ZAIRA CLEMENTE

ECOTOXICOLOGICAL EVALUATION OF TITANIUM DIOXIDE NANOPARTICLES, UNDER DIFFERENT ILLUMINATION CONDITIONS

AVALIAÇÃO ECOTOXICOLÓGICA DO DIÓXIDO DE TITÂNIO NANOPARTICULADO, SOB DIFERENTES CONDIÇÕES DE ILUMINAÇÃO

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ABSTRACT

The ecotoxicology of titanium dioxide nanoparticles (nano-TiO_2) has been extensively studied in recent years but the results are so far confusing. Hence, doubts remain about the applicability of current ecotoxicological protocols to evaluate the possible impacts of nanotechnology. Few toxicological investigations have considered the photocatalytic properties of the substance, which can increase its toxicity to aquatic biota. The aim of this work was to evaluate the effects on aquatic organisms exposed to different nano-TiO_2, under different illumination conditions. The interaction of variables as concentration, crystal phase (pure anatase – TA, or a mixture of anatase and rutile – TM) and illumination condition (visible light or ultraviolet and visible light) were investigated by observing lethal an sublethal parameters in juveniles fishes (pacu caranha, *Piaractus mesopotamicus*), fish embryos (zebrafish, *Danio rerio*) and microcrustaceans (*Daphnia similis* and *Artemia salina*). The acute and prolonged exposure of juvenile fishes caused no mortality neither Ti accumulation in fish muscle, but showed biochemical and genetic effects, which depends on the crystal phase and the illumination condition employed. The acid phosphatase activity (AP) as well as the metallothionein and protein carbonylation (PCO) levels and the micronucleus test were useful biomarkers of acute exposure of fish to nano-TiO_2. On the other side, the findings showed that for prolonged exposure, the specific activity of catalase (CAT), glutathione S-transferase (GST), PCO levels and comet assay were more useful as biomarkers. Nano-TiO_2 was also considered pratically non-toxic under visible light to *D. similis* and *A. salina*. Exposure to nano-TiO_2 under visible and ultraviolet light enhanced the toxicity of nano-TiO_2 to microcrustaceans. In the case *of D. similis*,

TM was more toxic than TA, showing values of $EC50_{48h}$ = 60.16 and 750.55 mg/L, respectively. *A. salina* was more sensitive than *D. similis*, with $EC50_{48h}$ = 4 mg/L for both products. At sublethal concentrations, the nano-TiO_2 did not show any negative impacts on the growth of *Daphnia* and *Artemia*. The specific activities of CAT, AP and superoxide dismutase were usefull biomarkers of nano-TiO_2 exposure in *Daphnia*. To embryos the nano-TiO_2 exposure caused early hatching. Under ultraviolet light, nano-TiO_2 caused reduction in larvae length. Also, an increase in larvae with alteration in equilibrium and larvae mortality was observed in groups exposed to TM under ultraviolet light. The specific activities of CAT and GST showed good answer in embryos exposed to nano-TiO_2. Determination of the nano-TiO_2 toxicity using bioassays depends on the organism, culture medium, and exposure time employed. It also depends on the crystal phase and the illumination condition. Exposure to ultraviolet light at minimal environmental levels increases the nano-TiO_2 toxicity. The results indicate the occurrence of oxidative stress in consequence of nano-TiO_2 exposure, but in general there was not a clear concentration-response relationship when considering sublethal parameters. This can be related to the instability of exposure systems, due to nanoparticles aggregation and precipitation. However, our results indicates that the influence of abiotic factors on nano-TiO_2 ecotoxicity must be explored in detail, in order to establish experimental models to study their toxicity to environmentally relevant species and contribute to nanotechnology development in a sustainable way.

Key words: aquatic toxicology, nanotechnology, water, fish, microcrustacean, embryo, biomarker.

