been idle since that time and the plant's buildings have been totally destroyed after 90'. When the plant was operating, it produced 6-10 tons of lindane per year. Technical HCH mixture is produced by the photochlorination of benzene. It contains 65-70% a-HCH; 7-10% b-HCH; 14-15% g-HCH (lindane); 7% d-HCH; 1-2% e-HCH and 1-2% of other chlororganic compounds, e.g., heptachlor. Lindane is separated by extraction with methanol. The mixture of the other HCH-isomers can be converted by thermal treatment to useful byproducts like tri-chlorbenzene and hydrogen chloride. Industrial wastes of chemical plant were discharged for many years directly to the sea.

The scale of pesticides use after 90' in agriculture has decreased, due the change of soil structure. Emigration of many peoples in western country and free movement inside the country were two main factors that impact directly in agriculture areas and it's developing. Use of pesticides generally has decreased, because of large areas were not using for agricultural purposes and other areas were used for building new houses and industries. Except this, many chemical industries, include Lindane Plant, were stopped or destroyed. Unfortunately families have been living in this area of plant after destroying. New houses were building near this territory. The former has generated the expired pesticides, which due to the inappropriate conditions of conservation and storage have been damaged. The other part of expired or out of use pesticides, to be disposed of, has been distributed in various districts of the country. Mismanagement of oddments pesticides, for some years after 90' was another source of pesticides contamination not only in area of chemical plant but in a diameter higher; include the waters of Adriatic Sea. This area is considered a "hot-spot" of pollution in Albania. Different studies and analyses showed extremely high levels of technical HCH mixtures in the area of the plant and in storage facilities located two kilometers away. Studies were conducted in this area in different samples (soil, water and milk). Levels of HCHs for soil sample in area of the former chemical plant in Porto-Romano were 8.79 mg/g. The sample also contained chlorinated benzenes. The HCH content in water sample was 4 µg/l. Milk samples showed a HCH content of 12.86 µg /kg of HCHs.

Study of pesticide residues in marine waters (Adraiatic Sea) located near ex-chemichal plant of Porto-Romano was carrying out using sentinel organisms. The mussel *Mytilus Galloprovincialis* were used because of their physiology and accumulation processes are well known, have a large geographic distribution in the Adriatic Sea and easy to find (Colombo *et al.,* 1995; Dame, 1996; Farrington *et al.,* 1983). The direct measurements of chlorinated contaminants need sophisticated and costly methods, with some difficulties for their application to the monitoring of Water Sea. Monitoring of organochlorinated pesticides were based on the ability of mussels to concentrate chemical contaminants in their tissues from filtering Water Sea. Mussels have been shown to concentrate many organic contaminants by a

factor of 10 above ambient sea water levels, providing a direct representation of pollutant in studding are. Contamination of the area from the remains of former Lindane plant was the main factor. Marine waters currents in the Adriatic Sea usually moving in the timetable direction. Several important rivers in the south of Porto-Romano station such as Shkumbi, Semani and Vjosa River can bring significant amounts of organic pollutants due to agricultural land rinsing. Water basins of these rivers covers wide are that was used and continues to be used for agricultural purposes.

Although in some European countries the use of organochlorinated pesticides was banned before 80' the presence of organochlorinated pesticides was reported in other studies of the last ten years realize in Adriatic and Mediterran Sea in different types of samples. Higher levels are reported for mussels and fish and other biota samples because of bioconcentration, bioaccumulation and biomagnifications processes. Habitat of these species and their place in the food chain are factors that explain found levels for organic pollutants. Levels of chlorinated organic pollutants (pesticides and PCBs) found in sediment coming next. Levels for organochlorinated pesticides vary according to geographic position of sediment sampling. Found levels in water are lower due to their dilution and the solubility of these compounds in water. It was noticed a movement of organic pollutants from the countries where they applied to other areas. This is related to the hydrological cycle of Adriatic Sea. Levels of organic pollutants in marine waters also depend on the sampling period, water column and the random flow mainly from rivers that discharge to the Adriatic Sea.

MATERIALS AND METHOD

Sampling of Mussel Galloprovincialus

Natural mussels *Mytilus Galloprovincialis* were used for this study. Samples were collected in November for years 2002-2008 in the same station near the old former of Chemical Plant, Porto-Romano, Adriatic Sea. The study included for each year the analyses of 75 mussel *Galloprovincialus* samples devised in 5 groups by 15 members. Division was based on mussel's length (indirectly the age of mussel samples). Sampling was made according to the MAP No. 7, rev. 2, 1984 and UNEP/MED Wg.128/2, 1997. The samples were transferred to an aluminum container and stored at -10°C.

Preparation of samples for organochlorinated pesticide analysis

The method used was based on EN 1258/1/2/3/4 for determination of organochlorinated pesticides in fat samples. Tissues of mussel samples were used for analytical procedure. A metallic knife was used. Tissues of a 15 members of each class were collected together in a beaker. The samples firstly were homogenized with anhydrous sodium sulfate after that extracted by water bath assisted extraction. The internal standard PCB 29 was added to the sample prior to extraction. The extract was purified by shaking with 15g silica gel with 45% sulfuric acid. The organic and acid phases were separated. After filtration, the extract was

concentrated in a Kuderna Danish to 5 ml volume and a second purification on a column of florisil with 5% water (particle size 0.063±0.2 mm; Merck, Darmstadt, Germany) was performed. The organ chlorines were eluted with 15 ml of a mixture of hexane/dichloromethane (5/1), (v/v). The extract was concentrated and transferred to hexane (Spectroscopy grade; Fluka, Germany), and analyzed by GC-ECD (Barcelo*, 1991;* Schantz *et al., 1993)*.
Procedural blanks were regularly performed and all results presented are corrected for blank levels. All glassware was rigorously cleaned with detergent followed by pyrolysis at 250°C. The sodium sulfate, florisil and silica gel were pre-extracted with hexane/dichloromethane (4/1) in a Soxhlet extractor, dried in 250°C for 12 hours and were rinsed with hexane/dichloromethane (4/1) just before utilization.

Apparatus and chromatography
Gas chromatographic analyses were performed with an HP 6890 Series II gas chromatograph equipped with a ^{63}Ni micro-electron capture detector and a split/splitless injector. The column used was a HP-5 [low/mid polarity, 5% (phenyl methyl siloxane)] (25 m x 0.33 mm I.D., 0.25µm film). The split/splitless injector and detector temperatures were set at 280^{0}C and 320^{0}C, respectively. Carrier gas was He at 1 ml/min and make-up gas was nitrogen with 25 ml/min. The initial oven temperature was kept at 60^{0}C for 4min, which was increased, to 200^{0}C at 20^{0}C/min, held for 7 min, and then increased to 280^{0}C with 4^{0}C min. The temperature was finally increased to 300^{0}C, at 10^{0}C/min, held for 7min. Injection volume was 2µl, when splitless injections were made. OCP quantification was performed by internal standard method. PCB 29 was used as internal standard. The following organchlorinated pesticides: Hexachlorobenzene (HCB), Dieldrin, Endrin, alfa-, beta-, delta- and gama-isomer (Lindane) of Hexachlorocyclohexane, Heptachlor, Heptachlor epoxide, DDT-related chemicals (*o,p*-DDE, *p,p*-DDE, *p,p*-DDD, *p,p*-DDT), Methoxychlor and Mirex were detected.

RESULTS AND DISCUSSION

Mussel *Mytilus Galloprovincialis* were used for evaluate the levels of organochlorinated pesticides in marine waters of Adriatic Sea. This station is considered a "hot-spot" area for organic pollutants in Albania. For all investigated years were detected the presence of organochlorinated pesticide residues in mussel samples indicates the occurrence of these contaminants in our coast. They are not in use in our country since 90'. Were expected that levels for this class of organic pollutants to be a regressive linear function. The levels must be in this direction because of their degradation process. The old industries such as Lindane chemical plant in Porto-Romano, Durres represent the major polluting factor for this area. Mismanagement of pesticides and other possible sources of contamination such as agricultural contribution, water-based contribution, atmospheric factor, etc.
Total of organochlorinated pesticides for years 2002-2008 was shown in Figure 1. Maximum of OCPs was for mussel samples for year 2007 with 611.3 ng/g fresh

weight sample. Minimum were shown for year 2003 (98.2 ng/g fresh weight) and for year 2008 (101.7 ng/g fresh weight). There is a comparability between our data obtain for other areas of Adriatic Sea (Andral *et al.,* 1997). We should emphasize that the schedule in years for station on study, is not a linear function as expected. Regression linear function noted if excludes the levels for organochlorinated pesticides for years 2006 and 2007. Higher levels of OCPs for these years could be because of new arrival from stocks of pesticides near this area. Rainsed of land, in this area or from other areas, marine waters currents and causality of levels in mussel samples could be other factor.

Distribution of studied organochlorinated pesticides was shown in Figure 2. The most frequently detected pesticides for Porto-Romano stations for all years were *p,p*-DDE followed by HCH (γ-isomer) and HCB. These would have principally agricultural component although there are also other possible sources of contamination such as mismanagement of oddments pesticides, irrigation of surfaces where these pesticides were used. Other sources could be rainwater, water-based contribution and atmospheric component. Note that HCHs levels were not higher than other pesticides. Lindane and other isomers were produced for many years near this area. These results could be because of HCHs and other pesticides degradation time, their physical-chemical properties and their biodisponibility to accumulate in mussel's tissue. DDT was used in a wide range and for almost all agricultural areas in all country before 90'. DDT degradation and stability for it and its metabolites could be the main factor for DDTs levels and distribution. Mirex was not in use in our country. It was found almost for all studied samples. The higher levels were for years 2006 and 2007. This is a clear connection between found levels and water currents in Adriatic Sea.

Figure 1. Total of organochlorinated pesticides in mussel samples for years 2002-2008

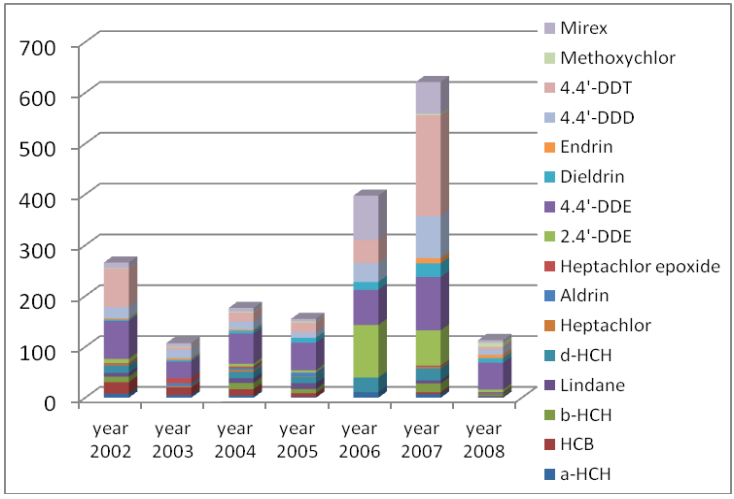
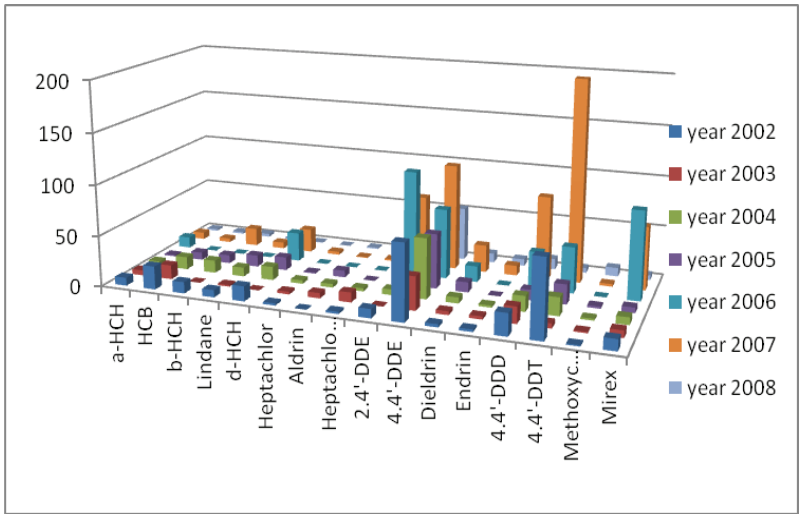


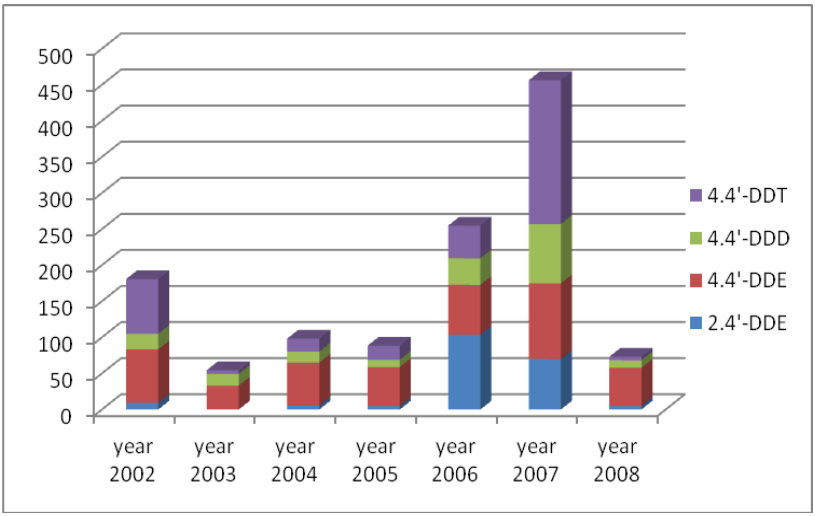
Figure 2. Distribution of organochlorinated pesticides in mussel samples



DDT and their metabolites

The concentrations for DDTs were presented in Figure 3. Mussels of 2007 year have the high concentration of DDT and its metabolite products. ∑DDT (o,p'-DDE; p,p'-DDE; p,p'-DDD and p,p'-DDT) was quantified in mussel tissues in concentrations between 45.35 and 448.7.31 ng/g fresh weight. Range of DDT levels found for each year was the same with total of organochlorinated pesticides in mussel samples. Levels of DDTs for years 2006 and 2007 were higher. It was noted that DDT contribute was higher than its metabolites for year 2007. This could be because of new DDTs arrival from mismanagements of stocks of this pesticide. p,p'-DDT was found in 93% of samples collect in concentration between 0-75.7 ng/g. p,p'-DDE was present in all samples, enormous contaminant too in each of them. Concentration of p,p'-DDE was in an interval between 9.04-175.13 ng/g. The banning of DDT use after 90' in Albania and it degradation process are both factor that explain fact that p,p'-DDE was found in elevated concentration. Note that p,p'-DDE has a great potential for magnification in the food chain and it is very persistent between other breakdown products of DDT. Unfortunately it was toxicity much higher than DDT. The low DDT/DDE ratios (exclude the found levels for year 2007) recorded in the current study indicates the lack of recent use for DDT formulations. Levels of other metabolites p,p'-DDD and o,p'-DDE were lower than p,p'-DDE. Their levels were elevated for years 2006 and 2007. Degradation process and their stability were the main factors. DDTs levels were higher than reported concentrations in other study for biota samples in Adriatic Sea.

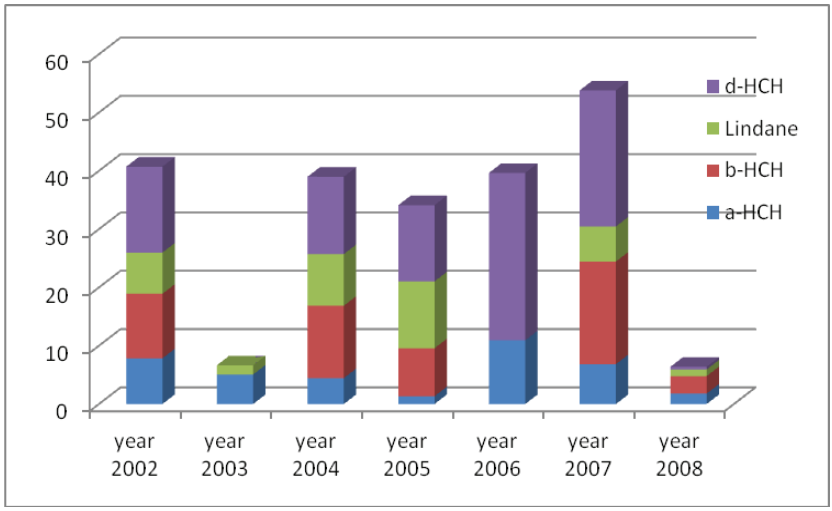
Figure 3. DDTs in mussel samples for years 2002-2008



Lindane and its isomers

Concentration of hexachlor cyclohexanes (HCH) found in musel samples was presented in Figure 4. ∑HCH ranging from 5.75 ng/g fresh weight sample (year 2008) to 52.82 ng/g fresh weight sample (year 2007).This sum is going on according a descending linear function besides the HCHs values found during the years 2006 and 2007. Technical HCH isn't in use in our country but the present concentrations are being influenced by past produce and a use of HCHs. Lindane was not the major contributor. Concentrations of other Lindane isomer were highest in mussel samples. These isomers and other compounds such as chlor-benzenes were considered waste of Lindane fabric. These wastes were discharged directly to the sea. Degradation process, physical-chemical properties for HCH isomers build their relative contribute. Concentration of b- HCH was higher than the other isomers' because of its higher tendencies to accumulate in fatty tissue. It was noted that beta isomer is missing for samples of years 2003 and 2006. Alfa and delta isomers of HCH were found almost for all analyzed samples. They come into in industrial process of Lindane produce. These isomers and other compound were separate from technical Lindane and were treated as waste. This was the main factor for their presence in mussel samples for Porto-Romano station. The α/γ HCH ratio, an indicator of current technical HCH application, was lower. Again this likely reflects recent use of HCH formulations. Discharging of waste in working period for Lindane chemical plant before 90' and after that mismanagement of waste repositories of ex-chemical plant were the main factors for found levels in this station. Found levels for Lindane and its isomers were in the same range with other reports for Adriatic and Mediterrean Sea.

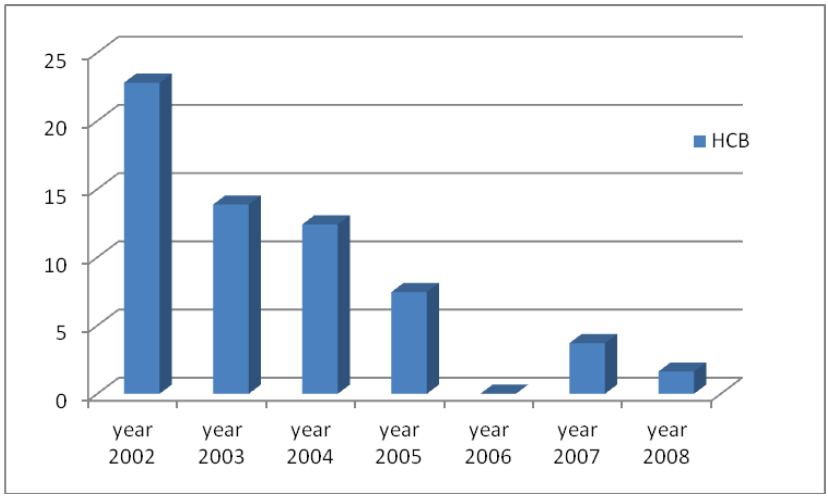
Figure 4. Lindane and its isomers in mussel samples for years 2002-2008



Hechachlorbenzene (HCB)

Level of HCB was shown to be a regressive linear function as expected for all pesticides (Figure 5). The higher level of HCB was for year 2002 with 22.4 ng/g fresh weight sample. Minimum was for year 2006 where HCB was not detected. HCB was used wide in Albania as insecticide in fruit trees. HCB concentration was in a regressive linear function because of recent use and it degradation process. Degradation period of HCB is shorter than main part of organochlorinated pesticides but it was found almost for all samples. Degradation of HCB and for other pesticides is slower notably in sediments. Sediments and water column interact between each other exchanging pollutants using physical process of absorption and desorption. Generation of HCB and other pesticides from sediment could be a significant factor for found levels. Levels of levels of Hexachlorbenzene could be because it's before use of discharging the waste from the chemical plant in Porto-Romano. Chlorinated benzenes could be secondary product of Lindane production. Chlorobenzenes were reported in other studies for soil, water and milk samples in this area.

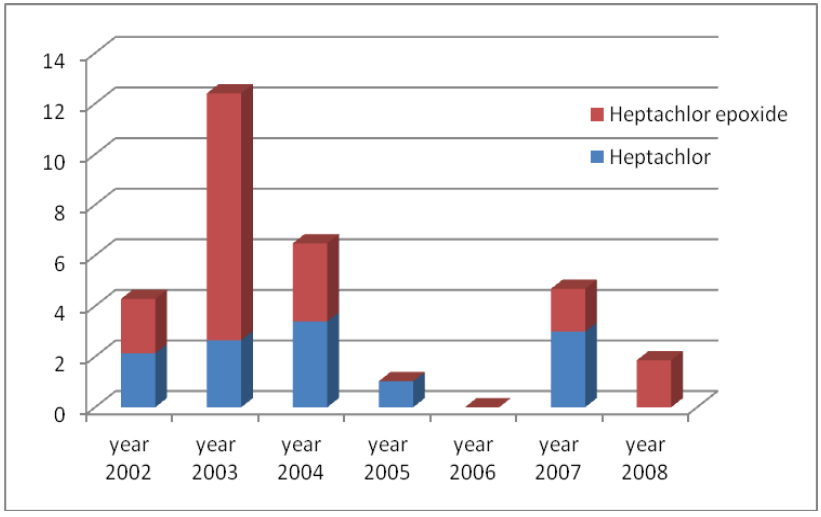
Figure 5. HCB in mussel samples for years 2002-2008



Heptachlor and Heptachlorepoxide

Heptachlor and its metabolite Heptachlorepoxide were found in a range from 0 (year 2006) to 12.4 ng/g fresh weight for year 2003 (Figure 6). Levels of Heptachlors were noted to be almost a regressive linear function. Noted that metabolite of Heptachlor, its epoxide was the main contributor. Their levels and distribution was because of recent use of Heptachlor for agricultural purposes.

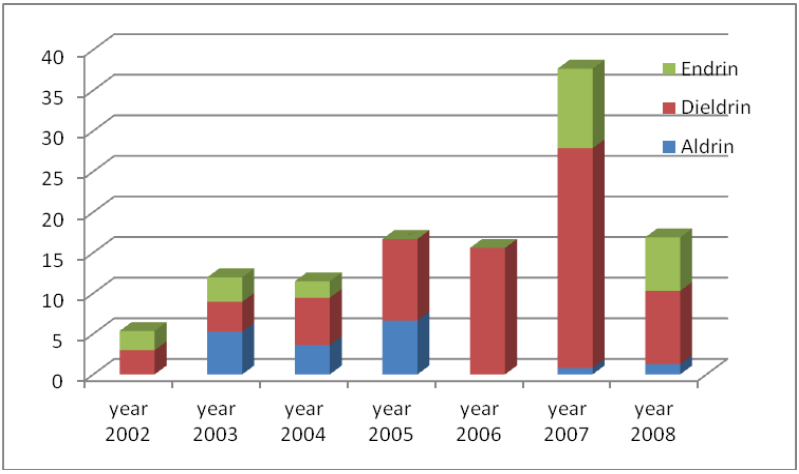
Figure 6. Heptachlor and Heptachlorepoxides in mussel samples for years 2002-2008



Endrin, Dieldrin and Aldrin

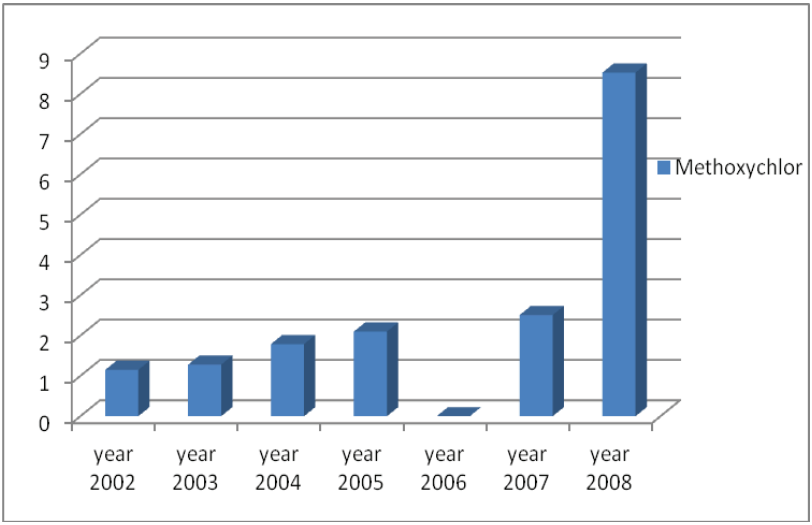
Levels of Aldrins (Figure 7) were shown to be a progressive linear function, the different what expected. This range was shown to build from levels of Dieldrin and Endrin that grow up from year to year (to year 2007). Concentrations for Aldrin were e regression linear function. The higher levels for Aldrins weas for year 2007 with 36,8 ng/g fresh weight and minimum for year 2002 with4,6 ng/g fresh weight. Found levels and their distribution could be because of their before use. Cyclodiene pesticides have been determined during the last 20 years in a large number of organisms, mainly bivalves and fish, in coastal areas of Spain, Egypt, Morocco, in the middle and north Adriatic Sea, Greece, Turkey, etc. (UNEP/FAO/WHO/IAEA, 1990). Found concentrations of Aldrin, Dieldrin, Endrin and Heptachlor were 10 times higher than reported values for Adriatic and Mediterrean Sea.

Figure 7. Endrin, Dieldrin and Aldrin in mussel samples for years 2002-2008



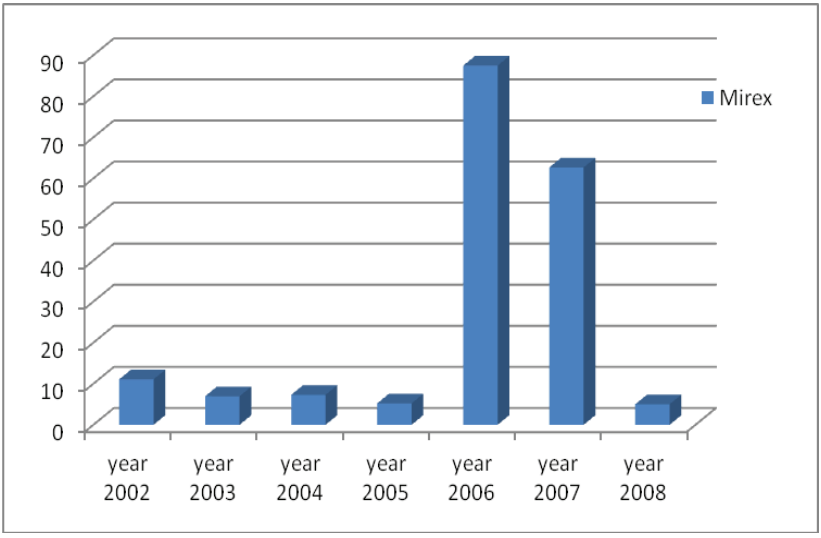
Methoxychlor. Methoxychlor was used wide in Albania. It was used for replacing DDT in their use because of its degradation. Period of degradation for Methoxychlor is shorter than DDT. It was used later than DDT but its levels were 50 times lower than DDTs. Maximum level of Methoxychlor for mussel samples was for year 2008 with 8.3 ng/g fresh weight. It was not detected for year 2006. These levels that grow from year to year could be because of new arrival from water rainfall for agricultural areas, from sediments or from stocks of pesticides. It's not excluded use of Methoxychlor or other pesticides hidden in different commercial names.

Figure 8. Methoxychlor in mussel samples for years 2002-2008



Mirex. Mirex was not produced or used never in Albania. Found levels for Mirex were in interval 3.1 ng/g fresh weight for year 2008 to 85.3 ng/g for year 2006. Found levels are a clear connection because of pollution and water sea currents in Adriatic Sea. Reported concentration for Mirex in other studies was in the same range with found levels.

Figure 9. Mirex in mussel samples for years 2002-2008



CONCLUSIONS

The occurrence of organochlorinated pesticides was studied in the Albanian coastal waters for a period of six years. For this study wild mussel *Muttilus Galloprovincialus* were used. Monitoring of organochlorinated concentrations based on the ability of mussels to concentrate chemical contaminants in tissues in relation to their biodisponibility. The aim of this study was to provide more information regarding the organochlorinated pollutants concentrations in Albanian coast. Porto-Romano station is considered a "hot-spot" area for organochlorinated pesticides in Albania because of ex-chemichal plant of Lindane in this area. For all investigated years were detected the presence of organochlorinated pesticide residues in mussel samples. They are not in use in our country since 90'. Levels of OCPs in years for station on study, is not a linear function as expected. The old industries such as Lindane chemical plant in Porto-Romano, Durres represent the major polluting factor for this area. Mismanagement of pesticides and other possible sources of contamination such as agricultural contribution, water-based contribution, atmospheric factor, etc. Generation of organochlorinated pesticides from sediment could be a significant factor for found levels. It's not excluded use of banned organochlorinated pesticides that hidden in different commercial names. Mirex was not produced or used never in Albania. Found levels for Mirex are a clear connection because of pollution and water sea currents in Adriatic Sea.

The most frequently detected pesticides for Porto-Romano stations for all years were *p,p*-DDE followed by HCH (γ-isomer) and HCB. These would have principally agricultural component although there are also other possible sources of contamination such as mismanagement of oddments pesticides, irrigation of surfaces where these pesticides were used. DDT degradation and stability for it and its metabolites could be the main factor for DDTs levels and distribution. p,p'-DDE was present in all samples, enormous contaminant too in each of them. The banning of DDT use after 90' in Albania and it degradation process are both factor that explain fact that p,p'-DDE was found in elevated concentration. Mirex was not in use in our country. It was found almost for all studied samples. This is a clear connection between found levels and water currents in Adriatic Sea. Present concentrations of HCHs influenced by past produce and a use of Lindane and HCHs. Lindane was not the major contributor. Other HCH isomers and other compounds such as chlor-benzenes were considered waste of Lindane fabric. These wastes were discharged directly to the sea.

Impact of organochlorinated pesticides in this area will be evident for years to come because of their stability and slow degradation in marine waters, adding the shift of them between water column and respective sediment. Trend of pesticides in this area must continue because of pesticide presence in elevated concentrations. Study of levels for organochlorinated pesticides in Porto-Romano must continue in mussel samples combined with samples of sediment and marine waters for getting clear results.

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Pesticide Risk Index of Del Azul Water Creek (Argentina): Tool for Predicting Its Overall Environmental Hazard

Fabio Peluso, Fabián Grosman, José González Castelain, Natalia Othax, Lorena Rodriguez

INTRODUCTION

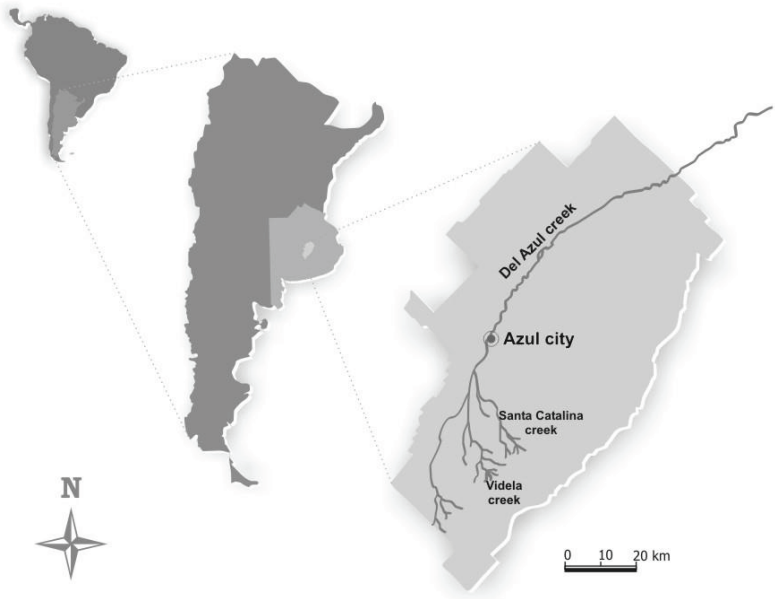
According to FAO, a pesticide is any substance or mixture of substances intended for preventing, destroying or controlling any pest, including vectors of human or animal diseases, unwanted species of plants or animals that cause damage or interfere of any kind in the production, processing, storage, transportation or marketing of food, agricultural products, wood and wood products or animal feeds or which may be administered to animals to control insects, arachnids or other pests on their bodies (FAO, 1986). It is now very intense the use of agrochemicals in the production systems in order to obtain higher yields, which directly results into higher incomes. The province of Buenos Aires is the major agricultural producing district of Argentina and accounts for 40% of national production (MAA, 2004). The main crops are soybeans ($3.7x10^6$ Ha. planted), wheat ($2.9x10^6$ Ha.), sunflower ($1.1x10^6$ Ha.), and maize ($0.8x10^6$ Ha.), according to data from the 2005/2006 campaign (CITAB, 2010). These volumes are the product of the agriculturization process, which started in 1970 in Argentina, which expanded the agricultural frontier at the expense of grasslands and other natural environments, and brought greater mechanization of the activity and the use of agrochemicals (Pengue, 2000; Pengue, 2001). The increased use of pesticides in the country (from 73 to 236 million kg. between 1995 and 2005 (CASAFE, 2010) reported the presence of these substances in different environmental compartments (soil, surface water and groundwater, biota, foods derived from agricultural activities, etc.) as mentioned in Peluso et al., 2011a.

In Argentina, local systems of environmental management (municipalities) are usually unaware of the type of substances used in its jurisdiction, their characteristics, volumes discharged to the environment and its potential environmental effects. This creates a situation of great uncertainty.

Water quality monitoring of Del Azul creek (36°00'/37º20'S - 60º12'/58º52'W, center of the Province of Buenos Aires, Argentina), has found different pesticides as a consequence of their wide use in the basin. This is a water course of plain terrain of 160 km long that, at about half its length passes through the city of Azul (60,000 inhabitants) (Figure 1). Upstream from this city, there has been a widening of the channel to create a bath resort that, in summer, can be visited by 10,000 people over the weekend. Many of these visitors are bathers. In addition to this use, people also

do fishing, boating and kayaking. In the area of the basin, upstream from the bath resort, intensive yields of wheat, corn, soybeans, and sunflowers are conducted. Given the possibility of soil washing or spray dispersion, product of the application in adjacent areas to the water course above mentioned, water quality of the Del Azul creek is periodically monitored by analyzing the presence of pesticides and heavy metals (Peluso et al., 2011a). However, these monitoring occur mainly in the context of scientific research projects funded by national or provincial agencies (Peluso et al., 2011a) with schedules and economic budgets adjusted to the objectives specific to such projects. One of their objectives was to conduct a toxicological risk assessment of pesticides founded in water considering the recreational use of the bath resort (Peluso et al., 2011b). However, other potential negative effects of the presence of pesticides were not considered such as persistence, bioaccumulation, mobility, or toxicity to biota.

Figure 1. Location of the study area (reproduced with permission of Taylor and Francis Publishing Co.)



In this regard, a methodology for assessing water quality that, as well as human or biota toxicity, considers other characteristics of the substances that create environmental hazards (i.e. persistence and bioaccumulation) can result in a highly useful tool. For example, this tool could be used to decide which substances are of higher priority either when monitoring, or when proposing remedial measures, or when regulating the use of such substances. Therefore, the objective of this paper is to present a methodological development called *DelAzulPestRisk* model that, based

on that presented in Swanson et al. (1997), allows ranking, in environmental priority order, all pesticides in waters of the Del Azul creek.

MATERIALS AND METHODS

Description of Hazard Levels of Pesticides: CHEMS Model

Literature shows that different methods have been developed to establish levels of environmental hazards in terms of the potential of the substances of having toxic effects on human health or biota, their mobility, persistence and bioaccumulation, etc. For example, Halfon and Reggiani, 1986; Gustafson, 1989; Kovach et al., 1992; Galassi et al., 1996; Halfon et. al., 1996; Swanson et al., 1997; Maud et al., 2001; Finizio and Villa, 2002; Grammatica and Di Guardo, 2002; Reus et al., 2002; Sanchez-Bayo et al., 2002; Russom et al., 2003; Ares, 2004; Padovani et al., 2004; Palma et al., 2004; Kookana et al., 2005; Yazgan and Tanik, 2005; Greitens and Day, 2007; Tixier et al., 2007; and Feola et al., 2011.

According to Feola et al. (2011), who are actually based on Reus et al. (2002), there would be two groups of ranking methods of environmental hazards of pesticides: those based on negative physicochemical and environmental properties and weighted by the rate of application of the substances, and those mainly based on the toxicity (to humans or to biota) by establishing a proportional relationship between that property and the concentration of substances in the environment (Reus et al., 2002), although in the latter group, many of them work with concentrations predicted or estimated by models (Tixier et al., 2007).

Swanson et al. (1997) introduced a system of ranking and scoring hazardous substances called “Chemical Hazard Evaluation for Management Strategies" (CHEMS-1), which would belong to the first group mentioned in the previous paragraph. On the one hand, the negative environmental properties of substances with direct relation to the possible toxic effects on human health and biota are considered (called Human Health and Environmental Effects within the model). On the other, some properties with indirect relation to the environmental effects mentioned above are also considered, such as the persistence of the substances and their bioaccumulation potential (Exposure Factor), as shown in Eq. 1.

$$TotalHazardValue = (HumanHealthEffects + EnvironmentalEffects) * ExposureFactor \quad (1)$$

Where:

Human Health Effects = $\sum$ scored hazard values for acute oral and inhalation toxicity, carcinogenicity and for chronic non-cancer toxicity.

Environmental Effects = $\sum$ scored hazard values for acute oral toxicity for rodents and acute and chronic toxicity for fish.

Exposure Factor = $\sum$ scored hazard value for biodegradation, hydrolysis degradation, and for aquatic bioconcentration.

The value of Eq. 1 is then multiplied by the scored value of the amount of the substance discharged to the environment obtaining the Release-Weighted Hazard Value (Swanson et al., 1997).

Description of the Del Azul Creek Pesticide Risk Index: The *DelAzulPestRisk* Model

The *DelAzulPestRisk* model is a tool for assessing the environmental hazards of pesticides in the aquatic environment, which is useful to rank and prioritize substances as a potential cause of a decline in the quality of that environment. Based on the model presented by Swanson et al. (1997), an algorithm was developed that, while respecting the structure of Eq. 1, it shows some modifications. Instead of using toxicity reference values for each substance (i.e. human oral LD50 or human inhalation LC50, as in CHEMS-1), the risk to human health and biota is estimated based on probabilistic USEPA methodology, starting with the concentrations of hazardous substances in the aquatic environment. In this way, the model would be part of the second group of ranking systems of hazardous substances mentioned in Reus et al. (2002), as it would be more appropriate a risk index rather than a dangerousness one (MacKay et al., 2001). Furthermore, the algorithm will be applied based on real measured concentrations and not the ones estimated by models (Tixier et al., 2007).

The main algorithm of the calculation respects the structure of Eq. 1, although changes were made in the estimation of the three components of the calculation of the equation and by changing the names of two of them. On the other hand, when considering the real concentrations of the substances in the environment, they are not considered as relevant the discharge rates of the substances (moreover, it is not easy to obtain this information). This term was calculated as multiplier of Eq. 1, so that the result obtained by the *DelAzulPestRisk* model is equivalent to a Total Hazard Value resulting from the CHEMS-1 model, not to a Release-Weighted Hazard Value.

Other important differences to note is that *DelAzulPestRisk* is estimated probabilistically and, as previously stated, only focuses on the aquatic environment disregarding the potential impacts on other media. Thus, the potential impact will be assessed by estimating the risk from exposure to hazardous substances in the water for recreational bathing, or by contact with representative organisms of aquatic biota.

Model of Calculation of the Effects to Human Health (Human Health Risk)

The risk to human health according to the USEPA model is quantified based on a relation between exposure to the hazardous substance and a toxicological safety

reference value for that substance. The exposure quantifies the contact dose between the hazardous substance and a human target considering the routes, scenarios and times of exposure (USEPA, 1992). The effect estimation to human health was performed by applying a probabilistic risk analysis by the exposure to the hazardous substance in the water during recreational bathing (Peluso et al., 2011b). The risk was quantified for a 10-year-old child as a human target, considering two possible routes of exposure: accidental water intake and dermal contact.

In both cases USEPA models were used (1992; 2007), applying Eq. 2 and 3, respectively.

$$ADDI = [Conc * Ir * EF * ED] / [Bw * AT] \quad (2)$$

$$ADDS = [DAevent * ESA * EF * ED * FC] / [Bw * AT] \quad (3)$$

Where
ADDI = Average daily dose by accidental intake (mg kg^{-1} day^{-1})
ADDS = Average daily dose by skin contact (mg kg^{-1} day^{-1})
Conc = Concentration of the hazardous substance in water (mg L^{-1})
Ir = Daily water intake rate (L day^{-1})
EF = Exposure frequency (day $year^{-1}$)
ED = Exposure duration (year)
BW = Weight of the exposed human (kg)
AT = Correction factors of average time for chronic exposure (ED * 365 days for non cancer risk estimation, 70 years * 365 days for cancer risk estimation)
DAevent = Absorbed dose per event (mg cm^{-2} $event^{-1}$);
ESA = Exposed Skin Area (cm^2)
FC = Correction factor of surface and volume units (10000 cm^2 m^{-2} * 0.001 L cm^{-3})

Both the carcinogenic and the non carcinogenic effects were considered, so that both types of risk were calculated. The non carcinogenic risk (NCR) calculation was performed based on the ratio between ADD and the chemical specific non cancer toxicological safe dose (RfD) for the route of exposure (USEPA, 1989). If the risk scores are less than 1.0, the NCR is assumed to be negligible (USEPA, 1989). The cancer risk (CR) (incremental lifetime cancer risk) was calculated multiplying ADD by the chemical specific cancer toxicological reference value, the Slope Factor (SF), also particular for each exposure pathway (USEPA, 1989). In the ADD for the cancer risk estimation, AT adopts 70 years as lifetime duration as stated by USEPA (1989).

The aggregated health risks (risk caused by the simultaneous exposure through different routes of contact to the same hazardous substance), were calculated using an additive model (Risk Index) (USEPA, 1989; 2007).

In this paper, it was assumed as safe criteria 1.0 and $10E^{-05}$ for aggregated NCR and aggregated CR respectively.

The value of the potential effects to human health from pesticides can then be estimated as the addition of the NC aggregated risk and the ratio between the C and their safe criteria, as shown in Eq. 4:

$$HumanHealthRisk = NCAgrR + CR / CSF \quad (4)$$

Where
Human Health Risk (dimensionless)
NCAgrR = Non carcinogenic aggregated risk
NCR = Carcinogenic risk
CSF = Carcinogenic slope factor

The RfDs and SFs used for accidental water intake risk calculation were obtained from USEPA IRIS database (2010). The RfDs and SFs for dermal risk calculation were estimated based on USEPA (2007), following Eq. 5 and 6, because chemical specific dermal toxicity factors are not still available.

$$RfDderm = RfDin * ABSGi \quad (5)$$

$$SDerm = SFin / BSGi \quad (6)$$

Where
RfDderm and SDderm: Dermal reference dose (mg kg^{-1} day^{-1}) and dermal slope factor (mg^{-1} kg day)
RfDin and SDin: Intake reference dose (mg kg^{-1} day^{-1}) and intake slope factor (mg^{-1} kg day)
ABSGi: Fraction of contaminant absorbed in gastrointestinal tract (dimensionless) in the critical toxicity study.
The RfD and SD values for all the substances are presented in Table 1.

Calculation Model for the Potential to Generate Effects on the Biota (Biota Health Risk)

In the *DelAzulPestRisk* model, the risk to biota is estimated based on the ratio between the pesticide concentration in water and the toxicological threshold concentration of safety for the organisms of the selected ecosystems. The Toxicity Exposure Ratio (Finizio et al., 2001), is also often referred to as the ratio between Predicted Environmental Concentration and Predicted Non Effect Concentration or PEC/PNEC (USEPA, 2004). If PEC/PNEC <1, negative effects should not occur on the organism from which the PNEC was chosen. The PNEC value selected in this work is LC50, or lethal concentration 50, which is the concentration that kills 50% of the exposed organisms in toxicological testing. The PNEC value was obtained with ECOSAR (USEPA, 2011a). This software uses the structure-activity relation to

predict the aquatic toxicity of a substance based on the structural similarity of the substance with similar substances for which toxicity data exist (USEPA, 2011a). In this case, the data consist of mortality trials by means of acute exposure to the active ingredient of the agrochemical.

The groups of selected organisms to be used as reference to assess the potential of generating effects to the freshwater biota are a microinvertebrate (*Daphnia sp.*, "Water fleas"; Arthropoda Crustacea, in LC50 toxicity test of 48 hours), and a fish (*Cyprinus carpio*, "Common carp"; Chordata Actinopteyigii, in 96 h. LC50 test). These organisms were selected because of their presence in the waters of the Del Azul creek. The PNEC values for all pesticides are presented in Table 2.

Table 1. Toxicological reference values used for oral Intake and Dermal contact exposure pathways of the Human Health Effects module of the *DelAzulPestRisk* calculation (based on Peluso et al., 2011b)

Substances	RfD [a]		SF [b]		ABS_{GI} [c]
	Int[d]	Derm[e]	Int	Derm	
2,4 D (2,4 Dichlorophenoxy Acetic Acid)	$1.00E^{-02}$	$9.00E^{-03}$	N.A.	N.A.	$9.00E^{-01}$
α – HCH (Hexachlorociclohexane)	$3.00E^{-04}$	$3.00E^{-04}$	$6.30E^{+00}$	$6.30E^{+00}$	$1.00E^{+00}$
β – HCH	$3.00E^{-04}$	$3.00E^{-04}$	$1.80E^{+00}$	$1.80E^{+00}$	$1.00E^{+00}$
δ – HCH	$3.00E^{-04}$	$3.00E^{-04}$	$1.30E^{+00}$	$1.30E^{+00}$	$1.00E^{+00}$
γ- HCH	$3.00E^{-04}$	$3.00E^{-04}$	$1.30E^{+00}$	$1.30E^{+00}$	$1.00E^{+00}$
Acetochlor	$2.00E^{-02}$	$2.00E^{-02}$	N.A.	N.A.	$1.00E^{+00}$
Aldrin	$3.00E^{-05}$	$3.00E^{-05}$	$1.70E^{+01}$	$1.70E^{+01}$	$1.00E^{+00}$
Cypermethrin	$1.00E^{-02}$	$1.00E^{-02}$	N.A.	N.A.	$1.00E^{+00}$
Chlorpyrifos	$3.00E^{-03}$	$3.00E^{-03}$	N.A.	N.A.	$1.00E^{+00}$
Endosulfan	$6.00E^{-03}$	$6.00E^{-03}$	N.A.	N.A.	$1.00E^{+00}$
Endosulfan Sulphate	$6.00E^{-03}$	$6.00E^{-03}$	N.A.	N.A.	$1.00E^{+00}$
γ – Chlordane	$5.00E^{-04}$	$4.00E^{-04}$	$2.50E^{-04}$	$4.38E^{-01}$	$8.00E^{-01}$
Glyphosate	$1.00E^{-01}$	$1.00E^{-01}$	N.A.	N.A.	$1.00E^{+00}$
Heptachlor	$5.00E^{-04}$	$5.00E^{-04}$	$4.50E^{+00}$	$4.50E^{+00}$	$1.00E^{+00}$

[a] Reference dose (mg Kg^{-1} d^{-1})

[b] Slope factor (mg^{-1} Kg d)

[c] Fraction of contaminant absorbed in gastrointestinal tract (dimensionless), based in USEPA, 2007.

[d] Oral intake

[e] Dermal

The value of the potential effects to the biota of the substances would then be estimated as the addition of the ratio PEC/PNEC for these two organisms, as it is presented in Eq. 7

$$BiotaHealthRisk = PEC / PNECD + PEC / PNECC \quad (7)$$

Where:
Biota Health Risk (dimensionless)
PEC = Predicted Environmental Concentration
PNECD = Predicted Non Effect Concentration for *Daphnia sp.*
PNECC = Predicted Non Effect Concentration for *Cyprinus carpio*

Later on, it is mentioned how the PEC value was obtained.

Both for the health and biological risk, environmental dangerousness increases when increasing its values. Following the CHEMS model, it is then conducted the addition of human health risk to the biota risk (Human + Biota Health Risk).

Table 2. Ecotoxicological reference values (Predicted Non Effect Concentration, in mg L^{-1}) used in the Biota Health Effect module of the *DelAzulPestRisk* model.

Substances	*Daphnia sp.*	*Cyprinus carpio*
2,4 D	303.96	506.72
α – HCH	1.56	2.24
β - HCH	1.56	2.24
δ - HCH	1.56	2.24
γ- HCH	1.56	2.24
Acetochlor	8.98	13.10
Aldrin	0.02	0.02
Cypermethrin	0.05	0.04
Chlorpyrifos	0.44	0.47
Endosulfan	10.64	15.13
Endosulfan Sulphate	8.50	11.77
γ - Chlordane	0.02	0.01
Glyphosate	2.46 E^{+07}	1.68 E^{+08}
Heptachlor	0.11	0.10

Calculation Model of Aggravating Factors

"Aggravating factors" was the name given to the "exposure factors" according to the CHEMS-1 model, and responds to the structure of the algorithm presented in Swanson et al. (1997). That is, it functions as a "worsening factor" for the potential effects to human or biota health that the substance may cause. It responds to Eq. 8:

$$AggravatingFactor = Persistence + Bioconcentration \quad (8)$$

Where
Persistence (Hs)
Bioconcentration (L kg^{-1})

In Swanson et al. (1997), the measure of the persistence of the substance in the environment was a scored hazard value for biodegradation and for hydrolysis degradation, as two separate parameters. In the *DelAzulPestRisk* model, the persistence was estimated as a single parameter, applying EPISUITE 4.1 (USEPA, 2011b). This digital tool has different calculation modules in which, from the identification of the molecule and the amount of substance released to the environment, it estimates quantitatively the mass percentage share based on the fugacity (Mackay and Paterson, 1981; 1991; Mackay et al., 1996a; Mackay et al., 1996b), and also the half-life in different environmental compartments based on a calculation module called BIOWIN. This model estimates the probability of rapid aerobic and anaerobic biodegradation of the compound in the presence of mixed populations of environmental microorganisms (Boethling and Sabljic, 1989; Boethling et al., 2004). This calculation module scales the results of half-life in each compartment as a series of decreasing values depending on the length of it. This would be approximately: 1 (half-life measured in years), 2 (in months), 3 (in weeks), 4 (in days) and 5 (in hours). Since the half-life results are obtained on a decreasing scale of severity (the higher value, the less potential environmental impact), the scale had to be reversed to fulfill its role as "enhancer". This was done according to Eq. 9, where half-life is the value calculated with EPISUITE for the aquatic compartment.

$$PersistenceValue = 5 - halflife \quad (9)$$

The potential for bioconcentration (BCF) was also estimated using EPISUITE. This has an estimation module called BCFBAF, which estimates BCF of an organic compound using the compound's log octanol-water partition coefficient (Kow), according to Meylan et al. (1999). Bioconcentration potential used in *DelAzulPestRisk* is the log BCF generated by EPISUITE for the substance. Thus, the aggravating factor was set as Eq. 10:

$$AggravatingFactor = (5 - halflife) + \log BCF \quad (10)$$

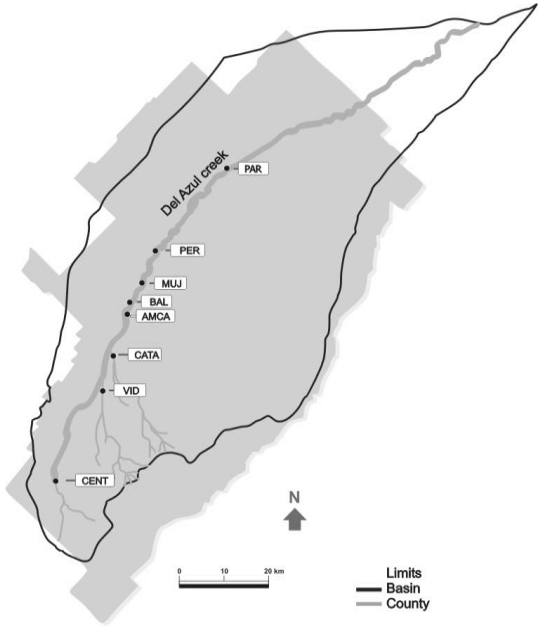
The Aggravating Factor is dimensionless. Given the role that the Aggravating Factor has in the model, it was decided to disregard the units, only considering the absolute valuesmaking it a risk multiplier. The maximum persistence possible value is 5 and 5 is an extreme value for log BCF. The Aggravating Factor, in situations of high persistence and high bioconcentration potential, could enhance up to 10 times or even more the health and the biota risk value. However, this factor only aggravates the situation if there is a risk to health or biota, for which the clause that will only work if Human Health Risk or Biota Health Risk ≥ 1 was established. Therefore, when Human and Biota Health Risk < 1, Aggravating Factor = 1.

Applying the *DelAzulPestRisk* model

Concentration of agrochemicals in water

A preliminary study on the presence of agrochemicals in the waters of the Del Azul creek (12 samples from eight sites in the stream, see Figure 2, taken bimonthly between December 2005 and December 2007), revealed the presence of organochlorine pesticides (*α-HCH*, *β-HCH*, *γ-HCH*, *δ-HCH*, *Aldrin*, *γ-Chlordane*, *Endosulfan*, *Endosulfan Sulphate*, *Heptachlor*), pyrethroids (*Cypermethrin*) and organophosphorus (*Chlorpyrifos*) and herbicides (*2,4-D*, *Acetochlor*, *Glyphosate*) (Peluso et al., 2011b).

Figure 2. Location of sampling stations in the waters of the Del Azul creek (reproduced with permission of Taylor and Francis Publishing Co.)



To estimate the values of Human Health Risk and Biota Health Risk, the probability distributions of the concentrations of the pesticides samples were used. The best fitted probability distribution models and their statistical descriptors can be seen in Table 3 and were obtained using Crystal Ball 7.1 (Decisioneering. 2007a). The following explains how the probability distributions were used in these estimates.

Estimation of Human Health Risk

It had previously been mentioned that the health risk was calculated based on the estimation of the average daily exposure dose by accidental intake (ADDI, Eq. 2) and by skin contact (ADDS, Eq. 3), considering as the exposed individual a child. The concentration values used in Eq. 2 (*Conc*) were the distributions of probability with its statistical descriptors presented in Table 3. The rest of the values used to outline the exposure scenario are presented in Table 4.

Table 3. Probability Distribution Model parameters and descriptive statistics of concentrations (in mg L^{-1}) used for Human and Biota Health Effect calculations (based on Peluso et al., 2011b).

Substance	Fitted Distrib.	Min[a]	Max[b]	Mean	SD[c]	CV[d]	Others	
2,4 D	T-student	2.35 E^{-03}	2.98 E^{-01}	7.93 E^{-02}	2.91 E^{-02}	3.59 E^{-01}	Mid.[e] 7.70 E^{-02}	Sc.[f] 2.32 E^{-02}
α – HCH	Beta	8.13 E^{-07}	6.60 E^{-05}	3.54 E^{-05}	2.01 E^{-05}	5.74 E^{-01}	α 0.71	β 0.52
β - HCH	Min. Ext[g]	1.77 E^{-06}	5.00 E^{-06}	2.80 E^{-06}	6.51 E^{-07}	2.37 E^{-01}	Like.[h] 2.00 E^{-06}	Sc. 1.00 E^{-06}
σ - HCH	T-student	6.64 E^{-08}	1.90 E^{-05}	5.04 E^{-06}	2.44 E^{-06}	4.90 E^{-01}	Mid. 4.74 E^{-06}	Sc. 2.21 E^{-06}
γ- HCH	Logistic	7.47 E^{-07}	9.50 E^{-06}	3.96 E^{-06}	8.53 E^{-07}	2.15 E^{-01}		Sc. 4.71 E^{-07}
Acetochlor	Min. Ext.	5.82 E^{-05}	7.10 E^{-02}	1.94 E^{-02}	1.24 E^{-02}	6.37 E^{-01}	Like. 1.05 E^{-02}	Sc. 2.42 E^{-02}
Aldrin	T-student	4.04 E^{-07}	1.80 E^{-05}	6.04 E^{-06}	2.15 E^{-06}	3.62 E^{-01}	Mid. 6.00 E^{-06}	Sc. 2.00 E^{-06}
Cypermethrin	Lognormal	4.00 E^{-04}	1.94 E^{+00}	4.74 E^{-01}	2.57 E^{+02}	3.21 E^{+00}	Loc.[i] 3.71 E^{-01}	
Chlorpyrifos	Gamma	2.00 E^{-04}	3.10 E^{-02}	4.53 E^{-03}	2.95 E^{-03}	6.53 E^{-01}	Loc. -3.13	Sc. 1.83

							E^{-04}	E^{-03}
Endosulfan	Min. Ext.	1.00 E^{-06}	7.50 E^{-06}	2.65 E^{-06}	1.07 E^{-06}	4.08 E^{-01}	Like. 1.85 E^{-06}	Sc. 2.11 E^{-06}
Endosulfan Sulphate	T-student	9.16 E^{-06}	3.70 E^{-05}	2.17 E^{-05}	4.01 E^{-06}	1.88 E^{-01}	Mid. 2.17 E^{-05}	Sc. 3.54 E^{-06}
γ - Chlordane	Logistic	1.53 E^{-06}	8.90 E^{-06}	2.05 E^{-06}	4.29 E^{-07}	2.05 E^{-01}		Sc. 4.89 E^{-07}
Glyphosate	Logistic	5.01 E^{-03}	1.11 E^{+00}	1.59 E^{-01}	1.22 E^{-01}	7.86 E^{-01}		Sc. 1.00 E^{-01}
Heptachlor	Logistic	1.55 E^{-05}	6.30 E^{-05}	2.04 E^{-05}	4.02 E^{-06}	2.03 E^{-01}		Sc. 3.39 E^{-06}

[a] Minimum value of concentration of the fitted distribution
[b] Maximum value
[c] Standard deviation
[d] Coefficient of variability
[e] Midpoint
[f] Scale
[g] Minimum extreme
[h] Likeliest
[i] Location

For the calculation of ADDI, the incidental ingestion rate for water during bathing was assumed as being 0.05 L per hour of the bath event duration according to USEPA (1989).

The duration of the bath event (*tevent*, relevant for the absorbed dose per event calculation —*DAevent* in Eq. 3) and its frequency during the year (*EF*, common to Eq. 2 and 3) were probabilistically estimated based on a questionnaire administered by the authors conducted in the Del Azul bath resort during the summer of 2010-2011 (Peluso et. al., 2011b).

The duration of exposure (*ED*, common to Eq. 2 and 3) was probabilistically treated, assuming a triangular probability distribution with the lower and upper limits equaling 1 and 30 years respectively, and mode equaling 15 (Peluso et al, 2011b).

The probability distribution model and its descriptive parameters of the Body Weight (*BW* of Eq. 2) and Body Height (necessary to estimate the Exposed Skin Area) were based on Lejarraga and Orfila (1987), which consisted of the anthropometric data specified for the population of Argentina.

The Exposed Skin Area (*ESA* from Eq. 3) was calculated based on the DuBois and DuBois (1916) equation corrected by a factor called bath pattern (Eq. 11). This factor expresses a measure of skin surface which is effectively submerged in the

water in comparison with the duration of the recreational event, considering how this is carried out (Peluso et al., 2011b).

$$ESA = (H^{0.725} * BW^{0.425} * 0.007184) * BP \quad (11)$$

Where
ESA: Exposed Skin Area (cm^2)
H: Body Height (cm)
BW: Body Weight (kg)
BP: Bath Pattern (dimensionless)

Table 4. Probability Distribution Model parameters and descriptive statistics of the exposure dose for the Human Health Effects estimation (based on Peluso et al., 2011b).

Param.	Fitted Dist.	Min	Max	Mean	SD	Others	
tevent[a]	Min.Ext.	0	300	188.16	25.38	Like. 218.15	Sc. 62.24
EF[b]	Gamma	0	54	19.91	14.34	Loc. -0.11	Sc. 34.58
BW[c]	Normal	23.5	44.5	33.56	5		
H[d]	Normal	125	149	136	0.5		
BSA[e]	Normal	9178.74	13341.96	11256.18	725.90		
BP[f]	Triangular	0.072	1	0.49	0.19	Mo[h] 0.401	
ESA[g]	Beta	838.35	12014.88	5526.35	2216.89	α 2.36	β 3.95

[a] Duration of the bath event (minutes)
[b] Annual frequency of bathing days (days)
[c] Body weight (Kg)
[d] Body height (cm)
[e] Body surface Area (cm^2)
[f] Bath pattern (dimensionless)
[g] Exposed skin area (cm^2)
[h] Mode

The Absorbed dose per event (*DAevent* from Eq. 3) was estimated based on a steady state approach from USEPA, which is a model that calculates the absorbed dose through the skin, relating the substance concentration, the permeability of the stratum corneum to the same event, and the duration of the recreational event (USEPA, 2007).

The ADDI and ADDS calculations were made with Crystal Ball 7.1 software (Decisioneering 2007a), applying Monte Carlo for simple random sampling for

5,000 trials based on the probabilities distribution of each variable. As previously stated, based on accidental ingestion dose and dermal contact dose, the simultaneous risk was calculated in both contact ways for each substance (aggregate risk) for the Non Cancer effects and for the Cancer ones. The process of addition is conducted iteration by iteration, so the results are a new distribution of values, for which descriptive statistics are obtained for later analysis.

Biota Health Risk Estimation

It was previously stated that the risk to biota is estimated based on the ratio PEC/PNEC for selected ecosystem organisms. The PEC value used is the distribution of probability of the concentrations of each pesticide, as performed for the calculation of Human Health Risk. The Health Risk Biota result is a distribution of values obtained by adding the PEC/PNEC ratios for the two obtained biological groups, iteration by iteration, by the application of Monte Carlo. From these, it was also obtained descriptive statistics to conduct analysis of the results.

Interpretation of the Results of the DelAzulPestRisk Model

The results offered by the model cannot be considered a formal measurement of risk but an indication of it, since the model includes variables that are risk measures (human and biological) with others that are not (the half-life and the bioaccumulation potential). The risk values are dimensionless while neither life nor the bioaccumulation potential are, so the integration of these terms ignoring the units of the latter is a methodological decision that makes the model not a " risk" model but a "risk-based" one.

Statistical Analysis

The statistical analysis (obtaining descriptive statistics of the probability distributions following the Monte Carlo application or determining the best fit model of probability distribution) was performed using Crystal Ball (Decisioneering, 2007), as well as the sensitivity analysis. This calculates sensitivity based on the rank correlation coefficients between every parameter of the model and the model results while the simulation is running (Decisioneering, 2007).

RESULTS

The risk to human health analysis results show that no substance by itself is capable of producing health effects to the population, not even considering the carcinogenic effects or the non carcinogenic ones, which had already been evidenced by Peluso et al. (2011b). However, in the *DelAzulPestRisk* model, the risks for carcinogenic effects are finally estimated as a proportion of the value used as reference ($10E^{-05}$), so the risk threshold value for each substance is 1 for each type of effect. If the substance has both types of effects (as it happens with the isomers of *HCH*, *Aldrin*, *Chlordane* and *Heptachlor*), the risk threshold value to health in the *DelAzulPestRisk* model is all the same 1. From the results presented in Table 5, it can be seen that the substance that creates the greatest risk to human health is

Cypermethrin (95th percentile (P^{95}) = $5.09E^{-01}$); a substance that produces only NC effects, but it is below the limit value. As shown in the table, not even substances that have both types of effects are close to the threshold value.

Table 5 also shows the biological risk values. In this case, only one substance exceeds the threshold value, and it is again *Cypermethrin* ($P^{95} = 1.36E^{+01}$). The P^{95} of the risk to *Daphnia sp*. is 17.10 and 20.91 for *Cyprinus sp*. This high value leads to a higher value of Biota + Human Risk, as shown in Table 6, where *Cypermethrin* has a value of $1.41E^{+01}$. The remaining substances are from 3 (*Heptachlor*, *Chloropyrifos*, *α-HCH*, *2.4-D* and *Acetochlor*) to 7 orders of magnitude (*Endosulfan*) below the value of *Cypermethrin*.

Table 5. Probabilistic results after applying the *DelAzulPestRisk* model for Human and Biota Health Risks.

Substance	Human Health Risk				Biota Health Risk			
	Mean	SD	Median	P^{95a}	Mean	SD	Median	P^{95}
2,4 D	$4.37E^{-03}$	$4.38E^{-03}$	$3.03E^{-03}$	$1.30E^{-02}$	$4.19E^{-04}$	$1.55E^{-04}$	$4.11E^{-04}$	$6.70E^{-04}$
α – HCH	$1.14E^{-02}$	$1.51E^{-02}$	$5.52E^{-03}$	$4.30E^{-02}$	$3.88E^{-05}$	$2.21E^{-05}$	$4.03E^{-05}$	$7.01E^{-05}$
β - HCH	$2.65E^{-04}$	$2.94E^{-04}$	$1.67E^{-04}$	$8.43E^{-04}$	$3.07E^{-06}$	$7.17E^{-07}$	$3.00E^{-06}$	$4.37E^{-06}$
σ - HCH	$3.57E^{-04}$	$4.52E^{-04}$	$1.95E^{-04}$	$1.20E^{-03}$	$5.47E^{-06}$	$2.68E^{-06}$	$5.27E^{-06}$	$1.01E^{-05}$
γ- HCH	$3.06E^{-04}$	$3.37E^{-04}$	$1.91E^{-04}$	$9.69E^{-04}$	$4.30E^{-06}$	$9.31E^{-07}$	$4.29E^{-06}$	$5.81E^{-06}$
Acetochlor	$8.29E^{-04}$	$1.05E^{-03}$	$4.34E^{-04}$	$2.95E^{-03}$	$3.66E^{-03}$	$2.35E^{-03}$	$3.40E^{-03}$	$7.92E^{-03}$
Aldrin	$9.41E^{-04}$	$1.08E^{-03}$	$5.75E^{-04}$	$3.04E^{-03}$	$6.60E^{-04}$	$2.36E^{-04}$	$6.51E^{-04}$	$1.06E^{-03}$
Cypermethrin	$1.14E^{-01}$	$5.28E^{-01}$	$4.43E^{-03}$	$5.09E^{-01}$	$2.72E^{+00}$	$8.37E^{+00}$	$2.22E^{-01}$	$1.36E^{+01}$
Chlorpyrifos	$4.22E^{-03}$	$5.39E^{-03}$	$2.34E^{-03}$	$1.43E^{-02}$	$1.98E^{-02}$	$1.30E^{-02}$	$1.70E^{-02}$	$4.44E^{-02}$
Endosulfan	$2.36E^{-07}$	$2.49E^{-07}$	$1.58E^{-07}$	$7.31E^{-07}$	$4.20E^{-07}$	$1.71E^{-07}$	$3.98E^{-07}$	$7.31E^{-07}$
Endosulfan Sulphate	$2.10E^{-06}$	$1.98E^{-06}$	$1.52E^{-06}$	$5.98E^{-06}$	$4.41E^{-06}$	$8.45E^{-07}$	$4.40E^{-06}$	$5.84E^{-06}$
γ - Chlordane	$1.11E^{-04}$	$1.21E^{-04}$	$7.13E^{-05}$	$3.52E^{-04}$	$3.21E^{-04}$	$6.63E^{-05}$	$3.04E^{-04}$	$4.45E^{-04}$
Glyphosate	$3.92E^{-04}$	$5.29E^{-04}$	$1.94E^{-04}$	$1.41E^{-03}$	$7.26E^{-09}$	$5.72E^{-09}$	$5.97E^{-09}$	$1.82E^{-08}$
Heptachlor	$1.88E^{-02}$	$2.11E^{-02}$	$1.15E^{-02}$	$6.06E^{-02}$	$3.76E^{-04}$	$7.46E^{-05}$	$3.60E^{-04}$	$5.19E^{-04}$

[a] P^{95} 95th percentile

Table 7 shows the values of the variables comprising the Aggravating Factor. There can be seen that the range of values is between 2.29 (*Glyphosate*) to 9.06 (*γ-Chlordane*). Since the only value of Biota or Human Health Risk greater than 1 corresponds to *Cypermethrin*, the only Aggravating Factor other than 1 that is applied is the correspondent to this substance, which has a value of 6.24. This value comes from a half life of the order of 1.74 (i.e. with half-life from months to years, i.e. recalcitrant) that generates a persistence value of 3.26, and also a high potential for bioaccumulation (log BCF = 2.99).

Table 6. Probabilistic results after applying the *DelAzulPestRisk* model for Human and Biota Health Risks.

Substance	Human + Biota Health Risks			
	Mean	SD	Median	P^{95}
2,4 D	4.73 E^{-03}	4.39 E^{-03}	3.34 E^{-03}	1.35 E^{-02}
α – HCH	1.14 E^{-02}	1.54 E^{-02}	5.56 E^{-03}	4.34 E^{-02}
β - HCH	2.70 E^{-04}	2.95 E^{-04}	1.73 E^{-04}	8.73 E^{-04}
σ - HCH	3.64 E^{-04}	4.47 E^{-04}	2.10 E^{-04}	1.25 E^{-03}
γ- HCH	3.10 E^{-04}	3.41 E^{-04}	1.97 E^{-04}	9.82 E^{-04}
Acetochlor	4.50 E^{-03}	3.09 E^{-03}	4.01 E^{-03}	1.01 E^{-02}
Aldrin	1.61 E^{-03}	1.18 E^{-03}	1.26 E^{-03}	3.94 E^{-03}
Cypermethrin	2.82 E^{+00}	8.97 E^{+00}	2.20 E^{-01}	1.41 E^{+01}
Chlorpyrifos	2.42 E^{-02}	1.61 E^{-02}	2.10 E^{-02}	5.45 E^{-02}
Endosulfan	6.56 E^{-07}	3.43 E^{-07}	5.90 E^{-07}	1.32 E^{-06}
Endosulfan Sulphate	6.46 E^{-06}	2.25 E^{-06}	5.96 E^{-06}	1.09 E^{-05}
γ - Chlordane	4.33 E^{-04}	1.48 E^{-04}	3.95 E^{-04}	7.21 E^{-04}
Glyphosate	3.90 E^{-04}	5.50 E^{-04}	1.84 E^{-04}	1.46 E^{-03}
Heptachlor	1.93 E^{-02}	2.17 E^{-02}	1.21 E^{-02}	6.26 E^{-02}

Table 8 shows the results of the *DelAzulPestRisk* model for *Cypermethrin* in descriptive statistics of the distribution of values obtained after the application of Monte Carlo. Figure 3 shows the probability distribution (lognormal) with a mean value quite low. Although the maximum value is around 90, the figure shows that this value has a negligible probability level. The P^{95} of the probability distribution has a certainty level greater than 90%.

The sensitivity analysis performed to find which variables are the ones that are affecting the most the total variance displays that the substance concentration represents 98.6 % of the variance. Given the importance of this variable (also reflected in the high value of the variability coefficient (2.40)), it is clear that potential dangerousness would merit increasing the sampling of water quality given the high dispersion of concentration data of this substance.

Table 7. Results of application of the *DelAzulPestRisk* model for the Aggravating Factor (AF).

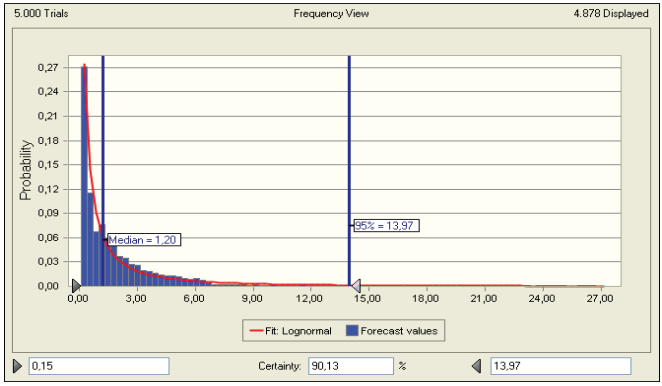
Substance	Per[a]	Log BCF	AF
2,4 D	2.40	0.50	2.90
α – HCH	3.48	2.40	5.88
β - HCH	3.48	2.40	5.88
σ - HCH	3.48	2.40	5.88
γ- HCH	3.48	2.40	5.88
Acetochlor	2.78	1.67	4.45
Aldrin	4.28	3.96	8.24
Cypermethrin	3.26	2.99	6.24
Chlorpyrifos	3.26	2.94	6.20
Endosulfan	4.38	2.19	6.57
Endosulfan Sulphate	4.41	2.08	6.49
γ - Chlordane	4.94	4.12	9.06
Glyphosate	1.79	0.50	2.29
Heptachlor	4.47	3.28	7.75

[a] Persistence value (measured as 5- halflife)

Table 8. Probabilistic results after applying the *DelAzulPestRisk* model.

Substance	Mean	SD	Median	Min.	Max.	P95	CV
Cypermethrin	3.54 E^{+00}	8.49 E^{+00}	1.20 E^{+00}	1.15 E^{-01}	9.12 E^{+01}	1.390 E^{+01}	2.40 E^{+00}

Figure 3. Probability distribution model and descriptive statistics of the application of the *DelAzulPestRisk* model for *Cypermethrin*.



ANALYSIS AND DISCUSSION

Levitan (1995) raises two issues that still remain in effect when designing the best index for the ranking of the environmental hazards of pesticides: how to include and weigh a wide range of environmental parameters when creating an integrated index and how to achieve a balance between the large amount of information demanded by the analysis of a complex system and the real possibilities of obtaining it. In this regard, due to cost and time required to achieve this wealth of information, many of the methodologies developed are considered expedient or "snapshot" assessments that, at least, allow providing fairly immediate preliminary answers, in many cases, within environmental management programs (Swanson et al., 1997; Finizio et al., 2001 Padovani et al. 2004; De Smet et al., 2005).

Maud et al. (2001) considers the wide dispersion of results that can be obtained from applying different rates to these pesticides. While acknowledging that an important element to consider in estimating the danger of pesticides is the volume of discharged substance into the environment, the complexity of the system subtracts "realism" by failing to incorporate the values of the site-specific variables. To this, it must also be added the unique assumptions often held in the development of each model, for example, in the allocation of weights to the variables (Finizio et al., 2001), causing the hazard ratings to be very little comparable.

An intensive property is one that depends on the nature of the substance and not the quantity, while the extensive property depends on the amount of substance (MacKay et al., 2001). Thus, in this study, persistence is clearly an intensive property and bioaccumulation potential semi intensive one (depends on the LogKow of the substance and the fat body contents of the organism). On the other hand, the concentration of the substance in water is a clearly extensive property. Today there

are many ranking systems of dangerous substances based primarily on the aggregation of intensive or semi intensive parameters. However, this approach tends to ignore a fundamental extensive parameter: the amount of substance in the environment as regards concentration. In the cases where it is considered, this parameter is very often estimated by models of fugacity (i.e. see Sanchez-Bayo et al., 2002; Ares, 2004; Padovani et al., 2004); based on the amounts of substances released into the environment (i.e. see Swanson et al., 1997; Finizio et al., 2001; De Smet et al., 2005; Yazgan and Tania, 2005) based on transport models (Kookana et al., 2005). Without this parameter, it is impossible to delineate the exposure, and thus, the analysis does not result in a risk analysis of substances, it is rather one about their dangerousness (MacKay et al., 2001), resulting in less analytical power for decision-making.

In a dangerousness index, the literature stated that is desirable it can handle the concept of "risk" within the meaning of "technical concept of risk"; that is, to consider the extent of damage depending on its occurrence likelihood (Maud et al., 2001). Comparative analysis systems of the dangers of pesticides based on risk only focus either on the ecotoxicological risk (i.e. Palma et al., 2004) or on human risk (i.e. Ares, 2004). The *DelAzulPestRisk* model focuses on both in an integrated manner and based on concentrations measured in situ. This draws to a flexible estimating system of the human exposure to substances deducted from recreational use of an affected, and thus, real environment and does not estimate the exposure indirectly, from the emission of data of substances to the environment. In addition to the uncertainties inherent in estimation models (of which the *DelAzulPestRisk* model is not exempt), the uncertainties of the information of the survey about the use of pesticides have to be added (formulations, emission rates, etc.). This information is not easily obtained locally in developing countries such as Argentina, as highlighted by Feola, et al. (2011) in his study on the selection of an index to be applied in Colombia. *DelAzulPestRisk* is also a snapshot methodology that integrates available information locally to respond to the guidelines set forth by Levitan (1995; 1997).

The probabilistic analysis, while some simplicity is subtracted (one more desirable characteristic of these systems explained by Maud et al. (2001)), provides a greater choice of estimation and uncertainty analysis. The results of applying the *DelAzulPestRisk* model showed that *Cypermethin* is the priority substance to study in the system, even though it is neither the most toxic to humans or biota considering toxicological reference values, nor the one that possesses the greatest persistence or the greatest bioaccumulation potential. Other methodologies exclusively based on intensive characteristics would have highlighted other substances that simultaneously meet higher levels of environmental dangerousness over *Cypermethrin*.

The sensitivity analysis showed the great importance of examining closely the evolution of the concentrations of this substance. Concentrations considered in the context of the analysis of human and ecological risk together with their persistence

and bioaccumulation potential is what allowed obtaining more integrated priority levels for the studied environment. On the other hand, the system could allow, for other pesticides, estimating the limit concentrations that would trigger hazardous situations for a bather or aquatic biota, which could be used as an early warning system.

CONCLUSIONS

To predict the overall environmental hazard of Del Azul creek, a screening risk based index called *DelAzulPestRisk* was developed, based on an index of the bibliography. It was based on an equation function of the toxicological health risk for bathing (considering the aggregated exposure to the accidental intake and dermal contact to the water for a 10 years old child) and the ecological risk (based on toxicological data of two representative species of the aquatic biota, a crustacean – *Daphnia sp.* – and a fish –*Cyprinus carpio*), and several pesticides characteristics that indicate their environmental fate and transport (bioaccumulation in biota and persistence in water compartment).

The pesticides determined in the Del Azul creek water samples were insecticides (*α-HCH*, *β-HCH*, *δ-HCH*, *γ-HCH*, *δ–Chlordane*, *Aldrin*, *Chlorpyrifos*, *Cypermethrin*, *Endosulfan*, *Endosulfan Sulphate* and *Heptachlor*) and herbicides (*2,4 D*, *Acetochlor*, and *Glyphosate*).

The results show that only *Cypermethrin* has an index value indicating a significant level of environmental hazard because of the potential toxicological impact to aquatic biota in addition to its characteristics of high half-life in the compartment and the bioaccumulation potential. However, the model managed to highlight it, though it is neither the most toxic to humans or biota considering its toxicological reference values, nor the one that possesses the greatest persistence or greatest bioaccumulation potential.

The described approach may provide a tool for the comparative study of the overall environmental hazard of the pesticides for surface water.

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Long-term Monitoring of Pesticides in Water from Lake Biwa and Rivers Flowing into Lake Biwa

Taizo Tsuda

ABSTRACT

Surveys of various pesticide contaminations were performed for water in Lake Biwa from 2000 to 2009 and in rivers flowing into Lake Biwa from 1988 to 2009. Eleven pesticides (fenobcarb, diazinon, fenitrothion, iprobenfos, isoprothiolane, chlornitrofen (CNP), thiobencarb, molinate, bromobutide, simetryne and pretilachlor) were selected and the concentration changes of the pesticides were evaluated based on their shipment amounts. Their concentrations in water of Lake Biwa and rivers flowing into Lake Biwa were compared with their no observed effect concentration (NOEC) and their predicted no effect concentration (PNEC) values and initial ecological risk assessment was conducted for the six pesticides (dichlorvos, diazinon, fenitrothion, iprobenfos, isoprothiolane and thiobencarb) by their PNEC values. Further, the concentrations of the 11 pesticides in the water from Lake Biwa and rivers were evaluated by their environmental water quality standard and guideline values in Japan.

INTRODUCTION

We have already reported various pesticide contamination of water from seven rivers flowing into Lake Biwa (Tsuda et al. 1991; 1992; 1994; 1996; 1997; 1998; Takino et al. 1998). Recently, we again performed the same survey in water from Yanamune River once or twice from April to August in 2007 (Tsuda et al. 2009a) and in water from 21 rivers around Lake Biwa from May to June in 2009 (Tsuda et al. 2011b). On the other hands, we have performed the same survey in water from Lake Biwa and Seta River since 2000 (Nakamura et al. 2008; Tsuda et al. 2009a; Tsuda et al. 2009b; Tsuda et al. 2011b).

Many survey data of various pesticide contamination could be collected in the water from Lake Biwa and the 21 rivers in 2009 (Tsuda et al. 2011b), from Lake Biwa and Seta River in 2000-2009 (Nakamura et al. 2008; Tsuda et al. 2009a; 2009b; 2011b) and from Yanamune River in 1988-2009 (Tsuda et al. 2011a). Five pesticides (fenobucarb, diazinon, iprobenfos, fenitrothion and isoprothiolane) were detected at relatively high frequency in Lake Biwa from 2000 to 2009 and 10 pesticides (diazinon, fenitrothion, iprobenfos, isoprothiolane, chlornitrofen (CNP), thiobencarb, molinate, bromobutide, simetryne and pretilachlor) were detected at high frequency and concentrations in Yanamune River from 1988 to 2009.

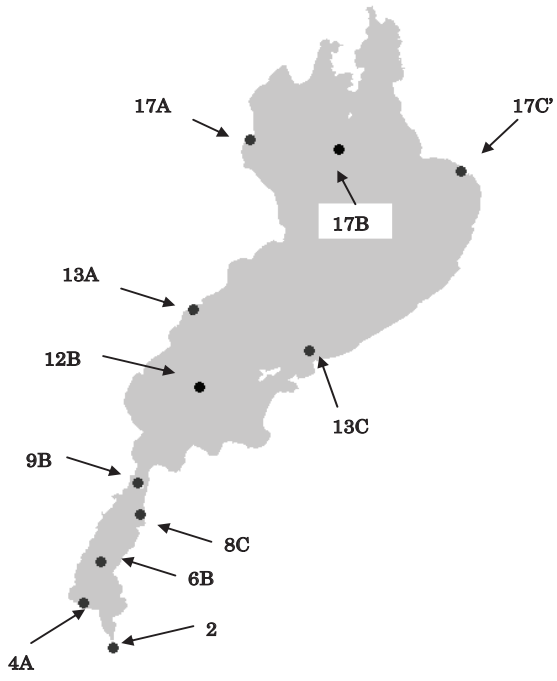
The five pesticides in Lake Biwa and the 10 pesticides in Yanamune River were selected and concentration changes of the pesticides were evaluated based on their shipment amounts (National Institute for Environmental Studies, Japan 2011).
The concentrations of pesticides in the water from Lake Biwa and the 21 rivers in 2009 and from Yanamune River in 1988-2009 were compared with their no observed effect concentration (NOEC) values in algae, water flea and fish (Ministry of the Environment, Japan 2011a; US Environmental Protection Agency 2011) as reference data for initial ecological risk assessment and the risk assessment (Ministry of the Environment, Japan 2011b) was conducted for the pesticides using their predicted no effect concentration (PNEC) values (Ministry of the Environment, Japan 2011c). Further, the concentrations of pesticides in the water from Lake Biwa in 2000-2009 and the 21 rivers in 2009 were evaluated by their environmental water quality standard and guideline values in Japan (Ministry of the Environment, Japan 2011d; 2011e; 2011f)

PESTICIDE CONTAMINATION IN WATER FROM LAKE BIWA AND RIVERS

Survey of pesticides in water from lake Biwa and rivers in 2009

Materials and methods

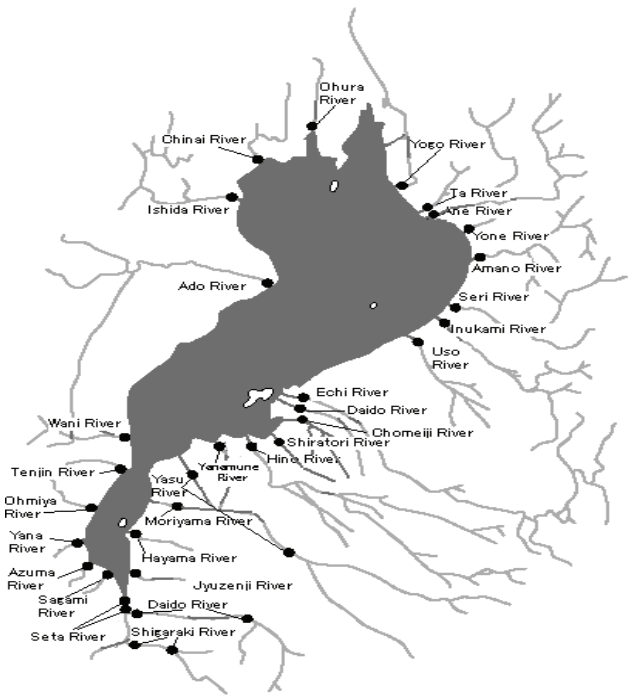
Fig. 1 Map of sampling sites in Lake Biwa and Seta River



Sampling sites of the survey in 2009 are shown in Fig. 1 for Lake Biwa and Seta River and in Fig.2 for rivers around Lake Biwa. Water samples were collected from Lake Biwa (17A, 17B, 17C', 13A, 13C, 12B, 9B, 8C, 6B, 4A) on June 1, Seta River (2) on June 2, Yone river, Amano River, Seri River, Uso River and Echi River on May 25, Shiratori river, Hino River, Yanamune River, Yasu River, Moriyama River and Jyuzenji River on May 26, Daido River, Shigaraki River, Sagami River and Azuma River on June 15, Tenjin River, Wani River, Ado River, Ishida River, Chinai River and Ohura River on June 17. Water samples were analyzed immediately after sample collection.

Analyses of 30 pesticides (Table 1) were performed by the following procedure (Nakamura et al. 2008). A measured volume (500 mL) of water sample was passed through a Aqusis PLS-3 column and eluted with 4 mL of dichloromethane after drying with air stream at room temperature for 40 min. Determination of the pesticides in the eluate was performed using selected ion monitoring (SIM) by gas chromatograpy-mass spectrometry (GC-MS) after evaporation to 0.5 mL. Average recoveries (n=5) were 52% for dichlorvos, 130% for iprobenfos, 124% for propiconazole-2 and 78-117% for the other 27 pesticides at a spiked level of 0.1 μg/L. Quantification limits were 0.1 μg/L for propiconazole-1 and propiconazole-2, 0.05 μg/L for cafenstrole and 0.01-0.02 μg/L for the other 27 pesticides.

Fig. 2 Map of sampling sites in rivers around Lake Biwa



Results of survey

Table 1 Concentrations of pesticides in water from Lake Biwa and rivers in 2009

Pesticide	Use	Lake Biwa and Seta River	River (1)	River (2)	Quantification limit
Dichlorvos	Insecticide	ND～ND	ND～ND	ND～0.06	0.02
Isoprocarb		ND～ND	ND～ND	ND～ND	0.01
Fenobucarb		ND～ND	ND～ND	ND～ND	0.01
Diazinon		ND～ND	ND～0.02	ND～ND	0.01
Fenitrothion		ND～ND	ND～0.08	ND～ND	0.01
Fenthion		ND～ND	ND～ND	ND～ND	0.01
Dichlobenil	Fungicide	ND～ND	ND～ND	ND～ND	0.01
Fthalide		ND～ND	ND～ND	ND～ND	0.01
Flutolanil		ND～ND	ND～ND	ND～ND	0.01
Propiconazole-1		ND～ND	ND～ND	ND～ND	0.1
Propiconazole-2		ND～ND	ND～ND	ND～ND	0.1
Iprobenfos		ND～0.01	ND～0.01	ND～ND	0.01
Isoprothiolane		ND～ND	ND～0.03	ND～0.02	0.01
Pyrokiron		ND～0.01	ND～0.52	ND～5.9	0.01
Simazine	Herbicide	ND～ND	ND～ND	ND～ND	0.01
Propyzamide		ND～ND	ND～ND	ND～ND	0.01
Alachlor		ND～ND	ND～ND	ND～ND	0.01
Thiobencarb		ND～ND	ND～ND	ND～ND	0.02
Dimethamethorin		ND～0.08	ND～0.02	ND～0.22	0.01
Dimepiperate		ND～ND	ND～0.02	ND～ND	0.01
Pyributicarb		ND～ND	ND～ND	ND～ND	0.01
Anilofos		ND～ND	ND～ND	ND～ND	0.02
Esprocarb		ND～ND	ND～0.10	ND～0.03	0.01
Thenylchlor		ND～0.04	ND～0.19	ND～ND	0.01
Molinate		ND～0.01	ND～0.54	ND～ND	0.01
Cafenstrole		ND～ND	ND～0.86	ND～0.05	0.05
Pretilachlor		0.02～0.28	ND～0.68	ND～4.4	0.01
Mefenacet		ND～0.36	ND～1.6	ND～0.25	0.02
Simetryne		0.03～1.1	0.03～5.9	ND～0.43	0.01
Bromobutide		0.09～3.1	0.02～21	ND～6.6	0.01

Results of the survey are summarized in Table 1 for Lake Biwa and Seta river, River (1) (Yone River, Amano River, Seri River, Uso River, Echi River, Shiratori River, Hiro River, yanamune River, Yasu River (2 sites), Moriyama River and Jyuzenji River) and River (2) (Daido River, Shigaraki River, Sagami River, Azuma River, Tenjin River, Wani River, Ado River, Ishida River, Chinai River and Ohura River). Detection of the six insecticides in Lake Biwa and Seta River, River (1) and River (2) are shown in Figs. 3-5, respectively. No insecticides were detected in Lake Biwa but diazinon, fenitrothion and dichlorvos were detected in the rivers. Detection of the eight fungicides in Lake Biwa and Seta River, River (1) and River (2) is shown in Figs. 6-8, respectively. For fungicides, iprobenfos and pyrokiron were detected at low concentrations in Lake Biwa. Iprobenfos was detected in Lake Biwa at the same concentration level as in River (1). The concentrations of pyrokiron in the rivers were considerably higher than those in Lake Biwa and isoprothiolane was detected only in the rivers. Detection of the 16 herbicides in Lake Biwa and Seta River, River (1) and River (2) is shown in Figs. 9-11, respectively. The kinds of herbicides detected in River (1) were almost the same as those in Lake Biwa and Seta River. Detection frequeny and concentrations of the herbicides in River (1) were higher

than those in Lake Biwa and Seta River. The concentration levels and the kinds of the herbicides detected in River (1) were different from those in River (2). This is probably due to the difference of sampling date. That is, the kinds and amounts of the used herbicides were gradually changing from May 25 and 26 to June 15 and 17.

Fig. 3 Detection of insecticides in Lake Biwa and Seta River water

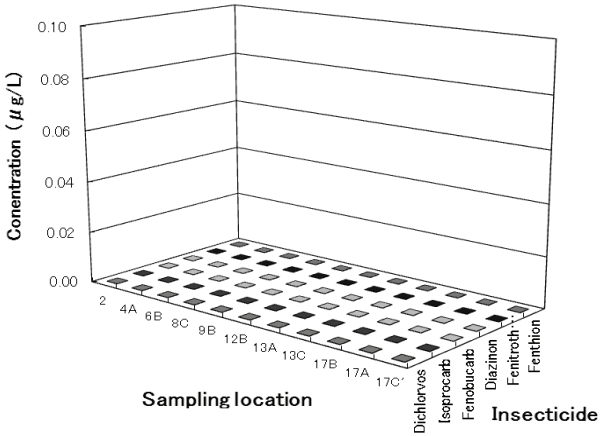


Fig. 4 Detection of insecticides in River (1) flowing into eastern area of Lake Biwa

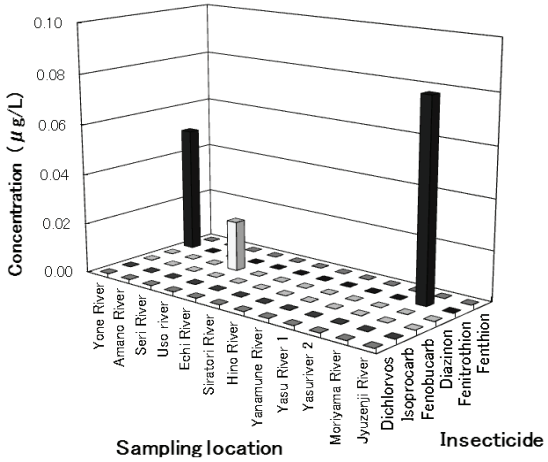


Fig. 5 Detection of insecticides in River (2) flowing into Seta River and western area of Lake Biwa

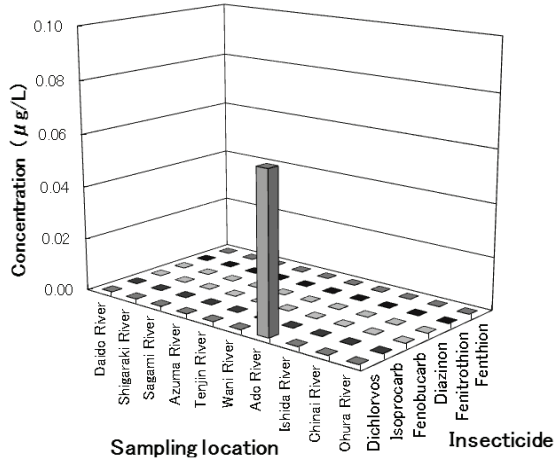


Fig. 6 Detection of fungicides in Lake Biwa and Seta River water

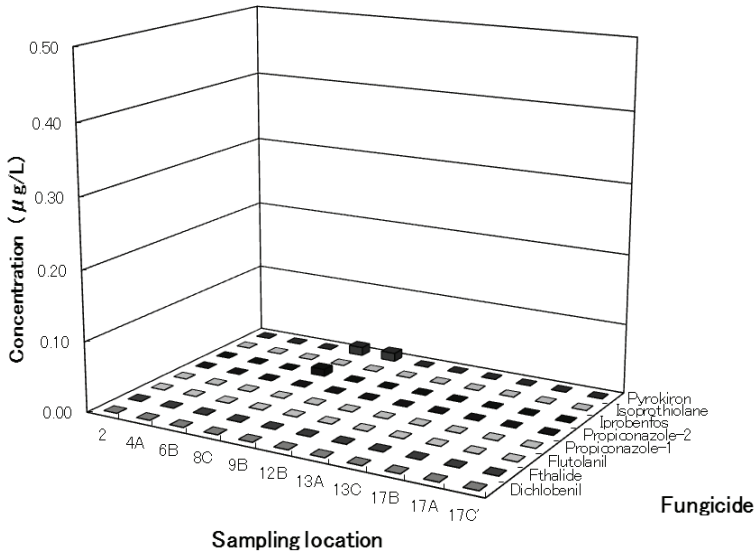


Fig. 7 Detection of fungicides in River (1) flowing into eastern area of Lake Biwa

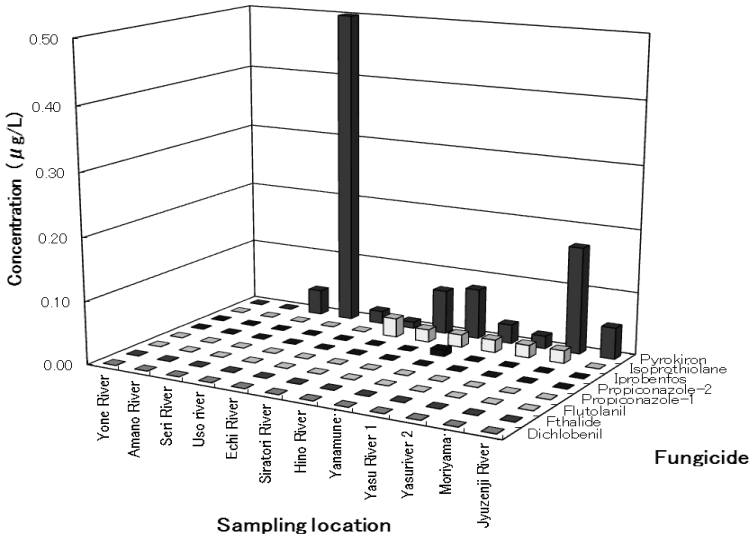


Fig. 8 Detection of fungicides in River (2) flowing into Seta River and western area of Lake Biwa

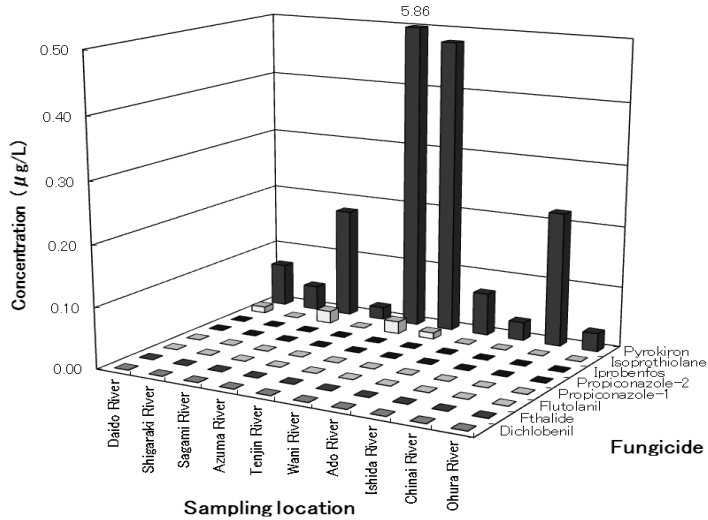


Fig. 9 Detection of herbicides in Lake Biwa and Seta River water

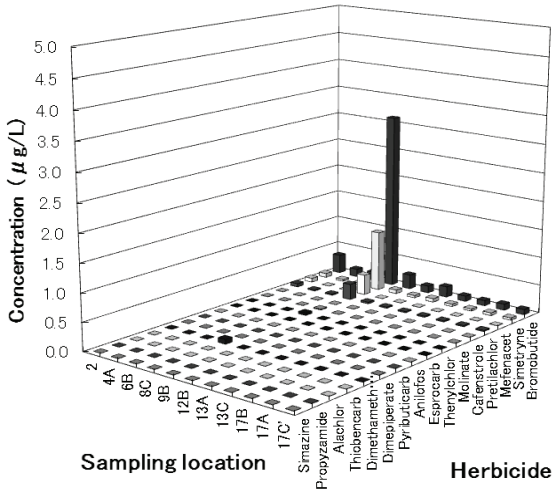


Fig. 10 Detection of herbicides in River (1) flowing into eastern area of Lake Biwa

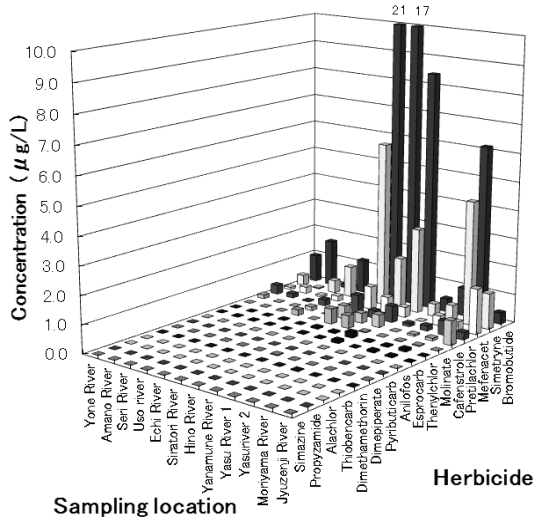
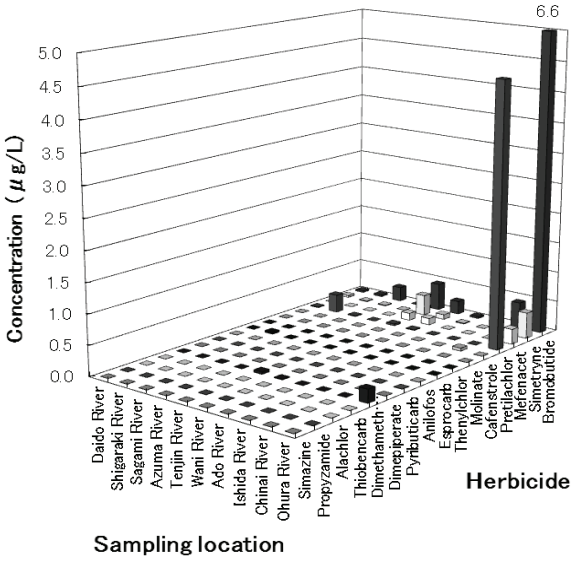


Fig. 11 Detection of herbicides in River (2) flowing into Seta River and western area of Lake Biwa



of pesticides in Lake Biwa and Seta River water from 2000 to 2009

Materials and Methods

Water samples were collected from Lake Biwa (17A, 17C', 13A, 13C, 9B, 8C, 6B, 4A) and Seta River (2) (Fig. 1) once in August from 2000 to 2009. Water samples were analyzed immediately after sample collection.