RESUMO

A ecotoxicologia de nanopartículas de dióxido de titânio (nano- TiO_2) tem sido amplamente estudada nos últimos anos, mas os resultados obtidos ainda são inconclusivos. Assim, permanecem dúvidas sobre a aplicabilidade dos atuais protocolos ecotoxicológicos para avaliação dos possíveis impactos do uso da nanotecnologia. Poucas investigações toxicológicas tem considerado as propriedades fotocatalíticas da substância, que podem aumentar a sua toxicidade para a biota aquática. O objetivo deste trabalho foi avaliar os efeitos em organismos aquáticos expostos a diferentes nano- TiO_2, sob diferentes condições de iluminação. A interação de variáveis como concentração, fase cristal (anatase puro - TA , ou uma mistura de anatase e rutilo - TM) e da condição de iluminação (luz visível ou luz ultravioleta e visível) foram investigadas através da observação de parâmetros de letalidade e de efeitos subletais em peixes juvenis (pacu caranha, *Piaractus mesopotamicus*), embriões de peixe (zebrafish, *Danio rerio*) e microcrustáceos (*Daphnia similis* e *Artemia salina*). A exposição aguda e prolongada de peixes juvenis não causou mortalidade nem acúmulo de Ti em músculo dos peixes, mas houve efeitos bioquímicos e genéticos, que dependeram da fase cristal e da condição de iluminação empregada. A atividade de fosfatase ácida (FA), bem como os níveis de proteínas carboniladas (PCO) e de metalotioneína foram biomarcadores úteis de exposição aguda ao nano -TiO_2. Por outro lado, os resultados mostraram que para a exposição prolongada, a atividade específica da catalase (CAT), glutationa S-transferase (GST), os níveis de PCO e o ensaio cometa foram os biomarcadores mais úteis. O nano-TiO_2 também foi considerado praticamente não tóxico sob luz visível para *D. similis* e *A. salina*. A exposição ao nano -TiO_2, sob luz visível e ultravioleta aumentou a toxicidade dos nano-TiO_2 para microcrustáceos. No caso de *D. similis*, TM foi mais tóxico do

que o TA , mostrando valores de $CE50_{48h}$ = 60,16 e 750,55 mg/L, respectivamente. A *A. salina* foi mais sensível do que *D. similis*, com $CE50_{48h}$ = 4 mg/L para ambos os produtos. Em concentrações subletais, o nano- TiO_2 não apresentou qualquer impacto negativo sobre o crescimento de *Daphnia* e *Artemia*. As atividades específicas de CAT, superóxido dismutase e FA foram biomarcadores úteis de exposição ao nano- TiO_2 em *Daphnia*. Para embriões, a exposição ao nano-TiO_2 causou eclosão precoce. Sob luz ultravioleta, o nano- TiO_2 causou redução no comprimento das larvas. Além disso, um aumento no número de larvas com alteração de equilíbrio e na mortalidade foi observado nos grupos expostos a TM sob luz ultravioleta. As atividades específicas de CAT e GST apresentaram boa resposta em embriões expostos ao nano-TiO_2. A determinação da toxicidade do nano- TiO_2 depende do organismo, meio de cultura e o tempo de exposição utilizado nos bioensaios. Depende também da fase cristal e das condições de iluminação. Verificou-se que a exposição à radiação ultravioleta a níveis ambientais mínimos aumenta a toxicidade do nano-TiO_2. Os resultados indicam a ocorrência de estresse oxidativo em conseqüência da exposição ao nano-TiO_2, mas em geral, não houve uma clara relação concentração- resposta ao considerar parâmetros subletais. Isto pode estar relacionado com a instabilidade dos sistemas de exposição, devido a agregação e precipitação das nanopartículas. Entretanto, nossos resultados indicam que a influência de fatores abióticos sobre a ecotoxicidade do nano- TiO_2 deve ser explorada em detalhes, a fim de estabelecer modelos experimentais adequados para estudar a sua toxicidade em espécies de relevância ambiental e contribuir para o desenvolvimento sustentável da nanotecnologia.

Palavras chaves: toxicologia aquática, nanotecnologia, água, peixe, microcrustáceo, embrião, biomarcador.

SUMÁRIO

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INTRODUÇÃO GERAL

As nanociência e nanotecnologia (N&N) têm atraído grande interesse em diversos setores industriais e acadêmicos devido aos benefícios que podem ser alcançados no desenvolvimento tecnológico e econômico. O grande diferencial desses materiais é potencializar propriedades físicas e químicas em concentrações extremamente reduzidas e conferir características antes não apresentadas por um dado produto. Este alcance de propriedades se deve basicamente ao fato de que tais estruturas possuem dimensões nanométricas, que resultam em uma área superficial elevada, maior grau de dispersão e funcionalidades que são dependentes do tamanho da estrutura (ABDI, 2010).

O mercado mundial de nanomateriais é estimado em 11 milhões de toneladas a um valor de € 20 bilhões (European Comission, 2013). O mercado brasileiro de produtos com base em nanotecnologia, desenvolvido originalmente no país, somou em 2010 cerca de R$ 115 milhões (Agência Brasil, 2011). Grande parcela do crescimento do mercado de nanotecnologia não provém da produção de nanomateriais básicos, mas sim da capacidade de transformar os nanomateriais básicos em produtos de alto valor agregado ou na melhoria de processos produtivos (ABDI, 2010). O material mais comumente utilizado em nanoprodutos é a prata, em segundo lugar apresenta-se o carbono, seguido pelo titânio, sílica, zinco e ouro (Project on Emerging Nanotechnologies, 2011).