A measured volume (500 mL) of water sample was passed through 3M Empore Extraction Disk (C18) after washing with 10 mL of acetone and 10 mL of dichloromethane and conditioning with 10 mL of methanol and 3×10 mL of distilled water. The disk was washed with 10 mL of water, dried 15 min under air stream at room temperature and eluted with 2 mL of acetone, 4 mL, 2 mL and 2mL of dichloromethane. The eluate was mixed with 20 mL of hexane, dehydrated with anhydrous Na_2SO_4 and rotary-vacuum evaporated nearly to 1 mL at 40℃. The solution was transferred to 5 mL graduated test tube by rinsing with hexane and evaporated to 0.5 mL under N_2 gas stream. Determination of 13 pesticides (dichlorvos, fenobucarb, simazine, diazinon, propyzamide, chlorothalonil, iprobenfos, fenitrothion, thiobencarb, isoprothiolane, isoxathion, chlornitrofen and EPN) in the hexane solution was performed using SIM by GC/MS (Nakamura et al. 2008). Average recoveries (n=3) were 71-132% at a spiked level of 0.1 μg/L and quantification limits were 0.01-0.02 μg/L for the 13 pesticides.

Results of survey

Table 2. Concentrations of pesticdes detected in water from Lake Biwa in August from 2000 to 2009

Pesticide	Sampling site									Quantification limit, μg/L
	2	4A	6B	8C	9B	13A	13C	17A	17C'	
	Concentration range, μg/L (Frequency of detection)									
Dichlorvos				0.21 (1/10)						0.02
Fenobucarb	0.02-0.09 (3/10)	0.08 (1/10)	0.05-0.09 (2/10)	0.02-0.08 (4/10)	0.05-0.09 (2/10)	0.05-0.09 (2/10)	0.06-0.09 (2/10)	0.06-0.09 (2/10)	0.06-0.09 (2/10)	0.01
Simazine	0.06 (1/10)			0.04 (1/10)						0.01
Diazinon	0.01-0.10 (4/10)	0.01-0.10 (2/10)	0.02-0.10 (4/10)	0.03-0.55 (8/10)	0.01-0.10 (7/10)	0.09 (1/10)	0.09 (1/10)	0.01-0.09 (2/10)	0.09 (1/10)	0.01
Propyzamide										0.01
Chlorothalonil										0.01
Iprobenfos	0.09-0.20 (3/10)	0.07-0.18 (3/10)	0.11-0.19 (3/10)	0.15-3.09 (7/10)	0.03-0.13 (3/10)		0.12 (1/10)	0.13 (1/10)	0.12 (1/10)	0.01
Fenitrothion	0.12-0.22 (2/10)	0.12-0.21 (2/10)	0.19 (1/10)	0.23 (1/10)	0.22 (1/10)	0.30 (1/10)	0.01-0.16 (2/10)	0.18 (1/10)	0.17 (1/10)	0.01
Thiobencarb	0.12 (1/10)	0.12 (1/10)	0.12 (1/10)	0.12 (1/10)	0.12 (1/10)	0.12 (1/10)	0.12 (1/10)	0.12 (1/10)	0.12 (1/10)	0.02
Isoprothiolane	0.05-0.23 (7/10)	0.03-0.21 (8/10)	0.04-0.21 (7/10)	0.06-9.86 (9/10)	0.04-0.24 (8/10)	0.01-0.16 (6/10)	0.03-0.16 (6/10)	0.01-0.15 (7/10)	0.01-0.16 (6/10)	0.01
Isoxathion										0.02
Chlornitrofen										0.01
EPN	0.11 (1/10)	0.11 (1/10)	0.11 (1/10)	0.11 (1/10)	0.11 (1/10)	0.11 (1/10)	0.10 (1/10)	0.10 (1/10)	0.10 (1/10)	0.01

Results of the survey are summarized in Table 2 for Lake Biwa and Seta River from 2000 to 2009 and environmental water quality standard and guideline values in Japan are summarized in Table 3 (Ministry of the Environment, Japan 2011d; 2011e; 2011f). All concentrations of the 13 pesticides in 2000-2009 were less than the standard and guideline values. Dichlorvos, simazine, propyzamide, chlorothalonil, isoxathion, chlornitrofen were detected at very low frequency or not at all. On the other hand, fenobucarb, diazinon, iprobenfos, fenitrothion and isoprothiolane were detected at relatively high frequency.

Yearly changes in the concentrations of iprobenfos and isoprothiolane in Lake Biwa water and those in their shipment amounts in Shiga Prefecture are shown in Figs. 12 and 13, respectively, as example data. In 2000-2009, the concentrations of iprobenfos and isoprothiolane decreased according to the decrease of their shipment amounts in the two sites (4A and 6B) and the four sites (4A, 6B, 13C and 17A), respectively.

Monitoring of pesticides in Yanamune River water from 1988 to 2009

Materials and Methods

Water samples were collected from Yanamune River flowing into Lake Biwa (Fig. 1) once or twice every month in 1988-1997 (Tsuda et al. 1991; 1992; 1994; 1996; 1997; 1998; Takino et al. 1998), once or twice from April to August in 2007 (Tsuda et al. 2009) and on May 29 in 2009. Water samples were analyzed immediately after sample collection.

Table 3 Environmental water quality standard and guideline values by Ministry of the Environment, Japan

Pesticide	Use	Standard value (μg/L)	Guideline value 1 (μg/L)	Guideline value 2 (μg/L)
Dichlorvos	Insecticide		8	
Fenobucarb			30	
Diazinon			5	
Fenitrothion			3	
Isoxathione			8	
EPN			6	
Iprobenfos	Fungicide		8	
Fthalide				100
Flutolanil				200
Isoprothiolane			40	
Chlorothalonil			50	
Molinate	Herbicide			5
Simazine		3		
Propyzamide			8	
Bromobutide				40
Simetryne				60
Esprocarb				10
Thiobencarb		20		
Thenylchlor				40
Mefenacet				9

Fig. 12 Variations in shipment amount of iprobenfos in Shiga Prefecture and its concentration in Lake Biwa water

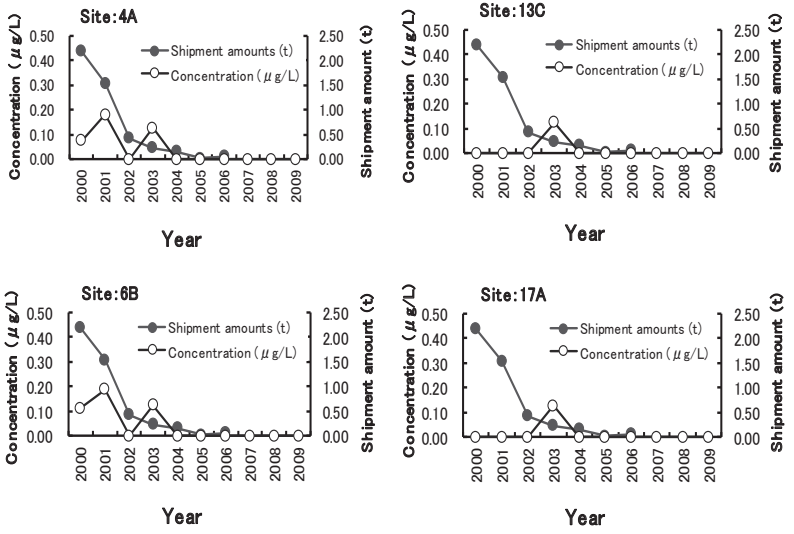
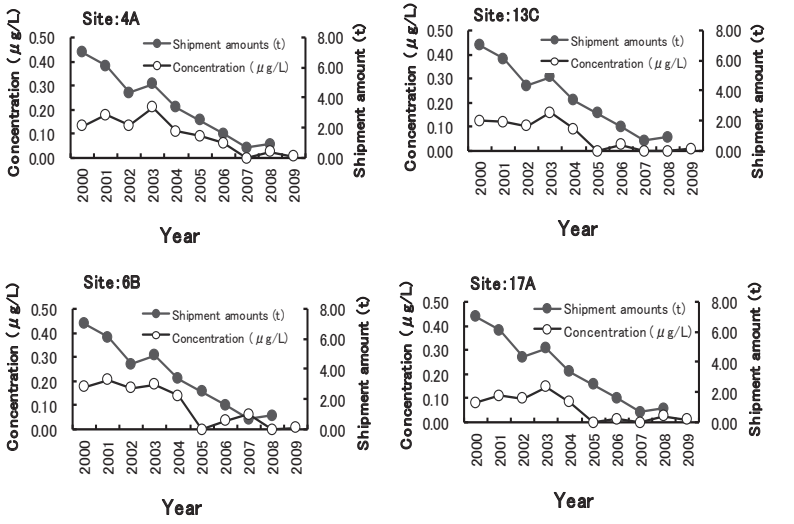


Fig. 13 Variations in shipment amount of isoprothiolane in Shiga Prefecture and its concentration in Lake Biwa water



In 1988-1997, analyses of 10 pesticides (diazinon, fenitrothion, iprobenfos, isoprothiolane, chlornitrofen (CNP), thiobencarb, molinate, bromobutide, simetryne and pretilachlor) in the water samples were performed by the following procedure.

A measured volume (1000 mL) of the water was shaken with 100 mL of dichloromethane after addition of 50 g of NaCl. The organic layer was again shaken and filtered through anhydrous Na_2SO_4 and the aqueous layer was again shaken and filtered in the same manner. The combined filtrate was rotary-vacuum evaporated just to dryness at 40℃ and the residue was dissolved in 1 mL of hexane. Determination of the pesticides in the hexane solution was performed using SIM by GC-MS. In 2007 and 2009, analyses were performed by the following procedure (Nakamura et al. 2008). A measured volume (500 mL) of water sample was passed through a Aqusis PLS-3 column and eluted with 4 mL of dichloromethane after drying with air stream at room temperature for 40 min. Detemination of the 10 pesticides in the eluate was performed using SIM by GC/MS after evaporation to 0.5 mL. Average recoveries (n=3) were 88-108% for the 10 pesticides at a spiked level of 0.1 μg/L and quantification limits were 0.01 μg/L for all of the 10 pesticides.

Results of survey

Variatons in yearly maximum concentrations of the two insecticides (diazinon and fenitrothion) in Yanamune River were compared with those in their shipment amounts (Fig. 14). Diazinon concentrations showed no tendency to increase or decrease from 1988 to 1996 but showed low values (0.01 μg/L and ND) in 2007 and 2009, respectively. The variations in shipment amounts of diazinon showed decreasing tendency after 1996 and this tendency was equal to those in the concentrations after 1996. On the other hand, fenitrothion showed decreasing tendency in both of the concentrations and the shipment amounts from 1990 to 2007.

Fig. 14 Variations in shipment amounts of two insecticides in Shiga Prefecture and their yearly maximum concentrations in Yanamune River water

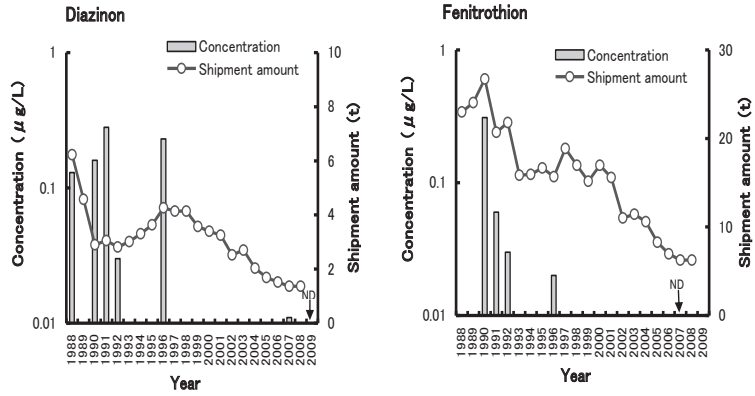
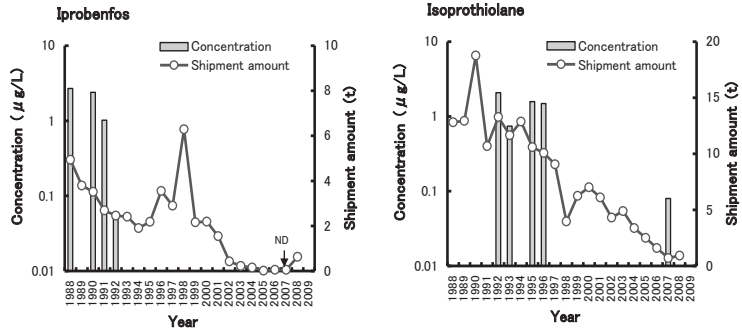


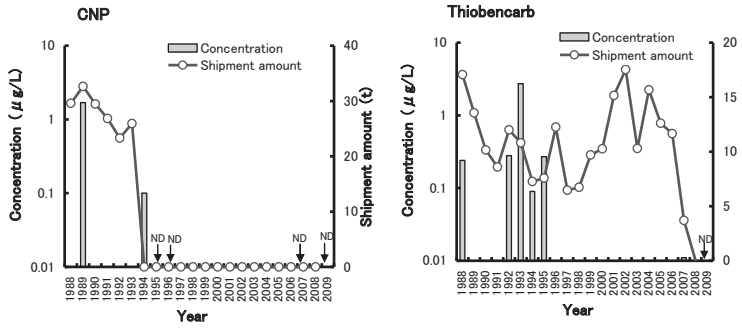
Fig. 15 Variations in shipment amounts of two fungicides in Shiga Prefecture and their yearly maximum concentrations in Yanamune River water

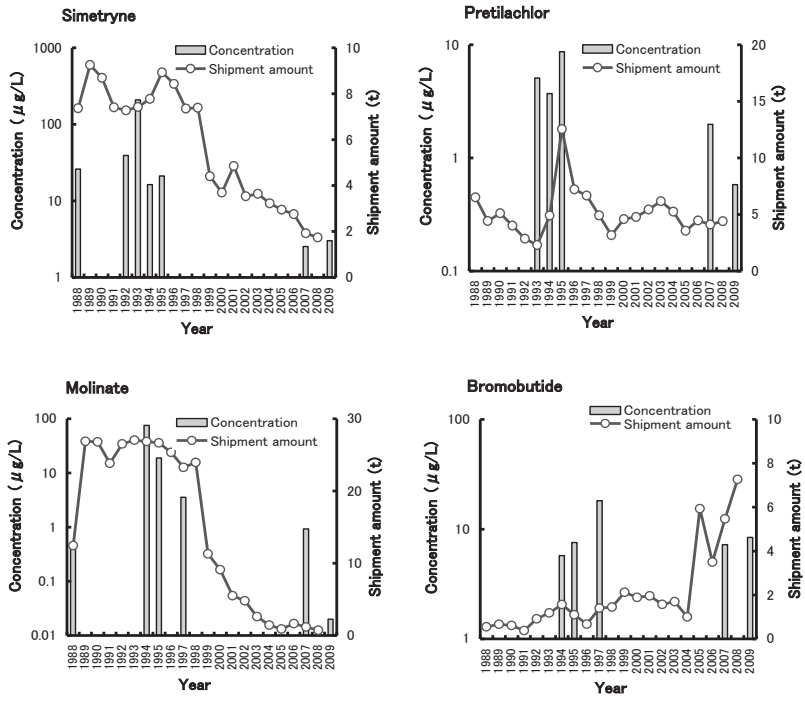


Similarly, variations in the concentrations of the two fungicides (iprobenfos and isoprothiolane) in Yanamune River were compared with those in their shipment amounts (Fig. 15). Iprobenfos and isoprothiolane showed decreasing tendency in both of the concentrations and the shipment amounts from 1988 to 2007 for iprobenfos and from 1992 to 2007 for isoprothiolane, respectively.

Variations in shipment amounts of six herbicides in Shiga Prefecture and their yearly maximum concentrations in Yanamune River water are shown in Fig. 16. For CNP, the use in Japan was inhibited in 1994 and the shipment amount has been reported to be 0 t since 1994. The CNP concentrations were 1.7 μg/L in 1989, 0.1 μg/L in 1994, ND in 1995, 1996, 2007 and 2009. The decreasing tendency in the CNP concentrations was Prefecture was 11.7 t in 2006 but decreased to 3.7 t in 2007 and 0 t in 2008 owing to self-control of the use in Shiga Prefecture equal to that in the shipment amount. For thiobencarb, the shipment amount in Shiga.

Fig. 16 Variations in shipment amounts of six herbicides in Shiga Prefecture and their yearly maximum concentrations in Yanamune River water





The thiobencarb concentrations were 0.09～2.7 μg/L from 1988 to 1995 and ND in 2009 in agreement with the shipment tendency. The shipment amounts of simetryne and molinate have decreased since 1995 and the decreasing tendencies in their concentrations have been equal for simetryne but were considerably deviated in 2007 for molinate. The shipment amount of pretilachlor was nearly constant except in 1995 and the pretilachlor concentrations were almost constant. For bromobutide, the shipment amount has increased since 2004 but the concentrations have been nearly constant.

ECOLOGICAL RISK ASSESSMENT OF PESTICIDES IN WATER FROM LAKE BIWA AND RIVERS

Initial risk assessment of pesticides in water from Lake Biwa and rivers in 2009

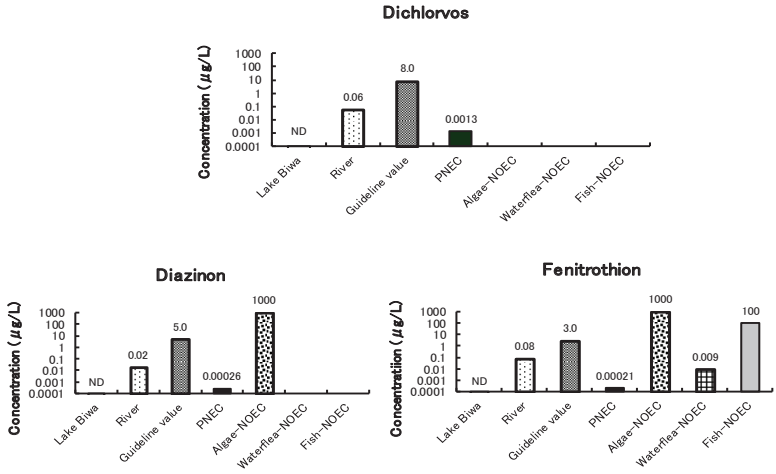
Methods

The concentrations of the 11 pesticides (dichlorvos, diazinon, fenitrothion, iprobenfos, isoprothiolane, esprocarb, molinate, pretilachlor, mefenacet, simetryne and bromobutide) in Lake Biwa and river water were evaluated by their environmental water quality guideline values (Table 3) in Japan (Ministry of the Environment, Japan 2011d; 2011e; 2011f). Further, their concentrations were compared with their NOEC values in algae, water flea and fish (Ministry of the Environment, Japan 2011a; US Environmental Protection Agency 2011) as reference data for initial ecological risk assessment and ecological risk assessment

was conducted for the five pesticides (dichlorvos, diazinon, fenitrothion, iprobenfos and isoprothiolane) by Guideline for Initial Environmental Risk Assessment of Chemicals (Ministry of the Environment, Japan 2011b). The pesticide concentrations were evaluated using their PNEC values in this risk assessment method (Ministry of the Environment, Japan 2011c).

Evaluation by guideline values and ecological risk assessment

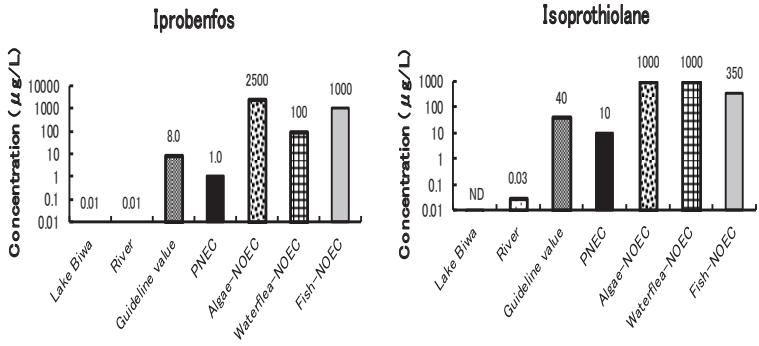
Fig. 17 Maximum concentrations of three insecticides in Lake Biwa and river water and their guideline, NOEC and PNEC values



Maximum concentrations of the 11 pesticides in in Lake Biwa and river water were compared with their guideline., NOEC and PNEC values in Figs. 17 – 19.

All of the dichlorvos, diazinon and fenitrothion concentrations were considerably less than the guideline values (Fig. 17). The diazinon and fenitrothion concentrations were considerably less than the NOEC values of algae and the fenitrothion concentration was considerably less than the NOEC value of fish. However, the fenitrothion concentration exceeded the NOEC value of waterflea. For initial ecological risk assessment by PNEC, all of the dichlorvos, diazinon and fenitrothion concentrations considerably exceeded the PNEC values (0.0013, 0.00026 and 0.00021 μg/L). Several survey data of dichlorvos, diazinon and fenitrothion in river water have been reported in Japan (Sakai 2009). For example, their concentrations in Tsurumi River water in Yokohama City were 0.06～0.14 μg/L for dichlorvos, 0.05 μg/L for diazinon and 0.05～0.82 μg/L for fenitrothion. All of the pesticide concentrations considerably exceeded the PNEC values.

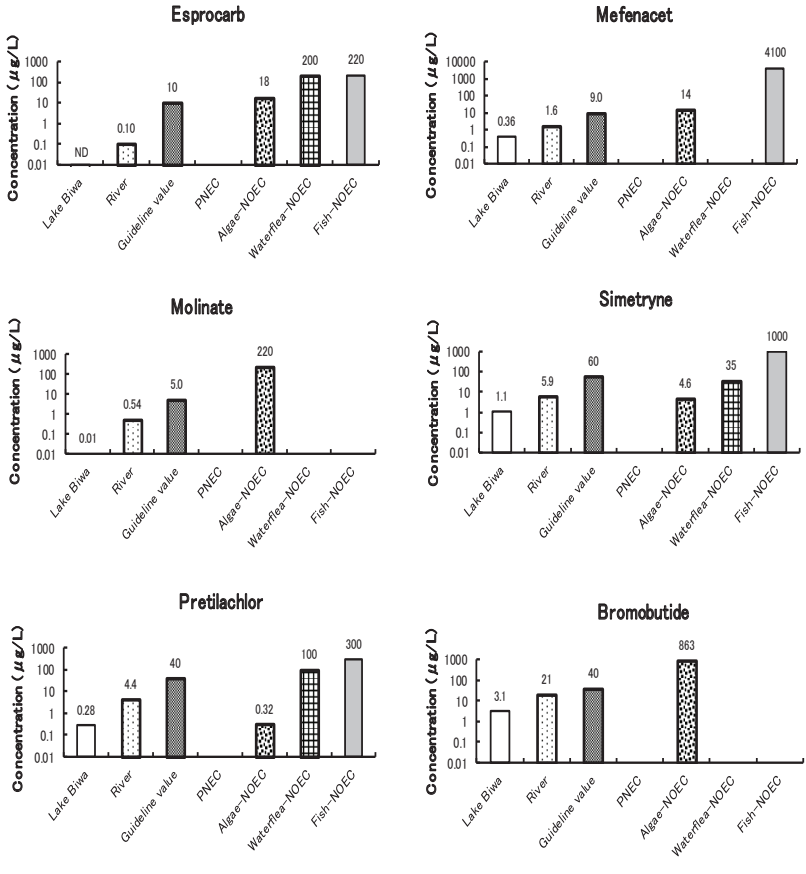
Fig. 18 Maximum concentrations of two fungicides in Lake Biwa and river water and their guideline, NOEC and PNEC values



All of the iprobenfos and isoprothiolane concentrations were considerably less than the guideline values (Fig. 18). Further, all of the iprobenfos and isoprothiolane concentrations were considerably less than the NOEC values of algae, waterflea and fish. For the initial risk assessment, all of the iprobenfos and isoprothiolane concentrations were considerably less than the PNEC values (1 and 10 μg/L).

All concentrations of the esprocarb, molinate, pretilachlor, mefenacet, simetryne and bromobutide were considerably less than the guideline values (Fig. 19). The pretilachlor concentration was 14 times higher than the NOEC value of algae and the simetryne concentration was nearly the same as the NOEC value of algae. All concentrations of the other four herbicides were considerably less than the NOEC values of algae. All concentrations of the esprocarb, pretilachlor, mefenacet and simetryne were considerably less than the NOEC values of waterflea and fish.

Fig. 19 Maximum concentrations of six herbicides in Lake Biwa and river water and their guideline, NOEC and PNEC values

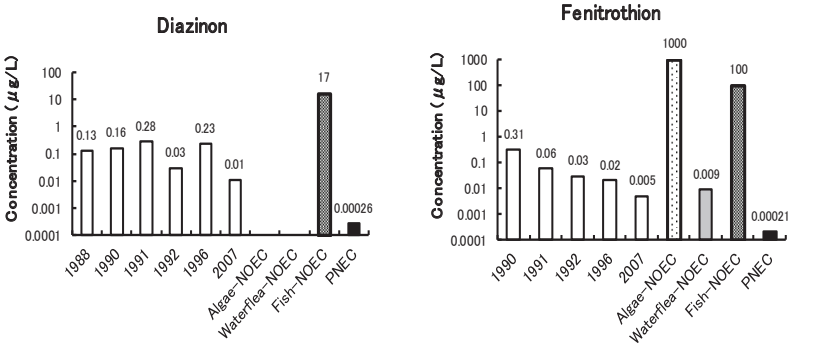


Initial risk assessment of pesticides in Yanamune River water from 1988 to 2009

Methods

Yearly maximum concentrations of the eight pesticides (diazinon, fenitrothion, iprobenfos, isoprothiolane, thiobencarb, molinate, simetryne and pretilachlor) in Yanamune River water were compared with their no observed effect concentration (NOEC) values in algae, water flea and fish (Ministry of the Environment, Japan 2011a; US Environmental Protection Agency 2011) as reference data for initial ecological risk assessment and ecological risk assessment was conducted for the five pesticides (diazinon, fenitrothion, iprobenfos, isoprothiolane and thiobencarb) by Guideline for Initial Environmental Risk Assessment of Chemicals (Ministry of the Environment, Japan 2011b). The pesticide concentrations were evaluated using their PNEC values in this risk assessment method (Ministry of the Environment, Japan 2011c).

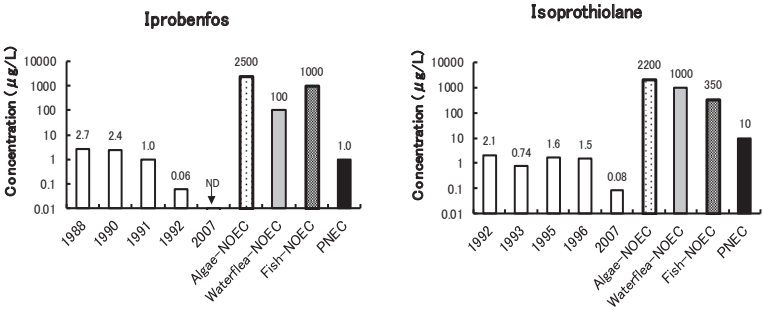
Fig. 20 Yearly maximum concentrations of two insecticides in Yanamune River water and their NOEC and PNEC values



Yearly maximum concentrations of eight pesticides (two insecticides, two fungicides and four herbicides) in Yanamune River from 1988 to 2009 were compared with their NOEC and PNEC values in Figs. 20 – 22.

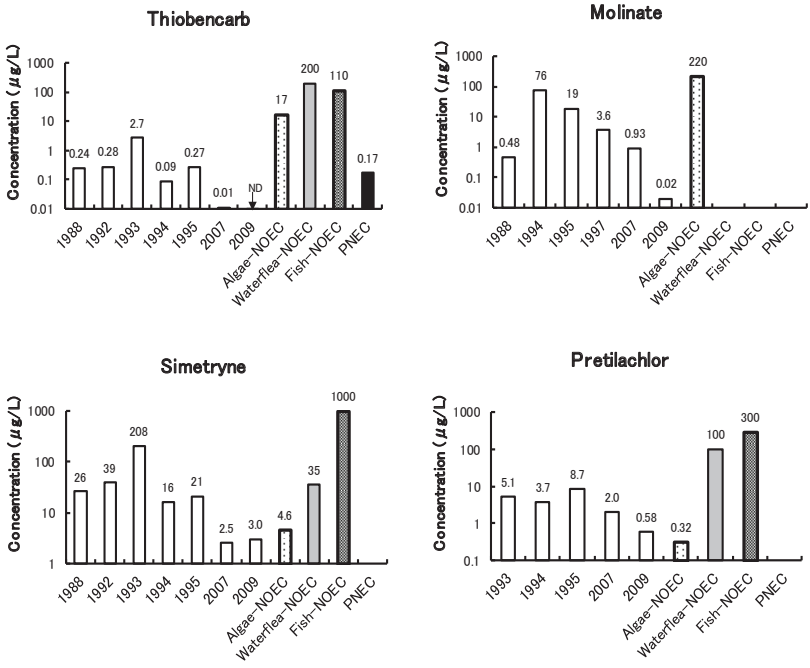
All of the diazinon concentrations in the river water were less than the NOEC value of fish and all of the fenitrothion concentrations were less than the NOEC values of algae and fish (Fig.20). However, the fenitrothion concentrations from 1990 to 1996 exceeded the NOEC value of waterflea. For initial ecological risk assessment by PNEC, all of the diazinon and fenitrothion concentrations from 1988 to 2007 exceeded the PNEC values (0.00026 and 0.00021 μg/L). Many survey data of diazinon in river water have been reported in the world. For example, the diazinon concentrations were <0.005～0.51 μg/L in Selangor River, Malaysia (Leong et al. 2007), <0.002～0.01 μg/L in Po River, Italy (Agradi et al. 2000) and ND～0.08 μg/L in Sacramento River, USA (Hall 2003). Many data of the diazinon concentrations exceeded the PNEC value (0.00026 μg/L) not only in Japan but also in the other countries.

Fig. 21 Yearly maximum concentrations of two fungicides in Yanamune River water and their NOEC and PNEC values



All of the iprobenfos and isoprothiolane concentrations in the river water from 1988 to 2007 were less than the NOEC values of algae, waterflea and fish (Fig.21). For the initial risk assessment, all of the isoprothiolane concentrations were less than the PNEC value (10 μg/L). However, the iprobenfos concentrations in 1988 and 1990 exceeded the PNEC value (1 μg/L) and those in 1992 and 2007 were less than the value.

Fig. 22 Yearly maximum concentrations of four herbicides in Yanamune River water and their NOEC and PNEC values



All of the thiobencarb concentrations in the river water were less than the NOEC values of algae, waterflea and fish and all of the molinate concentrations were less than the NOEC values of algae (Fig. 22). In the other countries, the molinate concentrations were <1～3.2 μg/L in Sacramento River, USA (California Environmental Protection Agency, 2002) and 0.004～0.36 μg/L in Po River, Italy (Agradi et al. 2000). Both data were similarly less than the NOEC values of algae. All of the simetryn concentrations were less than the NOEC value of fish. However, those from 1988 to 1995 exceeded the NOEC value of algae and those in 1992 and 1993 exceeded the NOEC value of the waterflea. All of the pretilachlor concentrations were less than the NOEC values of waterflea and fish but exceeded the NOEC value of algae. For the initial risk assessment, the thiobencarb concentrations in 1988, 1992, 1993 and 1995 exceeded the PNEC value (0.17 μg/L).

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Time Trend Variation of Selected Pesticides Residues in Soil from some Regions in Vietnam

Toan Vu Duc

ABSTRACT

This study was carried out to assess the time trend variation of selected organochlorine pesticides (OPCs = *p,p'*-DDT, *p,p'*-DDE, *p,p'*-DDD, α, β, γ and δ-HCH) residues in the soils from some regions of Vietnam. The levels of the selected OCPs were investigated by means of gas chromatography coupled with mass spectrometry. The obtained results show that remarkable residues of selected OPCs were found in most collected soil samples. This highlights the wide contamination of selected OPCs in the study areas. In general, the distribution of low level contamination of ΣHCH and high level contamination of ΣDDT were displayed. Investigation of the ratio of HCH isomers and DDT metabolites in the analysed soil samples indicates that there is no recent input of these compounds. The decreasing trends of ΣDDT and ΣHCH levels are observed.

INTRODUCTION

Of all the chemical compounds with potential environmental and human health impacts, persistent organochlorines pesticides (OCPs) have received the most attention. They are chemicals of high toxicity. They inflict mutagenic, teratogenic and carcinogenic effects on human beings and have significant impact on environmental ecosystems. These substances are transported over long distances, and accumulated in soil, marine environment, plants, and in tissues of living organisms. OCPs can be found actually everywhere on the earth even in regions located far away from emission sources. Strengthening the ability of developing countries to identify, reduce and finally eliminate sources of persistent toxic substances is a crucial step in reducing ambient levels of such chemicals in the air, water and soil, and so safeguarding public health and the environment everywhere.

Viet Nam is located entirely in the tropical zone. A considerable amount of selected pesticides residues such as Dichlorodiphenyltrichloroethane (DDT) and hexachlorocyclohexane (HCH) have been used in the country. These compounds seriously affected public health and environmental quality in Viet Nam (Thao et al., 2005). However, to our knowledge, few data are available for the contamination status, composition analysis and the temporal trend of DDT and HCH in the surface soils from Vietnam. The present study aims at assessing the residue of DDT and HCH in the surface soil from two typical areas in Vietnam (Hanoi city and Bacninh province) to fill this gap.

OVERVIEW

Chemical and physical information

Chemical and physical information of DDT

Chemical formula of DDT is $C_{14}H_9C_{15}$. Technical DDT is prepared by the Bayer condensation of chlorobenzene with trichloroacetaldehyde in oleum (fuming sulfuric acid), and the reaction is carried out with an excess of chlorobenzene (recommended molar ratio 3:1). In the exothermic reaction, oleum is added to the mixture of chlorobenzene and trichloroacetaldehyde under cooling, to maintain the temperature below 30°C. Crude DDT is filtered off, washed with alkali, and dried. Technical grade DDT is composed of up to fourteen chemical compounds, of which only 65–80% is the active ingredient, *p,p'*-DDT and included 15–21% of the nearly inactive *o,p'*-DDT. DDT is transformed by metabolism or by degradation in the environment. The most common metabolites are DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene) and DDD (1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethane) which usually are found together with DDT in environmental samples. Thus, actually, people and animal are poisoned by these compounds at the same time. Each compound has three isomers and their primary isomers are *p,p'*-DDT; *p,p'*-DDE; *p,p'*-DDD. This study will evaluate total concentration DDT which was the sum of *p,p'*-DDT; *p,p'*-DDE and *p,p'*-DDD.

Figure 1. Chemical structure of *p,p'*-DDT, *p,p'*-DDE and *p,p'*-DDD

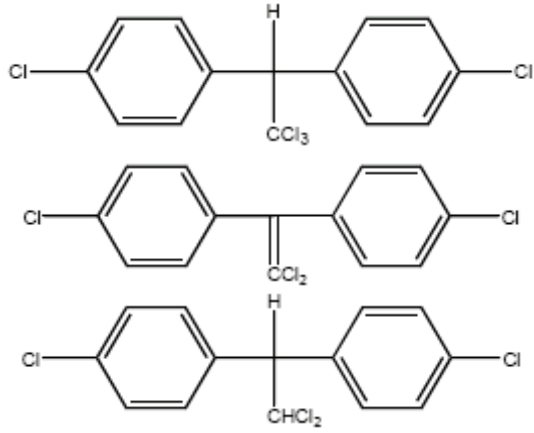


Table 1. Physical and chemical properties of DDT and its metabolites (ATSDR, 2001)

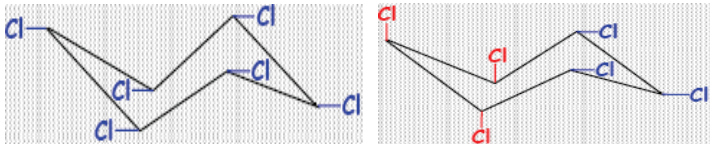
Property	p,p'-DDT	p,p'-DDE	p,p'-DDD
Color	Colorless crystals, white powder	White	Colorless crystals, white powder
Melting point (°C)	109	89	109 - 110
Boiling point (°C)	No data	336	350
Density (g/cm^3)	0,98 – 0,99	No data	1,385
Odor	Weak aromatic odor	No data	Odorless
Water solubility (mg/l)	0,025 at 25°C	0,12 at 25°C	0,09 at 25°C
Partition coefficients			
- Log K_{ow} (a)	6,91	6,51	6,02
- Log K_{oc} (b)	5,18	4,70	5,18
Vapor pressure (ton)	$1,6,10^{-7}$ at 20°C	$6,0,10^{-6}$ at 25°C	$1,35,10^{-6}$ at 25°C
K_H ($at.m^3/mol$) (c)	$8,3,10^{-6}$	$2,1,10^{-5}$	$4,0,10^{-6}$

(a) K_{ow}: Partition coefficient between n-octanol and water
(b) K_{oc}: organic carbon partition coefficients
(c) K_H: Henry's law constant

Chemical and physical information of HCH

1,2,3,4,5,6-hexachlorocyclohexane (HCH), also called benzene hexachloride (BHC), is an organochlorine insecticide used throughout the world. HCH was first synthesized in 1825 by Michael Faraday, and the insecticidal properties of the γ-isomer were discovered in 1942. HCH is available in two formulations: technical HCH and lindane. A total of eight HCH isomers have been identified in technical HCH; however, only the γ-HCH, α-HCH, β-HCH, and δ-HCH and ε-HCH isomers are stable and these are the ones commonly identified in technical formulations. Generally, technical HCH consists of approximately 60–70% α-HCH, 5–12% β-HCH, 10–15% γ-HCH, 6–10% δ-HCH, and 3–4% ε-HCH. Lindane contains more than 90% of γ-HCH but lindane used in many countries is almost pure γ-HCH.

Figure 2. Chemical structure of β-HCH and γ-HCH



β-HCH γ-HCH

This study will evaluate total concentration HCH which is the sum of γ-HCH, α-HCH, β-HCH and δ-HCH.

Table 2. Physical and chemical properties of HCH isomers (ATSDR, 2005)

Property	γ-HCH	α-HCH	β-HCH	δ-HCH
Color	White	Brownish to white	No data	No data
Melting point (°C)	112,5	159–160	314–315	141–142
Boiling point (°C)	323,4 °C at 760 mmHg	288°C at 760 mmHg	60°C at 0,5 mmHg	60°C at 0,36 mmHg
Density (g/cm^3)	1,89 at 19 °C	1,87 at 20 °C	1,89 at 19 °C	No data
Odor	Slightly musty odor	Phosgene-like odor	No data	No data
Solubility				
- Water (mg/l)	17	10	5	10
- Ethanol (mg/l)	6,4	1,8	1,1	24,4
Partition coefficients				
- Log K_{ow}	3,72	3,8	3,78	4,14
- Log K_{oc}	3,57	3,57	3,57	3,8
Vapor pressure (ton)	$4,2x10^{-5}$ at 20 °C	$4,5x10^{-5}$ at 25 °C	$3,6x10^{-7}$ at 20 °C	$3,5x10^{-5}$ at 25 °C
K_H ($at.m^3/mol$)	$3,5x10^{-6}$	$6,86x10^{-6}$	$4,5x10^{-7}$m,n	$2,1x10^{-7}$

Application of study compound

Application of DDT

DDT is a broad spectrum insecticide that was very popular due to its effectiveness, long residual persistence, low acute mammalian toxicity, and low cost. DDT was first synthesized in 1874, but its insecticidal properties remained unknown until reported in 1939 by Swiss chemist Paul Hermann Muller and widely used until about 1970. During Second World War, it was extensively employed for the control of malaria, typhus and other insect-transmitted diseases. DDT has been widely used in agriculture to control insects, such as the pink boll worm on cotton, codling moth on deciduous fruit, Colorado potato beetle, and the European corn borer. DDT has been used extensively to eradicate forest pests, such as the gypsy moth and spruce budworm. It was used in the home as a mothproofing agent and to control lice. In some regions of the world where malaria is endemic, such as South Africa, Swaziland, and Madagascar, DDT is sprayed onto the interior surfaces of homes to decrease the incidence and spread of the disease by controlling mosquitoes

History of global technical DDT usage

Products of DDT are available in several different forms: aerosols, dustable powders, emulsifiable concentrates, granules and wettable powders. DDT have been

marketed under variety of trade names such as Agritan, Anofex, Arkotine, Azotox, Bosan Supra, Bovidermol, Chlorophenothan, Chloropenothane, Clorophenotoxum, Citox, Clofenotane, Dedelo, Deoval, Detox, Detoxan, Dibovan, Dicophane, Didigam, Didimac, Dodat, Dykol, Estonate, Genitox, Gesafid, Gesapon, Gesarex, Gesarol, Guesapon, Gyron, Havero-extra, Ivotan, Ixodex, Kopsol, Mutoxin, Neocid, Parachlorocidum, Pentachlorin, Pentech, PPzeidan, Rudseam, Santobane, Zeidane, Zerdane.

The world production of DDT is not continuously recorded, and estimates of its usage vary. Konishi et al. (2001) reported that the worldwide cumulative output of DDT was about 3 milions tonnes in the 1970s, while Whylie et al. (2003) suggested that annual world consumption from 1971 to 1981 was 68800 tonnes. In 1990, the production of DDT was estimated at 2800 tonnes. The total usage of DDT in European countries decreased from approximately 28000 tonnes in 1970 to zero usage in 1996.

China and India have been suspected to be the biggest producers and users of DDT. China had been a significant producer and user of DDT since the 1950s (about 20% of the total world production). The total quantity of DDT used was estimated to be 43,520 tonnes from 1960 to 1983, with large scale usage in agricultural practices. The highest consumption of DDT was in 1970s close to 1.9 thousand tonnes.

In Thailand, from 23 tonnes to over 73 tonnes of DTT were used annually from 1988 to 1997. In Costa Rica, 128 tonnes of DDT and an additional 147 tonnes of an unspecified mixture of DDT and toxaphene were imported from 1977 to 1985. China, India, and Japan used, in total, 270, 330, and 30 kilo tonnes of DDT, respectively. The former USSR used about 10 kilo tonnes annually from 1950 to at least 1970 and the amount dropped to 300 tonnes in 1980 (Fiedler, 2003).

Application of HCH

Products of HCH are available in emulsifiable and flowable concentrates, soluble concentrates/liquids, wettable powders, dusts, ready-to-use liquids, pressurized liquids and impregnated materials, oil base and aerosol sprays, granules, and as a smoke generator. HCH has been used as a broad spectrum contact and ingested insecticide against grasshoppers, cohort insects, rice insects, wireworms and other soil pests. It has also been used for seed protection, poultry and livestock treatment, household vector control, lumber protection, and even for rodent baits. γ-HCH is also available for the pharmaceutical treatment of scabies and head lice. A 1% γ-HCH lotion is available for the treatment of scabies, and a 1% shampoo is available for the treatment of head lice. It was used as a smoke bomb during First World War.

History of global technical HCH usage

In order to estimate the global technical HCH usage, one should know the amount of this insecticide used in each country. Various production and usage statistics have been reported for different countries. Some of these data, however, are unreliable or inconsistent. It was necessary to undertake an intensive review and comparison in order to select reasonable data. According the research of Y.F. Li et al, the results of the temporal interpolation give the top 10 countries with highest usage listed in table 3.

Table 3. Top 10 countries with highest technical HCH use (Li et al., 1999)

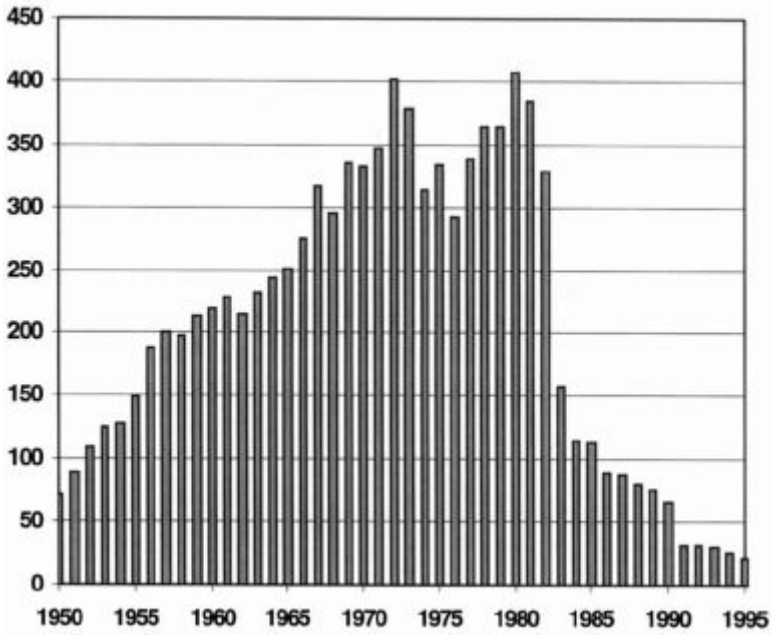
Number	Country	Total usage (kilo tonnes)
1	China	4464
2	India	1057
3	Soviet Union	693
4	France	520
5	Egypt	479
6	Japan	400
7	United State	343
8	Germany East	142
9	Spain	133
10	Mexico	132

China started production and use of technical HCH in 1952. For the 31 years from 1952 to 1983, the total amount of technical HCH produced reached more than 4 million ton in China, almost half of the total global usage. Production and usage of technical HCH in Japan began after World War II and accelerated steadily until 1971 when a ban was implemented on this chemical. Accumulative production and consumption of this insecticide up to the ban has been estimated at 400 kilo tonnes. Total HCH residue concentration in different environmental compartments in China and Japan were comparable, and among the highest level in the world. This is not surprising since the total average usage of technical HCH over arable land in China was similar to that in Japan. In the former Soviet Union, technical HCH was one of the most widely used insecticides from 1940s to 1980s. The most extensive areas of arable lands on the territory of the former Soviet Union were in Russia, Ukraine, and the middle Asian republics.

Global technical HCH usage trends

Global technical HCH use trends from 1950 to 1995 are depicted in Figure 3. Three significant decreases of global technical HCH usage are apparent in the figure. The first one started in 1973 after Japan and other countries, mostly the developed ones, banned the use of this pesticide. The second started in 1983 when China banned the use of technical HCH, and the third one occurred around 1990 when India banned technical HCH usage in agriculture and the former Soviet Union banned the usage of this chemical (Li et al., 1999).

Figure 3. Long-term trends of global technical HCH usage from 1950 to 1995



TOXICOLOGY

Toxicology of DDT

Distribution and excretion of DDT

DDT enters rivers and streams mainly through industrial point sources, runoff from agricultural fields and from atmospheric deposition due to volatilization. People who live in the environment with contamination of DDT would most likely be exposed by accidentally swallowing soil, having skin contact with the soil, inhaling DDT vapor, or breathing in DDT in dust.

The distribution and storage of DDT in humans and animals has been extensively studied. DDT and its metabolites, DDE and DDD, are lipid-soluble compounds. Once absorbed, they are readily distributed via the lymph and blood to all body tissues and are stored in these tissues generally in proportion to organ tissue lipid content.

Morgan and Roan (1971, 1974) evaluated the distribution of orally administered DDT, DDE and DDD in volunteers (ATSDR, 2001). The administered doses ranged from 5 to 20 mg DDT/kg/day for up to 6 months. The ratio of concentration of DDT stored in adipose tissue to that present in blood was estimated to be 280:1.

DDT uptake into tissues is a function of the blood flow, lipid content of that tissue, and the partition coefficient for DDT between the blood and lipids in specific organs. DDT, DDE, and DDD have been reported to be distributed to and retained in the adipose tissue of humans. The affinity for storage in adipose tissue is related

to each chemical's lipophilicity and increases in the order *p,p'*-DDD, *o,p,'*-DDT < *p,p'*-DDT < *p,p'*-DDE. DDT and DDE selectively partition into fatty tissue and into human breast milk. The *p,p'*-isomer of DDT and DDE was found in 100% of the samples tested, with mean concentrations of 0.19 and 1.9 ppm (lipid-basis), respectively. In Finland, samples taken between 1973 and 1982 indicate a reduction of more than 50% in total DDT concentration in human milk (ATSDR, 2002).

Excretion of DDT has been studied in humans and a variety of animals. The major route of excretion of absorbed DDT in humans appears to be in the urine, but some excretion also occurs by way of feces and breast milk. Results of studies with mice, rats, and hamsters indicate that the metabolites of DDT are excreted primarily in the urine and feces.

The metabolism of DDT, DDE, and DDD has been studied in humans and a variety of other mammalian species. The metabolism in rats, mice, and hamsters is similar to that in humans; however, not all of the intermediary metabolites identified in animals have been identified in humans. It has been proposed by a number of investigators that in mammals, the major urinary metabolite of DDT, 2,2-*bis*(*p*-chlorophenyl) acetic acid (DDA), is produced by a sequence involving reductive dechlorination, dehydrochlorination, reduction, hydroxylation, and oxidation of the aliphatic portion of the molecule.

Followed the model metabolic scheme for DDT of Peterson and Robison 1964, DDT is initially metabolized in the liver to two intermediary metabolites, DDE and DDD (ATSDR, 2001). In rats, DDE is slowly converted in the liver to 1-chloro-2,2-*bis*(*p*-chlorophenyl)ethene (DDMU), and then to DDA in the kidney by way of 1,1-*bis*(*p*-chlorophenyl)ethene (DDNU). DDD is rapidly detoxified by way of DDMU to 1-chloro-2,2-*bis*(*p*-chlorophenyl) ethane (DDMS) and then to DDNU. Metabolism of DDMS to DDNU occurs in both the liver and kidney, but the kidney is the primary site. DDNU is then further metabolized, primarily in the kidney, to 2,2-*bis*(*p*-chlorophenyl)ethanol (DDOH) then to 2,2-*bis*(*p*-chlorophenyl)ethanal (DDCHO), which is further oxidized to DDA (ATSDR, 2002).

Health effects of DDT

The International Agency for Research on Cancer, which is part of the World Health Organization (WHO), measures the carcinogenic risk of various chemicals and places them in two groups:

- Those which are "carcinogenic to humans" (group 1);
- Those which are "probably carcinogenic to humans" (group 2).

The latter group is further subdivided into groups A and B:

- For group 2 A, evidence of carcinogenicity is "fairly well established";
- For group 2 B, evidence is "less well established".

In 1991, the International Agency for Research on Cancer classified DDT as a possible human carcinogen, based on the strong evidence of cancer in laboratory animals and the limited evidence of higher cancer risk in humans exposed to DDT. The United Nation Environmental Protection Agency (US EPA) classified DDT as a Class B2 probable human carcinogen.

DDT can cause cancer in laboratory animals including rats, mice or hamsters. Tumors have been found in the liver, adrenal glands, lung, or lymphatic tissues of

laboratory animals fed or injected with DDT. In contrast, there is little evidence that DDT can cause cancer in monkeys or dogs. There is some evidence of a higher risk of lung or pancreatic cancer in men who worked in DDT manufacturing plants. A greater number of deaths from liver cancer and multiple myeloma (cancer of cells in the bone marrow) were seen in a study of male pesticide applicators that had sprayed DDT for mosquito control. More studies are needed to evaluate cancer risk of those who were exposed to DDT through their occupations.

There is evidence that DDT causes reproductive effects in test animals. In rats, oral doses of 7.5 mg/kg/day for 36 weeks resulted in sterility. In rabbits, doses of 1 mg/kg/day administered on gestation days 4-7 resulted in decreased fetal weights and 10 mg/kg/day on days 7-9 of gestation resulted in increased resorptions. In mice, doses of 1.67 mg/kg/day resulted in decreased embryo implantation and irregularities in the estrus cycle over 28 weeks. It is thought that many of these observed effects may be the result of disruptions in the endocrine system.

Available epidemiological evidence from two studies does not indicate that reproductive effects have occurred in humans as a result of DDT exposure. No associations between maternal blood levels of DDT and miscarriage or premature rupture of fetal membranes were observed in two separate studies.

There is evidence that DDT causes teratogenic effects in test animals as well. In mice, maternal doses of 26 mg/kg/day DDT from gestation through lactation resulted in impaired learning performance in maze tests. In a two-generational study of rats, 10 mg/kg/day resulted in abnormal tail development. Epidemiological evidence regarding the occurrence of teratogenic effects as a result of DDT exposure is unavailable. It seems unlikely that teratogenic effects will occur in humans due to DDT at likely exposure levels.

The evidence for mutagenicity and genotoxicity is contradictory. DDT show positive results in only 1 out of 11 mutagenicity assays in various cell cultures and organisms. Results of in vitro and in vivo genotoxocity assays for chromosomal aberrations indicated that DDT was genotoxic in 8 out of 12 cases, and weakly genotoxic in 1 case. In humans, blood cell cultures of men occupationally exposed to DDT showed an increase in chromosomal damage. In a separate study, significant increases in chromosomal damage were reported in workers who had direct and indirect occupational exposure to DDT. Thus it appears that DDT may have the potential to cause genotoxic effects in humans, but does not appear to be strongly mutagenic. It is unclear whether these effects may occur at exposure levels likely to be encountered by most people (ATSDR, 2002).

The nervous system appears to be one of the primary target systems for DDT toxicity in humans after acute, high exposures. A number of investigators conducted experimental studies on humans in the 1940s and 1950s at controlled doses that produced effects. Other data come from accidental poisonings where dose levels were crudely estimated. Persons exposed to 6 mg DDT/kg administered orally by capsule generally exhibited no illness, but perspiration, headache, and nausea have been reported. Convulsions in humans have been reported at doses of 16 mg DDT/kg or higher (ATSDR, 2002).

Toxicology of HCH

Distribution and excretion of HCH

People will be directly exposed to HCH if they need to use a prescription medication that contains this compound in order to treat control scabies and head lice. They can also be exposed to small amounts of γ-HCH and the other isomers (α-, β-, and δ-HCH) by eating foods that may be contaminated with these compounds. Low levels of exposure to the HCH isomers are also possible from ingesting contaminated drinking water, breathing contaminated air, or having contact with soil or water at hazardous waste sites that may contain these compounds.

The distribution of HCH in humans has been well documented. HCH isomers have been detected in breast milk, adipose tissue and blood samples of humans. A study of adipose tissue samples of 25 persons (19 males and 6 females) from Texas in the mid-1980s found lindane residues in 96% of the samples, with a mean level of 0.20 ppm. The beta-isomer of HCH is more persistent and was determined in 27% of human milk samples tested from Arkansas women. Studies of human milk samples from most European countries indicate low levels of HCH in general, average around 0.2 ppm in fat, with relatively higher levels in Czechoslovakia, France and Italy (ATSDR, 2005).

Humans excrete γ-HCH and its metabolites in urine, feces and milk. Lindane-fed rats were found to excrete 39% of the insecticide in an unmodified form in urine and feces. Angerer et al. (1983) determined that chlorophenols were the primary urinary metabolites of γ-HCH excreted by workers involved in γ-HCH production (ATSDR, 2005). The metabolites 2,3,5-, 2,4,6-, and 2,4,5-trichlorophenol accounted for almost 57.7% of the γ-HCH metabolites identified in the urine collected during the last 2 hours of the workers' shifts. Other urinary metabolites identified included other trichlorophenols, dichlorophenols, tetrachlorophenols, and dihydroxychlorobenzenes. Pentachlorophenol has also been identified as a urinary metabolite in humans following occupational exposure. In vitro investigations indicate that human liver microsomes convert γ-HCH by dechlorination, dehydrogenation, dehydrochlorination, and hydroxylation to five primary metabolites: 3,6/4,5-hexachlorocyclohexene, pentachlorocyclohexene, 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol, and pentachlorobenzene. Similar *in vitro* studies have demonstrated that an epoxide forms during the metabolism of pentachlorocyclohexene. This stable halogenated hydrocarbon epoxide metabolite may be responsible for the mutagenic and carcinogenic effects of γ-HCH (ATSDR, 2005).

Health effects of HCH

Lindane has been classified in Group 2B, possibly carcinogenic to humans as judged by the International Agency for Research on Cancer. The evidence for carcinogenicity in humans has been considered to be inadequate. The evidence for carcinogenicity to animals is considered limited for lindane, but sufficient for technical-grade HCH. The carcinogenicity of lindane in experimental animals is low. Mice fed 100-500 mg/kg diets for 24 weeks showed no signs of tumors. Rats

fed for a lifespan at 5-1,600 mg/kg diet with a mean age at death of 58 weeks, had no increase in tumor incidence. One of the confounding factors in establishing a link between the insecticide and carcinogenicity is the presence of three different dimensional forms of HCH isomers. Each form has a slightly different toxicity. The International Agency for Research on Cancer has concluded that there is sufficient evidence to show that one of the lindane isomers is carcinogenic and limited evidence to establish the carcinogenicity of the beta and gamma isomers.

Female rats experienced a disturbance of their reproductive cycle and inhibited fertility with doses of 0.5 mg/kg for four months. Treatments of 0.05 mg/kg did not produce these effects. Lindane was found to be slightly estrogenic to female rats and also caused the seminiferous tubules in male rats to become atrophied at doses of 8 mg/kg/day over a ten day period. These tests suggest that the compound may have reproductive effects in human populations.

Beagles given 7.5 or 15 mg/kg from day five throughout gestation did not produce pups with any noticeable birth defects. Pregnant rats given small amounts of lindane in their food had offspring unaffected by the pesticide. Lindane, however, can be passed from the mother to the developing fetus. It appears that lindane will not cause developmental effects at low levels of exposure and causes reproductive effects at levels approaching the acute toxicity doses. These effects have not been observed in human populations.

A variety of tests on mice and on microbes have shown no mutagenicity in the cells tested. It has been shown to induce some changes in the chromosomes of cultured human lymphocytes during cell division at fairly low doses. It is unlikely that lindane would pose a mutagenic risk in humans at very low exposure levels (ATSDR, 2005).

Current situation of study compounds in Vietnam

Current application

DDT usages for malaria control in Vietnam begun in 1954 and officially ended in 1995. Before 1993, DDT was even sprayed onto the walls for mosquito prevention in malaria prevention programs. From 1962 to 1981, DDT was widely used in great amounts. Basing on World Bank's documents, in 1962, 1963 and 1981, the amount of DDT for health service reached 1000 tonnes annually (Buxton et al., 2001). In 1990, malaria broke out in large scale, therefore the import of DDT into Viet Nam increased considerably from 1992. DDT was also used for malaria control during the time of war. It is estimated that Vietnam imported approximately 447 tonnes of DDT for malaria control purpose.

DDT and HCH were once used as pesticides in large amounts. Since 1995, DDT and HCH were wide applied for soil treatment before cultivations, harmful insect prevention and farm product preservation after harvest. At present, the official ban on DDT, HCH import and usage is in place. However, there are still high risks of illegal import of these substances in the border between Vietnam and China.

Situation of study compounds in Vietnam

DDT and HCH are not included in the list of monitored items in Vietnam's monitoring system due to certain constraints: finance, equipment and laboratory capabilities. Most statistics about the contents of DDT and HCH in samples have been provided by projects from Institutes, universities and some governmental laboratories, such as the Ministry for Agricultural and Rural development, the Ministry of Science and Technology. However, these statistics provide only limited data on the pollution situation of the studied substances in some provinces of Viet Nam.

STUDY AREAS AND SAMPLING

Study areas

This study selected Hanoi city and Bacninh province of Vietnam to research and collect soil samples.

Hanoi

Hanoi city, located in the Red River Delta in the North Vietnam, is the centre of culture, politics, economy and trade of the whole country. Hanoi comprises seven urban districts and five suburban districts, occupying an area of 927 km^2. Hanoi has many factories in the urban districts and agricultural areas in the surrounding suburbs. Some Persistent organochlorines pesticides (OCPs) such as dichlorodiphenyl trichloroethane (DDT), hexachlorocyclohexane (HCH) were widely used here as pesticides. Although these compounds were banned for a long time, their existences seem to be everywhere. The residues of DDTs and HCHs are also detected in agricultural areas in Hanoi (Thao el al., 2005). Due to the important role of Hanoi, the safety of public health and environmental quality, an assessment of the content and distribution of these compounds in soil is therefore essential.

Bacninh

Bacninh province, located at the east of Hanoi city, is typical centre of industrial, agriculture and traditional village in Viet Nam. Bacninh comprises seven urban districts, occupying an area of 804 km^2. The provincial capital, also known as Bacninh, is a separate municipality.

Collected data showed that DDTs and HCHs have been used in large quantities in this region for a long time. Data about the residues of OCPs in the surface soils here are rarely reported. Understanding the content and distribution of these compounds is therefore essential to determine the utilization of soil for multi-purposes. In the present study, we attempted to elucidate the recent contamination levels and temporal trend of OCPs in surface soil from Hanoi and Bacninh.

Soil sampling

Soil sampling was followed Vietnamese standards (TCVN). These standards compose:

● TCVN 4046 – 85: Method of soil sampling in agricultural areas

● TCVN 5297 – 1995: Soil quality - Sampling - General requirements

● TCVN 5960 – 1995: Soil quality – Sampling: Guidance on the collection, handling and storage of soil for the assessment of aerobic microbial processes in the laboratory.

● TCVN 4047 – 85: Method for the preparation of soil sample for analysis.

● TCVN 6857 - 2001: Soil quality − Simplified soil description.

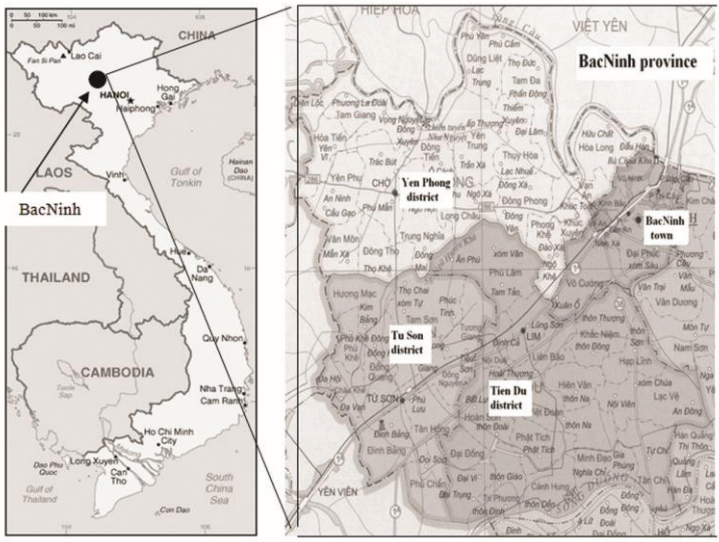
The sampling of soil samples from Bacninh province and Hanoi city according to those standards is shortly described below.

Soil sampling in Bacninh

40 soil samples were collected at 11 typical traditional villages and towns from Bacninh province in February 2006. The sampling-locations were chosen at random, with an attempt to get them evenly distributed over the region (804 km^2, about 1 million people, Figure 4). Among the forty soil samples, most of which were collected from agriculture areas and traditional villages, seven were from industrial areas and five were from urban areas. At each sample location, five samples were collected from a 10 x 10 m^2 plot (located on the crossing diagonals: four in the corners and one in the crossing point), and then thoroughly mixed to form a composite sample.

The samples were taken with an acid-cleaned stainless steel scoops from the upper 5 cm of the soil and then transferred to precleaned polyethylene bags. The collected samples were air dried at room temperature (22–25^oC), sieved to <2 mm, and maintained at 4^oC prior to chemical analysis.

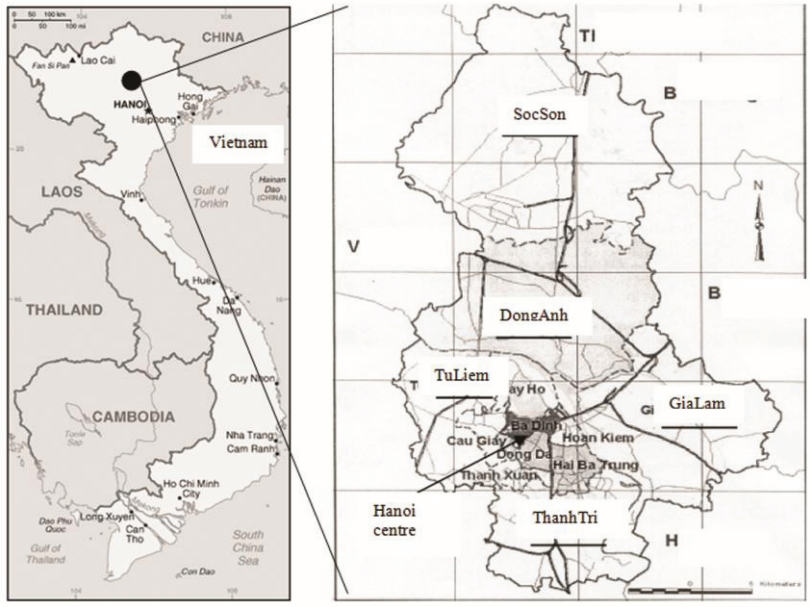
Figure 4. Map showing sampling areas of surface soil from Bacninh, Vietnam



Soil sampling in Hanoi

The sampling campaign for Hanoi was carried out in February 2006 (60 soil samples), during the dry season. Soil samples were collected from agricultural, industrial areas and towns of all five suburban districts, as well as the centre of Hanoi to compare. The sampling sites were chosen at random, with an attempt to get them evenly distributed over Hanoi city (approximately 921km^2, about 3 million people, Figure 5). The sampling square is similar as those from Bacninh. The samples were taken with solvent-rinsed stainless steel scoops from the upper 5cm of the soil and then transferred to pre-cleaned polyethylene bags. The pre-treatment and storage of soil samples from Hanoi prior to analysis are similar with those from Bacninh.

Figure 5. Map showing sampling areas of surface soil in Hanoi, Vietnam



RESULTS AND DISSUSION

Contamination status

Contamination status of DDT

The concentrations of ΣDDT (*p,p'*-DDT, *p,p'*-DDE, *p,p'*-DDD) in soil samples from Hanoi in 2006 are shown in table 4. DDT and its metabolites were detected in 47 of 60 soil samples.

Table 4. Concentrations of DDT and its metabolites (ng g^{-1} dw) in soil samples from Hanoi

Location	*p,p'*-DDE	*p,p'*-DDD	*p,p'*-DDT	ΣDDT
A. Agricultural areas				
SocSon 1 [(a)]	48.85 – 93.16 (67.93) [(c)]	23.42 – 45.75 (32.68)	17.75 – 24.42 (22.73)	94.25 – 163.75 (123.33)
DongAnh 1	32.86 – 49.78 (41.06)	15.43 – 25.87 (21.09)	13.7 – 23.49 (18.24)	62.27 – 97.66 (80.39)
GiaLam 1	35.58 – 47.92 (42.23)	16.38 – 21.44 (19.75)	15.47 – 18.98 (17.53)	67.43 – 88.34 (79.47)
Hanoi centre 1	14.19 – 28.17 (22.36)	8.12 – 13.15 (11.46)	0.06 – 5.38 (3.53)	22.36 – 46.44 (37.35)
TuLiem 1	29.94 – 83.75 (46.56)	15.74 – 45.29 (23.83)	12.83 – 40.96 (21.43)	58.34 – 168.27 (91.83)
ThanhTri 1	N.D [(d)] – 97.54 (48.83)	N.D – 49.36 (21.98)	N.D – 24.93 (12.26)	N.D – 171.83 (81.06)
B. Industrial and urban areas				
SocSon 2 [(b)]	N.D – 36.82 (24.53)	N.D – 17.88 (11.68)	N.D – 13.93 (7.65)	N.D – 67.82 (43.85)
DongAnh 2	N.D – 19.43 (7.59)	N.D – 9.82 (3.89)	N.D – 9.18 (3.43)	N.D – 38.24 (19.93)
GiaLam 2	N.D – 35.19 (13.63)	N.D – 16.83 (6.18)	N.D – 15.45 (5.43)	N.D – 67.47 (25.23)
Hanoi centre 2	N.D – 13.68 (5.04)	N.D – 5.92 (1.99)	N.D – 5.16 (1.75)	N.D – 24.76 (8.79)
TuLiem 2	14.84 – 19.48 (17.23)	5.88 – 8.59 (7.46)	5.62 – 7.72 (6.85)	26.34 – 35.18 (31.53)
ThanhTri 2	N.D – 13.44 (8.83)	N.D – 4.92 (3.23)	N.D – 4.39 (2.93)	N.D – 22.57 (14.95)

[(a)] SocSon 1: agricultural areas of SocSon; [(b)] SocSon 2: industrial and urban areas of SocSon. It is similar with DongAnh, GiaLam, Hanoi centre, Tu Liem and ThanhTri; [(c)] min – max (mean) value. [(d)] N.D: not detected.

ΣDDT was detected in relatively high concentrations in Hanoi agricultural areas. The ΣDDT concentrations ranged from N.D to 171.83 ng g^{-1}. The mean ΣDDT concentration (89.86 ± 47.17 ng g^{-1}) was a little lower than the maximal allowable

concentration (MAC) in surface soil according to the Vietnamese standard 5941-1995 (ΣDDT<100 ng g^{-1}). However, the ΣDDT concentrations in some soil samples were still higher than MAC. The results point out the common usage of DDT as pesticides for crop protection in these sites. Because the usage of DDT in Vietnam was banned in 1994, this clearly indicates that the residues of DDT are a result of the use of such compounds over the last decades. In non-agricultural areas such as industrial areas, the centre of Hanoi and the towns of 5 surrounding suburban districts, ΣDDT was also detected, which ranged from N.D to 67.82 ng g^{-1} (mean 21.22 ± 22.67 ng g^{-1}). This reflects the use of DDT as a vector control for public health purposes in the city.

In order to compare the level of contamination and the pattern of selected OCPs between the densely industrial city like Hanoi with typical agricultural area like Bacninh province, the study about selected OCPs in Bacninh is implemented. The ΣDDT concentrations of soil samples from Bacninh were detected in 29 of 40 soil samples. Obtained results are given in Table 5. Noticeable concentrations of ΣDDT were found in most agricultural areas in Bacninh. The ΣDDT concentrations ranged from N.D to 160.86 ng g^{-1} with a mean 106.79 ± 37.12 ng g^{-1}. The mean ΣDDT concentration in agricultural areas in Bacninh was higher than those in Hanoi as well as the MAC-value of ΣDDT (ΣDDT<100 ng g^{-1}).

Table 5. Concentrations of DDT and its metabolites in the soil from Bacninh (ng g^{-1} dw)

Location	*p,p'*-DDE	*p,p'*-DDD	*p,p'*-DDT	ΣDDT
A. Agricultural areas				
TuSon 1	N.D - 79.68 (53.19)	N.D - 45.49 (28.79)	N.D - 36.29 (24.69)	N.D - 160.86 (106.69)
TienDu 1	42.56 – 70.45 (61.32)	24.04 – 39.63 (33.39)	20.07 – 36.18 (30.19)	86.67 – 146.26 (124.92)
Bacninh town 1	14.49 – 52.48 (41.17)	7.24 – 35.43 (26.13)	4.89 – 29.76 (21.47)	26.62 – 114.56 (89.07)
YenPhong 1	44.29 – 53.65 (49.49)	19.76 – 28.25 (25.64)	18.29 – 22.85 (20.68)	82.34 – 104.75 (95.79)
B. Towns and traditional villages				
TuSon 2	N.D - 19.98 (9.45)	N.D - 17.38 (4.35)	N.D - 8.23 (2.95)	N.D - 39.45 (16.75)
TienDu 2	N.D - 34.84 (20.38)	N.D - 17.86 (10.49)	N.D - 9.89 (5.58)	N.D - 34.84 (20.38)
Bacninh town 2	N.D - 12.86 (2.57)	N.D - 6.24 (1.25)	N.D - 5.37 (1.07)	N.D - 24.47 (4.89)
YenPhong 2	N.D	N.D	N.D	N.D

[(a)] TuSon 1: agricultural areas of TuSon; [(b)] TuSon 2: Towns and traditional villages of TuSon. It is similar with TienDu, Bacninh town and YenPhong; [(c)] min – max (mean) value. [(d)] N.D: not detected.

In general, agricultural scale production in Bacninh is higher than Hanoi. Therefore, the larger quantity of DDT used for crop protection from pest is possible. This might be the reason for higher levels of DDT contamination. In non-agricultural areas such as town and traditional village, ΣDDT was also detected, which ranged from N.D to 39.45 ng g^{-1} (mean 12.09 ± 16.42 ng g^{-1}). It might be as results of DDT used as a vector control for public health purposes in these areas. Unfortunately, the official quantitative information on the cumulative use of DDT in Hanoi and Bacninh over the years is lacking. Thus, it is impossible to calculate their possible quantity which penetrated into the soil. According to Hung et al. (2002), 24,042 tonnes of technical DDT were used against malaria and mosquitoes from 1957 to 1994 in Vietnam.
When compared with other regions in the world (Table 6), the analysed concentrations of ΣDDT in Hanoi are comparable with those of Shanghai (China), lower than those in Tasman (New Zealand) or Beijing (China) and higher than residues found in others regions.

Table 6. ΣDDT concentration in soil from other regions in the world (ng g^{-1})

Locations	ΣDDT	References
Shanghai (China)	18 – 142	Nakata et al. (2005)
Guanting (China)	N.D – 94.07	Zhang et al. (2005)
Tasman (New Zealand)	30 – 34500	Gaw et al. (2006)
Beijing (China)	0.77 – 2178	Zhu et al. (2005)
Tanzania	<0.1 – 97	Kishimba et al. (2004)
South California (USA)	0.11 – 44.8	Kannan et al. (2003)
Georgia (USA)	0.34 – 33.6	Kannan et al. (2003)
Sao Paulo State (Brazil)	0.12 – 11.01	Rissato et al. (2006)
Hanoi (Vietnam)	N.D - 171.83	This study

Contamination status of HCH

The concentrations of ΣHCH (α, β, γ and δ-HCH) in soil samples from Hanoi are shown in table 7. Low concentrations were detected in 47 of 60 soil samples. The ΣHCH concentrations in Hanoi agricultural areas ranged from N.D to 20.57 ng g^{-1} (mean 8.03 ± 3.55 ng g^{-1}), while those from industrial and urban areas ranged from N.D to 7.75 ng g^{-1} (mean 3.23 ± 2.85 ng g^{-1}). In comparison with the Vietnamese standard 5941-1995, γ-HCH concentrations in the analysed soil samples were much lower than MAC (γ-HCH<100 ng g^{-1}). At present, the Vietnamese standard has no MAC of ΣHCH in soil. Similar to DDT, the contamination of HCH originated from their use as pesticides for crop protection and as vector control for public health purposes. Low HCH concentrations suggest that HCH was used in small quantities in agricultural areas and was probably deposited into the urban areas by atmospheric transport. From the surface soil in the agricultural areas, HCH may be accumulated in the food chain and then in human body. Due to this reason, although the analysed ΣHCH concentrations are low, its presence in most soil samples is clearly marked.

Table 7. Concentrations of HCH isomers (ng g^{-1} dw) in the surface soil from Hanoi

Location	α-HCH	β-HCH	γ-HCH	δ-HCH	ΣHCH
A. Agricultural areas					
SocSon 1	2.57 – 4.08 (3.35)	2.25 – 4.26 (3.65)	0.65 - 1.08 (0.89)	0.29 - 1.18 (0.59)	6.56 – 10.39 (8.49)
DongAnh 1	1.68 – 2.66 (2.28)	2.35 – 3.67 (2.89)	0.56 – 0.76 (0.67)	0.15 – 0.59 (0.46)	5.06 – 7.66 (6.28)
GiaLam 1	2.26 – 3.19 (2.59)	2.48 – 3.96 (3.05)	0.56 – 0.87 (0.75)	0.37 – 0.68 (0.49)	5.67 – 8.69 (6.88)
Hanoi centre 1	1.48 – 2.68 (2.27)	2.48 – 2.96 (2.79)	0.28 – 0.86 (0.59)	0.25 – 0.59 (0.47)	4.96 – 6.89 (6.15)
TuLiem 1	3.06 – 6.07 (4.15)	3.48 – 6.68 (4.48)	0.75 – 1.46 (0.99)	0.18 – 0.96 (0.38)	7.39 – 14.68 (9.99)
ThanhTri 1	N.D [(a)] – 8.56 (3.89)	N.D – 9.28 (4.25)	N.D – 1.86 (0.87)	N.D – 0.87 (0.35)	N.D – 20.57 (9.35)
B. Industrial and urban areas					
SocSon 2	N.D – 2.37 (1.58)	N.D – 2.39 (1.48)	N.D – 0.58 (0.38)	N.D – 0.57 (0.28)	N.D – 5.59 (3.75)
DongAnh 2	N.D – 2.28 (0.89)	N.D – 2.36 (0.86)	N.D – 0.77 (0.27)	N.D – 5.37 (0.09)	N.D – 5.37 (2.15)
GiaLam 2	N.D – 3.27 (1.39)	N.D – 2.89 (1.35)	N.D – 0.77 (0.39)	N.D – 7.76 (0.35)	N.D – 7.76 (3.47)
Hanoi centre 2	N.D – 2.36 (0.95)	N.D – 2.08 (0.85)	N.D – 0.77 (0.28)	N.D – 5.79 (0.19)	N.D – 5.79 (2.25)
TuLiem 2	1.98 – 2.36 (2.15)	1.96 – 2.37 (2.14)	0.56 – 0.78 (0.59)	4.77 – 5.78 (0.44)	4.77 – 5.78 (5.29)
ThanhTri 2	N.D – 2.36 (1.55)	N.D – 2.08 (1.29)	N.D – 0.76 (0.48)	N.D – 5.66 (0.31)	N.D – 5.66 (3.65)

[a] min – max (mean) value

In Bacninh, low concentrations of ΣHCH were also detected in 29 of 40 soil samples (Table 8). The ΣHCH concentrations in agricultural areas ranged from N.D to 9.54 ng g^{-1} (mean 7.38 ± 2.17 ng g^{-1}), while those from towns and traditional villages ranged from N.D to 5.86 ng g^{-1} (mean 1.58 ± 2.18 ng g^{-1}). γ-HCH concentrations in the analysed soil were also much lower than MAC (γ-HCH < 100 ng g^{-1}). Similar to the explanations for Hanoi, the contamination of HCH originated in the usage as pesticides for crop protection and as vector control for public health purposes. Obtained results show that the level of HCH contamination in Bacninh is similar with those from Hanoi. At present, official information on HCH consumption in Hanoi and Bacninh is not available. Thus, it is not possible to assess in detail the relation between the really used HCH quantity and the contamination status. Results of table 7 and table 8 shows that HCH mean concentrations in agricultural areas from Bacninh are higher than those in other areas in Hanoi but the difference is not remarkable.

Table 8. Concentrations of HCH isomers (ng g^{-1} dw) in the surface soil from Bacninh

Location	α-HCH	β-HCH	γ-HCH	δ-HCH	ΣHCH
A. Agricultural areas					
TuSon 1	N.D – 3.97 (2.85)	N.D – 4.56 (3.35)	N.D – 0.88 (0.59)	N.D – 0.93 (0.45)	N.D – 9.45 (7.19)
TienDu 1	3.09 – 4.05 (3.55)	3.35 – 4.07 (3.95)	0.57 – 1.17 (0.86)	0.65 – 0.99 (0.76)	8.36 – 9.58 (9.08)
Bacninh town 1	1.47 – 2.36 (1.89)	2.05 – 2.96 (2.35)	0.45 – 0.55 (0.49)	0.46 – 0.98 (0.59)	4.69 – 6.78 (5.35)
YenPhong 1	2.08 – 3.08 (2.55)	2.86 – 3.46 (3.09)	0.57 – 0.85 (0.66)	0.38 – 0.89 (0.58)	5.97 – 8.28 (6.88)
B. Towns and traditional villages					
TuSon 2	N.D - 3.65 (0.89)	N.D - 2.58 (1.05)	N.D - 0.66 (0.26)	N.D - 0.35 (0.15)	N.D - 5.86 (2.35)
TienDu 2	N.D - 1.79 (0.88)	N.D - 1.98 (1.05)	N.D - 0.45 (0.25)	N.D - 0.35 (0.18)	N.D - 4.57 (2.35)
Bacninh town 2	N.D - 1.56 (0.29)	N.D - 1.75 (0.35)	N.D - 0.39 (0.08)	N.D - 0.49 (0.09)	N.D - 4.19 (0.85)
YenPhong 2	N.D	N.D	N.D	N.D	N.D

When compared with other regions in the world (Table 9), the analysed concentrations of ΣHCH in Hanoi and Bacninh are comparable with those of

Shanghai and Guanting Reservoir (China). Some regions such as Beijing (China) and Tanzania showed higher results.

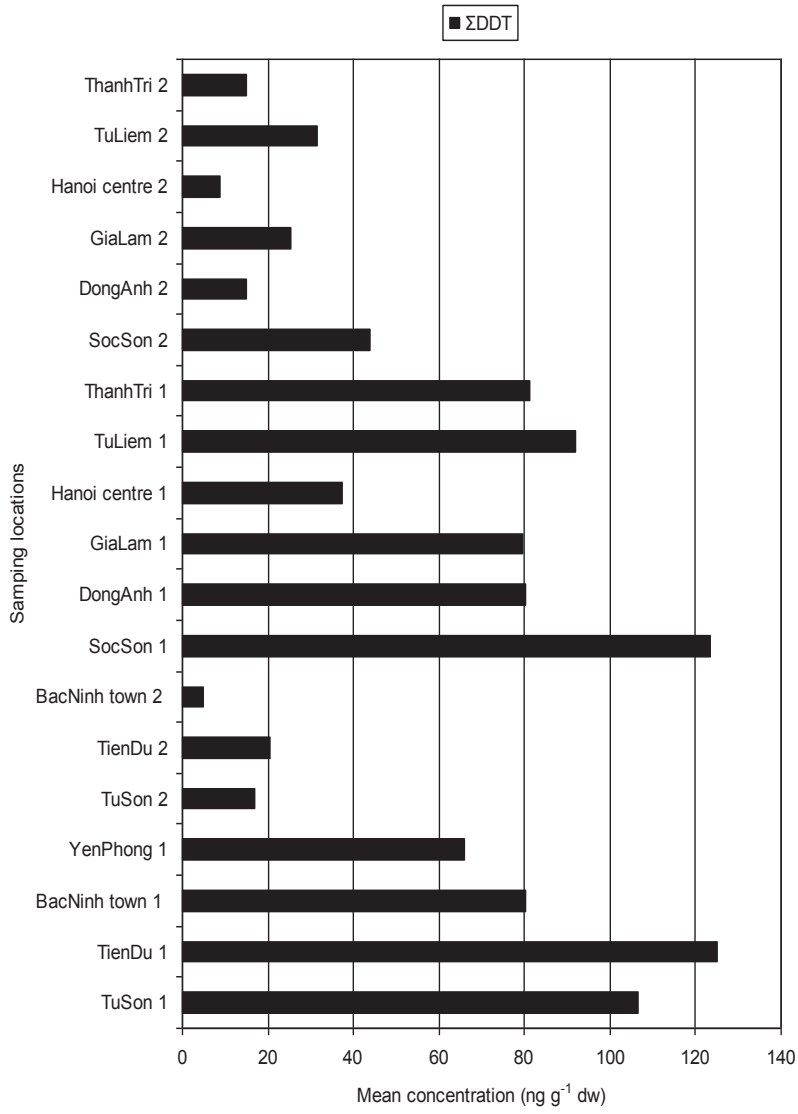
Table 9. Concentration of ΣHCH (ng g^{-1} dw) in soil from other regions in the world

Locations	ΣHCH	References
Shanghai (China)	<0.03 – 2.4	Nakata et al. (2005)
Guanting (China)	N.D – 8.96	Zhang et al. (2005)
Tasman (New Zealand)	–	Gaw et al. (2006)
Beijing (China)	1.36 – 56.61	Zhu et al. (2005)
Tanzania	<0.1 – 59	Kishimba et al. (2004)
South California (USA)	<0.1 – 0.54	Kannan et al. (2003)
Georgia (USA)	<0.1 – 0.54	Kannan et al. (2003)
Sao Paulo State (Brazil)	0.05 – 0.92	Rissato et al. (2006)
Hanoi (Vietnam)	N.D - 20.57	This study

Spatial distribution of DDT and HCH

The distributions of ΣDDT and ΣHCH in the soil samples from Hanoi and Bacninh are shown in Figure 6 and Figure 7. In Hanoi, it is evident that the surface soil is remarkably contaminated with ΣDDT. This suggests that a high quantity of DDT was possibly used. Analytical results show that the ΣDDT concentrations were highest in agricultural areas, followed by those in urban and in industrial areas. This applies for the DDT usage in Vietnam. Between agricultural areas, the mean ΣDDT concentrations followed the order SocSon 1 (123.32 ng g^{-1}) > TuLiem 1 (91.82 ng g^{-1}) > ThanhTri 1 (81.06 ng g^{-1}) > DongAnh 1 (80.39 ng g^{-1}) > GiaLam 1 (79.47 ng g^{-1}) > Hanoi centre 1 (37.35 ng g^{-1}). In fact, agricultural areas in Hanoi are mainly situated in SonSon, ThanhTri and TuLiem district, whereas the industrial Parks are mainly situated in DongAnh and GiaLam district. This can be one element to explain this order and may also suggest that the used quantity of DDT in SonSon, ThanhTri and TuLiem were probably higher than in the other areas in Hanoi. The mean ΣDDT concentrations in these areas are close to MAC-value of ΣDDT in soil and thus, need further studies to assess their contamination status in more detail.

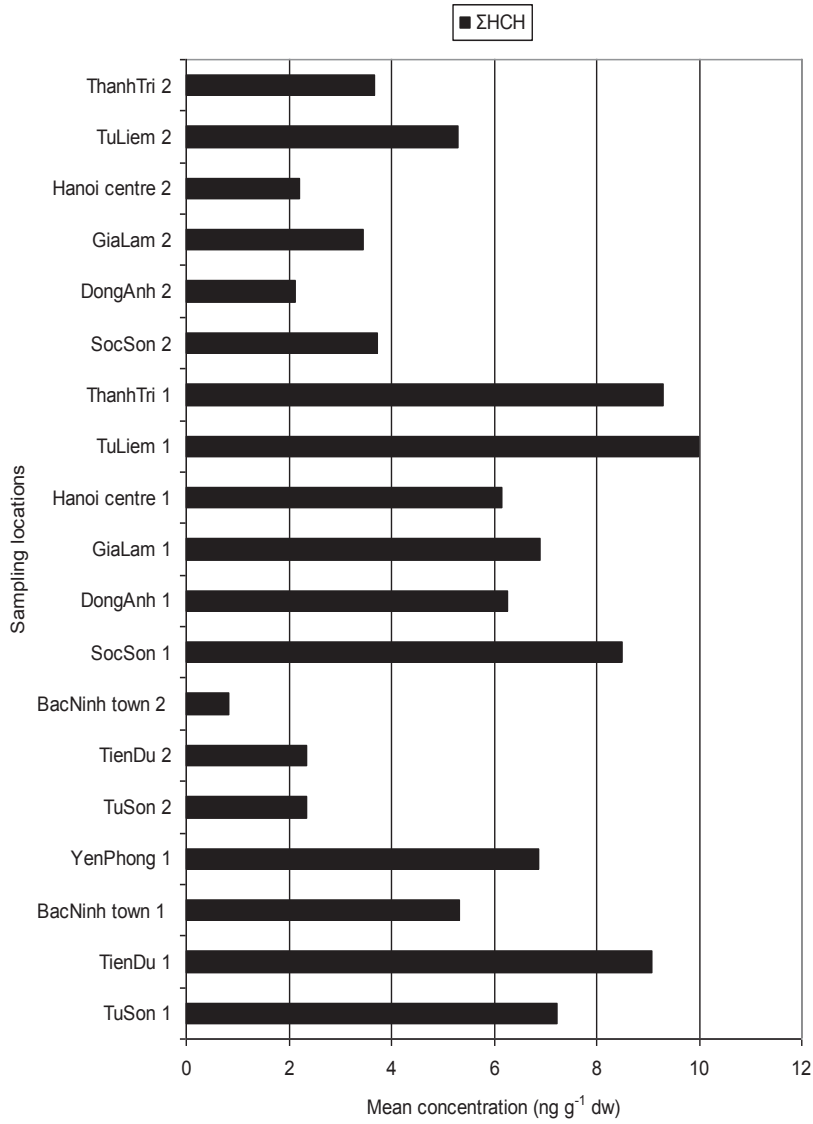
Figure 6. Distribution of ΣDDT concentrations in surface soil from study areas



In Bacninh, the distribution of significant ΣDDT levels is found in agricultural areas. The contamination levels were followed the order TienDu 1 (mean 124.92 ng g^{-1}) > TuSon 1 (mean 106.69 ng g^{-1}) > Bacninh town 1 (mean 80.07 ng g^{-1}) > YenPhong 1 (mean 65.81 ng g^{-1}). TuSon and TienDu district are typical areas where inhabitants live mainly from agriculture. Bacninh town is new town with agricultural areas in the suburb, while in YenPhong there is a concentration of many traditional villages. These characteristics may be related with the quantity of DDT used and explain found order.

Contrary to the results of ΣDDT, the distributions of low level contamination of ΣHCH from Hanoi and Bacninh are displayed.

Figure 7. Distribution of ΣHCH concentrations in surface soil from study areas



The most likely explanation for the their current distribution is the relatively short environmental half-lives of HCH in soil, lower log K_{ow} and higher vapour pressure than that of DDT. Due to the influence of a tropical climate, it is possible that the major loss of HCH occurs through volatilisation. It was reported that ninety days after HCH application, the HCH concentration decreased to 99.1% for cultivated

soil and to 96.8% for non-cultivated soil measured in 7.5 cm upper layer (Kaushik, 1989). The losses are mainly due to the volatility of HCH under subtropical conditions. Moreover, a significant quantity of HCH could be washed out by rain and irrigation of the fields.

Besides the contamination of soil in Hanoi, DDT and HCH also found ways to penetrate into the human body and other environmental compartments in Hanoi. According to Nhan et al. (2001), the concentration of ΣDDT and ΣHCH in sediments from canals in the downtown area and in the suburbs of Hanoi city in August 1997 ranged from 7 to 80 ng g^{-1} and 0.1 to 3.1 ng g^{-1} dry weight, respectively. With regard to the research by Hung et al. (2002), ΣDDT and ΣHCH were detected on the surface of four lakes in the centre of Hanoi, as well as in six irrigation canals, and two nearby rivers. It has been reported that the mean concentrations of ΣDDT and ΣHCH in the lakes (August 1999) were 5.07 ± 6.88 ng l^{-1} and 31.7 ± 60.4 ng l^{-1}, respectively. In addition, the research by Minh et al. (2004) found that the mean concentrations of ΣDDT and β-HCH in 42 human breast milk samples in Hanoi were 2'100 and 58 ng g^{-1} lipid weight, respectively. The results above are really high and clearly reflect the presence of a local contaminant source. These results, together with our study, highlight the wide extent of contamination of DDT and HCH in Hanoi.

Composition analyses

Besides the contamination status of selected OCPs, the analysis of their composition is useful to understand their pattern as well as possible contaminant sources.

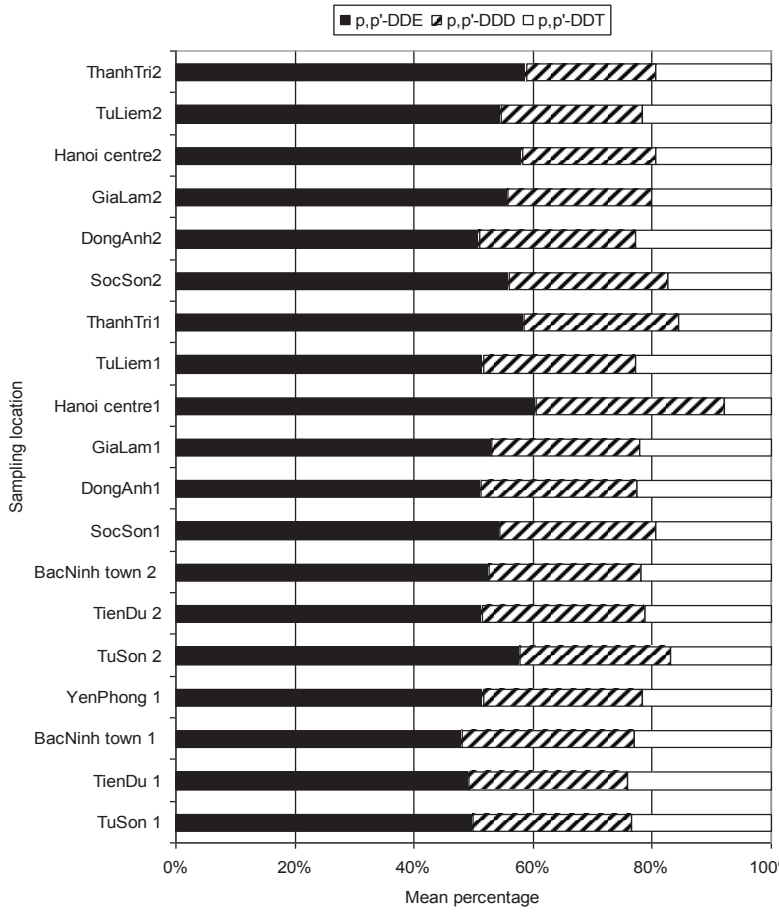
Composition analyses of DDT

DDT has been imported and used in Vietnam from 1957 up to 1994. It was banned in Vietnam due to its high toxicity and its persistence in the environment (Hung et al., 2002). In general, technical grade DDT is composed of up to fourteen chemical compounds, of which only 65–80% is the active ingredient, *p,p'*-DDT and includes 15–21% of the nearly inactive *o,p'*-DDT (ATSDR, 2002). The order of mean percentages of DDT and its metabolites in soil samples from Hanoi was *p,p'*-DDE (54.4%) > *p,p'*-DDD (25.5%) > *p,p'*-DDT (20.1%). A similar order was found in Bacninh with little different percentages (*p,p'*-DDE 50.5%, *p,p'*-DDD 29.4%, *p,p'*-DDT 20.1%). It shows a similar pattern of DDT and its metabolites in samples from Hanoi and from Bacninh. It can be explained by the fact that Bacninh province is close to Hanoi City. Therefore, the climate condition and the soil types are similar. Even thought, the quantity of DDT used is not the same, the DDT pattern in Hanoi and in Bacninh are comparable.