O desenvolvimento da N & N é considerado um assunto estratégico por diversas nações, inclusive o Brasil (NAE, 2004; NETS, 2010; NNI, 2011), pois pode contribuir para o desenvolvimento das indústrias de energia, farmacêutica, agrícola, eletrônica, automobilística, têxtil, entre tantas outras. O MCTI (Ministério da Ciência, Tecnologia e Inovação) lançou em

2013 um programa que prevê o investimento de R$ 450 milhões em dois anos para estimular a ligação entre universidade e empresa na área da nanotecnologia (Garcia, 2013). Em termos relativos, os dados revelam que a presença de publicações em nanociência tem quase triplicado durante a última década, confirmando-se que a nanociência, como campo de conhecimento, está se desenvolvendo muito mais rápido do que o conhecimento científico nos demais campos (ABDI, 2010).

O crescente desenvolvimento da N&N, bem como o uso de bens contendo nanopartículas (NPs) geram efluentes e resíduos, acendendo preocupações sobre os riscos ambientais e à saúde humana que podem estar envolvidos. Além disso, têm surgido métodos de remediação de água e de solos contaminados utilizando nano-óxidos como alternativas com maior custo–eficiência do que os tradicionalmente utilizados. Apesar da nanotecnologia se apresentar com uma alternativa de substituição benéfica às técnicas atuais de remediação ambiental, os riscos potenciais são ainda pouco compreendidos.

Uma das principais missões da ecotoxicologia é compreender os mecanismos pelos quais os contaminantes perturbam o desempenho biológico normal (refletindo-se no mecanismo de ação), para desenvolver medidas apropriadas à prevenção de efeitos adversos resultantes de contaminantes ambientais (Connon et al., 2012). A completa elucidação dos efeitos adversos de contaminantes a organismos ecologicamente relevantes faz-se extremamente necessária para avaliação de risco ambiental, direcionamento das políticas públicas e determinação de limites permissíveis. Os fatores e processos que afetam a ecotoxicidade dos nanomateriais manufaturados são complexos e o conhecimento sobre os riscos ambientais e à saúde humana ainda é limitado (Project on Emerging Nanotechnologies, 2013). Iniciativas de normalização e de regulamentação no contexto das nanotecnologias ganham importância a cada dia, na perspectiva

de se assegurar à sociedade que seu desenvolvimento industrial seja conduzido no futuro segundo um marco seguro, responsável e sustentável (ABDI, 2010).

Muitos estudos vêm mostrando o potencial uso das nanopartículas de dióxido de titânio (nano-TiO_2) em processos de fotocatálise heterogênea, para degradação de compostos orgânicos e inorgânicos (Chatterjee e Dasgupta, 2005; Fujishima e Zhang, 2006). Tal propriedade encontra aplicabilidade na remediação de solo e águas contaminadas, além de produção de superfícies autolimpantes, produtos de limpeza, ou mesmo na desodorização de ambientes e destruição de compostos voláteis em fase gasosa. O nano-TiO_2 também tem a propriedade de absorção de radiação ultravioleta (UV) e tem sido utilizado como pigmento em tintas e alimentos. Diversas NPs de TiO_2 têm sido produzidas atualmente (Xiaobo, 2009), com variações no tamanho das partículas, área de superfície, pureza (devido a dopagem, cobertura ou controle de qualidade), características de superfície, forma cristalina, reatividade química e outras propriedades. De acordo com um esboço de revisão da *Environmental Protection Agency of the United States* a produção anual de nano-TiO_2 foi estimada em 2000 toneladas métricas por volta de 2005, sendo 65% dessa produção utilizada em produtos como cosméticos e protetores solares (USEPA, 2009).

Tem sido observada uma grande variabilidade de resultados na literatura com relação aos testes de ecotoxicidade do nano-TiO_2. Tal variabilidade pode ser decorrente de diferentes características dos nano-TiO_2 e tratamentos aplicados, assim como nos desenhos experimentais. A falta de informações em alguns trabalhos dificulta a comparação dos resultados (Warheit et al., 2008). Por isso, tem-se discutido amplamente a necessidade da caracterização apropriada das NPs estudadas, assim como a padronização dos métodos de avaliação nanoecotoxicológica. Atualmente há diversas diretrizes para a realização de ensaios ecotoxicológicos (OECD, USEPA, DIN-STANDARDS, IBAMA, etc). Há um consenso de que os métodos e estratégias de avaliação