It should be noted that DDT can be biodegraded in the environment to DDD under anaerobic conditions and to DDE under aerobic conditions. During the dry season, in northern Vietnam, with aerobic conditions, the active oxidative transformation of *p,p'*-DDT to *p,p'*-DDE is facilitated and creates a larger percentage of *p,p'*-DDE than the other components of ∑DDT. This is in good agreement with the research by Ramesh et al. (1991) in tropical regions reporting that *p,p'*-DDE was a major breakdown product of DDT in soil from different places in India. With regard to

DDT metabolites, the ratio of (*p,p'*-DDE + *p,p'*-DDD)/ΣDDT in the soil samples from Hanoi ranged between 0.75 and 0.99 (mean 0.79), whereas those from Bacninh ranged between 0.74 and 0.87 (mean 0.77). This indicates that the degradation of DDT occurred significantly and there is no recent input of DDT in the study areas. Mean percentages of DDT and its metabolites in surface soil from the study areas are showed in Figure 8. Because ΣDDT has not been detected in soil samples from YenPhong 2, this location is absent in the figure.

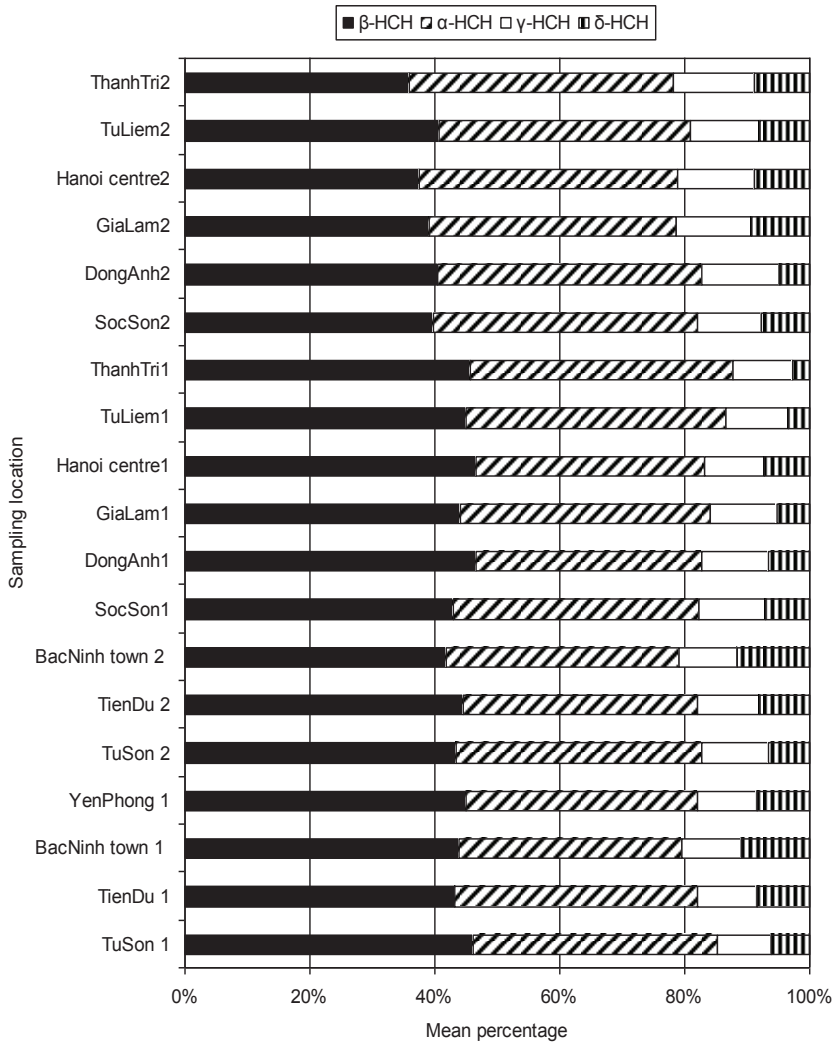
Figure 8. Mean percentages of DDT and its metabolites in surface soil from the study areas



Composition analyses of HCH

Technical HCH and lindane (>99% γ-HCH) have been officially banned since 1994 in Vietnam. The typical technical HCH generally contains 55–80% of α-HCH, 5–14% of β-HCH, 8–15% of γ-HCH and 2–16% of δ-HCH, respectively (Lee et al., 2001).

Figure 9. Mean percentages of HCH isomers in surface soil from the study areas



The mean percentages of HCH isomers in analysed soil samples from Hanoi followed the order β-HCH (42.8%) > α-HCH (39.7%) > γ-HCH (10.9%) > δ-HCH (6.6%). A similar order was also found in Bacninh with a little different mean percentage (β-HCH 44.4%, α-HCH 37.9%, γ-HCH 9.4%, δ-HCH 8.3%). It shows a similar pattern of HCH isomers in samples from Hanoi and from Bacninh. The explanation is the same as for DDT.

Among the isomers, β-HCH has the lowest water solubility and vapour pressure. It is the most stable isomer and is relatively resistant to microbial degradation. Besides that, there is the isomerisation of α- to β-HCH and of γ- via α- to the more stable β-HCH, which is energetically more favourable in the environment (Manz et al.,

2001). Therefore, the predominance of β-HCH reflects an old source of input of HCH in the environment. Low ratios of α-HCH/γ-HCH may represent the use of lindane, whereas high ratios of these isomers may depict the use of technical HCH. Measured results of McConnell et al. (1993) show that the ratio of α-HCH/γ-HCH in areas where lindane has been typically used ranges from 0.2 to 1, compared to a range of 4–15 for technical HCH. Here, the mean ratios of α-HCH/γ-HCH in the analysed soil samples from Hanoi and Bacninh were 3.7 and 4.1, respectively. This result confirmed the use of technical HCH as major source and lindane as minor source in the study areas. According to Nhan et al. (2001), the proportion of β-HCH, α-HCH and γ-HCH in sediments which were collected from 12 locations in canals in the downtown area and in the suburbs of Hanoi city are approximately similar to those of the technical HCH mixture. This observation also corresponds with a above results above of our study.

Temporal trends of selected OCPs concentration

With regard to concentrations of selected OCPs in soil samples from the study areas reported in the other studies, their temporal trend could be shown. The analytical results of this study were compared with those of Thao el at., (2005). Sixty four samples of various soil types from Hanoi and Bacninh were randomly collected during the period from 1992 to 2001. Details of the sample collection time and sampling locations are given in Table 10.

Table 10. Collection time and sampling locations of colleted and analysed soil samples (Thao el al., 2005, this study)

Location	Number of samples						Total
	1992	1995	1998	2001	2005	2006	
Bacninh							
Rural soil	3	6	6	6	-	24	45
Urban soil	1	2	2	2	-	16	23
Hanoi							
Rural soil	3	6	6	6	8	31	60
Urban soil	9	2	2	2	8	29	52

Their study was implemented from 1992 to 2001 and the measured concentrations of selected OCPs are presented in Table 11.

Table 11. Selected OCPs concentrations from 1992 to 2001 (ng g^{-1} dw)

Location and soil type	1992	1995	1998	2001
A. ΣDDT				
Bacninh Rural soil Urban soil	 1.4 – 273.8 0.9 – 32.7	 1.4 – 297.6 0.9 – 42.8	 1.5 – 269.5 0.8 – 51.9	 0.8 – 168.7 0.7 – 36.7
Hanoi Rural soil Urban soil	 498.8 – 896.5 50.6 – 171.6	 396.7 – 875.7 151.5 – 162.6	 504.8 – 796.5 41.6 – 147.7	 398.7 – 796.7 43.8 – 106.8
B. ΣHCH				
Bacninh Rural soil Urban soil	 0.15 - 24.23 9.42 - 23.88	 0.23 - 28.41 12.26 - 21.79	 0.16 - 19.87 6.79 – 20.76	 1.12 - 28.96 5.38 – 19.78
Hanoi Rural soil Urban soil	 8.21 - 36.15 17.91 – 38.79	 5.61 - 40.73 23.48 – 36.78	 9.03 - 33.75 14.32 – 32.47	 3.28 - 24.62 11.37 – 29.48

Thao et al., (2005) used the same method for sampling, pretreatment and storage for the soil samples collected from 1992 to 2001. The sampling sites of the campaign in 2006 are the same as from 1992 to 2001. The same analytical procedure (GC/MS) was used in the present study, to be able to compare the data with those of Thao el al., (2005). Relative standard deviations of all collected and analysed soil samples were less than 15%, and thus, stand for the comparison as well as assessment of temporal trend of selected OCPs in the same diagram.

Regarding the value in Table 11, it clearly indicates that these compounds have found ways to penetrate in Vietnam soil for a long time. The ΣDDT concentrations ranged from 0.8 to 896.5 ng g^{-1}. The ΣDDT concentrations were highest in agricultural soil and then, reduced in industrial and urban soil. The maximal values of ΣDDT in agricultural soil in Hanoi in 1992, 1995, 1998, and 2001 are significant higher than MAC-value of ΣDDT in 2006. In the case of ΣHCH, the concentrations in soil samples during the period from 1992 to 2001 ranged from 0.15 to 38.79 ng g^{-1} (Table 11). Similar to ΣDDT, ΣHCH concentrations in agricultural soil samples were also higher than those in industrial and urban soil. The residual levels of ΣHCH were all in low range. Though the ΣHCH levels were not so high, the presence of ΣHCH in most soil samples was clearly shown.

Figure 10. Temporal trends of ΣDDT from surface soil in Hanoi and Bacninh (Thao el al., 2005, this study)

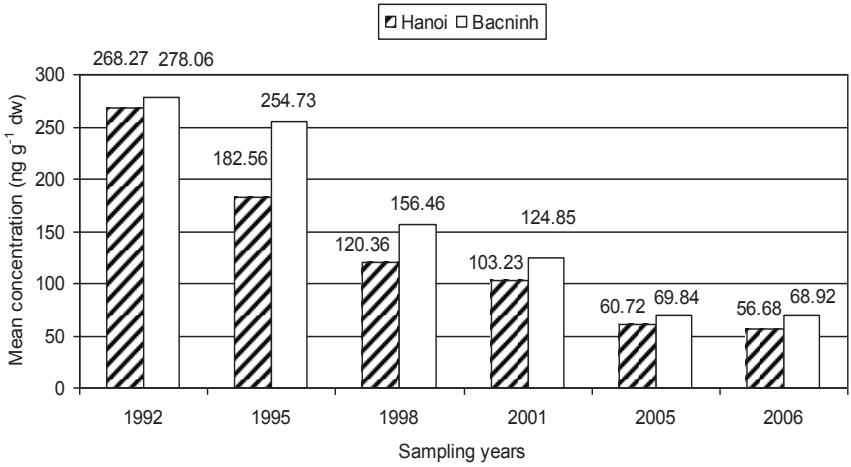
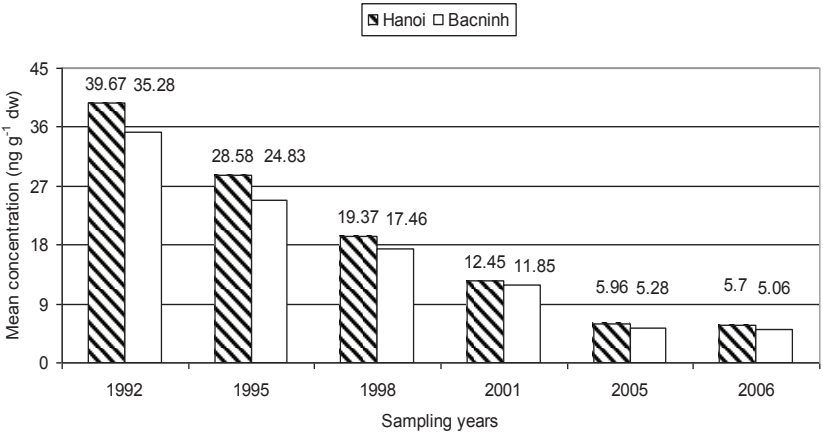


Figure 11. Temporal trends of ΣHCH from surface soil in Hanoi and Bacninh (Thao el al., 2005, this study)



Regarding figure 10 and figure 11, both ΣDDT and ΣHCH showed a decreasing trend. Levels of soil ΣDDT contamination in Hanoi decreased from the mean concentration of 268.27 ng g^{-1} in 1992 to 182.56 ng g^{-1} in 1995, to 120.36 ng g^{-1} in 1998, to 103.23 ng g^{-1} in 2001, to 60.72 ng g^{-1} in 2005 and to 56.68 ng g^{-1} in 2006 (Thao et al., 2005; this study). With regard to ΣHCH, the mean concentrations in Hanoi decreased from 39.67 ng g^{-1} in 1992 to 28.58 ng g^{-1} in 1995, to 19.37 ng g^{-1} in 1998, to 12.45 ng g^{-1} in 2001, to 5.96 ng g^{-1} in 2005 and to 5.7 ng g^{-1} in 2006 (Thao et al., 2005; this study).

In general, the total temporal trend of selected OCPs can be divided into two trends. The first trend is an increase of the concentrations of selected OCPs when their sources continue to penetrate in the environment. The main contaminative sources

are DDT and HCH products used by humans in the study areas. Besides, the deposition of these compounds by atmosphere transportation from other regions is also remarkable. The second trend is a decrease of the concentrations of selected OCPs when the dissipation of study compounds took place. Dissipation comprises two main types of processes: transport processes, such as volatilisation, leaching, plant uptake, runoff or erosion that transfer substances to different environmental compartments; and transformation processes such as microbial degradation, hydrolysis and photolysis that produce transformation products. These processes led to a decrease of their concentration during the time. Therefore, the significant decreasing trend of ΣDDT and ΣHCH reflects that the dissipation of these compounds took place for a long time, whereas the new contaminative sources are limited. It is possible that their use for crop protection and vector control for public health purposes took place during the period from 1992 to 1998, and then was discontinued from 1998 to 2006. According to Thao et al. (1993), the analyses of collected soil samples from Hanoi and Bacninh in 1992 showed that *p,p'*-DDT and α-HCH are predominant in comparison with the other DDT metabolites and HCH isomers, respectively. It clearly indicates their new inputs in study areas in 1992. This could relate to the DDT and HCH history of use. After the announcements by the Ministry of Agriculture and Rural Development 1994, their use was banned. However, there are likely illegal supplies of DDT and HCH in use in 1998. DDT and HCH can be brought to Vietnam by illegal way from China. In many case, farmers and other pesticide users ignored the risks of using these products. Another contaminative source could be the products which have been stored in stocks in other provinces before proper disposal. The poor control and unregulated storage can lead to their release into the soil environment. But after 6-8 years of the ban, this situation seems to be under control. Composition analyses of soil samples in 2006 show that there is no recent input in the study areas.

The decrease of ΣHCH during the period from 1992 to 2006 is faster than ΣDDT. Between 1992 and 2006, ΣDDT and ΣHCH concentrations in soil from Hanoi decreased by factor 0.21 and 0.14, respectively (Table 11). It can be explained by the relatively shorter environmental half-lives of HCH in soil, lower log K_{ow} and higher vapour pressure than that of DDT. Besides, HCH may have been used to a lesser extent as compared to DDT.

Table 11. Factors of declining trends of ΣDDT and ΣHCH

Location	Ratios of mean concentration over the sampling years				
	1992/1995	1992/1998	1992/2001	1992/2005	1992/2006
A. Hanoi					
ΣDDT	0.68	0.45	0.39	0.22	0.21
ΣHCH	0.72	0.49	0.31	0.15	0.14
B. Bacninh					
ΣDDT	0.91	0.56	0.45	0.25	0.24
ΣHCH	0.70	0.49	0.34	0.15	0.14

CONCLUSIONS

An evaluation of selected organochlorine (*p,p'*-DDT, *p,p'*-DDE, *p,p'*-DDD, α, β, γ and δ-HCH) residues in the soils was implemented in 2006 in Hanoi city and Bacninh province of Vietnam. The soil samples were collected from agriculture areas as well as from urban areas with an attempt to get them evenly distributed over selected regions. The analysed results indicated the wide extent of remarkable contamination of DDT's metabolism and HCH's isomer in study areas. Assessment of composition analyse indicated that there is little recent input of DDT and HCH. The decreasing trend of DDT and HCH levels from 1992 to 2006 is observed.

Observations for future research

- Continue to research of the fate of selected OCPs in soil in the study area. Collect soil samples from different depths to investigate the vertical distribution of selected OCPs. Construct dot maps and raster maps about residual distributions of selected OCPs in the study area as a tool for environmental management.
- At present, selected OCPs modeling has received the attention of scientists in the world. Selected OCPs modeling can provide estimates of the major pollution pathways, contamination levels in main environmental compartments and source-receptor relationships. Regarding to the necessity of selected OCPs modeling, this study could be continued with time trend variations in different environmental media. These data would be useful for OCPs modeling, such as half life time of selected substances in water and air. It will also be useful to assess the contamination status and to find the relation between selected OCPs in soil, water and air in the study area.

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List of Acronyms and Abbreviations

DDT	Dichlorodiphenyltrichloroethane
DDE	1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene
DDD	1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethane
IARC	International Agency for Research on Cancer
HCH	Hexachlorocyclohexane
OCPs	Persistent organochlorines pesticides
NEA	National Environmental Agency
POP	Persistent organic compound
USEPA	United State Environmental Protection Agency
WHO	World Health Organization

DDT Residues in Breeding Population of Booted Eagle (Aquila pennata) Associated with Agricultural Land Practices

Emma Martínez-López, Pedro María-Mojica, Pilar Gómez-Ramírez, José F. Calvo, José E. Martínez and Antonio J. García-Fernández

ABSTRACT

In this study we have used blood of Booted eagle (Aquila pennata) nestlings (n=62) to evaluate the exposure to p,p'-DDT, p,p'-DDD and p,p'-DDE in an agricultural area from Southeastern Spain in 1999-2003 breeding seasons. Our results showed than the trans-Saharan migratory factor cannot account for the results obtained for DDT and its analogues in the blood samples and, therefore, other factors directly associated with the study zone must be considered. Perhaps, the recommended use of dicofol (a product in whose manufacture DDT is used) against specific plagues to the more important crops in the study area, may account for the levels of DDT and its analogues present in the blood of forest raptor chicks. On the other hand, we corroborated that the blood of Booted eagle chicks may be taken into account as a control unit for monitoring or evaluating situations associated with changes in agricultural land use or practice.

INTRODUCTION

The dichlorodiphenyltrichloroethane (DDT) is an organochlorine compound which was synthesized in 1874. Although technical DDT consists of several compounds, only p,p'-DDT (DDT), p,p'-DDD (DDD), and p,p'-DDE (DDE) have been related to adverse environmental effects (Blus, 1996). They were widely used in agricultural and malarial control programs from 1940s to 1960s with dramatic benefits, but they fell into disfavour because of their persistence in the environment, wildlife and humans (Smith, 2004).

Studies indicate that these compounds are directly correlated with impaired reproductive success, embryonic deformities, and mortality in wildlife (Custer et al., 1999; Konstantinou et al., 2000). There have been documented deleterious effects of DDE in wild populations in birds (Helberg et al., 2005; Jiménez et al., 2005). DDE exposure is able to affect both adults and embryos resulting in mortality, reduced fertility, and suppression of egg formation, eggshell thinning and impaired incubation (Fry, 1995). In this sense, Mura et al. (2009) demonstrated that a single exposure to low levels of p,p'-DDE during embryonic development may have important neuroendocrine effects in Japanese quail (Coturnix coturnix japonica).

In Spain, the use of DDT insecticide for agricultural practices was banned in 1994 (Orden 4 de Febrero de 1994). However, it and its metabolites are still frequently

found in tissues or fluids samples from several species of birds (Espín et al., 2010; Martínez-López et al., 2007; Piqué et al., 2006; Van Drooge et al., 2008). In our country, unhatched egg has been the most utilized type of sample to assess organochlorine exposure in raptors, including DDT. These studies have been conducted from 1973 through to the year 2004, although not constantly over time and with considerable variation in terms of areas (García-Fernández et al., 2008).

In general, concentrations of DDE in eggs of raptors (including globally threatened species such as the Spanish imperial eagle –Aquila adalberti-) have decreased in Spain since 1997 probably due to the legal restrictions in its use (González and Hiraldo, 1988; Hernández et al., 2008). However, increases in levels of DDTs in eggs have been associated with illegal spraying (Hernández et al., 1986, 2008). González et al. (1983) observed increases in DDTs concentrations in eggs of Spanish imperial eagles and Eurasian buzzards (Buteo buteo) from Doñana (South Spain) in the early of 1980s, a few years after its prohibition, probably due to a rebound effect because of the need to finish off the backlog of pesticide stocks (Hernández et al., 2008). Recent studies in this area (Doñana National Park) showed that levels of DDTs in eggs have not decreased, and in some cases, they were greater in the 1990s than in the 1980s (Gómara et al., 2008; Jiménez et al., 2005). Similar findings were described in studies on raptors from other parts of Spain. Jiménez et al. (2007) observed that the levels of DDE did not vary over time in Osprey (Pandion haliaetus) and Red kites (Milvus milvus) from the Balearic Islands; similarly, Mañosa et al. (2003) observed no decrease in DDE concentrations in the eggs of Goshawks (Accipiter gentilis) and Buzzard collected in the 1980s and 1990s in Catalonia (Northeast Spain). In this sense, the fact that the prohibition and legal restrictions on the use of organochlorine pesticides in Spain did not reduce levels in eggs, especially at Doñana, leads one to think that the migration of these species towards Africa was responsible for the presence of these compounds in said specimens (Gómara et al., 2004; Negro et al., 1993).

There are very few bibliographic references regarding concentrations of these compounds in blood of wild birds. However, high levels of blood residues of different organochlorines have been linked to impaired behaviour, reduced reproductive performance, poor survival, and other adverse effects in glaucous gull (Larus hyperboreus) (Bustnes et al., 2001, 2003, 2005; Sagerup et al., 2000; Verreault et al., 2004). Female Great black-backed gulls (Larus marinus) showed a significant relationship between DDE blood concentrations and egg size, with the second and third laid egg being smaller than the first one (Helberg et al., 2005). Furthermore, the measurement of organochlorines in plasma has proven to be an effective monitor for residual levels (Donaldson et al., 1999), which may even be useful for identifying exposure in migratory species, as concluded by Johnstone et al. (1996). Likewise, blood plasma is shown to be a good, non-destructive indicator, which makes it particularly valid for studying species of a delicate conservation status (Donaldson et al., 1999). Besides, the blood of Booted eagle (Aquila pennata) nestlings has demonstrated to be an excellent sampling unit for monitoring the use of lindane and endosulfan, reflecting changes in agricultural practices in breeding areas in the studied area (Martínez-López et al., 2009).

The Booted eagle is a trans-Saharan migratory species. Little is known for this species about the routes used during migration to Africa. The satellite tracking of three female adults from a French and Spanish population showed that the wintering areas used by these individuals extended to Niger, Mali, Burquina Faso and Senegal (Chevallier et al., 2010; Díaz, 2006). However, in the studies on Booted eagles ringed in Spain and recovered between 15 November and 14 February, only five individuals were recovered in African countries (Morocco, Togo, Mali, Algeria, and Nigeria) and the rest birds wintered in Spain (García-Dios, 2004). This fact, supports the idea suggested by Martínez and Sánchez-Zapata (1999) and Sunyer and Viñuela (1996) of a positive tendency of wintering in Mediterranean areas instead of migrating to Africa. In a previous study on these species in Southeastern of Spain, we concluded that the eggs of Booted eagle were contaminated with OCs in concentrations lower than those associated with adverse effects. However, the 15% of the Booted eagle eggs examined in that study contained DDE residues that would be expected to cause significant (15%) eggshell thinning (Martínez-López et al., 2007).

The study area (Murcia Region) has one of the highest sales rates of pesticides in Spain (10.14% of the nation-wide total) (AEPLA, 2010). During the period of time studied in this work, the use of DDT was permitted in the manufacture of insecticides commonly used in Spain such as dicofol. In that period, there were 62 products registered in Spain which contained dicofol (MARM, 2005).

The aim of this study was to analyze DDT and its metabolites in the blood of Booted eagle nestlings in order to evaluate the exposure to these compounds and to check the possible influence of the migratory process on these concentrations.

MATERIAL AND METHODS

To carry out the sampling it was necessary to obtain permits from the Autonomous Regional Government of Murcia.

Species

The Booted eagle (Aquila pennata) is a trans-Saharan medium-sized raptor species, which usually nests in trees and, more rarely on cliffs (del Hoyo et al., 1994). Although it is a common species of the forests and woodland areas of the Iberian Peninsula, several authors have shown that Booted eagle selects areas with a mixture of woodlands and open lands, suggesting the importance of extensive crops adjacent to the nesting woodland patches as foraging habitats for the species (Sánchez-Zapata and Calvo, 1999; Suárez et al., 2000). In terms of its conservation, one of the threats highlighted for this species is the potential accumulation of organochloride pesticides, possibly affecting reproductive success, as has been described for other migratory species (Muñoz and Blas, 2003).

Study area and Sampling

This study was carried out in the northwest of the Region of Murcia which is located in southeastern Spain (Figure 1). The study area covers about 250,000 ha and elevation ranges from 550 to 1,521 m. The climate is Mediterranean with a mean annual rainfall of 400 mm. The landscape is characterized by mountain slopes covered by Aleppo pine forests (Pinus halepensis) interspersed with farming crops (cereals, vineyards, olive and woody crops) and far from any urban, industrial or mining zones. The nearest industrial area, urban area (population >350,000 h), and highway are situated 70, 60, and 70 km away, respectively. The study area has one of the highest densities of Booted eagle and Short-toed eagle (Circaetus gallicus) in Europe (Martínez, 2002) with one pair per 3-4 km^2 (Martínez et al., 2006). The Booted eagle population in the area was estimated at 50 breeding pairs between 1999 and 2004 (Martínez et al., 2006; unpublished results).

From the 1999-2003 breeding seasons, the reproductive behaviour of the Booted eagle was observed from the beginning of courtship (March-April) until nestlings left their nests (June-July). Close to the predicted hatching date, nests were visited twice weekly. The same nest was visited each year. A total of 62 nestlings were studied and blood samples (2.0 ml) were taken by puncturing the radial vein using a hypodermic needle and syringe and taken immediately to the laboratory in refrigerated conditions and frozen at –40ºC until processing. Nestlings were sampled at between 35 and 45 days old in order to obtain sufficient blood samples without damage the nestlings' health. Careful steps were taken in order to avoid stressing the nestlings. The head of the nestlings was covered before the animal was put into a bag and brought down to the ground, where a blood sample was taken by the veterinarian from the "Santa Faz" Wildlife Recovery Centre (Alicante, Spain), who also evaluated the health status of the nestlings. All individuals were marked with a numbered steel band. Finally, nestlings were returned to the nest. These nests were monitored until the chicks commenced flying. No chicks were injured during the sampling and all were able to fly satisfactorily.

Organochlorine Analysis

Blood samples were analyzed for dichlorodiphenyltrichloroethane (p,p'-DDT), dichlorodiphenyldichloroethane (p,p'-DDD), and dichlorodiphenyldichloroethylene (p,p'-DDE).

Reagents and Standards

All reagents used for the analysis were of a trace analysis grade. Hexane, acetone, petroleum ether, diethyl ether were supplied by Lab-scan Analytical Sciences and anhydrous sodium sulphate by Merck Co. (Darmstadt). SepPak® Florisil columns were supplied by Waters®. Pesticide standard (Pesticide Mix 4-8858 dissolved in methyl alcohol-methylene chloride 98:2) was procured from Supelco (USA). Prior to analytical procedures, all glassware was rinsed several times with acetone and hexane.

Analytical Procedure

Samples were analyzed according to the method described by Martínez-López et al. (2009). A volume of 200 µl blood was sonicated and homogenized using hexane:acetone (3:1 v/v) as an extract solvent. The samples were filtered using anhydrous sodium sulphate and then the solvent collected was evaporated until dry. After redisolution in 5 ml hexane, samples were cleaned up via Florisil column chromatography (SepPak, Watersâ) using a petroleum ether-diethyl ether mix (21:4) as the elution. The solvent collected was evaporated until dry.

The final volume was adjusted to 1 ml with n-hexane. One microlitre was injected into a gas chromatograph with electron capture (GC-ECD 17 Shimadzu) for the detection of organochlorine compounds. The SPB-608 capillary column (Supelco) was 30 m long, 0.25 mm i.d. with a 0.25 mm-thick coating. Helium was used as the carrier gas. The injector was set at the splitless mode; the injector temperature was 220 ºC. The column program was: 2 min 50ºC, from 50 to 150 ºC at 40ºC/min, 2 min 150 ºC, from 150 to 290ºC at 8ºC/min, 10 min 290ºC. The detector temperature was 300ºC and the gas make up was nitrogen. Quantification was based on an external standard. The standard solution marked in mixture was prepared by dissolving the references substances in n-hexane (1/50) at the following concentrations: 60 µg/ml for p,p'-DDT and p,p'-DDD; and 20 µg/ml for p,p'-DDE. Detection limits ranged from 0.20 to 0.70 mg/l. The percentage of variability between duplicates varied from 0.8 to 12 % and mean recovery in spiked samples ranged from 85.8 to 146.0 %. Quality assurance criteria were based on the application of quality controls which included the analysis of blank and duplicate samples covering the complete analytical procedure. Concentrations of organochlorine compounds (OC) were expressed as µg/l.

Statistical Analyses

All analyses were carried out using the SPSS v.11.5 statistical package. Reported OC values provide the geometric mean; arithmetic mean ± standard deviation, median and range. DDT/DDE and DDD/DDE ratios were calculated for each individual chick. For this analysis, those samples where both DDE and DDT or DDE and DDD were not detected, were excluded. Since residues were not normally distributed, non-parametric Kruskal–Wallis test was used in order to detect differences between sampling years, followed by Mann–Whitney tests when differences were found. Non-detected values were assigned 1/2 the detection limit to perform mean comparison tests. The level of significance for these tests was set at α=0.05.

RESULTS AND DISCUSSION

P,p'-DDT p,p'-DDE and p,p'-DDD levels are showed in the table 1. Similarly to others studies about DDT concentrations in blood samples from raptors (Donaldson et al., 1999; Van Wyk et al., 2001), p,p'-DDE was the most frequent compound detected, followed by p,p'-DDT and lastly, p,p'-DDD. The DDT is metabolized in

the liver, mainly to p,p'-DDE and p,p'-DDD (Gold and Brunk, 1982). DDE is the most stable and toxic metabolite of DDT and the most frequently found in tissues. In Spain DDT was widely used in agricultural applications but, similarly to other European countries, its use was restricted in the late 1970s and finally banned in 1994 (Orden de 22 de Marzo de 1971 and Orden 4 de Febrero de 1994). However, indoor residual spraying (IRS) with DDT are still being used in certain endemic malarial areas to minimize the incidence and spread of this disease by controlling mosquitoes (Bouwman, 2004). Most of these countries are localized in Africa, where there is a high incidence rate of malaria cases. In 2004, an estimated 350–500 million people contracted malaria globally, of whom more than a million died (80–90% in Africa) (WHO and UNICEF, 2005; WHO, 2007). In this sense, the fact that the prohibition and legal restrictions on the use of organochlorine pesticides in Spain did not reduce levels in eggs, especially at Doñana, leads one to think that the migration of these species towards Africa was responsible for the presence of these compounds in said specimens (Gómara et al., 2004; Negro et al., 1993). However, several authors have demonstrated that levels are similar in migratory and sedentary species (Hernández et al., 1986, 1988; Van Drooge et al., 2008).

Donaldson et al. (1999), found DDE concentrations in plasma of prefledged bald eagle (Haliaeetus leucocephalus) from Canadian Great Lakes ranging from 25 to 60 µg/l. In that study, there were no indications that concentrations of contaminants detected were adversely affecting productivity in the populations. In our study, DDE blood concentrations were similar to those found by Van Wyk et al. (2001) in blood of African whitebacked vulture (Pseudogyps africanus) (Table 1). On the other hand, these concentrations have been considered as low concentrations, since the overall mean for the whole study period were 4-5 times lower than those described by Donaldson et al. (1999). As such, and unlike other studies, DDT concentrations were similar or higher to those for DDE. In general, no significant differences were found between years (χ^2=7.64; p=0.106), although a general tendency to increase the ∑DDT levels was observed throughout the studied period (Table 1). This increase was mainly due to increased concentrations of DDD in 2003 without statistically differences. The major detoxification pathway of DDT is via dechlorination to DDD which readily degrades to 2,2-bis(p-chlorophenyl) acetic acid (DDA) and is rapidly excreted as detoxification product (Baselt, 1982),

Since the biological half-lives for elimination of these compounds are ranked as follows: DDE > DDT > DDD, detection of higher ratios of DDD or DDT to DDE is believed to indicate more recent exposure while lower ratios are believed to correlate with long-term exposure and storage capacity (Morgan and Roan 1971). The mean p,p´-DDT/p,p´-DDE ratio in our samples was 1.83 ± 0.78 meanwhile that p,p´-DDD/p,p´-DDE was 1.92 ± 1.2 (Table 1). The Booted eagle is considered a trans-Saharan migratory species which overwinters in African countries, from mid October to mid March (Martínez, 2002), where malaria and the use of DDT is common (Del Hoyo et al., 1994). Although it seems there is a positive tendency of wintering in Mediterranean areas instead of migrating to Africa (Martínez and Sánchez-Zapata, 1999; Sunyer and Viñuela, 1996).

There may be doubts if the concentrations of these compounds found in the blood of nestlings might partly reflect prior exposure of adults in the African stage (as there is a mother transfer of these compounds to eggs). However, p,p'-DDT is metabolized by the liver by dehydrodechlorination to DDE, although at a much slower rate than the DDT-to-DDD pathway (Gold and Brunk, 1982; Morgan and Roan, 1971). DDD is rapidly detoxified to 1-chloro-2,2-bis(p-chlorophenyl) and eliminated mainly by urine (ATSDR, 2002). However, metabolism of DDE is apparently slow, and DDE is retained in adipose tissue (Hayes et al. 1971; Morgan and Roan 1971). Therefore, the data obtained in this study can only be explained if DDT is still present in the environment. It has been suggested that the recent increase in the detection of DDT in Spain, is due to the use of other pesticides that leave traces of DDT (Hernández et al., 2008; Jiménez et al., 2005; Martínez-López, 2005). In this sense, dicofol is the only organochlorine insecticide whose use was permitted in Spain during the period of study, having been recommended for a wide variety of agricultural products until 2009 (Commission Decision 2008/764/EC; Resolución de 28 de marzo de 2007). In Spain, its use has been estimated on 100-150 tons/year (OSPAR, 2002). Dicofol is produced by hydroxylation reaction of DDT, which is an emulsifiable concentrate and can be commercialized as wettable tablets or water-soluble (Van de Plassche et al., 2002). Spain is the only country, within the UN-ECE region, where dicofol produced by certainly one and maybe two other chemical companies (Van de Plassche et al., 2002). Besides their frequency of detection, the concentrations of these pesticides have also been subjected to variations over time. In that period, 62 products containing dicofol were registered in Spain (MARM, 2005). Because DDT was used in the synthesis of dicofol, a small fraction of DDT could be found in the formulations of this insecticide (Council Directive 90/533/EEC) and therefore in the environment. In several countries or international organisations, dicofol has to meet standards with respect to the minimum content of the p,p'-isomer and the maximum content of DDT and related substances (DDTr) (Van de Plassche et al., 2002). For this reason, EU Council Directive 79/117/EEC prohibited use and marketing of products containing less than 78% p,p'-dicofol or more than 1 g/kg (equal to 0.1%) of DDTr. Dicofol was purified to meet the stringent specifications set in Spain, but the DDTr content of 'raw' dicofol produced by companies as feedstock for purification is unknown (Van de Plassche et al., 2002). Similarly to other organochlorine compounds occurs (Martínez-López et al., 2009), it is possible to assume that changes in the use of the agricultural fields could also reflect variations on the dicofol blood concentrations of the nestling, especially if we take into account that in our study area specimens of this species tend to look for their preys around these agricultural areas.

This fact could provide information on the exposure of nestlings to contaminants via their diet (immediate dietary intake). A few years ago, the main crop in the study area was cereal, however, in the last years, the low yields of these crops has induced their replacement by more productive crops for the dry climate of this area, such as olives, grapes and woody crops (CARM, 2005). According to the data gathered by the Autonomous Government of Murcia Region (CARM, 2005), from 1999 to 2003, the surface area occupied by cereal crops in the study area decreased from 44,200 to

33,370 ha, whilst that of woody crops went from 40,150 ha to more than 46,000 ha, among which the plum tree was to be highlighted (Figure 2).
Using agricultural production data available from the Murcia Regional Autonomous Government from 1999 to 2003 (CARM, 2005) on crops mostly associated with dry farming areas (grains, vineyards, almonds and olives) and fruit trees from market garden areas and matching them against concentrations of the different compounds, an increase in DDTs concentrations in the blood of Booted eagle chicks was observed over the period which followed a progression coinciding with the increase of surface devoted to olive, grape and plum tree production, whilst displaying tendencies to the contrary of those for cereal grain production (Figure 3).
In view of the results, it seems more likely that the use of dicofol, but not the use of DDT in African wintering retreats, would explain the levels of diphenyl aliphatics in the blood of chicks.

CONCLUSIONS

The trans-Saharan migratory factor cannot account for the results obtained for DDT and its analogues in the blood of forest raptors, and as such, other factors directly associated with the study zone must be considered. Assuming that DDT is not actually being used in agricultural practices, then the recommended use of dicofol (a product in whose manufacture DDT is used), against plagues specific to the more important crops in the study area, may account for the levels of DDT and its analogues present in the blood of forest raptor chicks. In this sense, the increase in DDT concentrations in the blood of Booted eagle chicks throughout the study period was parallel to the increase of surface dedicated to vineyards, citrus orchards and olive groves production in the study area, for which dicofol was the recommended pesticide against certain plagues. Finally, we believe that the blood of Booted eagle chicks may be taken into account as a control unit for monitoring or evaluating situations associated with changes in agricultural land use or practice.

TABLES AND FIGURES

Figure 1. Maps showing the geographical location of the Murcia Region (SE Spain) and the study area

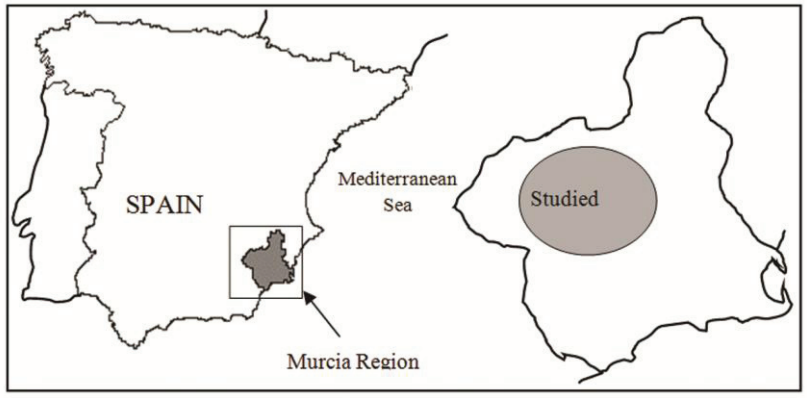


Figure 2. Changes in the surface area dedicated to crops in the study area over the period from 1999-2003.

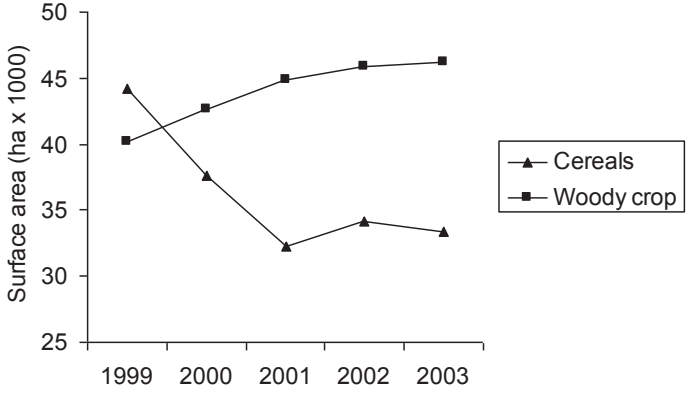
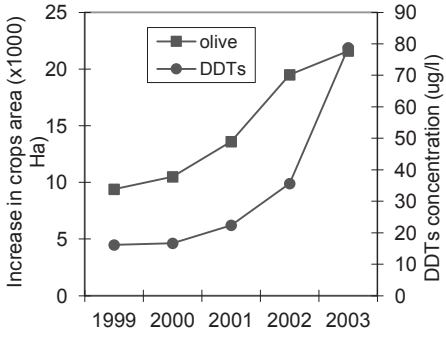
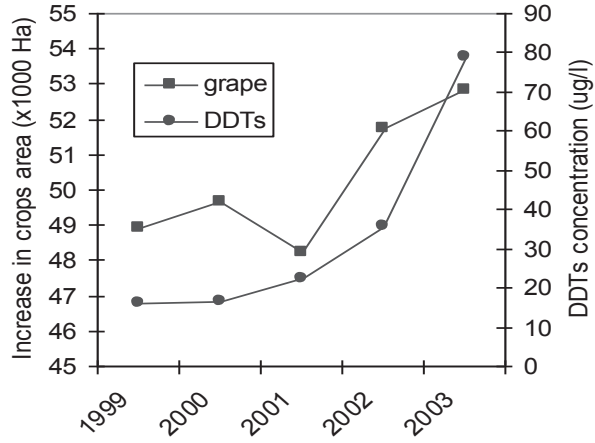
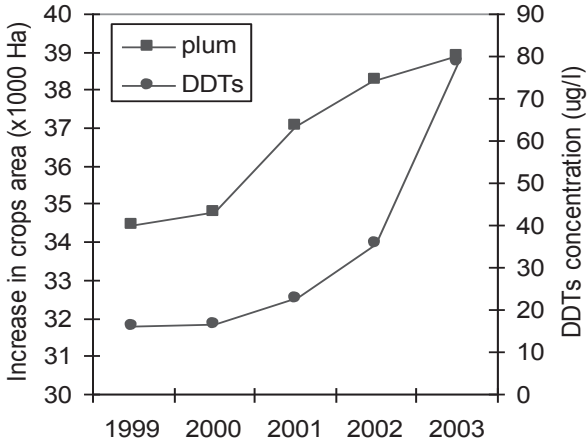


Figure 3. Mean yearly blood DDTs (p,p'-DDT, p,p'-DDE and p,p'-DDD sum) concentrations (μg/l) in Booted eagle nestlings matched with the increase in surface area of a range of crops in the Region of Murcia.


Increase in crops area (x1000) Ha)
DDTs concentration (ug/l)
olive
DDTs
25
20
15
10
5
0
90
80
70
60
50
40
30
20
10
0
1999
2000
2001
2002
2003


Increase in crops area (x1000 Ha)
DDTs concentration (ug/l)
grape
DDTs
55
54
53
52
51
50
49
48
47
46
45
90
80
70
60
50
40
30
20
10
0
1999
2000
2001
2002
2003


plum
DDTs
Increase in crops area (x1000 Ha)
DDTs concentration (ug/l)
40
39
38
37
36
35
34
33
32
31
30
90
80
70
60
50
40
30
20
10
0
1999
2000
2001
2002
2003

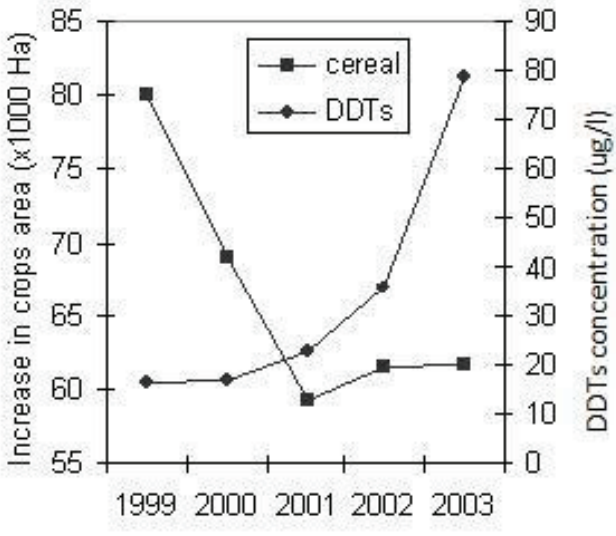

cereal
DDTs
Increase in crops area (x1000 Ha)
DDTs concentration (ug/l)
85
80
75
70
65
60
55
90
80
70
60
50
40
30
20
10
0
1999
2000
2001
2002
2003

Table 1. Blood concentrations of organochlorine insecticides (μg/l) for Booted eagle nestlings over five breeding seasons (1999-2003).[a]

	1999 **N = 10**	**2000** **N = 18**	**2001** **N = 14**	**2002** **N = 10**	**2003** **N = 10**	**1999-2003** **N = 62**	**% not** **detect** **ed**
P,p'- DDE	4.08; 4.69 ± 2.33 1.30-9.10	3.31; 4.23 ± 2.65 nd(1)- 10.00	2.13; 4.90 ± 6.68 nd(3)-25.00	8.30; 8.49 ± 5.96 nd(1)-17.00	4.05; 7.06 ± 5.40 nd(2)-16.27	1.21; 5.60±4.93 nd(7)– 25.00	11.3
P,p'- DD D	3.36; 5.71 ± 7.09 nd(3)-24.00	4.98; 5.93 ± 3.46 nd(1)- 13.00	2.00; 8.12 ± 15.56 nd(8)-46.00	12.50; 17.58 ± 18.43 nd(3)-50.00	43.00; 65.42 ± 77.42 nd(4)- 207.11	1.62; 7.89 ±38.28 nd(19)– 207.11	30.6
P,p'- DDT	3.94; 5.75 ± 4.09 nd(2)-11.00	5.26; 6.51 ± 4.15 nd(1)- 15.00	2.75; 9.38 ± 17.39 nd(6)-63.00	6.50; 9.58 ± 10.60 nd(3)-30.00	1.67; 6.29 ± 11.36 Nd(7)- 34.60	1.59; 7.50 ± 10.48 nd(19)– 63.00	30.6
Σ **DD** **Ts**	**13.61;** **16.15 ±** **11.27** **4.15-44.10**	**14.02;** **16.67 ±** **9.38** **nd(1)-** **38.00**	**8.54;** **22.40 ±** **37.96** **nd(1)-** **134.00**	**19.82;** **35.64 ±** **32.13** **nd(1)-77.00**	**46.10;** **78.78 ±** **89.28** **nd(1)-** **257.40**	**22.18;** **30.99 ±** **46.57** **nd(7)–** **257.40**	**12.9**

[a]Values area presented as geometric mean; arithmetic mean ± standard deviation
Range (min-max)
Nd: not detected in parenthesis

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Dynamic and Batch Adsorption Studies of Isoproturon on Activated Carbon

José Luis Sotelo, Juan García, Araceli Rodríguez, Silvia Álvarez

ABSTRACT

The presence of endocrine disruptors as pesticides in water sources is becoming a concern in drinking water production, wastewater treatment, and water reuse applications due to potentially adverse health effects associated with these compounds. Numerous experimental studies with animals have shown that a number of pesticides are involved in endocrine disruption, while many of the well known herbicides (such as isoproturon) or insecticides are blamed for long term toxicity and carcinogenicity.

In this sense, isotherm experimental runs are performed in batch system to estimate the type of isotherm and their parameters under different operation conditions. The effect of chemical composition and structure of isoproturon were investigated. Adsorption isotherms seemed generally approach to the Freundlich isotherm model with an excellent fit to experimental data.

In a second part, experimental breakthrough curves of isoproturon are assessed in fixed-bed columns. Several experiments were conducted using aqueous synthetic solutions under different initial concentrations, flow rates and bed weights of adsorbent. Different parameters of the column, such as adsorption capacities at breakthrough time and saturation time, length of the mass transfer zone (MTZ), fractional bed utilization and removal percentage have been calculated. Several dynamic adsorption models were applied obtaining a good fit to experimental data. The theoretical breakthrough curve has been obtained.

INTRODUCTION

The potential presence of pharmaceuticals, pesticides, personal care products (PPCPs) and other endocrine disrupting chemicals (EDCs) in the wastewater is an emerging concern among the public, and thus an appropriate consideration for a planned indirect potable water reuse project. These compounds have been detected in a wide variety of environmental water samples including sewage flows, surface and groundwater, with concentrations generally ranging from traces to ppb levels. The lack of validate of analytical methods, non-uniform monitoring data, and the lack of definite information about the fate and effects of these compounds and/or their metabolites and transformation by-products in the aquatic environment makes accurate risk assessments problematic (Stackelberg et al., 2004; Youbin et al., 2010).

Pesticides are used in order to protect plants or plant products against harmful organisms or prevent the action of such organisms. Many of them are not fatal for the target organisms but may also harm organisms in the environment and in this way affect the natural balance. Pesticides have been detected ubiquitously in the aquatic environment, with great presence in urban and industrial wastewaters. A wide variety of pesticides have been developed to protect plants. Among these, herbicides are quantitatively the most important class (more than 50% of all pesticides used). Herbicides, as isoproturon, can be classified under different aspects such as selectivity, chemical structure, the mode and mechanism of the biological action and the types of the plants to be controlled (Sorensen et al., 2011). They are used wherever the growth of undesired weeds has to be inhibited and are used extensively in North America, Western Europe, Japan and Australia (Glassmeyer et al., 2005).

Adsorption technology is currently being used extensively for the removal of organic micropollutants from aqueous solutions and is proved to be one of the most effective techniques for separation and removal of organic pollutants from wastewater. In a little over two decades, granular activated carbon (GAC) was extensively used for waste water treatment.

Most of the studies for isoproturon removal have been conducted in batch operation that is usually done to investigate the type of isotherm as well as to determine the maximum adsorption capacity of the system. So the continuous adsorption in fixed-bed column is often desired from an industrial point of view. It is simple to operate and can be scaled-up from a laboratory process. A continuous packed bed adsorber does not run under equilibrium conditions and the effect of flow conditions (hydrodinamics) at any cross-section in the column affects the flow behavior downstream. The flow behaviour and mass transfer aspects become peculiar beyond a particular length to diameter ratio of the column. In order to design and operate fixed-bed adsorption process successfully, the breakthrough curves under specified operating conditions must be predictable.

Several models have been used for the prediction of the breakthrough curves and the breakthrough time. Some of these are presented in Table 1. Thomas model assumes plug flow behavior in the bed, and uses Langmuir isotherm for equilibrium, and second-order reversible reaction kinetics. This model is suitable for adsorption processes where the external and internal diffusion limitations are absent. Application of this model leads to some error in adsorption processes where first-order reaction kinetics is followed (Singh et al., 2009).

Yoon and Nelson developed a model based on the assumption that the rate of decrease in the probability of adsorption of adsorbate molecule is proportional to the probability of the adsorbate adsorption and the adsorbate breakthrough on the adsorbent (Chen and Chien, 2003).

Clark used the mass transfer coefficient concept in combination with the Freundlich isotherm to define a new relation for the breakthrough curve (Singh et al., 2009).

Wolborska gave a relationship describing the concentration distribution in the bed for the low concentration region of the breakthrough curve. β, a constant in Wolborska model, is an effective coefficient which reflects the effect of both the mass transfer in the liquid phase and the axial dispersion (Goel et al., 2005).

In this work we have studied the removal of isoproturon in water by adsorption in fixed-bed column with GAC as adsorbent. In order to analyze the column dynamics in the adsorption process, the influence of the inlet isoproturon concentration (C_0) and volumetric flow rate (Q) on breakthrough curves has been investigated. Breakthrough curves have been modeled using various equations, and the theoretical breakthrough curve has been obtained.

Table 1. Models and error functions for prediction of breakthrough curves.

Model	Equations
Thomas	$\ln\left(\frac{C_0}{C}-1\right)=\frac{k_T \cdot q_0 \cdot m_c}{Q}-k_T \cdot C_0 \cdot t$ (1)
Yoon and Nelson	$\ln\left(\frac{C}{C_0-C}\right)=k_{YN} \cdot t-\tau \cdot k_{YN}$ (2)
Clark	$\ln\left[\left(\frac{C_0}{C}\right)^{n-1}-1\right]=-r \cdot t+\ln A$ (3)
Wolborska	$\ln\left(\frac{C}{C_0}\right)=\frac{\beta \cdot C_0}{N_0}t-\frac{\beta \cdot Z}{U}$ (4)
Error equations	
$MPSD=100 \cdot \sqrt{\frac{1}{N-P}\sum_{i=1}^{n}\left(\frac{(C/C_0)_{\exp}-(C/C_0)_{cal}}{(C/C_0)_{\exp}}\right)_i^2}$ (5)	
$SE=\sqrt{\sum\frac{(q_{0(\exp)}-q_{0(cal)})^2}{N}}$	(6)
$SE=\sqrt{\sum\frac{(\tau_{\exp}-\tau_{cal})^2}{N}}$	(7)

EXPERIMENTAL

Materials

Isoproturon was purchased from Sigma-Aldrich (Steinheim, Germany), in analytical purity and used as received in the experiments directly. Solutions of isoproturon of appropriate concentration were prepared by diluting a stock solution. The main characteristics of isoproturon are shown in Table 2.

Table 2. Main characteristics of isoproturon.

Compound	Use/category	CAS number	Molecular weight ($g.mol^{-1}$)	[a]Log K_{ow}	[a]pKa	[a]Water solubility ($mg.L^{-1}$)	Molecular formulae	Size (Å)
[b]Isoproturon	Herbicide	34123-59-6	206.3	2.87 (exp) 2.87 (calc)	n.a.	65	$C_{12}H_{18}N_2O$	7.4

[a] SRC Physical Properties Database. *Interactive PhysProp Database Demo*, 2011; http://esc.syrres.com/interkow/physdemo.htm.
[b]Known or suspected endocrine disrupting chemical.
n.a.: Information not available (no dissociation).
KowWin estimates the log octanol-water partition coefficient, log P, of chemicals using an atom/fragment contribution method developed at SRC.

Granular activated carbon, Calgon Filtrasorb 400, having a specific area of 997.0 $m^2.g^{-1}$, external area of 384 $m^2.g^{-1}$, microporous volume of 0.26 $cm^3.g^{-1}$ and pH_{PZC} = 7.6 has been used as adsorbent (ρ_P= 453.6 $g.L^{-1}$, ε_P=0.410). Before use, the adsorbent was washed with water to remove surface impurities, followed by drying at 100 ºC for 48 hours. The size fraction between 0.5 to 0.589 mm was selected by sieving.

METHODS

To obtain adsorption equilibrium isotherm data with granular activated carbon, aqueous phase adsorption experiments were performed in 250 mL glass vials. The suspensions containing different doses of adsorbent and the solutions of isoproturon were shaken with a magnetic stirrer. In all experiments, the vials were agitated on a fixed speed rotator at room temperature (25±1 ºC).

Fixed bed column experiments were conducted using borosilicate glass columns of 6 mm i.d. and 30 cm length. The column was packed with the granular activated carbon and then, the column was filled with a layer of glass balls (1 mm in diameter) to compact the mass of adsorbent and to avoid dead volumes. The influent solution to the column was pumped using a Dinko multichannel peristaltic pump, model D25V.

Fixed bed experiments were carried out under the operational conditions shown in Table 3. The influent solutions were passed in the down-flow mode through the bed. The effluent was collected at time intervals and its concentration was determined by HPLC, using a Varian Chromatograph equipped with a 335 UV-Vis Photodiode Array Detector (PAD) and an ACE 5 C18 PFP column (5µm, 250 mm x 4.6 mm i.d.). An isocratic elution of acetonitrile (A) and aqueous solution (B) was used (40% A, 60% B v/v)), with a mobile phase flow rate of 1.0 mL min^{-1}, and an injection volume of 100 µL.

Table 3. Operation conditions in fixed bed column experiments.

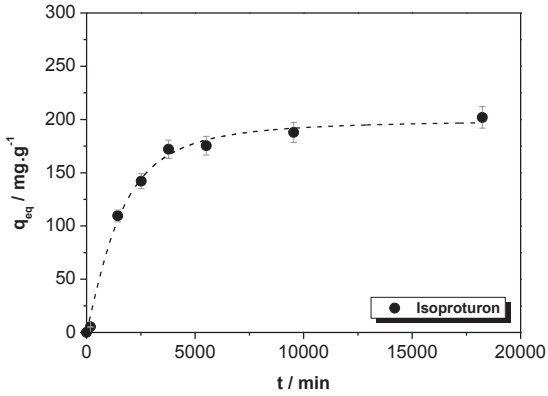
	Bed depth (cm)	Initial concentration ($\mu g.L^{-1}$)	Volumetric flow rate ($mL.min^{-1}$)
Different initial concentration	2.0	50.0	1.0
	2.0	150.0	1.0
Different volumetric flow rates	2.0	100.0	2.0
	2.0	100.0	3.0

RESULTS AND DISCUSION

Equilibrium isotherm

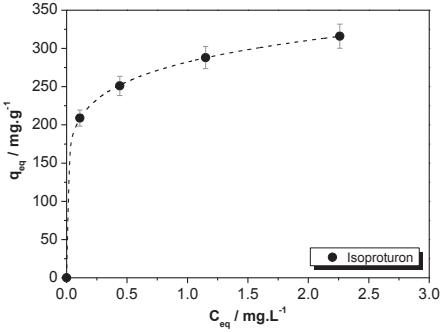
In order to obtain the adsorption isotherm, additional batch experiments were carried out to find out the equilibrium data. In this sense, experiments were conducted for various time intervals to determine the time required to reach adsorption equilibrium (Fig. 1). The equilibrium state was considered reached after about 300 hours, since the variations in equilibrium adsorption capacity did not change more than 5%.

Fig.1. Equilibrium time of isoproturon.



This contaminant, as seen in Fig. 2, presents a type of isotherm can be fitted to Freundlich model (the R^2 value for linear fit is 0.9999). This isotherm is L1-type according to the Giles classification (Giles and Nakhwa, 1960), indicating a high affinity adsorbent-adsorbate and suggesting that isoproturon molecules are adsorbed in parallel to the carbon surface and that there is no major competition between adsorbate and water molecules for the active adsorption sites on the activated carbon.

Fig.2. Adsorption isotherm of isoproturon.



ISOPROTURON ADSORPTION ON FIXED BED

Breakthrough curves for isoproturon adsorption at different conditions were obtained.

Effect of the Initial Influent Concentration

This variable was investigated by using two different initial concentrations, 50.0 and 150.0 $\mu g.L^{-1}$. The bed weight used was of 0.2 g and the flow rate 1.0 $mL.min^{-1}$. The breakthrough curves obtained are shown in Figs. 3a-6a. It is observed that curves have similar shape, although the breakthrough occurred faster at high concentration of isoproturon. A higher concentration leads to steeper slope of the breakthrough curve and a smaller breakthrough time, t_b. As the value of C_0 increased, sharper breakthrough curves were obtained. These results demonstrate that the change in the initial concentration affected the mass transfer velocity and the breakthrough time.

Effect of the Volumetric Flow Rate

The effect of flow rate on isoproturon removal was tested at 2.0 and 3.0 $mL.min^{-1}$, using initial influent isoproturon concentration of 100.0 $\mu g.L^{-1}$ and bed weight 0.2 g. The breakthrough curves are shown in Figs. 3b-6b. It is seen from this figure that the breakthrough occurred faster with higher flow rate. Therefore, the time required for reaching saturation increased significantly with the decrease in the value of the volumetric flow rate.

ADSORPTION PARAMETERS

As it was observed, in general, in the isoproturon adsorption studies, the operational conditions influence the mass transfer process and change the mass transfer zone and bed utilization values. Different parameters, such as adsorption capacities at breakthrough time (q_r) and saturation time (q_s), length of the mass transfer zone (L_{MTZ}) and fractional bed utilization (FBU) have been calculated and are shown in Table 4.

Table 4. Adsorption capacities (qr, qs), MTZ and FBU of isoproturon.

Parameter	Initial concentration ($\mu g.L^{-1}$)		Volumetric flow rate ($mL.min^{-1}$)	
	50.0	150.0	2.0	3.0
q_r (mg isoproturon/gGAC) (C/Co=0.06)	3.09	0.45	13.43	0.86
q_s (mg isoproturon/gGAC) (C/Co=0.88)	16.51	16.75	19.97	13.69
MTZ (cm)	1.81	1.98	1.34	1.96
FBU	0.19	0.03	0.67	0.06

As seen in Table 4, as the concentration increases, the length of the mass transfer zone (MTZ) increases and the fractional bed utilization (FBU) decreases. Therefore, in general terms, as the initial concentration increases, the driving force or mass transfer increases, and results in a decrease in the mass transfer zone (MTZ). In laboratory experiments, the fixed beds are not used long enough to ensure a fully developed profile (a constant pattern behaviour); where the same operating conditions, the value of MTZ remains constant. In this case, when working with very short columns, the mass transfer zone moves to non-constant velocity, since the constant pattern is not fully developed. In this case, an approximate expression for estimate MTZ parameter has been used (constant velocity supposed), so results that may not match those expected can be obtained (Wankat, 1990).
Mass transfer zone increased with increasing flow rate, leading to faster saturation at a higher value of the volumetric flow rate. In this case, as the volumetric flow rate increases, the mass transfer zone (MTZ) and the fractional bed utilization increases. If the concentration profile, C/Co versus length, is studied, an increase of the volumetric flow rate leads to an increase of the mass transfer zone and worsens the use of the bed.

BREAKTHROUGH MODELLING

The estimation of error between the experimental and predicted values of C/Co was done by using modified form of the Marquardt's percent standard deviation (MPSD) represented by Eq. (5) in Table 1. Therefore, adsorption capacity obtained by Thomas and Wolborska model and τ value obtained by Yoon-Nelson model were compared with experimental parameters using standard error of estimate (SE) method: Eq (6) and (7), in Table 1.

The Thomas Model

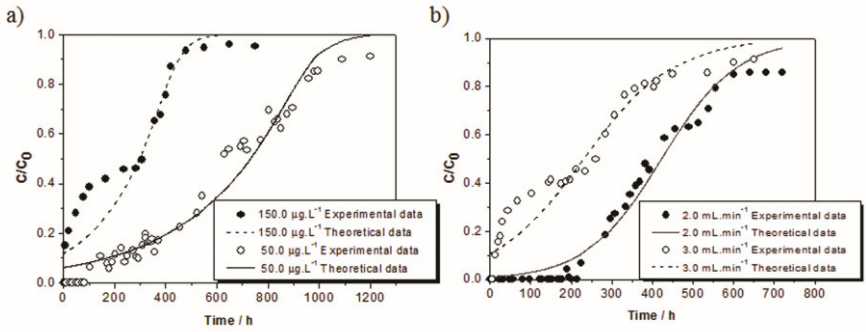
Thomas model parameters, k_T and $q_{0,}$ were determined by not linearized expression of Eq (1). K_T and q_0 values are shown in Table 5. It can be observed from Table 4 that the values of q_0 increased with an increase in the value of C_0 and with a decrease in the value of the volumetric flow rate.

Table 5. Predicted parameters for Thomas model and model deviations for isoproturon adsorption on activated carbon.

C_0 ($\mu g.L^{-1}$)	Q ($mL.min^{-1}$)	Z (cm)	K_T ($L.h^{-1}.mg^{-1}$)	q_0 exp ($mg.g^{-1}$)	q_0 cal ($mg.g^{-1}$)	MPSD	SE
50	1.0	2.0	0.10	16.51	15.57	62.16	0.13
150	1.0	2.0	0.06	16.75	18.86	21.46	0.42
100	2.0	2.0	0.11	19.97	25.31	203.13	0.75
100	3.0	2.0	0.09	13.69	20.75	40.87	1.21

Fig. 3 (a-b) show the experimental and theoretical breakthrough curves obtained at different operating conditions using Thomas model.

Fig.3. Breakthrough curves of isoproturon removal by granular activated carbon packed columns using Thomas model of a) different initial concentrations (bed weight = 0.2 g, initial, flow rate = 1.0 mL.min^{-1}), b) different flow rates (bed weight = 0.2 g, initial isoproturon conc. = 100.0 µg.L^{-1}).



The Yoon-Nelson Model

The values of k_{YN} and τ were determined by not linearized expression of Eq (2). These values are listed in Table 6.

Table 6. Predicted parameters for Yoon-Nelson model and model deviations for isoproturon adsorption on activated carbon.

C_0 (µg.L^{-1})	Q (mL.min^{-1})	Z (cm)	K_{YN} (h^{-1})	τ exp (h)	τ cal (h)	MPSD	SE
50	1.0	2.0	0.005	620.14	677.92	60.03	7.86
150	1.0	2.0	0.008	306.50	261.98	31.33	8.90
100	2.0	2.0	0.011	405.11	421.90	139.17	2.35
100	3.0	2.0	0.009	260.50	230.59	40.87	5.13

Fig.4. Breakthrough curves of isoproturon removal by granular activated carbon packed columns using Yoon-Nelson model of a) different initial concentrations (bed weight = 0.2 g, initial, flow rate = 1.0 mL.min^{-1}), b) different flow rates (bed weight = 0.2 g, initial isoproturon conc. = 100.0 µg.L^{-1}).

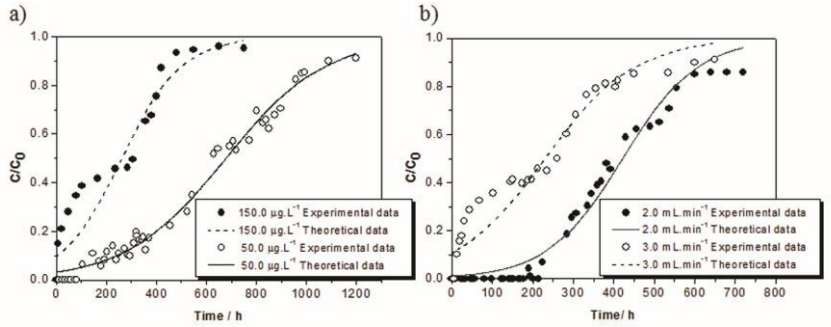


Fig. 4 (a-b) show the experimental and theoretical breakthrough curves obtained at different concentrations and flow rates using Yoon-Nelson model. Fig 4 and the data in Table 6 indicated that the τ values as predicted from the Yoon-Nelson model are predicted relatively well to the experimental results.

The Wolborska Model

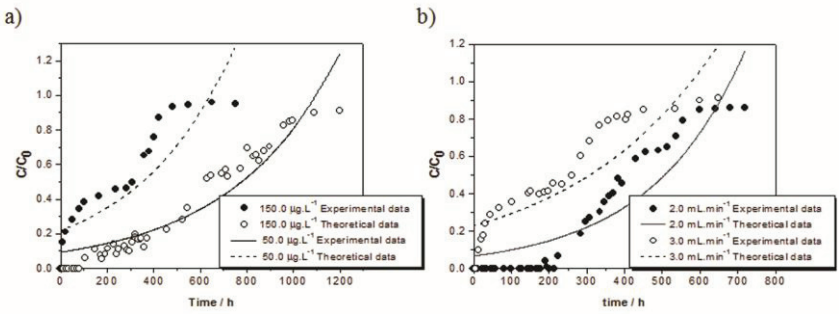
For the determination of the fit of the Wolborska model, not linearized form of Eq (4) was applied to the experimental data. The kinetic coefficients of mass transfer as well as the bed capacity (N_0) were determined from this Equation. Table 7 shows the values obtained along of MPSD and SE values. In general, there is no good fit in N_0 values between experimental and theoretical data obtained.

Table 7. Predicted parameters for Wolborska model and model deviations for isoproturon adsorption on activated carbon.

C_0 (µg.L^{-1})	Q (mL.min^{-1})	Z (cm)	β (h^{-1})	N_0 exp (mg.g^{-1})	N_0 cal (mg.g^{-1})	MPSD	SE
50 69	1.0	2.0	0.04	16.51	0.0021	88.63	2.25
150 67	1.0	2.0	0.03	16.75	0.0036	42.33	3.35
100 63	2.0	2.0	0.06	19.97	0.0032	253.58	2.80
100 68	3.0	2.0	0.05	13.69	0.0041	60.74	2.35

In Fig. 5 (a-b) is shown the experimental and theoretical breakthrough curves obtained at different operating conditions for Wolborska model.

Fig.5. Breakthrough curves of isoproturon removal by granular activated carbon packed columns using Wolborska model of a) different initial concentrations (bed weight = 0.2 g, initial, flow rate = 1.0 mL.min^{-1}), b) different flow rates (bed weight = 0.2 g, initial isoproturon conc. = 100.0 µg.L^{-1}).



The Clark Model

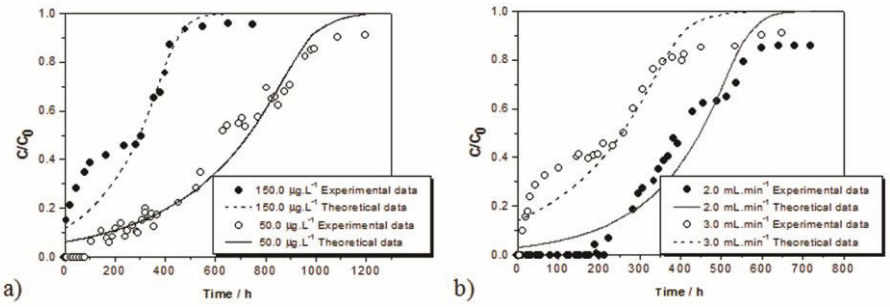
Values of r and A in the Clark equation were determined from not linearized expression of Eq (3), using the Freundlich constant n = 7.3. Table 8 shows the values obtained for MPSD parameter.

Table 8. Predicted parameters for Clark model and model deviations for isoproturon adsorption on activated carbon.

C_0 (µg.L^{-1})	Q (mL.min^{-1})	Z (cm)	r (h^{-1})	A	MPSD
50 69	1.0	2.0	0.018	42138071.16	69.73
150 67	1.0	2.0	0.03	661544.75	28.76
100 63	2.0	2.0	0.039	3376557626	181.02
100 68	3.0	2.0	0.031	219995.10	43.66

Fig. 6 (a-b) show the experimental and theoretical breakthrough curves obtained at different operating conditions for Clark model. In this Figure, a good fit between experimental and predicted data can be observed.

Fig.6. Breakthrough curves of isoproturon removal by granular activated carbon packed columns using Clark model of a) different initial concentrations (bed weight = 0.2 g, initial, flow rate = 1.0 mL.min^{-1}), b) different flow rates (bed weight = 0.2 g, initial isoproturon conc. = 100.0 µg.L^{-1}).



THEORETICAL BREAKTHROUGH CURVE

1. This contaminant presents a Freundlich adsorption isotherm which follows the Eq. 8:

$$q_e = K_f \cdot C_e^n \qquad (8)$$

where, q_e is the amount of isoproturon (mg) adsorbed per unit weight of GAC (g), C_e is the equilibrium concentration of isoproturon remaining in the solution (mg.L^{-1}), K_f and n are Freundlich Equation parameters.

2. According to Weber, the rate of transfer of solute from solution over a differential depth of column, dh, is given by Eq. 9:

$$F_w \cdot dC = K_a \cdot (C - C^*) \cdot dh \qquad (9)$$

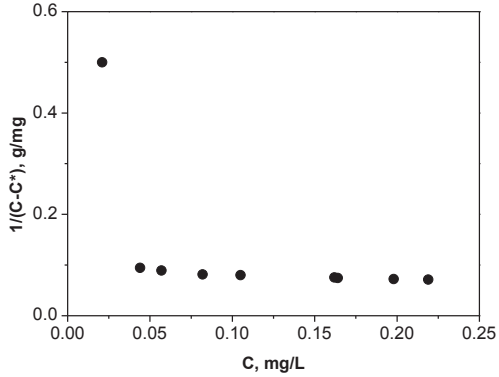
where, F_w = wastewater flow rate; K_a = overall mass transfer coefficient, which includes the resistances offered by film diffusion and pore diffusion; C^* = equilibrium concentration of solute in solution corresponding to an adsorbed concentration, q_e. The term (C-C^*) is the driving force for adsorption and is equal to the distance between the operating line and equilibrium curve at any given value of q_e. Integrating Eq. 9 and solving for the height of the adsorption zone.

$$h_z = \frac{F_w}{K_a} \cdot \int_{C_B}^{C_S} \frac{dC}{(C - C^*)} \qquad (10)$$

The plot of $(C\text{-}C^*)^{-1}$ vs. C is shown in Fig. 7. The area under the curve represents the value of the above integration. For any value of h less than h_z, corresponding to a concentration C between C_B and C_S, Eq. 10 can be written as:

$$h = \frac{F_w}{K_a} \cdot \int_{C_B}^{C} \frac{dC}{(C - C^*)} \qquad (11)$$

Fig.7. Curve to evaluate the integral in Equation (10) for drawing theoretical breakthrough curve of isoproturon removal by activated carbon packed column.



Dividing Eq. 11 by Eq. 10 results in:

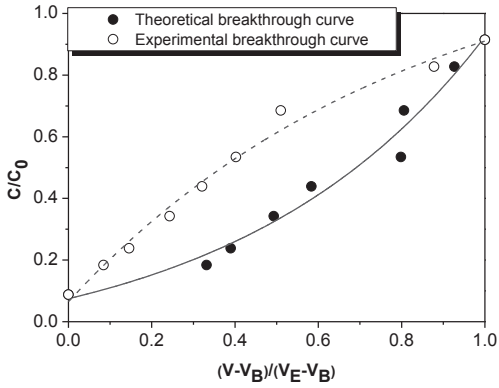
$$\frac{h}{h_z} = \frac{\int_{C_B}^{C} \frac{dC}{(C - C^*)}}{\int_{C_B}^{Cs} \frac{dC}{(C - C^*)}} = \frac{V - V_B}{V_s - V_B} \qquad (12)$$

where V_S and V_E are total volume of water treated till breakthrough and up to saturation point, respectively, and V is the volume of water treated within V_S for effluent concentration C within C_S. Dividing the values of both integrals, Eqs. 10 and 11, the term $V\text{-}V_B/V_S\text{-}V_B$ can be evaluated.

3. Now the plot of C/C_0 vs. $(V\text{-}V_B)/(V_S\text{-}V_B)$ represents the theoretical breakthrough curve.

Fig. 8 shows the experimental and theoretical breakthrough curves. The experimental and theoretical breakthrough curves follow a similar trend, but there is difference between these. The reason behind this is that it was assumed that the isotherm followed the Langmuir model, but originally it was not so.

Fig.8. Experimental and theoretical breakthrough curve of isoproturon removal by activated carbon packed column.



CONCLUSIONS

The present investigation illustrates that the adsorption of isoproturon from aqueous solutions on a fixed bed of granular activated carbon is an interesting and effective treatment for the removal of this micropollutant from wastewaters. The shape of breakthrough curves and front of adsorption obtained is strongly dependent on operation parameters, as initial adsorbate concentration and volumetric flow rate.

It is found that the breakthrough time decreases when initial isoproturon concentration and flow rate increase; showing that a change in the initial concentration or the flow rate affected the breakthrough time and the mass transfer velocity of the process. Parameters as adsorption capacity at breakthrough time (q_r) and saturation time (q_s), length of the mass transfer zone (MTZ) and fractional bed utilization (FBU) were obtained for different conditions.

The removal of isoproturon can be described by empirical models as Thomas, Yoon-Nelson, Wolborska and Clark model. Therefore, the theoretical breakthrough curve was obtained.

ACKNOWLEDGEMENTS

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Section 3

PESTICIDE ANALYSIS

Modern Sample Preparation Methods for Pesticide Multiresidue Determination in Foods of Animal Origin by Chromatographic-mass Spectrometric Techniques

Renato Zanella, Osmar Damian Prestes, Caroline do Amaral Friggi, Manoel Leonardo Martins and Martha Bohrer Adaime

ABSTRACT

Pesticides are widely utilized during cultivation to protect against pests and in postharvest treatments to prolong storage. During the last years much attention has been directed to control pesticide in food samples, especially of animal origin. Considering low concentration levels of pesticide residues in food matrices, the determination of these residues often requires extensive sample extraction and purification prior the analysis. In addition, a multiresidue analytical strategy is often necessary to facilitate the quantitative determination of individual pesticide residues due to the differences among their chemical and physical properties. Nowadays, during the sample preparation step there is a need to reduce time, costs of labor and materials, sources of error and risk of interference. This work summarizes the analytical characteristics of the modern different sample treatment methods available for pesticide residue determination in food matrices of animal origin, covering the evaluation and improvement of sample extraction techniques and clean-up procedures. Sample extraction and purification procedures are critical steps but also the instrumental method is, and cannot be treated separately from sample treatment. Factors that contribute to the matrix effect and some attempts, like additional clean-up steps, to overcome this effect were discussed. Modern sample preparation possibilities are discussed and their most recent applications for pesticide residues in various food matrices of animal origin in combination with chromatographic-mass spectrometric analysis are highlighted.

Key words: Food Analysis, Animal Products, Sample Preparation, Pesticides, Chromatography

INTRODUCTION

Pesticides are widely used and play a key role in pest management and preventing human and domestic animals from infectious diseases. They can be transferred to animals in a number of ways. Consequently, their residues may be found in animal tissues, milk, honey or eggs following the application and medicated premixes, or the consumption of feeds previously treated with agricultural chemicals. In recent years, food safety problems have become a frequently recurring phenomenon. Also as a result of media attention, expressions such as ‘mad cow disease’, ‘dioxin chickens’, ‘medroxy progesterone acetate (MPA) crisis’ and ‘chloramphenicol

scandal' are familiar to the general public. To reach the required level of protection, reliable data about residues in food samples have to be made available to enable adequate risk evaluation and subsequent action. However, it is important to remember that any pesticide should be considered an active poison. The use of pesticides varies greatly among different parts of the world in types and quantities. Consequently, many international organizations such as the Codex Alimentarius Commission and European Union as well as different countries have issued their own pesticide maximum residual limits (MRLs) in the international trade. Analysis of pesticide residues is carried out by using many different methods for extraction and clean-up, followed by a final analysis typically with chromatographic measurements. Extraction of pesticide residues from samples produces complex mixtures that often require sample purification and preparation steps to isolate the targeted pesticides for analysis. In other words, sophisticated and robust analytical methods have to be developed for a wide variety of, primarily organic, micro-contaminants. This chapter addresses one highly relevant problem, the analysis of pesticide residues in food of animal origin. A multiresidue analytical strategy is often necessary to facilitate the quantitative determination of individual pesticide residues due to the differences among their chemical and physical properties and incompatible detection techniques. The cost of labour and materials, and long turnaround times could be significantly reduced if sample preparation and clean-up procedures were performed by automated methods. Many traditional procedures used for extraction of fatty samples are time consuming and solvent intensive. The present chapter covers published methods and research articles, in which pesticide residues have been extracted from animal food origin, then cleaned-up, and isolated by chromatographic techniques to be identified and quantified by mass spectrometric methods. Lastly, future developments and perspectives in this field are outlined.

SAMPLE EXTRACTION TECHNIQUES

Nowadays, in the determination of pesticides residues, pre-concentration and isolation of the analytes from the food samples of animal origin by some types of sample preparation process would be critical for obtaining correct results. Various sample preparation techniques have been employed for this purpose. However, some of these methods are time consuming, have complicated procedures and use a large amount of organic solvents which are often toxic and flammable. Recently, chlorine solvent free and excellent performance techniques have been extensively studied as the substitution to this type of sample preparation processes. This chapter showed an overview about these modern sample extraction techniques.

Manual Sample Extraction Techniques

A comprehensive control of pesticide residues in food with multiresidue methods is needed to protect people effectively. While the basic determination methods LC and GC allow a high sample throughput with determinations up to 100 analytes, conventional sample extraction methods are often demanding in terms of labour,

time and cost. In the past few years, the manual sample extraction methods are easy to use and envolved an economical and environmentally compatible sample extracts for GC-MS and LC-MS. The advantages of these methods in comparison with classical clean-up methods are: high sample throughput due to a quick and easy few-step procedure, low need of laboratory glasses, bench space and equipment, low consumption of solvents, broad range of pesticides can be determined and rugged method with high and safe recovery rates.

Solid-Liquid and Liquid-Liquid Extraction

In a recent review about pesticides analysis in foods of animal origin, LeDoux (2011) showed that in the last two decades, the most widely used pesticide extraction techniques from these samples was solid–liquid extraction (SLE) and liquid–liquid extraction (LLE). The SLE procedure consists in grinding chopped samples or extracted fats several times at high speed in selected organic solvents. This technical procedure has been applied to meat and meat products animal fat, offal, eggs and fish for extracting different kinds of pesticides. Similarly, LLE procedure consists in shaking liquid samples several times in selected organic solvents for extracting pesticide residues.

QuEChERS

The QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method was originally developed for extracting a wide range of pesticides in fruit and vegetables has become very popular since it was introduced in 2003 by Anastassiades and coworkers. The method is characterized by using the polar solvent acetonitrile for extraction of water containing matrices with addition of salts in order to get phase separation. Since its introduction, this method has been readily accepted by many pesticide residue analysts because of its low cost, fast, the accurate procedures are no time consuming. Evaporation steps or clean-up using traditional SPE in cartridges no are required (Sack et al., 2011; Kolberg et al., 2010; Payá et al., 2007).

The original QuEChERS method version used neutral extraction conditions by single phase extraction of multiple analytes with a small volume (10 mL) of acetonitrile followed by liquid-liquid partitioning with the addition of 4 g of anhydrous $MgSO_4$ plus 1 g of NaCl. Removal of residual water and clean-up of polar residues are performed simultaneously using a dispersive solid-phase (d-SPE) clean-up. The d-SPE clean-up carried out by just adding a primary secondary amine sorbent (PSA) (Anastassiades et al., 2003), C-18 material (Lehotay et al., 2005a) or other adsorbents directly to the acetonitrile extract. A quick shaking followed by centrifugation removes several interferences.

A limited number of GC-amenable pesticides were evaluated in the original QuEChERS study, but in the last years this version has been demonstrated excellent results for hundreds of pesticides in different commodities (Wilkowska et al., 2011; Prestes et al., 2011; Prestes et al., 2009). However, for some pesticides, this method gave lower stability and/or recoveries depending on pH of the matrix (Anastassiades et al., 2003). The original approach was modified with add a buffering step at pH-5 during extraction, this gave the optimum balance to achieve acceptably high

recoveries (>70%) for certain pH-dependent pesticides (e.g. pymetrozine, imazalil, thiabendazole) independent of the fruit/vegetable matrix (Lehotay et al., 2005b).

Lehotay et al. (2005b) modified the method to use relatively strong acetate buffering conditions and Anastassiades et al. (2007) chose to use weaker citrate buffering conditions in terms of ionic strength. Both versions of these methods went through extensive interlaboratory program (Lehotay et al., 2007) for dozens of pesticides at fortified and incurred at different levels in different matrices and using different types of GC–MS and LC–MS/MS conditions and instruments. Both methods successfully met statistical criteria for acceptability from independent scientific standards organizations, with the acetate-buffering version becoming AOAC Official Method 2007.01 (AOAC, 2007) and the citrate-buffering version being named European Committee for Standardization (CEN) Standard Method EN 15662 (CEN, 2008).

The QuEChERS approach is very flexible and it serves as a template for modification depending on the analyte properties, matrix composition, equipment and analytical technique available in the laboratory. The template is also very rugged in that high recoveries will be achieved for many pesticides in many matrices even if different ratios and types of sample size, solvent, salts and sorbents are used in modifications. The ruggedness characteristics of the QuEChERS approach have been thoroughly evaluated in the original (Anastassiades et al., 2003) and subsequent publications (Lehotay et al., 2010; Stubbings and Bigwood, 2009; Aguilera-Luiz et al., 2008; Cunha et al., 2007; Díez et al., 2006; Lehotay et al., 2005c). In multiclass, multiresidue pesticide analysis, the sample preparation method inherently necessitates broad analytical scope which makes it impossible to obtain a high degree of clean-up without reducing recoveries for some pesticides. However, greater clean-up can be achieved by using different sorbents in d-SPE if the application has reduced analytical scope (Li et al., 2011; Koesukwiwat et al., 2010; Wong et al., 2010).

Sample preparation is always the major bottleneck in any analytical procedure for the determination of chemical residues in food products (Díez et al., 2006). Since 2003 many papers have been published where QuEChERS or related methods are successfully applied for foods of animal origin. A modified QuEChERS extraction method was developed and validated to detect 38 compounds (benzimidazoles, avermectins and flukicides) in milk samples by UHPLC-MS/MS. The compounds residues were extracted into acetonitrile using magnesium sulphate and sodium chloride to induce liquid–liquid partitioning followed by d-SPE clean-up. The extract was concentrated into dimethyl sulphoxide, which was used as a keeper to ensure analytes remain in solution. Using rapid polarity switching in electrospray ionisation, a single injection was capable of detecting both positively and negatively charged ions in a 13min run time. The method was validated at two levels: the unapproved use level and at the maximum residue level (MRL). The decision limit (CCα) of the method was in the range of 0.14–1.9 and 11–123 μg kg^{-1} for drugs validated at unapproved and MRL levels, respectively. The performance of the method was successfully verified for benzimidazoles and levamisole by participating in a proficiency study (Whelan et al., 2010).

An analytical method based on QuEChERS approach was refined for the extraction and determination of neonicotinoid pesticide residues and their metabolites in honey bees and bee products. Samples were extracted with 2% triethylamine in acetonitrile followed by salting out, solid phase extraction clean-up, and detection using LC-MS/MS. The method was validated in triplicate at three fortification concentrations in each matrix. Good recoveries were observed for most analytes and ranged between 70 and 120% with relative standard deviations between replicates of <20% in most cases. The method limits of detection were 0.2 μg kg^{-1} for the parent neonicotinoid pesticides and ranged between 0.2 and 15 μg kg^{-1} for the neonicotinoid metabolites. This refined method provides lower detection limits and improved recovery of neonicotinoids and their metabolites, which will help researchers evaluate subchronic effects of these pesticides, address data gaps related to colony collapse disorder, and determine the role of pesticides in pollinator decline (Kamel, 2010).

The QuEChERS method combines salting-out liquid-liquid extraction with acetonitrile and a dispersive-SPE clean-up. It was adjusted to determine organohalogens, organophosphorous, pyrethroids, insect growth regulators and synergists in honey and especially to honeybee and pollen, by adding a small fraction of hexane in acetonitrile to eliminate lipids that interfere with mass spectrometry analysis. This method, combined with accurate and sensitive detection, allowed quantification and confirmation at levels as low as 10 μg kg^{-1}, with recoveries between 60 and 120%. Application to more than 100 samples of each matrix was achieved for a global view of pesticide presence in the honeybee environment. Relatively high percentages of honeys, honeybees and pollens were found to be contaminated by pesticides like carbendazim and ubiquitous contaminants (Wiest et al., 2010).

The QuEChERS method was successfully applied to the determination of the natural pyrethrins (cinerin I and II, jasmolin I and II, and pyrethrin I and II), as well as two pyrethroid insecticides, cypermethrin and deltamethrin, in fin and non-fin fish products. Individual samples (5 g) were weighed into 50-mL fluorinated ethylene propylene tubes. Samples were allowed to sit for approximately 45 min, after which 5 mL of 1% glacial acetic acid in acetonitrile was added, and the samples were sonicated for 10 min prior to the addition of 2 g $MgSO_4$ and 0.5 g sodium acetate to the sample tubes. The tubes were capped tightly and shaken vigorously manually for 1–2 min. Samples were then centrifuged at 3,000 rpm for 5 min. Approximately 1 mL of the crude acetonitrile extract was removed to performed d-SPE clean-up. Analysis of these compounds was performed using GC-MS/MS. Cypermethrin concentrations ranged from 0.3 to 6.5 μg kg^{-1} in the positive samples. It was not, however, observed in any imported fish product or any other domestically produced fish product (Rawn et al., 2010). Adapted QuEChERS method, coupled to liquid chromatography tandem mass spectrometry, was also developed to quantify 13 pesticides (azoxystrobin, clomazone, diflufenican, dimethachlor, carbendazim, iprodion, isoproturon, mesosulfuron-methyl, metazachlor, napropamid, quizalofop and thifensulfuron-methyl) in muscle of fish. Quantification limits were below 1 μg kg^{-1} except for clomazone (1.8 μg kg^{-1}) and quizalofop (7.4 μg kg^{-1}) (Lazartigues et al., 2011).

Due the versatility of the QuEChERS method many versions have been published also for determination of other residues and contaminants (e.g. mycotoxins and veterinary drugs) in animal food origin samples. Garrido-Frenich et al. (2011) reported a reliable and rapid method for the determination of 10 mycotoxins in eggs at trace levels. UHPLC–MS/MS has been used for the analysis of these compounds in less than 7 min. Mycotoxins have been extracted from egg samples using a QuEChERS-based extraction procedure without applying any further clean-up step. Matrix-matched calibration was used for quantification. Blank samples were fortified at 10, 25, 50 and 100 μg kg^{-1}, and recoveries ranged from 70 to 110%. Relative standard deviations were lower than 25% in all the cases. Limits of detection ranged from 0.5 to 5 μg kg^{-1} and limits of quantification ranged from 1 to 10 μg kg^{-1}. Seven samples were analyzed and aflatoxins B1, B2, G1, G2, and beauvericin were detected at trace levels. Martinez-Vidal et al. (2011) published two rapid multiresidue screening methods for the determination of 21 veterinary drugs in milk by UHPLC-MS/MS. Milk samples were extracted using a rapid extraction procedure based on the modification of the QuEChERS method, and no further clean-up steps were necessary. One screening method was based on the selection of a characteristic neutral loss or production of the various families of compounds, whereas another one was based on the choice of a selected reaction monitoring (SRM) for each compound. These methods were compared with regards to false negatives, cut-off values and the unreliability region. The total run time for both methods was 3 min, allowing quick selection of samples that contained veterinary drugs.

To sum up, the QuEChERS method is very adaptable and provides the following advantages over traditional techniques: (1) significant reduction use of organic solvent; (2) complete removal of the use of chlorinated solvents; and (3) removal of the use of mechanical homogenizers or blenders. The combination of this extraction procedure with GC-MS/MS or LC-MS/MS allowed a batch of extracts in less than 30 min by a single analyst and up to hundreds of compounds can be analyzed simultaneously in a sample (Wilkowska et al., 2011; Prestes et al., 2009; Prestes et al., 2011).

Matrix Solid-Phase Dispersion (MSPD)

The MSPD technique was developed by Barker and coworkers in 1989. MSPD involved a modification of the SPE technique and is based on the use of a sorbent, which acts as an abrasive in order to produce a modified "opening" of the solid matrix, facilitating the extraction process when using a suitable solvent for eluting the analytes (Capriotti et al., 2010). The strategy of this technique is based on a mixer of fine dispersion of the matrix with a sorbent material (C18, alumina, silica, etc.). Usually, solid samples are prepared for subsequent extraction and/or clean-up by a stepwise process that begins with the disruption of the sample. After blending, the sorbent material is often packed into a minicolumn, where the analytes are eluted by a relatively small volume of a suitable eluting solvent. Many MSPD procedures also employ “co-columns” to obtain further fractionation and to achieve a better removal of matrix interferences (Gilbert-López et al., 2009). The MSPD has advantages over conventional extraction techniques because it employs small

amounts of sample and solvent, and the extraction procedure consists of only a few experimental steps (Capriotti et al., 2010).

MSPD is an attractive extraction technique which allows direct handling of solid, semisolid, and viscous samples, markedly reducing sample manipulation and, consequently, the likelihood of errors. Moreover, its wide acceptance is owed to its ease of implementation, reduced solvent consumption, low overall cost, and the possibility, in some cases, of integrating extraction and clean-up in a single step thus avoiding the need for additional manipulation of sample extracts (García-Lopez et al., 2008).

A rapid, sensitive and accurate MSPD method combined with accelerated solvent extraction has been developed by for selective determination of sixteen organochlorine pesticide residues in fish samples by GC-MS. 2 g fresh fish muscle was dispersed with 10 g anhydrous sodium sulfate and 2 g acid alumina thoroughly, and loaded into the stainless-steel extraction cell containing 6 g of acid alumina and 10 g anhydrous sodium sulfate. The adequate extraction efficiency was showed using dichloromethane-hexane (3:7, v/v) mixture as solvent. Not only the lipids, but also other co-extracts, which peaks mostly located in the forepart of chromatograms and maybe interfere the identification or quantitation of analytes, were eliminated exhaustively, while analytes were extracted selectively. The performance of proposed method was evaluated and validated: the detection limits were 0.008-0.05 μg kg^{-1} (1.9-5.0, RSD%) and recoveries were 91.0-104.1% spiked at 10 μg kg^{-1} level.

A method based on MSPD was developed by García de Llasera and Reyes-Reyes (2009) for the quantitative extraction of five organophosphorus pesticides from bovine samples. The determination was carried out by HPLC-UV detection. The MSPD extraction with C18 sorbent combined with a silica gel clean-up and acetonitrile elution was optimised for chlorpyrifos, chlorfenvinphos, diazinon, fenitrothion, and parathion-methyl. The method was validated, yielding recovery values higher than 94%, except for chlorfenvinphos in liver (55%), and precision values, expressed as relative standard deviations (RSDs), which were less than or equal to 15% in liver and 11.5% in muscle at spiking levels of 0.25, 2.5 and 5 mg kg^{-1}. Linearity was studied from 0.5 to 15 mg kg^{-1}, and the limits of detection (LODs) were found to be lower than 0.1 mg kg^{-1}. This method was applied to the analysis of real samples with confirmative analyses performed using GC–MS in selected ion monitoring mode (SIM).

A MSPD and gas chromatography-mass spectrometry were combined by Bezerra et al. (2010) to determine procymidone, malathion, bifenthrin and pirimicarb in honey. The best results were obtained using 1.0 g of honey, 1.0 g of silica-gel as dispersant sorbent and acetonitrile as eluting solvent. The method was validated by fortified honey samples at three concentration levels (0.2, 0.5 to 1.0 mg kg^{-1}). Average recoveries (n=7) ranged from 54 to 84%, with relative standard deviations between 3.7 and 8.5%. Detection and quantification limits attained by the developed method ranged from 0.02 to 0.08 mg kg^{-1} and 0.07 to 0.25 mg kg^{-1} for the honey, respectively.

In general, MSPD applications show evidence of a steady broadening of employed extraction conditions, as regards the type of dispersant and elution solvent, and field

of application, with some still scarce but promising results (García-Lopez et al., 2008). Automation of the whole extraction process, miniaturization (with the consequent reduction of sample, sorbent, and solvent consumption), and the use of cost-effective and harmless solvents are also current trends, which will keep on going.

Instrumental-based Sample Extraction Techniques

An important step in the preparation of food samples prior to final analysis is isolation and/or enrichment. The procedures consist of the transfer of analytes from the primary matrix into the secondary one with a concurrent purging of interfering substances (isolation) and increasing the analytes concentrations to a level above the detection limit for a given analytical technique (enrichment) (Beyer and Biziuk, 2008).

A number of instrumental-based extraction procedures have been developed to isolate pesticides residues from food of animal origin, including microwave-assisted extraction (MAE), supercritical fluid extraction (SFE), pressurised liquid extraction (PLE), solid-phase extraction (SPE) and solid-phase micro-extraction (SPME) systems. Advantages in using such technology include the potential for automation, more selective isolation of residues through tuning of instrument parameters and on-line clean-up of samples. Disadvantages include the limited number of commercially available instruments, additional extraction costs and instrumental downtime (Kinsella et al., 2009).

Microwave-Assisted Extraction (MAE)

MAE uses microwave energy to cause molecular movement and liquid rotation with permanent dipole, leading to very fast heating of the solvent and the sample in order to partition analytes of interest from sample matrix into the solvent (Beyer and Biziuk, 2008; Kinsella et al., 2009). Using microwave energy allows the solvent to be heated rapidly: an average extraction takes 15 to 30 min (Camel, 2001). MAE offers high sample throughput (several samples can be extracted simultaneously) with low solvent consumption (10 to 30 mL) (Kinsella et al., 2009). This technique is only applicable to thermally stable compounds due to the increase in temperature during extraction. As non-polar solvents do not absorb microwave energy, at least some polar solvent, such as water, must be used (Ridgway et al., 2007).

MAE systems can operate in two modes, open (focused MAE) or closed (pressurized MAE) vessels. Open vessels operate at atmospheric pressure, while closed vessels are sealed and operate under higher pressures. Closed vessel MAE operates somewhat like PLE, since the temperature of the solvent can be increased by increasing the pressure (Kinsella et al., 2009).

The main advantages of microwave pretreatment are the low temperature requirement, high extraction rate, automation and the possibility of simultaneously extracting different samples at the same time without interferences (Camel, 2000). However, solvent choice is limited, care must be taken not to overheat the sample,

additional clean-up of the samples is generally necessary prior to analysis and MAE is not amenable to automation (on-line extraction and detection) (Camel, 2001).

The main applications of MAE are as an alternative to Soxhlet extraction because good extraction efficiencies can be achieved using less solvent and shorter extraction times. Few papers has been found in the literature for the application of MAE to the extraction of pesticides residues from food of animal origin using mass spectrometry detection. This is generally due to the limited diffusion of the solvent in samples containing more than 30% of water (as it is the case of food samples), resulting in low analyte recovery. This problem can be circumvented by prior drying of samples by lyophilization (Marazuela and Bogialli, 2009).