de risco existentes atendem genericamente às necessidades de avaliação de risco das N & N, mas a utilização destas para a realização de ensaios nanoecotoxicológicos enfrenta diversos questionamentos e os detalhes para cada teste ou grupo de testes requer modificações/validações para trabalhar adequadamente com cada nanomaterial (Stone et al., 2010, Handy et al., 2012 a,b). Em especial nos ensaios de toxicologia aquática, tem sido discutidos fatores como: i) a dificuldade na manutenção da estabilidade das suspensões, ii) a agregação das NPs à matéria orgânica (alimento, muco, fezes) e consequente alteração das características da exposição, iii) a adesão das NPs à superfície dos organismos e consequente alteração na mobilidade dos mesmos e confusão na interpretação de resultados referentes ao acúmulo do material no organismo. Ainda, as características do meio de exposição, como pH e força iônica combinadas às características das NPs, como área e carga de superfície, dopagem etc, afetam consideravelmente o comportamento das NPs em suspensão, podendo ser motivo de variabilidade nos resultados. Alguns esforços têm sido feitos para avaliar a interferência dos diversos fatores nos bioensaios e aprimorar os protocolos. Experimentos com embriões e larvas de peixe, por exemplo, mostraram que o problema da produção de muco e perda do material teste é menor do que com experimentos usando peixes maiores. Por outro lado, os protocolos atuais para avaliação de bioconcentração em peixes mostraram-se inadequados para estudos com nanomateriais, devido à instabilidade das suspensões e a que provavelmente, na maioria dos casos, o maior tamanho das NPs (1-100 nm) com relação a moléculas (angstroms,< 1 nm) pode limitar sua absorção pelos peixes. Em vista destas questões, defende-se uma abordagem científica racional, onde as propriedades de cada nanomaterial sejam criticamente consideradas com relação à execução do método, e onde surgirem propriedades comuns para diferentes nanomateriais deverão surgir também soluções comuns em termos de modificação da metodologia (Handy et al., 2012b). Assim, a aplicabilidade

de cada bioensaio em estudos nanoecotoxicológicos precisa ser avaliada em detalhe. A realização de ensaios com nano-TiO_2 apresenta diversas particularidades, como as propriedades fotocatalíticas e de absorção da radiação ultravioleta, além do comportamento de agregação e sedimentação na água.

Nos bioensaios com organismos aquáticos, geralmente utilizam-se lâmpadas fluorescentes comuns para estabelecer o ciclo circadiano, as quais emitem basicamente luz visível. Em condições naturais, os organismos aquáticos estão expostos a diversos fatores que não são incluídos nos bioensaios laboratoriais, como a radiação solar. Aproximadamente 44% da energia emitida pela radiação solar se concentram entre 400 e 700 nm, denominado espectro de luz visível. O restante é dividido entre radiação ultravioleta (UV, < 400nm) com 7%, infravermelho próximo (entre 700 e 1500nm) com 37% e infravermelho (IF, > 1500nm) com 11% (Nesme-Ribes e Thuillier, 2000 citado por Corrêa, 2003). No que se refere aos efeitos à saúde humana e ao ambiente, a radiação ultravioleta pode ser classificada como UVA (400 – 320 nm), UVB (320–290 nm) e UVC (290 - 200 nm).

O valor real de radiação solar recebido à superfície do planeta depende de fatores como latitude, altitude, época do ano, refletância da superfície, estado de transparência da atmosfera sobre o lugar, entre outros (Corrêa, 2003; Oliveira, 2010). Segundo o Atlas Solorimétrico do Brasil (Tiba, 2000), a média anual típica de radiação solar global diária (IF, luz visível e UV) no estado de São Paulo é de 16 MJ/m^2. Ao passar pela atmosfera terrestre, toda a radiação UVC e aproximadamente 90% da radiação UVB é absorvida pelo ozônio, oxigênio e dióxido de carbono. A radiação UVA é menos afetada pela atmosfera (WHO 2002). Em um dia de sol no verão, a radiação UVB constitui aproximadamente 6% da radiação UV terrestre e a UVA, os restantes 94% (Diffey, 2002; Corrêa, 2003). Os valores de atenuação exponencial da radiação UV (200-

400 nm) em água destilada são menores do que na água do mar e vão de 10/m a 200nm até um mínimo de 0,05/m a 375nm (Stewart e Hopfield 1965, citado por Acra et al.1990). Há ampla evidência de que ocorre formação de espécies reativas de oxigênio quando o TiO_2 é exposto à radiação UV (Brezová et al., 2005). As propriedades fotocatalíticas do nano-TiO_2 podem elevar seus efeitos tóxicos a organismos aquáticos em condições ambientais e até agora poucos estudos têm considerado esse aspecto.

Este estudo foi conduzido dentro das necessidades e expectativas da Rede de Pesquisa em Nanotecnologia Aplicada ao Agronegócio (Rede AgroNano), coordenada pela Embrapa Instrumentação (São Carlos – SP); cujo objetivo é explorar as aplicações da nanotecnologia no agronegócio em vários estágios, desde a produção agrícola até o beneficiamento e desenvolvimento de produtos para o consumo. Surgiu assim, a exigência da investigação dos possíveis impactos da aplicação da nanotecnologia ao ambiente. A Rede conta com a parceria de vários pesquisadores de 20 centros da Embrapa e de várias Universidades e Institutos de pesquisa (http://www.redeagronano.cnptia.embrapa.br/).

Como discutido anteriormente, os métodos atuais de avaliação ecotoxicológica possuem lacunas para testar os nanomateriais devido à complexidade do seu comportamento em sistemas naturais. Dessa forma, o presente estudo teve por objetivo avaliar os efeitos tóxicos da exposição à NPs de TiO_2 sob diferentes condições de iluminação, utilizando para isso ensaios *in vivo* com organismos aquáticos. O trabalho avaliou a aplicabilidade dos ensaios com organismos aquáticos comumente utilizados, na avaliação da ecotoxicidade de nano-TiO_2. A interação de variáveis como concentração, fase cristal (anatase puro ou uma mistura de anatase e rutilo) e da condição de iluminação (luz visível ou luz ultravioleta e visível) foram investigadas através da observação de parâmetros de letalidade e de efeitos subletais em peixes juvenis (*Piaractus mesopotamicus,*

nome comum pacu-caranha), embriões de peixe (*Danio rerio*, nome comum zebrafish ou paulistinha) e microcrustáceos (*Daphnia similis* e *Artemia salina*). Pretendeu-se com estes ensaios gerar dados para a avaliação de risco do uso e da contaminação ambiental por tal material além do estabelecimento de possíveis biomarcadores utilizáveis em estudos de nanoecotoxicologia. O uso neste projeto de espécies nativas de peixe (*P. mesopotamicus*) e de microcrustáceos (*D. similis*) fornece bases adicionais para o desenvolvimento da nanoecotoxicologia no Brasil, além de dados experimentais para comparação com resultados obtidos na avaliação de áreas possivelmente contaminadas.

A presente tese foi estruturada em capítulos, cada um correspondendo a um artigo já publicado ou submetido em periódico científico internacional. Esta introdução contextualiza a temática do estudo. O primeiro capítulo apresenta uma revisão bibliográfica sobre as propriedades e usos do nano-TiO_2 à luz do conhecimento existente sobre a ecotoxicologia das NPs na época da concepção e início do projeto. Atualizações sobre esses assuntos são apresentadas nos capítulos seguintes, que apresentam o estudo propriamente dito. O segundo capítulo discorre sobre a avaliação dos efeitos tóxicos da exposição aguda de peixes juvenis ao nano-TiO_2, sob diferentes condições de iluminação. Os resultados desse trabalho levaram ao desenvolvimento do segundo estudo, que é apresentado no terceiro capítulo, e no qual se avaliou os efeitos da exposição prolongada de peixes ao nano-TiO_2, em duas diferentes apresentações de fase cristal, também sob diferentes condições de iluminação. Em linhas gerais, os resultados iniciais indicaram que os efeitos tóxicos do nano-TiO_2 dependem da formulação e da condição de iluminação empregados nos bioensaios. Assim, decidiu-se aprofundar o conhecimento dos efeitos ecotoxicológicos através de outros modelos experimentais, com organismos mais sensíveis. Para isso foi realizado um estudo com microcrustáceos, cujos resultados são apresentados no quarto

capítulo; e com embriões de peixes, cujos resultados são apresentados no quinto capítulo. A metodologia utilizada no trabalho é descrita brevemente nos artigos, mas alguns protocolos são apresentados em detalhes nos Anexos V a XVI. Por fim, são apresentadas as considerações finais da tese.

CAPÍTULO I

REVISÃO BIBLIOGRÁFICA

Artigo publicado: Clemente, Z et al. Ecotoxicology of nano-TiO_2 – an evaluation of its toxicity to organism of aquatic ecosystems. International Journal of Environmental Research. 6 (1): 33-50, 2012.