MAE was tested and compared with soxhlet extraction (SE) and accelerated solvent extraction (ASE) to extraction of persistent organic chemicals in fish samples in order to evaluate their performances by Wang et al. (2010). For MAE 2.0 g fish were weighed and mixed with 3–4 g anhydrous sodium sulphate powder, followed by loading into the extraction cylinders. 30 mL n-hexane/acetone (1:1, v/v) was added and the extraction was performed. The condition was as follows: the temperature was ramped to 115 °C in 10 min and held for 15 min, then cooled down in 20 min; microwave power was 1200 W. For both polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs), the two more recent developed techniques (ASE and MAE) were in general capable of producing comparable extraction results as the classical SE. This relatively uniform extraction results from ASE and MAE indicated that elevated temperature and pressure are favourable to the efficient extraction of PCBs from the solid matrices. For PBDEs, difference between the results from MAE and ASE (or SE) suggests that the MAE extraction condition needs to be carefully optimized according to the characteristics of the matrix and analyte to avoid degradation of higher brominated diphenyl ethers (BDE) congeners and improve the extraction yields.

Supercritical Fluid Extraction (SFE)

Hannay and Hogarth (1879) in experiments for dissolution of solutes in supercritical fluid media introduced the possibility of a new solvent medium. However, it was only around 1960 that commercial process applications of supercritical fluid extraction have been extensively examined (Herrero et al., 2010).

Before the development of commercial supercritical fluid extraction (SFE) instruments in the late 1980s, few researchers investigated the use of supercritical fluids in analytical, nonchromatographic applications. Only in the 90 years began to be published studies using SFE as extraction technique in combination with chromatographic techniques for analytical applications for analysis of pesticide residues (Lehotay, 1997).

The unique properties exhibited by supercritical fluids have been applied for the analysis of pesticide residues in solid samples SFE is selective and less-solvent-consuming, thus it is environmental friendly. The most serious problem of off-line SFE methods is evaporation of collecting solvent at the end of extraction to acquire high preconcentration factor. However, this procedure is a time-consuming step and contaminates the environment and collected analytes may be lost or degradated in this step (Naeeni et al., 2011).

The main supercritical solvent used is carbon dioxide (critical conditions = 30.9 ºC and 73.8 bar) that is cheap, environmentally friendly and generally recognized as safe. Supercritical CO_2 ($SCCO_2$) is also attractive because of its high diffusivity combined with its easily tuneable solvent strength. Another advantage is that CO_2 is gaseous at room temperature and pressure, which makes analyte recovery very simple and provides solvent-free analytes. Also, important for food and natural products sample preparation, is the ability of SFE using CO_2 to be operated at low temperatures using a non-oxidant medium, which allows the extraction of thermally labile or easily oxidized compounds. The main drawback of $SCCO_2$ is its low polarity, problem that can be overcome employing polar modifiers (co-solvents) to change the polarity of the supercritical fluid and to increase its solvating power towards the analyte of interest. The modifiers can also reduce the analyte–matrix interactions improving their quantitative extraction (Herrero et al., 2010).

In the SFE commercially available equipment, the fluid is pumped, at a pressure above its critical point, with the sample placed in an inert extraction cell. The temperature of the cell is increased to overcome the critical point of the fluid. After depressurization, the analytes are collected in a small volume of organic solvent or on a solid-phase filled cartridge (solid adsorbent trap). Extraction can be performed in the static, dynamic or recirculating mode: in the static extraction mode, the cell containing the sample is filled with the supercritical fluid, pressurized and allowed to equilibrate; using the dynamic mode, the supercritical fluid is passed through the extraction cell continuously; finally in the recirculating mode the same fluid is repeatedly pumped through the sample and, after the required number of cycles, it is pumped out to the collection system (Stoytcheva, 2011).

Pesticide supercritical fluid extraction (SFE) has been attempted for extracting contaminants from meat products. SFE is usually an efficient extraction method, primarily applicable to solid samples. However, as well as its numerous advantages (efficacy, selectivity, short extraction times, low solvent volumes) it also has serious drawbacks (difficult optimisation, high apparatus and maintenance cost, high blank and noise levels). In the case of pesticide residue analysis, recoveries for several compounds were unacceptable. Indeed, SFE techniques have not tended to be widely used for pesticide analysis in food from animal origin (LeDoux, 2011).

An analytical procedure using supercritical fluid extraction (SFE) and capillary gas chromatography with electron-capture detection and confirmation of pesticide identity was performed by gas chromatography–mass spectrometry in selected-ion monitoring mode was developed to determine simultaneously residues of different pesticides (organochlorine, organophosphorus, organonitrogen and pyrethroid) in honey samples. Best efficiency was achieved at 400 bar using acetonitrile as modifier at 90 ºC. Compared with the conventional methodology, the main advantages of SFE are that the chances of sample contamination are greatly diminished as sample handling is minimized and the use of organic solvents is reduced. A much lower solvent evaporation, a simplified clean-up step, higher power diffusion and solubility are the other advantages of SFE (Rissato et al., 2004).

Pressurised Liquid Extraction (PLE)

PLE has received numerous names, such as accelerated solvent extraction (ASE), pressurized fluid extraction (PFE), pressurised hot solvent extraction (PHSE), subcritical solvent extraction (SSE) and hot H_2O extraction (HWE) (Carabias-Martinez et al., 2005). This technique utilizes solvents that are raised to the near supercritical region, where they show better extraction properties. At high temperatures, the rate of extraction increases because the viscosity and the surface tension decreases, while its solubility and diffusion rate into the sample increase. Pressure keeps the solvent below its boiling point and forces its penetration into the pores of the sample (Beyer and Biziuk, 2008). The combined use of high pressures (500-3000 psi) and temperatures (50-200 °C) provides a faster extraction process (5-10 min) that requires smaller amounts of solvent compared with traditional extraction, thus decreasing the dilution of the sample (Richter et al., 1996). The time required for extraction is practically independent of the sample mass and the efficiency of extraction is mainly dependent on temperature (Beyer and Biziuk, 2008).

PLE can be performed in both static and dynamic (flowthrough) modes, or a combination of both. In static mode, the sample is enclosed in a stainless steel vessel filled with an extraction solvent, and following extraction the remaining solvent is purged with N_2 into a collection vial. Flow-through systems continuously pump solvent through the sample, but this has the disadvantage of using larger volumes of solvent and of diluting the extract. A desiccant, such as sodium sulphate, diatomaceous earth or cellulose can be added directly to the extraction cell or sorbent materials can be used to provide in situ clean-up. The extraction conditions must be optimised and this can be done using statistical "experimental design" procedures to minimize the number of experiments (Pallaroni and von Holst, 2003; von Holst et al., 2005).

Comparing PLE to LLE or Soxhlet extraction, the advantage of reducing solvent consumption and extraction time contrast with the disadvantage of using very expensive and specialized equipment. However, the major problem with fatty matrices is the presence of large amounts of co-extracted lipids, which means that post-clean-up of the extract is required to carry out lipid elimination (Beyer and Biziuk, 2008).

Frenich et al. (2006a) optimized and validated a method for the simultaneous determination of residues of organochlorine (OCPs) and organophosphorus (OPPs) pesticides in meat samples from chicken, pork and lamb. The method is based in the extraction of homogenized meat mixed with sodium sulphate and ethyl acetate in polytron, although accelerated solvent extraction (ASE) and the determination was performed by gas chromatography (GC) coupled to a triple quadrupole (QqQ) mass spectrometry (MS) detection system. Recoveries and precision values were 70.0–90.0% and 15%, respectively, for the bulk majority of pesticides. Values of LOD < 2.0 and LOQ < 5.0 μg kg^{-1} were obtained for all the target compounds, except for acephate.

A new analytical method was developed by Wu et al. to simultaneously determine residues of 109 pesticides (including isomers) in the foods of animal origin. Acetonitrile was selected for accelerated solvent extraction (ASE) for effectively

extracting the pesticides from the fatty samples. The prepared samples were analysed with GC–MS in the selected ion monitoring mode (SIM) using one target and two qualitative ions for each analyte. Chlorpyrifos-d_{10} was used as an internal standard. The lowest limit of detection was 0.3 μg kg^{-1} for some pesticides. The recoveries and relative standard deviations (RSDs) were checked by spiking untreated samples with pesticides at 0.05, 0.1 and 0.2 mg kg^{-1}. The average recoveries of most pesticides were from 62.6 to 107.8%. The precision values expressed as RSD were all 20.5% (n = 6). Good linearity ($r^2 \geq 0.99$) was observed between 0.05 and 1.5 μg mL^{-1} (Wu et al., 2011).

Zhang et al. compared PLE, selective pressurized extraction (SPLE), soxhlet and ultrasonic and heating extraction methods for the simultaneous extraction and clean-up of PBDEs and PCBs in sheep liver tissue samples followed by gas chromatography–mass spectrometry (GC–MS). Overall the mean percentage recoveries for all target chemicals using SPLE were 86–103% (n = 3, SD < 9%), and compared favourably with the Soxhlet (63–109%, n = 3, SD < 8%), off-line PLE (82–104%, n = 3, SD < 18%), ultrasonic (86–99%, n = 3, SD < 11%) and heating (72–102%, n = 3, SD < 21%) extraction methods. The limits of detection of the proposed method were 5–96 pg g^{-1} and 2–29 pg g^{-1} for the different PBDE and PCB chemicals studied, respectively. The outputs of the proposed method were linear over the range from 0.02 to 30 ng g^{-1}, for all PCB and PBDE congeners except for PBDE 100 and 153 (0.05–30 ng g^{-1}) and PBDE 183 (0.1–30 ng g^{-1}). The method was successfully applied to sheep liver samples for the determination of the target PBDE and PCB compounds (Zhang et al., 2011).

The feasibility of different extraction procedures (PLE, QuEChERS, SPE, and SPME) was tested and compared by Blasco et al. (2011) for the determination of 12 organophosphorus and carbamates insecticides in honey samples. The main aim of this work was to maximise the sensitivity of pesticides and to minimise the presence of interfering compounds in the extract. All curves were linear in the range from CC_β to 1000× CC_β for the four extraction methods (three orders of magnitude). Detection capabilities (CC_β) were 0.007–0.595 mg kg^{-1} with PLE, 0.024–1.155 mg kg^{-1} with QuEChERS, 0.010–0.646 mg kg^{-1} with SPE, and 0.001–0.060 mg kg^{-1} with SPME. All the target compounds could be recovered by any of the methods, at a CC_β fortification level ranged from 28 to 90% for the SPME. In comparison, the PLE method was the most efficient extraction method with recoveries from 82 to 104%. It was followed by the QuEChERS method with recoveries between 78 and 101% and the SPE method with recoveries between 72 and 100%. The repeatability expressed as relative standard deviation (RSDs) was below 20% for all the pesticides by any of the tested extraction methods. Results obtained applying the four extraction techniques to real honey samples are analogous.

Many applications have been described in the literature using pressurized liquid extraction systems applied to organochlorine pesticide extraction from animal internal organs, adipose tissue (Saito et al., 2004), eggs (Wang et al., 2005), milk (Brutti et al., 2010) and shellfish (Buisson et al., 2008).

Solid-phase Extraction (SPE)

In this technique sorbent with strong affinity towards some target analytes will retain and concentrate those compounds from the sample solution. Widely applied to many matrices, including food, solid-phase-based extraction techniques are matrix solid-phase dispersion (MSPD), solid-phase extraction (SPE), solid-phase microextraction (SPME) and stir-bar sorptive extraction (SBSE) (Beyer and Biziuk, 2008).

Solid-phase extraction involves the use of disposable cartridges and disks to trap analytes. As the sample solution passes through the activated sorbent bed, analytes concentrate on its surface, while the other sample components pass through the bed (or vice versa, if necessary for clean-up) (Zwir-Ferenc and Biziuk, 2006).

This technique offers many improvements over LLE and permits simultaneous removal of interfering substances and concentrations of analytes. In SPE, multiple samples can be treated in parallel with an relatively small quantities of solvent (Beyer and Biziuk, 2008).

Before SPE can be applied to a solid matrix (soil, vegetables and fruits), a separate homogenization step and, often, filtration, sonication, centrifugation, and liquid/ liquid clean-up are required. However, the presence of interfering substances, such as salts, humic acids, and other humic substances in water; or proteins, lipids, and carbohydrates in food; makes the determination of polar or early-eluted pesticides, difficult or almost impossible (Picó et al., 2007).

Dual-layer solid-phase extraction, a primary–secondary amine (PSA) in combination with graphitized carbon black (GCB), was evaluated for sample clean-up during multiresidue pesticide screening of agricultural and food products. The determination was made by GC-MSD. The retention of fatty acids by the PSA sorbent was quantified and the effect of the elution solvent on the retention of fatty acid on the SPE cartridge was evaluated. The use of stronger elution solvents to elute certain pesticides from graphitized carbon was shown to interfere with the capacity of PSA to bind fatty acids. The proposed method was tested using GCB/PSA dual-layer SPE to clean-up food matrices (milk, orange juice spinach and bacon) and to simultaneously screen multiple fortified pesticides with a wide range of physico-chemical properties. With a few exceptions, pesticide recoveries were between 85 and 110%, and sample-to-sample differences of less than 5% were achieved, demonstrating the versatile suitability of the dual-layer SPE to sample clean-up (Shimelis et al., 2007).

An analytical method for simultaneous quantification of seven neonicotinoids in food by ultra-performance liquid chromatography combined with electrospray ionization triple quadrupole tandem mass spectrometry (UPLC-MS/MS) under the multiple reaction monitoring (MRM) mode. The extraction and, clean-up, was made by QuEChERS-SPE approach, using comecial HLB cartgrides, SPE HLB cartridge (3 cm^3/60 mg) (Waters; Milford, MA). The low limits of quantification (LOQs) of neonicotinoids ranged from 0.1 to 6 μg kg^{-1}. Meanwhile, reasonable recoveries (65-120%) of seven neonicotinoids for food including apple, cabbage, potato, rice, tea, milk, chicken, pork and egg were demonstrated in different spiked levels within their respective linear range (0.025-150 μg kg^{-1}). The developed analytical method

would be appropriate for the routine, high throughput, high sensitivity quantification of seven neonicotinoids using simple sample pretreatment (Liu et al., 2010).

Solid Phase Microextraction (SPME)

SPME is a solvent free sample preparation technique that uses a fused silica fiber coated with an appropriate stationary phase attached to a modified microsyringe. It was originally developed by Pawliszyn and coworkers in 1990. SPME is essentially a two step process, firstly the partitioning of analytes between the sample matrix, which can be a liquid sample or headspace vapour, and the fiber coating, and then the desorption of the (concentrated) extract from the fiber into the analytical instrument, usually a GC, where the sample components are thermally desorbed. The fiber can also be extracted (desorbed) into an LC eluent using a static or dynamic mode (Ridgway et al., 2007).

The extraction temperature, time and sample agitation must be optimised for each application and operating conditions must be consistent. Another issue with SPME is the limited volume of stationary phase that can be bound to the fiber, which also may lead to incomplete extraction and limits the sample enrichment capabilities. Matrix effects can be an issue and quantitation generally requires matrix matched standards or the method of standard additions. The use of an isotopically labeled internal standard should be considered. The presence of high concentrations of matrix components or other compounds can result in competitive binding and displacement and potentially large errors can occur (Ridgway et al., 2007).

The main advantages of SPME extraction compared to solvent extraction are the reduction in solvent use, the combination of sampling and extraction into one step and the ability to examine smaller sample sizes. It can also have high sensitivity and can be used for polar and non-polar analytes in a wide range of matrices with linking to both GC and LC. Some disadvantages of SPME include batch to batch variation and robustness of fiber coatings (Ridgway et al., 2007).

A method based on solid-phase microextraction in mode headspace (HS-SPME) coupled to gas chromatography–mass spectrometry (GC–MS) was developed and optimized through multivariate factorial design to determine residues of organophosphorus pesticides in cow's milk. Under the optimized conditions, the proposed methodology was able to determine all of the pesticides with variation coefficients between 6.1% and 29.5%. Detection and quantification limits ranged from 2.16 to 10.85 μg L^{-1} and from 6.5 to 32.9 μg L^{-1}, respectively (Rodrigues et al., 2011).

SAMPLE CLEAN-UP

Matrix constituents can be co-extracted and later co-eluted with analysed components and can consequently interfere with analyte identification and quantification (LeDoux, 2004). Moreover, co-extracted compounds, especially lipids, tend to adsorb in GC systems such as injection port and column, resulting in poor chromatographic performance (Hong et al., 2011). A thorough clean-up minimizes such matrix issues, improves sensitivity, permits more consistent and

repeatable results, and extends the capillary column lifetime (Diaz et al., 1997; Rimkus et al., 1996). Several approaches have been attempted to eliminate co-extracted interference from extracts, including SPE, d-SPE, GPC and ultra-filtration (UF).

Solid-phase Extraction (SPE)

Efficient clean-up is indispensable for preventing matrix effects in multiresidue analysis of pesticides in food by liquid and gas chromatography coupled to mass spectrometry. Different than traditional detector systems, liquid chromatography (LC) and gas chromatography (GC) coupled to mass spectrometry (MS) provide a high degree of selectivity and sensitivity. Co-eluting matrix components may result in (i) false negatives, (ii) false positives, or (iii) inexact quantification caused by ion suppression or ion enhancement, depending on the matrix. The same problems hold true for GC–MS, but different matrix compounds can interfere and different mechanisms are responsible for these matrix effects (Oellig and Schwack, 2011).

Solid-phase extraction (SPE) has been used as a purification step to remove interfering compounds co-extracted with all classes of pesticides from a large selection of foods of animal origin (LeDoux, 2011).

The extraction clean-up typically requires, after the extraction, a multistep purification based on the use of various adsorbents, such as Florisil, C18, Alumina, and Silica gel, in order to eliminate interfering substances and matrix fatty materials, which can decrease rapidly the column efficiency, and affect severely the accurate identification and quantification of the analytes (Nardelli et al., 2010).

For analysis of food a widely used strategy is the combination of SPE-QuEChERS, with the advantage the relatively low solvent consumption, at least as compared to previous liquid partitioning based methods. In the case of fatty matrices, the clean-up was performed using a combination of PSA, graphitized carbon black (GCB) and C18. Recently, an alternative procedure similar to QuEChERS, but performed the clean-up step using SPE in cartridges (so that it can be easily automated) has been reported. It offers advantages in terms of solvent consumption and increased automation of the procedure (Gilbert-López et al., 2009; Beyer and Biziuk, 2010).

A multiresidue method for the determination of organochlorine pesticides in fish feed samples was developed and optimized. The method is based on a clean-up step of the extracted fat, carried out by liquid–liquid extraction on diatomaceous earth cartridge with n-hexane/acetonitrile (80/20, v/v) followed by solid phase extraction (SPE) with silica gel–SCX cartridge, before the identification and quantification of the residues by gas chromatography–triple quadrupole tandem spectrometry (GC-MS/MS). No matrix effects or interfering substances were observed in fish feed analyses. The proposed method allowed high recoveries (92–116%) of spiked extracted fat samples at 100 μg kg^{-1}, and very low LODs (between 0.02 and 0.63 μg kg^{-1}) and LOQs (between 0.05 and 2.09 μg kg^{-1}) determined in fish feed samples (Nardelli et al., 2010).

An analytical method was developed to determine the phenoxyacid herbicides 2,4-D, MCPA and mecoprop in kidney tissue from animals. Samples were Soxhlet extracted using diethyl ether and the extracts cleaned-up using anion. Exchange solid phase extraction cartridges. Analysis was performed using liquid

chromatography with negative-ion electrospray tandem mass spectrometry (LC-MS/MS). The method was evaluated by analysing control kidney samples fortified at 1 and 5 mg kg^{-1}. Mean recoveries ranged from 82 to 93% with relative standard deviations from 3.2 to 19%.The limit of detection was estimated to be 0.02 mg kg^{-1} (Charlton and Stuckey, 2009).

Dispersive Solid-phase Extraction (d-SPE)

The amount of co-extractants obtained after the extraction process selected for pesticide determination is a relevant parameter in routine laboratories not only because it can affect the performance of the method but also for the maintenance of the analytical equipment (Pareja et al., 2011). The complexity of food matrices decrease the life time of chromatographic columns and can even cause problems in the ionization and detection systems of the analytical instrument (Wilkowska et al., 2011; Prestes et al., 2009; Prestes et al., 2011). It is therefore necessary to select a methodology that allows the analysis of the largest number of analytes but not disregarding this factor (Pareja et al., 2011).

During the QuEChERS development, (Anastassiades et al., 2003; Ma et al., 2010) proposed a powerful clean-up method, called dispersive solid phase extraction (d-SPE), which is based on a clean-up with primary secondary amine (PSA). PSA is a good adsorbent for organic acids, pigments, and other polar impurities from the samples. So it shows great beneficial potentiality in detection of pesticides residues in food stuffs (Furlani et al., 2011; Cieslik et al., 2011; Lee et al., 2011). Dispersive-SPE is a clean-up technique that involves mixing sorbent with a sample that has been pre-extracted with acetonitrile solvent. It is typically part of the QuEChERS method where it follows the bi-polarity extraction step. The appropriate sorbent adsorbs matrix co-extractives on to its surface, leaving analytes of interest in the solvent. $MgSO_4$ is added to provide additional clean-up by removing residual H_2O and some other compounds via chelation. Afterwards, the mixture is centrifuged and the resulting supernatant can be analyzed directly or can be subjected to a concentration and/or solvent exchange step if necessary (Kinsella et al., 2009).

Lehotay and co-worker (2005a) published a rapid method of sample preparation and analysis of fatty foods (e.g., milk and eggs) and evaluated the performance for 32 pesticide residues representing a wide range of physicochemical properties. The method for pesticide residue analysis, entailed extraction of 15 g sample with 15 mL acetonitrile (MeCN) containing 1% acetic acid followed by addition of 6 g anhydrous magnesium sulfate ($MgSO_4$) and 1.5 g sodium acetate. After centrifugation, 1 mL of the buffered MeCN extract underwent a clean-up step (d-SPE) using 50 mg each of C18 and primary secondary amine sorbents plus 150 mg MgSO4. The extracts were analyzed concurrently by GC-MS and LC-MS. The recoveries of semi-polar and polar pesticides were typically 100%, but recovery of nonpolar pesticides decreased as fat content of the sample increased. Chen et al. (2009) concluded that when the acetonitrile extract was stored in the freezer at -24 °C for 20 min, most lipid components in the extract solution were precipitated as pale yellow lump on the flask surface, owning to their low solubility in acetonitrile. On the other hand, the interesting pesticides were soluble even in cold acetonitrile

solvent. The cold extract containing precipitated lipids was promptly filtered with filter paper to prevent melting lipids.

Nowadays, most analytical methods for pesticide residues in fatty foods (e.g. food of animal origin) are designed predominantly for the out-moded organochlorine compounds and employ solvents such as hexane, acetone, ethyl acetate, and dichloromethane for extraction in order to dissolve the lipids (LeDoux, 2011). However, intensive and time-consuming clean-up, such as gel permeation chromatography (GPC), is usually needed to remove the co-extracted fat from the extracts prior to the analytical step. From the possibilities of the QuEChERS method optimization, especially the d-SPE step, this method appears as an option to substitute a nonpolar solvent during extract the pesticide residues.

Gel Permeation Chromatography (GPC)

GPC is a method based in the principle size exclusion. This mode of purification is widely used in the area of pesticide residue analysis, wich is considered a good technique for the separation of low molecular mass compounds (up to 400 μm) such as pesticides from high molecular mass compounds such as lipids (600 to 1500 μm) (Buldini et al., 2002; Diaz et al., 1997; Rimkus et al., 1996).

GPC systems comprises a LC pump a fraction collector and a detector (optional). The columns are made from polymeric porous microspheres, which enables the separation of compounds according to their molecular weights. Using this principle, pesticide fraction is separated from the high molecular weight lipids fractions (Gilbert-López et al., 2009). In order to reach a higher sample throughput, Focant et al. (2001) replaced slow GPC purification with high-capacity disposable silica (HCDS) columns containing 28 g of acidic, 16 g of basic, and 6 g of neutral silica; this allowed up to 4 g of lipids for each sample to be retained. The HCDS column is added to the classic set of columns and is the first one in contact with the sample. Such a column system has found application in purifying extracts from samples characterized by a high lipid content, e.g., poultry, fish and eggs (Beyer and Biziuk, 2008).

GPC has therefore been widely used for cleaning up extracts from foods of animal origin with a high fat content. However, co-extracted compounds, including remaining trace amounts of lipids, can reach the GPC eluate and then interfere with the subsequent analysis. Complex samples such as fish, meat, and other fatty matrix extracts often require a two-step clean-up combining gel permeation chromatography and adsorption chromatography in series (Diaz et al., 1997; Rimkus et al., 1996).

A clean-up step by GPC was applied to remove fat and other matrix compounds from meat samples from chicken, pork and lamb (Frenich et al., 2006a), liver (Frenich et al., 2007) and meat (Frenich et al., 2006b) to determine pesticides organochlorine (OCPs) and organophosphorus (OPPs). Clean-up was done by GPC. Ethyl acetate–cyclohexane (1:1, v/v) was used as the mobile phase of the GPC system at a column flow of $5 mL min^{-1}$. The determination was carried out by gas chromatography with electron impact ionization tandem mass spectrometry (GC–EI-MS/MS) using a triple quadrupole (QqQ) analyzer.

GPC was applied as a nondestructive and semiautomatic clean-up method to determine residues of 109 pesticides (including isomers) in the samples of pork, beef, chicken and fish matrices by Wu et al. In this study, 300 mm x 10 mm i.d glass columns packed with Bio-Beads S-X3 were used. The flow rate was 5 mL min^{-1} and the first fraction from 0 to 8 min of the eluant (about 40 mL) contained the lipids and was discarded. The samples were analysed with GC–MS in the selected ion monitoring mode (SIM) using one target and two qualitative ions for each analyte (Wu et al., 2011).

Tsiplakou et al. investiged the presence of pesticide residues in milk from dairy sheep and goats, fed mainly with supplementary feed during the winter months. GPC system was applied as clean-up method using ethyl acetate:cyclohexane (1:1, v/v) as the mobile phase with column flow of 5 mL min^{-1}. No pesticide residues were also detected in milk samples of sheep and goats (Tsiplakou et al., 2010).

CONCLUSIONS

In this chapter we reviewed the recent progress in modern sample extraction techniques for the determination of pesticides residues in food from animal origin. Progress made over the past few years in topics like mass spectrometers design, innovative chromatographic technologies, laboratory automation /miniaturization and more efficient materials for SPE drive the trend towards sensitive detection together with high sample throughput and less time devoted to sample clean-up. But still in many cases, sample preparation remains as the crucial step in food residue determination. Conventional sample preparation techniques such as LLE and SPE are still the most widely used in routine laboratories. However, their performance should be surpassed by new modern approaches, e.g. QuEChERS, and their development should proceed further, following high-throughput, low volume, ease of use, automated and environmental trends in order to reduce the contrast with fast LC and GC approaches.

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Analytical methods to assess the impact of pesticides on human from coffees

Claudia M. D. Paes, Helena L. Capobiangoand Zenilda L. Cardeal

Abstract

Food production quality has shown increasing in recent decades and this increase in productivity leads to the need for greater control of unwanted species of plants or animals that cause damage or interfere with the production, storage, transport or marketing. Considering this, and from the perspective of food security and on the increasing number of pesticides is a pressing need to establish methodologies able to investigate the presence of pesticide residues. Thus, the development of analytical methodologies that provide reliable results in the analysis of this residues takes leading role to ensure quality and food safety. Given the large number of existing pesticides and their use in various products, analytical methods must: consider the different characteristics of matrices and analytes; be able to unambiguously detect and quantify low levels in the largest possible number of analytes; involved, preferably a small number of steps to provide better reproducibility and accuracy and time savings. Regarding the validation of methods for analysis of pesticide residues, there are guidelines that provide guidance on how the work should be conducted. The European Commission, through the Directorate General Health and Consumer Protection (DG SANCO/10684/2009), prepares recommendations for such quality control and validation of analytical methods for analysis of pesticide residues. It is also available to the Codex Alimentarius guidelines, good laboratory practice in the analysis of waste, which addresses the parameters to be evaluated in the validation of the method. It has been a tendency among the reference laboratories in this area, the adoption of these recommendations in their validation work.

INTRODUCTION

Coffee plant belongs to the Rubiaceae family and has more than seventy species. Among these species, two are of significant economic importance, known as the arabic (*Coffeaarabica*) and robusta species (*Coffeacanephora*). The coffee beverage is produced from the roasted beans of the coffee fruit. It is one of the most popular beverages in the world due to its specific flavor and aroma. This product is an economically important tropical agricultural commodity having a significant fraction exported to many countries. It is marketed globally and at times was the second most traded product in the world, second only to oil (Chanakya and De Alwis 2004). Table 1 shows the major world exporters of *arabica* and *robusta* coffee (www.ico.org 2011).

Coffee contains many beneficial antioxidants and is one of the richest sources of chlorogenic acid (Yang, Wang et al. 2011). Epidemiological studies have associated the consumption of this acid to prevention of diseases such as diabetes mellitus, cardiovascular disease and other diseases related to oxidative stress(Garambone 2007).

The coffee culture, due to its long permanence in the field, is subject to different environmental conditions which make it susceptible to the occurrence of many pests

and diseases caused by fungi, insects, nematodes and weeds. These are groups of organisms that harm the development, production and quality of the fruit (Andalo, Moino et al. 2004).
To reduce undesirable species of animals or plants from the coffee crop different insecticides, fungicides and herbicides may be used (Andalo, Moino et al. 2004). However, these products are toxic to humans and the environment, therefore their use and residues should be controlled.

PESTICIDES AND THEIR IMPACT ON HEALTH AND THE ENVIRONMENT

Pesticides become both environmental problem and risk to human health when applied indiscriminately. In terms of health, these products can affect the applicators of products, community members and consumers of foods contaminated with residues. At the same time, the farmer who does not understand the harmful effects that pesticides have on health may overestimate its benefits and use higher doses than necessary.Studies conducted in Cameroon, Africa by Food and Agriculture Organization of the United Nations (FAO) showed that overall contamination sources of the application of pesticides in coffee cropsare usually linked to inadequate handling and storage and the lack of personal protective equipment (PPE).The main herbicides, fungicides and insecticides used in coffee plantations in Cameroon country were: paraquat, glyphosate, bentazone, metalaxyl, cuprous oxide, copper hydroxide, copper oxychloride, maneb, captan, mancozeb, pirimiphos methyl, cypermethrin, chlorpyrifos, endosulfan, HCH , methyl parathion, carbofuran, carbaryl, dieldrin, ethoprophos, diazinon, dimethoate, acephateandfenobucarb. Coffee producers in Cameroon reported that the sprinklers of pesticides used in this culture were archaic,exposing the operator and the environment. Additionally, PPEwas not used at the moment of preparation and use of the chemical as a result of misinformation, discomfort and/or lack of economic resources. It was observed that the higher the education level, the greater the possibility of workers using protective equipment, and consequently lower the risk of exposure to pesticides (Matthews, Wiles et al. 2003).
In a study conducted in a family farms region, a major coffee producer in the state of Espírito Santo, Brazil, contamination by pesticides was observed in the residential environment, particularly dust, soil, air and food surrounding the home. This contamination originates from proximity of the home to crop fields where the pesticideswere applied. Moreover, in most cases the pesticides are stored in the place of residence and the farmers utilized contaminated clothes even while in their homes (Jacobson, Hacon et al. 2009).
In the study performed by Mathews et al. (2003), Cameroon,it was also observed that pesticides used in coffee plantations were stored into the home. Farmers reported that they stored the containers containing pesticides indoors and specifically within rooms due to fear that these products may be stolen because of their high prices (Matthews, Wiles et al. 2003).
Organophosphorus are the most utilized pesticides in the coffee crop (Rama and Jaga 1992; Oh-Shin, Ko et al. 1997; Lacorte, Vreuls et al. 1998; Cardeal, Rabelo et al. 1999; Robinson and Mansingh 1999; Matthews, Wiles et al. 2003; Capobiango and Cardeal 2005; Chung and Chan 2010; Yang, Wang et al. 2011) .These compounds are less persistent in the environment and more potent in relation to pests than organochlorine pesticides, although they are neurotoxic. Organophosphoruspesticides, as well as carbamates, inhibit action of the enzyme acetylcholinesterase. This enzyme is important in regulating the levels of

acetylcholine, a neurotransmitter that acts in the transmission of nerve impulses in muscle fibers (Casaret, Doull et al. 2001).The presence of acetylcholinesterase is used as a biological index of exposure to organophosphorus and carbamate pesticides (Rama and Jaga 1992).In a study conducted in northern South Africa, it was evaluated the exposition of coffee producers to the pesticides: parathion, monocrotophos, dicrotphos, trichlorfon, disulfoton, benomyl, mancoseb, maneb, zineb, bipyridals, paraquat-p-butyl fluazfop, glyphysate, copperoxychloride, miname and chlordane (Rama and Jaga 1992), by determining enzyme activity of acetylcholinesterase in red blood cells and blood plasma. The results obtained from analysis of red blood cells showed that 77% of workers presented acetylcholinesterase levels below those considered normal by legislation of the Republic of South Africa. There were also decreased levels of plasma acetylcholinesterase in 27% of the blood samples analyzed (Fuller and Berger 1990; Rama and Jaga 1992).

The study of Jacobson et al. (2009), state of Espírito Santo, Brazil, also showed a relationship between the use of organophosphorus pesticides and neurological problems, since workers have reported problems with depression and memory loss (Jacobson, Hacon et al. 2009).

Abusive use of pesticides can contaminate air, soil, water, as well as fauna and flora. This was reported in the study performed by Garcia et al.(2006), where the application of pesticides in coffee plantations of the Sierra Madre de Chiapas, Mexico, resulted in a reduction in the number of bats. In this region there is a variety of bat species due to tree cover and shading of coffee plantations that provide conditions similar to original forest. However, in coffee plantations sprayed with large amounts of the insecticide Thiodan (endosulfan) used in the chemical control of the Coffee Berry Borer (Hypothenemushampei) a reduction in the number of bats was observed, since these insects are important components of their diet (Garcia Estrada, Damon et al. 2006).

Similarly, Ellis-Tabanore and Hyslop (2005) reported that in Jamaica, farmers used organochlorine insecticides to combat the Coffee Berry Borer. Results of the study showed a reduction in the number of snails of the species *Thiara granifera* (family Thiaridae) living in rivers of the coffee producing regions. These sites were contaminated with endosulfam. This led the authors to propose that snails of the species Thiara can be used as an indicator of polution levels resultant of pesticides present in rivers or streams (Ellis-Tabanor and Hyslop 2005).

ANALYTICAL METHODS TO ASSESS EXPOSURE TO PESTICIDES USED IN COFFEE

Sample Preparation

The methods for extraction of pesticides and clean-up of food and environmental samples are extremely important for their quantitative determination in the matrices of interest. Extraction techniques used to concentrate the analytes include liquid-liquid extraction (LLE), solid phase extraction (SPE), single-drop microextraction (SDME) and solid phase microextraction (SPME) (Souza, Amorin et al. 2010).

The LLE extraction is based on the partition of the sample between two immiscible phases (organic and aqueous) and is often used in the analysis of pesticides in water and food samples.

Solid phase extraction (SPE) was introduced in 1978 as an alternative to LLE. The analytes contained in an aqueous matrix are extracted for their acquisition with the interfering compounds after passage through a sorbent cartridge. A selective organic

solvent is commonly used to extract the analytes of interest. These steps of the SPE method depend on the physic chemical properties of the pesticides and their concentrations, so as to utilize the ideal volume of solvent.

Single-drop microextraction (SDME)is a simple and inexpensive extraction technique that involves the consumption of small quantities of the solvent.This technique consists of the addition of a small drop (8.0μL)of the organic solvent(n-octane) to the aqueous sample containing the analyte. The solution is agitated for a short period (approximately 5 min)at constant velocity.Then,an aliquot of 1.0 μL of the organic phase is injected in the chromatograph for analysis (Jeannot and Cantwell 1996).

Solid phase microextraction (SPME) is a technique for sample treatment published in 1989 by Pawliszyn and Belardi (Zhang, Yang et al. 1994), applied in the extraction of organic compounds indifferent matrices, such as air, water, soil and food. The SPME technique is promising and has the advantage of not using solvents for extraction. It basically consists of partitioning compounds between the sample and the stationary phase (fiber of fused silica covered with polymeric film and adapted to a syringe) followed by thermal desorption of these compounds in an analytical instrument for qualitative and quantitative determination (Cardeal, Rabelo et al. 1999).

Other extraction methods have been used in order to develop multiresidue methods for determination of pesticides in food. Among these, highlighted is the method developed in 2003 by Anastassiades et al. (Lehotay, Michelangelo et al. 2010), referred to as QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe). This method involves initial extraction with a solvent such as acetonitrile, followed by a liquid-liquid partition.It comprises the addition of some salts (responsible for the separation of the aqueous phase from the organic phase and removal of significant amounts of polar compounds in the matrix) and buffering agents (for protection of analytes susceptible to degradation in alkaline or acidic conditions). The extract obtained is subjected to an extraction process in dispersive solid phase for “clean up”. The sorbent used varies in function of the matrix nature. This method requires a small sample size, consumes small amounts of solvent, produces satisfactory results in terms of accuracy and repeatability, allows the preparation of a considerable number of samples in a short time and is suitable for analyzing a wide range of pesticides by both gas chromatography and high performance liquid chromatography.

Analytical Determinations of Pesticides in Coffee

Several analytical methods were developed for identification and quantification of pesticides in food and the environment. Among the main analytical techniques used are gas chromatography (GC) equipped with an electron capture detector (ECD) or nitrogen and phosphorus detector (NPD) or mass spectrometry (MS) detector and high performance liquid chromatography (HPLC) using UV or MS detection, since these methods possess the selectivity, sensitivity and high capacity necessary for identification and quantification of pesticides.

Yang et al. (2011) developed a multiresidue method for determination of 69 pesticides (2,6-dinitroaniline, amide, azole, carbamate, chloroacetamide, diphenyl ether, organochlorine, organophosphates thio and dithiocarbamates, triazine and triazole)in coffee beans by GC/MS. The sample preparation used SPE techniques with Envi-carb cartridges, and gel permeation chromatography (GPC) with ethyl acetate-n-hexane as mobile phase. The method showed good linearity with correlation coefficients (R^2) above 0.99. Recovery in samples spikedwith 0.05 to 1.00 μg kg^{-1}presented values between 60-120%. The precision (RSD) was between

1.3 to 22.3% for all pesticides. Limits of detection for the method ranged from 10.0 to 150.0 μg kg^{-1}. Six commercial samples were analyzed and low levels of pesticides were detected in all samples(Yang, Wang et al. 2011).

Chung and Chan (2010) developed a multiresidue method for the determination of 98 analytes, belonging to the classes of organophosphorus pesticides (OPP) and carbamates in various foods, including coffee. This author used the modified QuEChERS method, involving a simple solvent extraction of pesticides followed by clean-up with secondary amine (PSA) and carbon C_{18} and/or graphitized carbon black (GCB) as sorbents. Analysis was performed by LC/MS/MS with electrospray ionization operated in multiple reaction monitoring (MRM) acquisition mode. The method showed good linearity (R^2> 0.995) in the range of 1.0 to 20.0 μg $L^{-1}$for all compounds studied. Average recovery for levels of 10.0 and 200.0 μg kg^{-1}were within the range of 70-120% and RSD was less than 20%. The value of 10.0 μg $kg^{-1}$was established as the limit of quantification (LOQ) for all analytes tested (Chung and Chan 2010).

Also analyzing OPP, Capobiango and Cardeal (2005)reported a GC/NPD method for determination of co-ral, diclorvós(DDVP), di-siston, ethion, phorate, fosdrin, gution, malathion and methyl parathion in samples of fish, potatoes, guava and coffee. Also analyzed were water samples from rivers neighboring fields where these crops were analyzed. Samples were collected between October 2002 and April 2003 in the city of Patos de Minas, Brazil. A SPME method was developed with a micro fiber polydimethylsiloxane(PDMS) of 100 μm, with optimization of time and temperature during extraction, sample volume in the flask and desorption time. Under optimized conditions the method was linear with correlation coefficients (R^2) ranging from 0.997 to 0.999.The precision was adequate with RSD ranging from 4.40-15.13%. Detection limits ranged from 0.05-8.37 μg $L^{-1}$and the limit of quantification from 0.09-8.70 μg L^{-1}. The coffee samples contained DDVP residues of 0.23 μg $kg^{-1}$and phorate residues of 0.02 μg kg^{-1}. The values encountered were below the limits permitted by Brazilian legislation (0.05 μg $kg^{-1}$for phorate and 2 μg $kg^{-1}$for DDVP) and within the limit prescribed by the Environmental Protection Agency (EPA) of the United States, which is of 0.02 μg $kg^{-1}$of phorate(Capobiango and Cardeal 2005).

The authors Diez-Rodriguez, De Baptista et al. (2006) studied the effect on coffee leaves of the insecticides thiamethoxamand aldicarbused for control of the coffee leaf miner (*Leucopteracoffeella*). Aldicarb concentrations were determined along with its active metabolites;aldicarbsulfoxide and sulfone by GC/NPD and thiamethoxam by GC/MS. Leaves collected from different heights of the plant were analyzed. The samples were extracted by LLE and GPC methods. Recovery rates and the LOQ of the method were determined. The leaf samples were spiked at several levels: 10.0, 2.0 and 0.5 μg kg^{-1} for aldicarb and its metabolites, and 1.00, 0.20 and 0.02 μg kg^{-1} for thiamethoxam.The LOQ of aldicarbsulfoxide and sulfone was 0.5 μg kg^{-1}, with recovery percentages of 101-118%, 88-101% and 98-105%, respectively. For thiamethoxam, the LOQ was 0.02 mg kg^{-1} with recovery percentages between 79-88%.The results indicated uniform translocation of both insecticides in the three thirds of height of the coffee plants, when the pesticides were applied to the soil. Greater persistence of thiamethoxam was also reported, whose residues were found up to eight months after application, while the sulfoxide and sulfone metabolites were present between four and six months after application (Diez-Rodriguez, De Baptista et al. 2006).

In Romania, a study was performed to investigate residues of nine organochlorine pesticides (OCP), HCH, lindane, heptachlor, p,p'- DDT, p,p'- DDE, p,p'-DDD, aldrin, dieldrin and endrin in samples of different types of coffee: green coffee

beans, roasted coffee, instant or granulated coffee, all of the same producer. The samples were treated by LLE and OCP pesticides were determined by GC/ECD. They contained pesticide residue levels between 1.0 and 7.0 μg kg^{-1},where the lowest MRL of pesticides analyzed is 10 μg $kg^{-1}$for the pesticide endrin (ec.europa.eu/sanco pesticides 2005).Of the pesticides analyzed, lindane was found in all samples. HCH and aldrin (7.0 μg kg^{-1}) were detected in samples of roasted coffee beans. Heptachlor was present in green coffee beans. DDT and its metabolites were not detected, probably because the green coffee beans, rich in nutrients, promote the growth of microorganisms capable of degrading DDT and its metabolites(Stanciu, Dobrinas et al. 2008).

Some studies show that spraying of pesticides on the coffee crop cause contamination of surface water, drinking water and agricultural soils. European legislation (ec.europa.eu/environment/water 1998) requires that pesticide concentration levels in surface water is up to 1.0 μg $L^{-1}$and in drinking water 0.1 μg L^{-1}.

López-Blanco et al. (2002 and 2003) studied different analytical methods for detection of endosulfan in water samples collected near coffee plantations. This pesticide is persistent in soil and can be transported towater through the flow of particles, usually determined as a mixture of isomers α- and β-endosulfan. The authors reported different extraction techniques, SPE, SPME and SDME, for the analysis of α-and β-endosulfan in samples of surface water and drinking water by GC/ECD. Conditions were optimized for analysis of each extraction method, followed by comparative determination of some parameters of merit.The SPE method used a C_{18} cartridge and hexane as solvent; the SPME method was selected with direct immersion of the fiber of divinylbenzene-Carboxen-polydimethylsiloxane (DVB/CAR/PDMS); and SDME method using isooctane as organic solvent. The results showed that the SPE method requires significantly longer analysis time (25 min) than necessary for SDME (20min) and SPME (15 min). Moreover, SPE requires a high organic solvent volume (50 mL). However, SDME and SPME showed much lower recoveries (10% and 0.1%, respectively) than SPE (100%). The precision and reproducibility (RSD%) were very similar for both isomers, being less than 14.4% regardless of the extraction technique used. All three methods showed good linearity (R^2>0.995). The three pre-concentration methods showed low limits of detection compared with those established by European legislation. However, the SDME technique was most sensitive. Effects of the matrix did not impair correct quantification of the SDME, SPE and SPME methods. Therefore, the three analytical methods were compared, where in terms of cost the SDME method is most favorable because the cost of organic solvent is insignificant compared to the cost of the SPE cartridge and SPME fiber (López-Blanco, Reboreda-Rodríguez et al. 2002; López-Blanco, Blanco-Cid et al. 2003).

Lacorte, Vreuls et al. (1998) reported a program called SAMOS (System for the Automated Monitoring of Organic pollutants in Surface water) to determine pesticide residues in rivers. The system was programmed so that eight samples were analyzed every day and could be operated for at least five days. These authors analyzed a mixture of triazines and other OPP. The samples were extracted by SPE method with a C_{18} column and analyzed by HPLC/UV. The method showed good linearity (R=0.99) in the range of 0.3-1.5 ng L^{-1} and LOD ranging from 30 to 100 ng L^{-1}.The authors reported that Terbuthylazine, a herbicide used in coffee plantations, was detected at the concentration of 31 μg L^{-1}in the Llobregat Riverin Catalonia, Spain. This analysis was also performed by liquid chromatography with chemical ionization at atmospheric pressure coupled to mass spectrometry (LC-APCI/MS) and found the same results using this technique (Lacorte, Vreuls et al. 1998).

Malathion, parathion, fenitrothion, diazinon and carbarylare pesticides used in the control of insects in coffee plantations. Oh-Shin, Ko et al.(1997)proposed the methods GC/NPD and GC/ECD to determine these pesticides derived from pentafluoropropionic acid anhydride (PFPA) in drinking water samples. The method showed to be linear (R^2=0.998 to 1.000), sensitive (LOD <0.1 ng mL^{-1}) and precise (RSD<11% for 10 ng $mL^{-1}$and< 2% for 1.0 ng mL^{-1}). Recovery of the method for 10 ng ml^{-1} ranged from 76 to 87% for the compounds analyzed (Oh-Shin, Ko et al. 1997).

The coffee berry borer (*Hypothenemushampei*) was controlled in Jamaica (1991-92) withtwo sprayings per year, usingendosulfan mixed with the OPP isazofos, dimethoateor profenofos, or with the pyrethroidspermethrin or cypermethrin. Carbamatefuradanwas applied to the soil for protection against nematodes. The pesticides paraquat and glyphosate were sprayed 4 to 5 times a year. Robinson and Mansingh utilized GC/NPD and GC/ECD techniques to determine pesticide residues in coastal waters of the rivers (Rio Grande, Swift and Espanhol in Jamaica). Two sampling stations were established in each of the three streams studied. OCP residues were found in the Swift and Spanish rivers, but residues of OPP, carbamates, and pyrethroids were not detected in any sample collected (Robinson and Mansingh 1999).

The same authors also reported a study of soil analysis. Soil samples were collected beginning in December 1991, April 1992 and May 1992 in five randomly selected locations. Samples were extracted with hexane or dichloromethane followed by clean-up with a florisil column. The results showed that endosulfan residues remain in soil for up to three or four months after the last application. Moreover, it was found that three days after the first spraying the levels of α and β-endosulfan were quite high, while its sulfate was encountered in low concentrations.Four weeks later, the levels of α and β-endosulfan were reduced by about 63%, but endosulfan sulfate increased by six times, indicating a conversion of the isomers in the ratio 70:30 of sulfate isomers. The conversion occurred to a lesser extent in soil under the shade of a tree, indicating a photolysis reaction (Robinson and Mansingh 1999).

The herbicidestriazine, simazineand ametryne, are used to combat caterpillars in leaves of coffee plants and to control weeds. These can persist for months in soil and contaminate water supplies by leaching. Pinto and Jardim (2000) developed a HPLC/UV method (λ=230nm) to determine residues of ametryne and simazine in water; andsamples were treated via SPE with C_{18}cartridges. The method showed good linearity with R^2 = 0.999 for both herbicides. For samples spiked with concentrations of 1 μg L^{-1} recovery of 97.4% was obtained for ametryne and 77.8% for simazine with precision less than 3%. The LOD/LOQ were 0.034/0.1 μg L^{-1} and 0.018/0.055 μg L^{-1}respectively, for simazine and ametryne(Pinto and Jardim 2000).

CONCLUSION

Different pesticides have often been used on coffee crops to control pests or weeds. Many of these are not only toxic but also persistent and their residues may contaminate coffee fruits, soil and water. Therefore, a surveillance system of environmental vigilance is necessary, involving guidance to limit the indiscriminate and/or unnecessary use of pesticides in coffee plantations.