ABSTRACT

The production and use of synthetic nanoparticles is growing rapidly, and therefore the presence of these materials in the environment seems inevitable. Titanium dioxide (TiO_2) presents various possible uses in industry, cosmetics, and even in the treatment of contaminated environments. Studies about the potential ecotoxicological risks of TiO_2 nanoparticles (nano-TiO_2) have been published but their results are still inconclusive. It should be noted that the properties of the diverse nano-TiO_2 must be considered in order to establish experimental models to study their toxicity to environmentally relevant species. Moreover, the lack of descriptions and characterization of nanoparticles, as well as differences in the experimental conditions employed, have been a compromising factor in the comparison of results obtained in various studies. Therefore, the purpose of this paper is to make a simple review of the principal properties of TiO_2, especially in nanoparticulate form, which should be considered in aquatic toxicology studies, and a compilation of the works that have been published on the subject.

1. INTRODUCTION

Nanotechnology is a rapidly expanding area of research which already has a wide variety of commercially available products. The material most commonly utilized in nanoproducts is silver, followed by carbon, titanium, silicon, zinc and gold (Meyer et al., 2009, Project on Emerging Nanotechnologies, 2011). An initial estimate indicates that nanotechnology may lead to a revolution in the development and fabrication of products that could contribute with up to one trillion dollars to the global economy by 2015 (Roco, 2001).

Nanomaterials have dimensions of less than 100 nanometers (nm), while nano-objects have dimensions smaller than 100 nm and nanoparticles (NPs) have three dimensions with less than 100 nm (Stone et al., 2010). However, the literature often describes NPs as particles that possess at least one dimension in the order of 1 to 100 nanometers (nm). The Royal Society of Chemistry suggests that 100 nm is the cut-off point above which particles will not enter cells through receptor-mediated processes (Royal Society of Chemistry and Royal Academy of Engineering, 2005), and some experimental evidence has emerged that corroborates this dimension (Chithrani and Chan, 2007, Clift et al., 2008). Another important cut-off dimension is particles smaller than 40 nm, which can enter the nucleus, while particles smaller than 35 nm can, potentially, cross protective barriers such as the hematoencephalic barrier (Oberdorster et al., 2004). However, these values should serve as guidelines, since the real size to be considered depends on other factors of the material and on details of its surface.

Titanium dioxide (TiO_2) has been used commercially since 1900, particularly in coatings and pigments. In 2002, the production capacity of this oxide was estimated at 4.6 million tons (Winkler, 2003). A review published by the United States Environmental Protection Agency

(USEPA) estimated the annual production of TiO_2 nanoparticles (nano-TiO_2) to be 2000 metric tons in around 2005, with 65% of this production used in products such as cosmetics and sunscreen lotions (USEPA, 2009).

The growing use of NPs generates effluents or wastewaters, raising concerns about the environmental risks and impacts of nanotechnology. Due to the wide utilization and promising uses that have emerged from nano-TiO_2, this material has been the target of several ecotoxicology studies. Based on a compilation of publishes works that evaluate the toxicity of nano-TiO2 to aquatic organisms, the article reviews the main properties of TiO_2, especially in nanoparticulate form, which should be considered in aquatic toxicology studies.

2. PROPERTIES AND USE OF TITANIUM DIOXIDE

In nature, TiO_2 occurs only in the form of oxide or oxides mixed with other elements. Mineral deposits are usually of volcanic origin, but are also found in beach sand (Winkler, 2003). TiO_2 can be found in three crystalline forms: anatase (tetragonal), rutile (tetragonal) and brookite (orthorhombic), and its main reserves are located in Canada, the US, Scandinavia, South Africa, the Mediterranean Sea, and Australia (Titaniumart, 2010).

Titanium dioxide, also known as titanium oxide (IV) or titania (molecular weight 79.88), is insoluble in water, chloric acid, nitric acid and ethanol, but is soluble in concentrated and heated sulfuric, hydrogen fluoride and alkaline media (NRC, 1999).

TiO_2 is obtained mainly from ore containing ilmenite ($FeTiO_2$), natural rutile (TiO_2) and leucoxene-like ilmenite. TiO_2 particles are referred to as primary, aggregates or agglomerates. Primary particles are individual crystals bound by crystal planes. Aggregates are sintered

primary particles connected by their crystal faces. Agglomerates are multiple primary particles and aggregates that are joined together by Van der Waal forces (IARC, 2010). Primary particles typically have a diameter of 0.2 to 0.3 μm, although larger aggregates are also formed (further details about bulk TiO_2 are given in Diebold, 2003).

Several TiO_2 NPs are produced today (Xiaobo, 2009), with variations in particle size, surface area, purity (due to doping, coating or quality control), surface characteristics, crystalline shape, chemical reactivity and other properties. One of the main differences between bulk TiO_2 and nano-TiO_2 is the larger surface area of a given mass or volume of NPs compared to an equivalent mass or volume of bulk TiO_2 particles (Shao and Schlossman, 1999). Approximately 35-40% of atoms are located on the surface of a 10 nm NP compared with less than 20% on particles larger than 30 nm. This higher surface area reinforces several properties, such as photocatalytic activity and ultraviolet absorption at given wavelengths (Shao and Schlossman, 1999).

Bulk TiO_2 absorbs ultraviolet radiation (<400nm). Because of its high refractive index, it is also very effective in dispersing radiation. Both dispersion and absorption are important in the attenuation of ultraviolet radiation (UV), making it an effective ingredient in sunscreen lotions (USEPA, 2009).

Small primary particles are less able to disperse visible light and are more transparent, while larger size particles are more opaque. Hence, sunscreen formulations containing nano-TiO_2 have become popular due to their greater transparency on the skin compared to the white appearance of formulations containing bulk TiO_2.

The theoretical calculations of Palmer et al. (1990) and experimental data of Sakamoto et al. (1995) showed that the UVB attenuation of submicrometric TiO_2 particles is predominantly

due to their absorption, while UVA attenuation is essentially due to their dispersion. The findings of Shao and Schlossman (1999) contribute to the idea that smaller particle sizes, and hence larger specific surface areas, are better for UVB attenuation. In contrast, the intensity of UVA dispersion is greater the larger the particle size (Shao and Schlossman, 1999).

TiO_2 is a semiconductor, i.e., a crystalline solid whose electrical conductivity is intermediate between that of conductors and insulators. Thus, an important application of this material is in the electronics industry and in processes of heterogeneous photocatalysis.

The principle of heterogeneous photocatalysis involves the activation of a semiconductor by solar or artificial radiation. A semiconductor is characterized by two energy regions: the region of lower energy is the valence band (VB), where the electrons cannot move freely, and the higher region is the conduction band (CB), where the electrons move freely through the crystal, producing electrical conductivity similar to that of metals. These two regions are divided by a "band-gap" zone. Figure 1 shows a schematic representation of a semiconductor particle. The absorption of photons with energy higher than the band-gap energy (E_G) causes the promotion of an electron from the VB to the CB, with the concomitant generation of a gap (h+) in the E_V. In the absence of suitable scavengers species, the stored energy is dissipated within milliseconds by recombination, with the formation of an unpaired electron. If a suitable scavenger or a surface defect is available to contain the electron or gap, recombination is prevented and redox reactions occur subsequently. E_V gaps are potent oxidants (potential of +1.0 to +3.5 V, depending on the semiconductor and pH) that are able to generate radical species (HO•, O_2•, HO_2•, etc.) from water molecules adsorbed on the semiconductor surface, which can subsequently oxidize other molecules (Nogueira and Jardim, 1998, Gaya and Abdullah, 2008, Malato et al., 2009). There are indications that the reaction occurs only in the adsorbed phase of the semiconducting particle,

hence, organic molecules that can effectively adhere to the surface of the photocatalyst are more susceptible to direct oxidation (Gaya and Abdullah, 2008).

The minimum E_G required for a photon to cause the photogeneration of charged species in TiO_2 (anatase form) is 3.2 eV, which corresponds to a wavelength of 388 nm. In fact, the photoactivation of TiO_2 occurs in the range of 300-388nm (Nogueira and Jardim, 1998, Gaya and Abdullah, 2008). Thus, the strong resistance of TiO_2 to decomposition and photocorrosion, its low cost, and the possibility of using solar UV radiation, makes it particularly interesting for processes of heterogeneous photocatalysis (Malato et al., 2009).

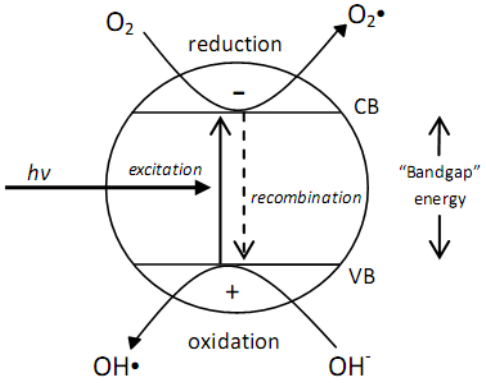

Figure 1. Schematic representation of a TiO_2 particle, where VB and CB are the Valence Band and Conduction Band, respectively (adapted from Nogueira and Jardim, 1998)

Many studies have demonstrated the potential use of heterogeneous photocatalysis with TiO_2 for the degradation of organic and inorganic compounds (Chatterjee and Dasgupta, 2005, Fujishima and Zhang, 2006). For the most part, photodegradation leads to the total mineralization of pollutants, generating CO_2, H_2O and inorganic acids (Malato et al., 2009). This property is applicable in the production of self-cleaning surfaces, cleaning products, in the remediation of contaminated soil and water, or even the deodorization of environments and the

destruction of gas-phase volatile compounds. The hydroxyl radicals generated during TiO_2 irradiation are also able to react with most biological molecules, resulting in bactericidal and virucidal activity (Nogueira and Jardim, 1998, Li et al., 2008).