Chromatographic techniques such as gas chromatography with nitrogen phosphorus,electron capture or mass spectrometer detectors are proposed for the determination of pesticide residues because of the selectivity, sensitivity and relative speed of analysis. However, liquid chromatographic techniques with UV/VIS or

mass spectrometer detectors are more suitable for analysis of more polar compounds because the samples are analyzed directly without derivatization.
Commonly used extraction processes involving analytical methods such as LLE, SPE, SDME or SPME are associated with the chromatographic techniques. Considering the use of solvents and the formation of residues by the LLE and SPE methods, the SPME and SDME methods are considered more advantageous. Moreover, SDME is of low cost.

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Table 1. Largest exporters of coffee arabica and robusta in the period November 2010 to October 2011.

Country	Amount of 60 kg bags exported
Brazil	33957546
Vietnam	16800000
Colombia	8034734
Indonesia	6056851
India	5934080
Honduras	3866371
Guatemala	3685714
Peru	3478612
Uganda	3177393
Ethiopia	2938802
Mexico	2794102
El Salvador	1926498
Nicaragua	1522809
Ecuador	1452805
Costa Rica	1218810
Papua New Guinea	1100373

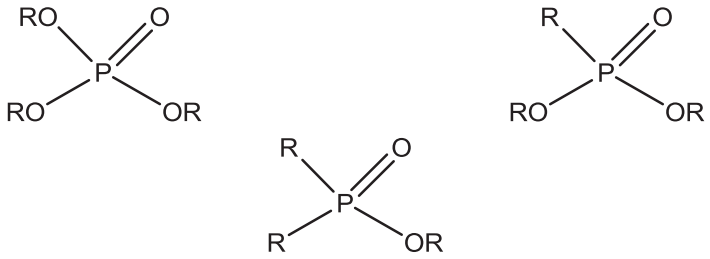
Sensors- A Nanotechnological Approach for the Detection of Organophosphorous Compounds/Pesticides

Sanjay Upadhyay, Mukesh K. Sharma, Mahabul Shaik, Ritu Das and V.K.Rao

INTRODUCTION

Pesticides are chemicals designed for preventing, destroying, repelling or mitigating any pest (insects, mice and other animals, unwanted plants, fungi, microorganisms). They are fall into three major classes: insecticides, fungicides, and herbicides (or weed killers). There are also rodenticides (for control of vertebrate pests), nematicides (to kill microscopic eelworms), molluscicides (to kill slugs and snails), and acaricides (to kill mites).Considering their chemical structure, the pesticides are organophosphorous, carbamates, organochlorines, and pyrethroid ones (U.S. EPA, 2009). OP compounds are widely used in the agriculture industry around the world as pesticides and insecticides. Phosphorous plays a central role in the living organism; it is sufficient to mention photosynthesis, metabolism, and involvement in coenzyme systems etc. It can have a variety of oxidation states 3 and 5, generally OP compounds based on their derivatives of phosphorous. Organophosphate trimesters, phosphonates, phosphonofluoridates and phosphonothioates comprise broad class chemical neurotoxins ((Pesticides action network, Ullman agrochemicals) (Fig 1). However, the high toxicity of the OP compounds had not been recognized until the 1930s, when Lange and Krűger described effects, which they noticed during synthesis of some OP with the P-F bond (Holmstedt, 1963). German Chemists subsequently became interested in synthesizing insecticides. G.Schrader, in 1936, synthesized highly toxic OP insecticide ethyl-N,N-Dimethylphosphor-amidocyanidate (tabun) and iso-propyl methylphosphonofluoridate (sarin) in 1937 (Robinson & Leitenberg, 1971). Schrader synthesized the toxic OP compounds in search of better insecticides. Nerve agents are also OP compounds such as sarin (GB), tabun (GA), soman (GD) and VX are categorized as chemical warfare (CW) agents. During World War II, the Germans possessed large quantities of tabun and sarin although they were not used in that conflict. Nerve agents are divided into two main groups: the G-agents and V-agents. The G-agents are nonpersistent (sarin, soman, & tabun) and cause casualties primarily by inhalation. Sarin is highly volatile compared to tabun and soman. The V-agents are persistent (VX) they can therefore cause casualties by both inhalation and absorption through the skin.

Fig. 1: Structures of (a) Phosphate, (b) Phosphonates and (c) Phosphinates (where R= alkyl, aryl, acyl)



. (a) (b) (c)

Organophosphorous (OP) compounds comprise a diverse group of chemicals which include parathion, malathion, methyl parathion, chlorpyrifos, diazinon, dichlorvos, phosmet, tetrachlorvinphos, triazophos, oxydemeton etc. OP compounds are the most often employed because of their low persistence and moderate toxicity for warm-blooded living organisms. Both their efficiency and acute toxicity stem from their ability to inhibit a group of hydrolytic enzymes called esterases. But over the years, more and more problems associated with the use of pesticides have shown up. They are easily accessible, thus they are a commonly associated with suicides and accidental poisoning. The general chemical structure of these types of deadly OP compounds consist of a tetrasubstituted phosphorous (v) center, an oxygen or sulfur atom double bonded to the phosphorous, a leaving group, and two substituents that vary widely depending on the subclass. Due to their widespread presence, great environmental concerns have recently arised around this type of pollution. These effective broad-spectrum compounds used against insect and arthropod, pests are highly toxic to humans by different routes of exposures, such as dermal absorption, ingestion or inhalation. These contaminants pose serious to fatal health hazards, such as asthma, birth defects and deaths. Therefore, environmental monitoring is required to protect the public and the environment from possible organic toxins released into the air, soil, and water. However, their intensive and indiscriminate application, as well as their high toxicity generated risks to human and environment. The European Union (EU) has strictly limited the emissions, discharges and losses of pesticides and has adopted under Decision No. 2451/2001/EC, a list of 33 priority hazardous substances wherein methyl parathion and chlorpyrifos are included (Decision, 2001). Therefore, rapid, sensitive, selective and reliable “in field” detection OP compounds is required to take necessary action. Nanomaterials provide a crucial platform for the determination of these compounds because of their unique properties such as catalytic, electronic, optical etc. Nanomaterials such as carbon nanotubes, metal particles, metal oxides, nano-structured conducting polymers and quantum dots have recently attracted much interest owing to their applications in nano-scaled devices, sensors and detectors.

This chapter is focused on the applications of nanomaterials in electrochemical biosensors and fluorescence based measurement for the detection of OP compounds/pesticides and nerve agents, albeit within certain limits. Biosensors are electronic devices that yield quantitative or semi-quantitative analytical information using a biological element. Electrochemical biosensors are finding numerous applications in the field of clinical diagnosis, drug discovery, and detection of environmental pollutants, biotechnology, military and civil defense due to their smart size, quick and dependable response compared to the conventional systems. Here, we will also discussed about mode of action of OP compounds on enzyme, a very brief discussion about electrochemical biosensor and their techniques which is used in biosensor application and nanomaterials which are mainly used to increase the sensitivity of biosensors.

MODE OF ACTION OF ORGANOPHOSPHOROUS COMPOUNDS

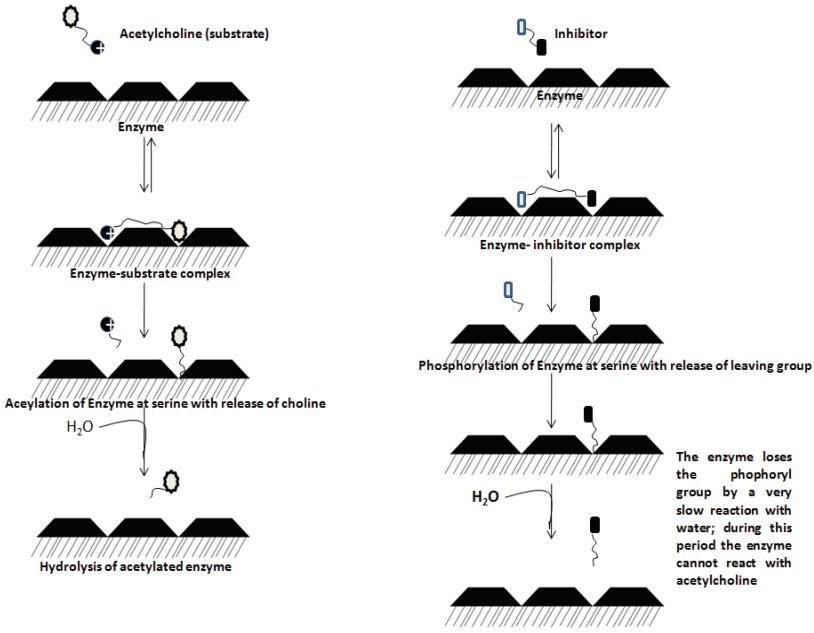
The toxicity or mode of action of OP compounds can be attributed to the inhibition of the enzyme acetylcholinesterase (AChE). AChE is a globular protein and its three-dimensional structure is known. Its physiological substrate is acetylcholine. The active site of AChE consists of two subsites, anionic and esteratic sites. The anionic site is represented by a glutamate ion. The esteratic site has serine moiety and histidine as well as tyrosine residue (Schumacher et al., 1986). This enzyme is essential for the central nervous system, and being present in both humans and insects. The normal function of AchE is hydrolyses the acetylcholine neurotransmitter in the synaptic membrane to prevent its accumulation, and as a result forming acetylated enzyme and releasing choline. Deacetyaltion occurs by the recation of water with the acetylated enzyme to form acetic acid and the original free enzyme. The high percentage of released choline is transported back into the nerve ending for reconversion to acetylcholine and storage. This degradation process results in a lowered level of acetylcholine, and ultimately the termination of nerve impulses.

OP compounds covalently block the active site of serine residue of AchE by undergoing nucleophilic attack to produce a serine-phosphoester adduct. This irrevrsible inactivation leads to an excess accumulation of acetylcholines in the peripheral and central nervous system causing cholinergic manifestations. Substrate inhibition of the enzyme may be caused by combination of substrate with the free anionic site of the acetylated enzyme accompanied by a reduction in the rate of deacetylation (Fig.2). At high doses, there is depression of the respiratory centre in the brain, followed by peripheral neuromuscular blocked causing respiratory paralysis and death (Baigar, 2004; Vijayaraghavan et al., 2010). The pharmacologic effects and toxicity of these OP compounds are dependent on their stability, rate of absorption by various routes, distribution ability to cross the blood-brain barrier, rate of reaction with AChE.

ELECTROCHEMICAL BIOSENSOR

Biosensors have recently been defined as analytical devices incorporating a biological material (e.g. enzymes, antibodies, nucleic acids, cell receptors, tissue, etc.), a biologically derived material (e.g. recombinant antibodies, engineered proteins, Aptamers, etc.) or a biomimic (e.g., synthetic catalysts, combinatorial ligands and imprinted polymers) intimately associated with or integrated within a physicochemical transducers which may be optical, electrochemical, thermometric, piezoelectric, magnetic etc. The biosensors can be classified according to their transduction methods and biological materials. There are three main classes of biological recognition materials which are used in biosensors applications. These are (i) enzymes, (enzyme based biosensors) (ii) antigen-antibodies (immunosensors) and (iii) nucleic acids (DNA based biosensors) (Sassolas et al., 2008). When the transducer used is based on electrochemical principle then it is called as electrochemical biosensor. These biosensors are mainly based on the observation of current, potential, impedance, conductance changes due to interactions occurring at the sensor (electrode) sample matrix interface. They are generally classified according to the observed parameter such as current (amperometric), potential (potentiometric), impedance (impedimetric) and conductance (conductometric).

Fig. 2**.** Pictorial presentation of the action of organophosphorous compounds on acetylcholiesterase enzyme



Amperometric biosensor

This is the most common electrochemical detection method used in biosensors. The basic principle of amperometric sensors is based on electrode reactions. It is a three electrode system consisting of a working electrode, a reference electrode and an auxiliary electrode. The working electrode, a polarizable electrode of a noble metal or carbon is used, where as Ag/AgCl electrode or calomel electrode (non-polarizable) is usually used as reference electrode. The auxiliary electrode should be much larger than the working electrode to make the reaction on the working electrode rate limiting. Amperometric biosensors operate by applying a constant potential and monitoring the current associated with the reduction or oxidation of an electroactive species involved in the recognition process. The current generated is linearly related to the analyte concentration. The solution in which the electrodes are immersed must contain sufficient amount of supporting electrolyte to minimize the Ohmic drop between the electrodes. The sensor potential is set at a value where the analyte, directly or indirectly, produces a current at the electrode.

Potentiometric sensor

The potentiometric sensors consist of an ion-selective membrane and some bioactive material, e.g. an enzyme and an internal reference electrode. The nature of the ion selective membrane determines the selectivity of the electrode it is also called as working electrode or ion selective electrode. Many types of electrodes are available and are classified by the properties and nature of the membrane. Representative membranes include pressed single crystals, sparingly soluble salts pressed into a pellet, and solvent polymeric membranes. For the polymeric membranes, polyvinylchloride (PVC) has been used as supporting matrix other polymers are also used such as polystyrene, polyamide etc. A plasticizer and an ionophore (ion-exchange) compound are incorporated there the potential difference across the ion-selective electrode is measured using an external reference electrode under the condition of zero current. The potential of the ion selective electrode (ISE) measured with respect to the reference electrode is linearly dependent on the logarithm of the activity or concentration of the analyte. Since potentiometry yields a logarithmic concentration response, the technique enables the detection of extremely small concentration changes. The widely used ion selective electrode is glass electrode for pH measurement.

Voltammetry

Voltammetric techniques involve the application of a potential (E) to an electrode and the monitoring of the resulting current (i) flowing through the electrochemical cell. It consists of three electrodes system working electrode, reference electrode and auxiliary electrode. The current will pass through in between working and auxiliary electrode and measured in between working and reference electrode. In many cases the applied potential is varied or the current is monitored over a period of time (t). Thus, all voltammetric techniques can be described as some function of E, i, and t. The analytical advantages of the various voltammetric techniques include excellent sensitivity with a very large useful linear concentration range for both

inorganic and organic species a large number of useful solvents and electrolytes, a wide range of temperatures, rapid analysis times (seconds), simultaneous determination of several analytes, the ability to determine kinetic and mechanistic parameters, a well-developed theory and thus the ability to reasonably estimate the values of unknown parameters, and the ease with which different potential waveforms can be generated and small currents measured.

Differential Pulse Voltammetry (DPV)

This is another common technique which is used under voltammetry; in this technique potential is applied in pulse form. This technique uses a series of potential pulses of increasing amplitude. The current measurement is made near the end of each pulse, which allows time for the charging current to decay. It is usually carried out in an unstirred solution at the electrodes. In DPV the potential is also scanned with a series of pulse and each potential pulse is fixed of small amplitude (10 to 100 mV) and is superimposed on a slowly changing base potential. Current is measured at two points for each pulse; the first point is just before the application of the pulse and the second at the end of the pulse. The difference between current measurements at these points for each pulse is determined and plotted against the base potential

Square Wave Voltammetry (SWV)

The excitation signal in SWV consists of a symmetrical square-wave pulse of amplitude Esw superimposed on a staircase waveform of step height ΔE, where the forward pulse of the square wave coincides with the staircase step. The net current, i_{net}, is obtained by taking the difference between the forward and reverse currents ($i_{for} - i_{rev}$) and is centered on the redox potential. The current difference between these two points is then plotted against the staircase potential in a square wave voltammogram. The peak height is directly proportional to the concentration of the electroactive species and direct detection limits as low as possible. Square-wave voltammetry has several advantages. Among these are its excellent sensitivity and the rejection of background currents. Applications of square-wave voltammetry include the study of electrode kinetics with regard to preceding, following, or catalytic homogeneous chemical reactions, determination of some species at trace levels, and its use with electrochemical detection in HPLC. SWV provides a more familiar peak-shaped signal for easy interpretation of analytical data.

NANOMATERIALS USED IN SENSORS/BIOSENSORS

Carbon Nanotubes

Carbon nanotubes (CNT) have been the subject of numerous investigations in chemical, physical and material science research since their introduction by Iijima (Iijima, 1991), owing their extraordinary mechanical, chemical and electronic properties. CNT can display metallic, semiconducting and superconducting electron transport, possess a hollow core suitable for storing guest molecules and have the largest elastic modulus of any known material (Davis et al., 2003). CNT can be

prepared by chemical vapor deposition, arc discharge, or laser ablation methods and can be divided into single-walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNT) depending upon the number of graphene layers rolled to form the side wall of the carbon nanotube. SWCNT possess a cylindrical nanostructure (with a high aspect ratio), formed by rolling up a single graphite sheet into a tube, whereas MWCNT comprise of several layers of grapheme cylinders that are concentrically nested like rings of a tree trunk (with an interlayer spacing of 3.4 A^o (Baughman et al, 2002). The unique properties of carbon nanotubes make them extremely attractive for the task of chemical sensors, in general, and electrochemical detection, in particular (Zhao et al, 2002). Recent studies demonstrated that CNT can enhance the electrochemical reactivity of important biomolecules (Zhao et al, 2002; Musameh et al, 2002) and can promote the electron-transfer reactions of proteins (Yu et al, 2003). In addition to enhanced electrochemical reactivity, CNT-modified electrodes have been shown useful to accumulate important biomolecules (Wang et al, 2003) and to alleviate surface fouling effects (such as those involved in the NADH oxidation process) (Musameh et al, 2002). The remarkable sensitivity of CNT conductivity to the surface adsorbates permits the use of CNT as highly sensitive nanoscale sensors. These properties make CNT extremely attractive for a wide range of electrochemical biosensors ranging from amperometric enzyme electrodes to DNA hybridization biosensors. To take advantages of the remarkable properties of these unique nanomaterials in such electrochemical sensing applications, the CNT need to be properly functionalized and immobilized.

Functionalization of CNTs: The pretreatment of the CNTs is performed in order to eliminate metallic impurities, and/or to improve the electron transfer properties and/or to allow further functionalization. The protocols are based on the oxidation of CNTs under different conditions. In all cases the ends and side-walls become rich in oxygenated functions, mainly carboxylic groups. Depending on how drastic is the treatment; it is possible not only to increase the density of oxygenated functions but also to break the tubes or even to shorten them (Rivas et al, 2007). Solutions of sulfuric, nitric, and hydrochloric acids and, either concentrated or diluted, alone or mixed have been extensively used for activation of CNTs. Vairavapandian et al. (2008) have reviewed the various strategies practiced in the preparation and modification of CNTs. They have concluded that incorporation of metal nanoparticles into CNT matrices lead to enhanced catalytic behavior. Other than CNTs, several nanomaterials and composites of CNTs and metal nanoparticles have been used to detect the pesticides.

Metal Nanoparticles

Transition metals such as gold, platinum, palladium, copper, silver and nickel are well known for their high catalytic activity. Nanoparticles (NPs) made from these transition metals have been widely utilized to enhance the performances of electrodes made of carbonaceous materials and in particular, to increase their sensitivity towards a specific analyte. Because nanoparticles can provide a larger

surface area and be easily modified with a wide range of biomolecules they enable the fabrication of biosensors with a plethora of sensing possibilities. On the nanoscale size (1–100 nm) many materials show interesting quantum effects. The observed strong Plasmon absorption bands, arising from the collective oscillation of "roving" electrons on the particle surface, often serve as a probe to monitor the interaction with surface-bound molecules. The nanoparticles in the diameter range of 1–10 nm would display electronic structures, reflecting electronic band structure of the nanoparticles, owing to quantum-mechanical rules (Alivisatos, 1996). The resulting physical properties are neither of the bulk metal nor of the molecular compounds but they strongly depend on the particle size, interparticle distance, and nature of the protecting organic shell and shape of the nanoparticles (Brust and Kiely, 2002). The gold nanoparticles (AuNPs) are the most stable metal nanoparticles and are expected to be key materials and building blocks for nanomaterials and nanodevices in the 21st century, because of their fascinating aspects in materials science, size-related electronics, magnetic and optical properties (quantum size effect), and their applications in catalysis and biology (Singh et al, 2010).

Nanostructured Metal Oxides

Nanostructured metal oxides such as CeO_2, SnO_2, Fe_3O_4, MnO_2, Sb_2O_3, TiO_2, ZnO and ZrO_2 have been found to exhibit interesting properties such as large surface-to-volume ratio, high surface reaction activity, high catalytic efficiency, and strong adsorption ability that make them potential candidate materials for the fabrication of a biosensor (Kerman et al, 2008; Chow et al, 2005; Li et al, 2006). The large surface area of nanomaterials is likely to provide a better matrix for the immobilization of desired enzyme, leading to increased enzyme loading per unit mass of particles. Moreover, the multipoint attachment of enzyme molecules to nanomaterials surfaces reduces protein unfolding resulting in enhanced stability of enzyme attached to the nanoparticles surface. The enzyme-attached nanoparticles facilitate enzymes to act as free enzymes in solution that in turn provide enhanced enzyme– substrate interaction by minimizing potential aggregation of the free enzyme (Pandey et al, 2007).

Quantum Dots

Quantum dots (QDs) or nanocrystals, are a special class of materials known as semiconductors, which are crystals composed of periodic groups of II-VI, III-V, or IV-VI materials. QDs, one of these nanomaterials, are nearly spherical semiconductor particles with diameters from 2 to 10 nm, comprising 200 to 10,000 atoms. QDs have size-controlled luminescence functions, which mean the same material with variable sizes can exhibit different colors under the excitation of an appropriate wavelength; broad absorption spectra; and narrow emission spectra, which mean simultaneous excitation of different colored QDs by a single wavelength (Han et al. 2001; Gill et al. 2008). The main advantages of QDs compared to the other nano materials is their nano scale size similar to the proteins,

broad excitation spectra for multi color imaging, robust, narrow band emission and versatility in surface modification.

NANOMATERIALS APPLICATION IN ELECTROCHEMICAL DETECTION OF ORGANOPHOSPHORUS COMPOUNDS AND PESTICIDES

Electrochemical biosensors have been the subject of basic as well as applied research for about last five decades. The most typical part of electrochemical biosensors is the presence of a suitable enzyme in the biorecognition layer providing electroactive substances for detection by the physico-chemical transducer providing the measurable signal. A native enzyme can be used as the biorecognition component; in this case the analyte is equal to the enzyme substrate; alternatively it may function as its inhibitor.

Stability, sensitivity, selectivity and other analytical characteristics of electrochemical biosensors are essential features to design desirable microenviroment for the direct electron transfer between the enzyme's active sites and the electrode. In recent years, due to the exceptional properties such as good conductivity, large surface area and extremely miniaturized size, nanomaterials such as carbon nanotubes, graphene, metal nanoparticles (gold nanoparticles, platinum nanoparticles etc.), nanostructured metal oxides (ZrO_2, Fe_2O_3, etc), and various composites of these nanomaterials have been widely employed in electrode modification and electrochemical sensor development (Guo and Wang, 2007; Kerman et al, 2008) Among the various nanomaterials, carbon nanotubes (CNTs) with excellent electrical conductivity, high mechanical strength and good stability have been used extensively in the development of electrochemical biosensors (Wang, 2005).

Electrochemical method based on chemically modified electrode has shown great potentials over other techniques for the detection of OPs because of its simplicity, fast responses, good sensitivity, high selectivity and excellent long-term calibration stability. Electrochemical biosensor technology emerged in the past decades is well suited for on-site environmental monitoring of OPs. Various inhibition and non-inhibition biosensor systems, based on the immobilization of acetylcholinesterase (AChE) or organophosphorus hydrolase (OPH) onto various electrochemical or optical transducers, have been proposed for field screening of OP neurotoxins.

Acetylcholinesterase Inhibition Based Biosensors

Recently biosensor techniques based on the inhibition of AChE activity have gained considerable attention due to the advantages of simplicity, rapidity, and reliability. These are referred to as inhibition biosensors since the quantification of OPs is based on the measurement of the decreased enzyme activity after exposure of enzyme to an inhibitor. These biosensors have used either AChE alone or combined with choline oxidase (ChO). The AChE inhibition in the single and bienzyme system is monitored by measuring the oxidation current of the product of the enzyme reaction.

$$\text{Acetylcholine} + H_2O \xrightarrow{\text{AChE}} \text{Choline} + \text{Acetate} \quad (1)$$

$$\text{Choline} + O_2 \xrightarrow{\text{ChO}} \text{Betaine Aldehyde} + H_2O_2 \quad (2)$$

$$\text{Acetylthiocholine} + H_2O \xrightarrow{\text{AChE}} \text{Thiocholine} + \text{Acetic Acid} \quad (3)$$

In the bienzyme system (equation 1 & 2) AChE catalyzes the hydrolysis of the neurotransmitter acetylcholine into acetate and choline. The choline is subsequently converted by ChO, producing hydrogen peroxide in the presence of oxygen. Hydrogen peroxide can be detected amperometrically with different electrochemical transducers. Whereas, in a single enzyme system (eq 3), thiocholine (TCh) ester, acetylthiocholine (ATCh), is preferred as substrate. Acetylthiocholine can be enzymatically hydrolyzed by AChE to TCh, which in turn is oxidized at constant potential at the electrochemical transducer, producing the initial biosensing response.

One approach to improve the performance characteristics of the AChE-based inhibitor biosensors would be to design and produce appropriate enzymes with characteristics more suitable for biosensor applications. Initial biochemical studies revealed that *Drosophila melanogaster* acetylcholinesterase (Dm. AChE) is the most sensitive enzyme toward OPs. The Dm. AChE-based inhibitor biosensors show great promise to improve the sensitivity of the biosensor system. Sotiropoulou et al.(2005) reported a Dm. AChE based inhibitor biosensor for the detection of dichlorvos with a detection limit of 10^{-17} M, which is 5 orders of magnitude lower than the *Electropharus electricus* AChE-based biosensor. However, this approach has been hampered by the fact that AChE from various sources was not easily available because of difficulties in isolation and purification procedures. Another possible approach for improving the performance characteristics of the AChE-based inhibitor biosensors is to improve the biosensor design and the electrochemical detection of the enzymatic product.

The immobilization of enzymes on solid electrode is largely influenced by method of deposition, which could significantly affect the electrocatalytic activity of the electrode. Various methods including physical adsorption, covalent attachment, layer-by-layer self-assembly, and electropolymerization method were reported for immobilizing AChE enzyme on electrode surfaces.

Physical Adsorption: The physical adsorption is one of the simple procedures to immobilize the enzyme onto the transducer (Bonnet et al, 2003). AChE was immobilized by adsorption on MWCNTs modified electrodes. In this way, few µL of AChE solution were dropped on the MWCNT modified electrode surface and allowed to evaporate at room temperature under a current of air. To remove losly bound enzyme molecules the AChE/MWCNT modified electrode was washed carefully with buffer solution (Joshi et al, 2005). The draw backs of physical adsorption are low quantity of adsorbed enzyme, leaching of the enzyme. Some of

these limitations can be overcome by adsorbing enzymes onto CNT-modified electrodes decorated with metallic nanoparticles, such as Pt-NP. By subsequently depositing a Nafion film onto the electrode, it is possible to reduce leaching of the enzyme and to improve the stability of the biosensor.

***Covalent Attachment*:** Covalent immobilization approach yields the direct anchoring of the enzymes to the carbon framework. One of the most used types of enzyme immobilization is the chemical immobilization by means of cross-linking with glutaraldeyde. Dan Du et al proposed the immobilization of AChE by using glutaraldehyde as cross-linker to MWNTs-chitosan (MC) composite, leading to a stable AChE biosensor for rapid determination of triazophos quantitatively (Du et al, 2007b).

Layer-by-layer self assembly: The Layer-by-layer (LBL) electrostatic self-assembly deposition method, initially reported by Decher, is one of the most convenient techniques for fabricating molecularly controlled ultrathin multilayer films (Decher and Hong, 1991). This technique based on the successive deposition of very thin layers of cationic and anionic species from a solution. The film fabrication is performed under mild conditions, which is particularly important for preserving activity of biomolecules. Guodong Liu et al reported the immobilization of AChE on the negatively charged CNT surface by alternatively assembling a PDDA layer and an AChE layer (Liu and Lin, 2006).

Electropolymerization: Electropolymerization is an attractive and well-controlled method for immobilizing enzymes onto electrodes. In this methodology, the enzyme is mixed with a monomer which is electropolymerized at a GCE or a metal electrode, whereupon the enzyme becomes embedded into the polymer matrix. The incorporation of the enzyme into the matrix is often promoted through electrostatic interactions. Numerous enzymes have been incorporated into electropolymerized films (Bartlett and Cooper, 1993). In many cases conductive polypyrrole (PPy) has been used as a polymer matrix. This choice relates to the fact that pyrrole can be electropolymerized at low oxidation potentials in aqueous solutions at neutral pH, which is compatible with a wide range of biological molecules. Recently, a simple method to immobilize AChE on PPy and polyaniline (PAn) copolymer doped with multi-walled carbon nanotubes (MWCNTs) was proposed (Du, et al, 2010b). The synthesized polyaniline/polypyrrole/MWCNTs copolymer presented a porous and homogeneous morphology which provided an ideal size to entrap enzyme molecules. The surface hydrophilicity was improved greatly after forming a complex structure instead of a separate layer. It provided an excellent environmental and chemical stability around the enzyme molecule to stabilize its biological activity to a large extent, resulting in a stable AChE biosensor for screening of organophosphates exposure.

As the oxidation of enzymatic product thiocholine occurs at a relatively high potential on conventional electrodes, mediators such as cobalt (II) phthalocyanine (Skladal, 1991; Hart and Hartley, 1994), prussian blue (Ricci et al, 2004; Sun and

Wang, 2010) and tetracyanoquinodimethane (Martorell et al, 1997) have been used to reduce the overvoltage of oxidation and enhance the sensitivity of the detection. The ideal biosensor fabrication method should employ mild chemical conditions, allow for large quantities of enzyme to be immobilized, provide a favorable microenvironment to maintain the enzyme activity, and provide a large surface area for enzyme-substrate contact within a small total volume. Barriers to mass transport of substrate and product should be minimized, and a chemically and mechanically robust system should be provided.

Joshi et al (2005) demonstrated a low cost disposable biosensor for the sensitive detection of OP pesticides by using CNTs. A film of acid functionalized MWCNT was cast on the surface of the screen-printed electrode by drop drying. Then AChE was immobilized on the CNT film by dropping 10 µL of AChE solution and drying at room temperature under a current of air and the electrode was carefully washed with buffer to remove loosely adsorbed enzyme molecules and bonded CNTs. To determine relative inhibition, the AChE-functionalized MWCNT-SPE electrode was immersed in a cell containing 2 mL of pH 7.4, 50 mM phosphate buffer with 0.1 M KCl under constant stirring. The potential was poised at 200 mV (vs. Ag/AgCl). After current stabilization, acetylthiocholine iodide substrate was added to a final concentration of 1.75 mM. This value of current corresponded to I_0, the current before inhibition. A known concentration of paraoxon was then dropped on to the electrode and incubated for 30 minutes. After incubation, the electrode was washed with the buffer and the response was measured again as described above, this second value corresponded to I_i, the current after inhibition. The relative inhibition (*RI*, %) was determined according to the following formula: RI (%) = $[(I_0-I_i)/I_0]*100$ and then related to the inhibitor concentration. The biosensor detected as low as 0.5 nM (0.145 ppb) of the paraoxon with good precision, electrode to electrode reproducibility and stability.

Upadhyay et al (2009) have proposed a sensitive amperometric biosensor by using gold–platinum bimetallic nanoparticles modified glassy carbon electrode for the sensitive detection of organophosphate pesticides, carbamates and nerve agent. The OP pesticide (paraoxon ethyl), carbamate (aldicarb) and nerve agent (sarin) were detected by utilizing the unique properties of Au–Pt bimetallic NPs, by using the bienzyme (AChE and ChOx) approach .This novel system has been developed by electrodeposition of the Au–Pt bimetallic NPs on the 3-aminopropyltriethoxy silane (3-APTES) modified glassy carbon (GC) electrode. Then AChE and ChOx are coimmobilized on the Au–PtNPs modified electrode by cross-linking enzymes and 3-APTES through glutaraldehyde. In this bienzyme system, AChE rapidly hydrolyzes acetylcholine into acetate and choline. The choline is subsequently oxidized by ChOx and H_2O_2 is produced, and finally H_2O_2 is detected amperometrically. By using this method paraoxon ethyl, sarin, and aldicarb could be detected up to 150–200 nM, 40–50 nM, and 40–60 µM respectively at 30–40% inhibition level of AChE enzyme and followed linearity in wide range concentration.

With their wide range of advantageous properties, nanomaterials have been extensively used as potent immobilization matrices for AChE. The various nanomaterials matrices used in AChE biosensors, the linear range, and the detection limit values obtained using various electroanalytical techniques have been presented in Table 1. From Table 1, it was obvious that nanomaterials based AChE sensors are suitable for OP pesticides determination in wide linear range with the detection limits in nM to pM range.

Table 1 Acetylcholinesterase inhibition based biosensors using nanomaterial modified electrodes

Electrode	Detected Agent	Method Applied	Linearity Range	LOD	Ref.
AChE/PANI/MWNT /GC	Carbaryl	SWV amperometry	9.9×10^{-9} -49.6 × 10^{-9} M 9.9×10^{-6} -49.6 × 10^{-6} M	4.6×10^{-9} M 1.4×10^{-6} M	(Cesario et al, 2010)
SPCE|CNTs/ZrO2/PB/Nf|GMP-AChE	Dimethoate	DPV	1.0 × 10^{-3}–10 ng·mL-1	5.6 × 10^{-4} ng mL^{-1}	(Gan et al, 2010)
AChE-MWCNTs/AuNPs-chitosan/GC	Monocrotophos	FFTCCV	0.1×10^{-6} - 10 $\times10^{-6}$ M	10×10^{-9} M	(Norouzi et al, 2010)
IL–MWCNT gel/CPE	Chlorpyrifos	Amperometry	10^{-8}–10^{-6} M	4×10^{-9} M	(Zamfirb et al, 2011)
AChE-AN-MWNTs/GCE	Dichlorvos		50 ngL^{-1}-1µgL^{-1} & 50 µgL^{-1}-5 mgL^{-1}	10ngL^{-1}	(Sun et al, 2011)
MWNTs–chitosan	triazophos	Amperometry	0.03 - 7.8 $\times10^{-6}$ & 7.8 - 32×10^{-6} M	0.01×10^{-6}	(Du et al, 2007)
AchE-SAM-Au	Parathion Carbaryl	Amperometry	-	-	(Pedrosa et al, 2008)
Au/ssDNA–SWCNT/PANI/AChE	methyl parathion chlorpyrifos	SWV	1.0×10^{-11} & 1.0×10^{-6} M	1×10^{-12} M	(Viswanathan et al, 2009)
CNTs-AChE	Chlorphenvinphos	SWV	4.9×10^{-7}- 7.46×10^{-6} M	1.15×10^{-7}M	(Oliveira and Mascaro, 2011)
Au–PtNPs/3-APTES/GC	Paraoxon ethyl Sarin aldicarb	CV	-	1.5–2 $\times10^{-11}$ M, 4–5 $\times10^{-10}$ M 4–6 $\times10^{-7}$ M	(Upadhyay et al, 2009)
AChE/MWCNT/SPE	Paraoxon	Amperometry	-	0.5×10^{-9} M	(Joshi et al, 2005)
ZrO_2 NPs/Au	Phosphorylated AChE adducts	Striping voltammetry	10 x 10^{-12} -4 x 10^{-9} M	8.0 x 10^{-12} M	(Liu et al, 2008)

PDDA/AChE/PDDA /CNT/GC	Paraoxon	FIA	1 x 10^{-12} - 0.1 x 10^{-9} M	0.4 x 10^{-12}	(Liu and Lin, 2006)
AChE–Au–PPy/GCE	methyl parathion	CV	0.005 - 0.12 & 0.5 - 4.5 μgmL^{-1}	2 ngmL^{-1}	(Gong et al, 2009)
AChE-CS/Au	malathion	CV	0.1–500 ng mL^{-1}	0.03 ng mL^{-1}	(Du et al, 2007a)
AChE-MWCNTs-Au-CHIT/GCE	malathion	CV	2 to 15 μg mL^{-1}	0.6 ng mL^{-1}	(Du et al, 2010a)
PDMS-PDDA/AuNPs/ChO/AChE	Paraoxon parathion	Amperometry	-	5.0×10^{-10} g/L 1.0×10^{-9} g/L	(Zhao et al, 2009)

OPH Based Biosensors

Organophosphorus hydrolase (OPH), an organophosphotriester-hydrolyzing enzyme discovered in soil microorganisms *Pseudomonas diminuta* MG and *Fla_obacterium* spp. The enzyme has a broad substrate specificity and is able to hydrolyze a number of OP pesticides such as paraoxon, parathion, coumaphos, diazinon, dursban, methyl parathion, etc., and chemical warfare agents, sarin and soman (Munnecke, 1980; Dumas et al, 1989a; Dumas et al, 1989b;). OPH selectively catalyzes a hydrolytic reaction in the P-O, P-S, P-F, or P-CN bonds in neurotoxins, resulting in the stoichiometric generation of two protons and an alcohol, which in many cases is electroactive. Direct pesticide detection is thus possible via 1) measurement of the pH change associated with enzyme activity by combining the OPH with potentiometric transducers such as a pH electrode or a field effect transistor, or a pH indicator dye to quantify protons produced (Lihong et al, 2006), 2) to monitor the oxidation or reduction current of the hydrolysis products by integrating OPH with an amperometric transducer.

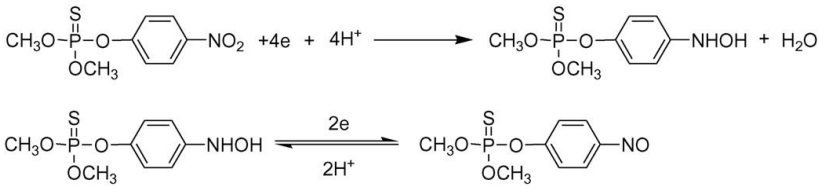
Since OPH utilizes OPs as substrates rather than inhibitors, as is in the case of cholinesterase, these biosensors are direct, have simple single-step protocol, and are reversible as compared to the inhibition-based format. Consequently, OPH-based biosensors demonstrate considerable potential for applications requiring repetitive/multiple and on-line analysis. As an alternative to purified enzyme, whole cells have also been applied as biological transducer. Several examples of microbial-based biosensors, including OPs, have been reported (Mulchandani et al, 2001). While these biosensors provided simple, rapid, and direct monitoring of OP compounds, either the detection limit or selectivity limited their applications.

Choi et al (2009) immobilized OPH on CNT/ionic liquid (IL) electrodes using three different intrinsic kinds of ILs, as binders for the detection of paraoxon. CNTs/ILs leads to dramatic electrochemical enhancements with respect to response time, stability, and sensitivity of composite electrodes. In addition, the electrochemical and biocatalytic properties of three-composite electrodes were strongly influenced by different types of ILs used, as verified by cyclic voltammetry and chronoamperometry. These results were attributed to the conformational changes of

the microenvironment between the OPH and the composite electrodes within three different types of ILs. In particular, the biocatalytic signals of three OPH/CNT/ILs-modified electrodes increased linearly to the concentration of paraoxon in a wide range of 2–20 μM. Choi et al (2010) used OPH as a model protein for convenient immobilization onto reduced graphene oxide/Nafion (RGON) hybrid films for the hydrolysis of paraoxon and obtained the excellent figure of merit as electrochemical biosensing platforms for organophosphate (OP) detection, that is, a sensitivity of 10.7 nA/ μM, detection limit of 1.37 $x10^{-7}$ M, and response time of <3 s. Although OPH-based non-inhibition biosensors provide a direct biosensing route for the detection of OPs, OPH is not commercially available, which limits widespread applications. A preferred indirect electrochemical biosensing route based on the inhibition of target enzymes has been widely used for the detection of OPs.

Non-enzymatic Biosensors

Although the enzyme (OPH & AChE) based detection of OP compounds and pesticides is highly sensitive and specific, the stability and availability of these enzymes hinders their potential applications in biosensors. Recently various mediators such as Poly(Safranine) (Liu, 2012) and phthalocyanines (Tapsoba et al, 2009) have been used to catalyze the electrochemical reduction or oxidation of OP compounds. The non-enzymatic detection of OP compound and pesticides widely reported by using various nanomaterials such as ZrO_2 nanoparticles, nanosized acetylene black (Liu and Lin, 2005; Yazen et al, 2010). In general OP compounds containing nitro- functional group such as parathion and malathion are detected by this procedure. The electrochemical reactions of parathion is given below (Yazen et al, 2010)



Liu and Lin (2005) described the electrochemical sensing nitroaromatic OPs based on a gold electrode modified with zirconia nanoparticles. This new ZrO_2 nanoparticle-based electrochemical sensing protocol involves electrodynamically depositing ZrO_2 nanoparticles onto a gold electrode surface, followed by OP adsorption (because of the strong affinity of zirconia for the phosphoric group, nitroaromatic OPs strongly bind to the ZrO_2 nanoparticle surface), and electrochemical stripping detection of adsorbed electroactive OPs. The electrochemical characterization and anodic stripping voltammetric performance of bound nitroaromatic OP compounds were evaluated using cyclic voltammetric and square-wave voltammetric (SWV) analysis. The promising stripping voltammetric performances open new opportunities for fast, simple, and sensitive analysis of OPs. The stripping voltammetric response is highly linear over the 5-100 ng/mL (ppb) methyl parathion range examined (2-min adsorption), with a detection limit of 3

ng/mL and good precision (RSD) 5.3%, n) 10). The detection limit was improved to 1 ng/mL by using 10-min adsorption time.

FLUORESCENCE BASED DETECTION OF ORGANOPHOSPHORUS COMPOUNDS AND PESTICIDES

After electrochemical approaches, optical devices are the second most commonly used transducers. A general optical sensor system consists of a light source, a number of optical components to generate a light beam with specific characteristics and to direct this light to a modulating agent, modified sensing head, and a photodetector. Optical immunosensors have been shown to be able to measure adsorbed molecular layers, which utilize the evanescent wave to form the sensing device. Different techniques can be used for creating an optical change, e.g., reflectometric interference spectroscopy, interferometry, optical waveguide light mode spectroscopy, total internal reflection fluorescence, and surface plasmon resonance.

Fluorescence occurs when a valence electron is excited from its ground state to an excited singlet state. The excitation is produced by the absorption of light of sufficient energy (Lazcka et al., 2007). The common principle of luminescence immunosensors is that an indicator or chemical reagent placed inside or on an immmunoreactor is used as a mediator to produce an observable optical signal. Typically, conventional techniques, such as spectrometers, are employed to measure changes in the optical signal. Neurotoxic organophosphates (OP) have found widespread use in the environment for insect control. In addition, there is the increasing threat of use of OP based chemical warfare agents in both ground based warfare and terrorist attacks. Together, these trends necessitate the development of simple and specific methods for discriminative detection of ultra low quantities of OP neurotoxins.

Recently, it was reported that a change in fluorescence properties of a fluorophore in the vicinity of gold nanoparticles might be used for detection of nanomolar concentrations of DNA oligonucleotides. The detection strategy was based on the fact that an enhancement or quenching of fluorescence intensity is a function of the distances between the gold nanoparticle and fluorophore. While these reports have demonstrated the use of nanoparticle-based sensors for the detection of target DNA. OPH-gold nanoparticle conjugates were prepared, then incubated with a fluorescent enzyme inhibitor or decoy. The fluorescence intensity of the decoy was sensitive to the proximity of the gold nanoparticle, and thus could be used to indicate that the decoy was bound to the OPH. Then different paraoxon concentrations were introduced to the OPH-nanoparticle-conjugate-decoy mixtures, and normalized ratios of fluorescence intensities were measured. The greatest sensitivity to paraoxon was obtained when decoys and OPH-gold nanoparticle conjugates were present at near equimolar levels. The change in fluorescence intensity was correlated with concentration of paraoxon presented in the solution (Simonian et al., 2005)

Based on the highly sensitive and selective fluorescence enhancement of water-soluble CdTe/CdS core-shell quantum dots (QD) by organophosphorus pesticides (OPs such as mevinphos, phosalone, methidathion and diazinon), a simple, rapid and selective method using CE with QD/LIF detection (473 nm excitation/532 nm fluorescence) to determine OPs in vegetable samples has been reported (Chen and Fung, 2010). The method enables the use of a simple pretreatment procedure based only on solvent extraction and eliminates the use of a time-consuming solid phase extraction step. The CE-QD/LIF method was shown to have a detection limit from 50 to 180 μg/kg, working ranges 0.1-30 mg/kg, recoveries 88.7-96.1% and repeatability (RSD, n=3) 0.36-0.75% for migration time and 2.9-5.7% for peak height. For tomato samples, the detection limits were more than ten times lower than maximum residue levels specified by the Codex Alimentarius Commission for all four OPs investigated.

Real-time detection of an organophosphorus compound using a sol–gel silica planar waveguide doped with a green fluorescent protein and an organophosphorus hydrolase on a yeast-cell surface display was reported (Enami et al., 2011). The waveguide was pumped at 488 nm, and it emitted green fluorescence at the far field. The green fluorescent light at 550 nm changed by 50% from the original power 1 min after application of the organophosphorus compound. The results enable the real-time detection of sarin and other biochemicals by using an in-line fiber sensor network.

Nanostructured biosensors for the determination of OPs are fabricated by a layer-by-layer assembly technique. In the biosensors, bi-enzymes of acetylcholinesterase (AChE) and choline oxidase (ChOx) are used as biological receptors, while CdTe quantum dots (QDs) are explored as fluorescent probes for optical transduction of the enzymatic activity. Increasing amounts of OPs lead to a decrease of the enzymatic activity and thus a decrease in the production of hydrogen peroxide, which can quench the fluorescence of the CdTe QDs. The decrease of quenching rate is relative to the concentration of OPs. Using this biosensor, monitoring of three types of commonly used OPs (paraoxon, dichlorvos and parathion) at picomolar levels is realized. The linear range of detection covers six orders of magnitude (10^{-12} to 10^{-6} M). In addition, the biosensors exhibit a similar limit of detection and calibration curves for these pesticides, which allow them to be used for the accurate determination of total OPs and carbamate content (not the sum of anti-acetylcholinesterase toxicity as obtained by standard cholinesterase inhibition assay) of mixtures of OPs and carbamate pesticides (Zheng et al., 2011].

A self-assembled multilayer (SAM) consisting of amino-silanized quartz functionalized with gold nanoparticles and coated with indole via a L-cysteine linker was reported (Sun et al., 2008). When the SAM sensor was exposed to the pesticide, the indole group of the sensor on the modified film was oxidized to a fluorescent indoxyl group. The oxidation process depended on the pesticide concentration and was reflected by changes in intensity. The sensor was capable of detecting methylparathion and monocrotophos in the ppm and ppb range, respectively. An advantage of the indole-based SAM sensor is that it could detect OP pesticides in ionic and other environmental species, but it was subject to interference at 20 equivalents of Fe^{3+} ions.

FUTURE PERSPECTIVES

The detection of pesticides and OP compounds are necessary because it is widely used in the field area to grow the crops and kill the insects. In many cases the toxicity is not acute, but it can exert its effect over many years at low concentrations of the pesticide. The contamination of aquatic systems has become a global problem, particularly as many of these pesticides are persistent in the environment and it causes many health problem and it also affect the food chain. Various methods are used for the detection of the pesticides and enzymatic electrochemical methods could play important role towards the development of rapid and field applicable system. By using of the nanomaterials, nanotubes, quantum dots etc one can achieve higher sensitive detection system. It can work colorimetric based detection because the nanoparticles have potential to change the colour in case of enzymatic inhibition measurements. It is clear that nanoparticles based detection approach is more powerful tool.

Significant progress has been achieved toward the development of fluorescent chemosensors for toxic OP pesticides. The chemosensors have been demonstrated to be time-effective and more robust that biosensors. It is clear that future improvements in this area will require the design of new fluorescent chemosensors with additional modes for signal transduction. Such sensors will play an important role in minimization or elimination of false-positives. Due to the structural similarity of OP compounds, it is also paramount that the designed sensors must be fabricated such that they are highly selective toward specific OP compounds.

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Milan Jokanović

Professor Milan Jokanović is employed at Faculty of Medicine, University of Nish, Nish, Serbia. He is a member of Academy of Sciences and Arts of the Republic Srpska in Banja Luka. He is also a member of Editorial Board of "Toxicology" published by Elsevier.

Professor Jokanović published more than 100 papers and book chapters on toxicology of organophosphorus compounds, antidotes and medical treatment of poisoning. He is also an expert for nonclinical and clinical drug development and regulatory affairs. More details about his work and expertise can be found at www.experta.co.rs.

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Prof. Jokanović from the University of Nish is one of the most prestigious experts in the fields of chemistry with over 100 published papers and book chapters covering toxicology of organophosphorus compounds, antidotes and medical treatment of poisoning. He is also an expert for nonclinical and clinical drug development and regulatory affairs.

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