Studies suggest that anatase and rutile have different photocatalytic properties, with anatase possessing the better combination of photoactivity and photostability (Gaya and Abdullah, 2008, USEPA, 2009). The rutile form is inactive for the photodegradation of organic compounds, although the reason for this is not completely clear (Nogueira and Jardim, 1998, Malato et al., 2009). However, the low adsorption capacity of O_2 on its surface is pointed out as one of the possible factors.

Among the different titanium oxide products, TiO_2 P25 fabricated by Evonik Degussa Corp. (Germany) is the one most commonly used because of its reasonably well defined nature (typically a mixture of 70:30 anatase:rutile, nonporous, surface area of about 50 m^2/g, and average particle size of 30 nm) and its high photoactivity when compared to that of other sources (Nogueira and Jardim, 1998, Malato et al., 2009).

Surface treatment of nano-TiO_2 can alter its light absorption and photocatalytic activity. In applications such as paints, coatings and cosmetics, which require chemical stability, the photocatalytic properties of TiO_2 are generally suppressed by coatings it with silica and aluminum layers (Diebold, 2003, Li et al. 2008). Doping of nanostructured TiO_2 materials has also often been employed to modify its band-gap energy and increase its photocatalytic activity.

TiO_2 is generally used in suspension (also called slurry), but can also be used immobilized in an inert matrix coating surfaces (Gelover et al., 2006, Gaya and Abdullah, 2008, Malato et al., 2009). Immobilized TiO_2 has been reported to have low catalytic activity when compared to systems in suspension (Gaya and Abdullah, 2008, Malato et al., 2009). The

mineralization rate generally increases with the concentration of the catalyst up to a limit of high concentration. Wei et al. (1994) used P25 for the disinfection of *E. coli* in water and reported that the disinfection rate depended mainly on two variables: the intensity of incident light and the TiO_2 dose.

In general, for any photocatalytic application, the optimal concentration should be determined in order to avoid an excess of catalyst and to ensure the total absorption of photons, i.e., to ensure the entire exposed surface of the particles is illuminated. When the concentration of TiO_2 is too high, the turbidity prevents radiation from penetrating and reaching all the particles (Herrmann, 1999). In photocatalysis studies, the optimal of TiO_2 have been a temperature of 20 to 80^{o}C, a concentration of 200-500 mg/L, oxygen concentration of $pO_2 \geq$ 0.21 atm and pH preventing pHzpc (Malato et al., 2009).

NPs tend to aggregate in the environment and can therefore be eliminated or captured by sedimentation. NP aggregates are generally less mobile and can interact with filtering organisms and with organisms that feed on sediment, or even with suspended organic matter. It is therefore important to understand the behavior of TiO_2 NPs in aquatic environments in order to understand their toxicology. The pH, ionic concentration and nature of the electrolytes in aqueous suspensions have been reported as important parameters in the aggregation of nano-TiO_2 (Sharma, 2009).

The pH of aqueous solutions significantly affects TiO_2, including the particle charge, the size of aggregates and the position of the VB and CB. The pH at which the surface of an oxide has no electrical charge is defined as the zero point charge (pHzpc). The pHzpc of nano-TiO_2 varies from 4.5 to 7, depending on the particle's size and crystal shape, with smaller particles presenting lower pHzpc (Kosmulski, 2002 cited by Sharma, 2009). Finnegan et al. (2007)

reports pHzpc values of ~5.9 for rutile and of ~6.3 for anatase. A pHzpc of 6.3 has been reported for Degussa P25 (Kosmulski, 2009).

The surface of titanium will remain positively charged in an acid medium and negatively charged in an alkaline medium (Gaya and Abdullah, 2008). The lack of surface charge renders an electrostatic potential null, because it does not produce the repulsive interaction needed to separate the particles in the liquid. Therefore, TiO_2 particles tend to aggregate close to the pHzpc.

Particle aggregation interferes in the ability of the suspension to transmit or absorb radiation. However, this variation in particle size may be an advantage when the objective is to separate TiO_2 from water (by sedimentation and/or filtration) at the end of a photocatalytic treatment (Malato et al., 2009).

Like other NPs, nano-TiO_2 can bind to organic matter, thus modifying its properties and behavior. The adsorption of acid fulvic and humic acid on nano-TiO_2 has proved to be pH-dependent and favors the dispersion and suspension of these particles in aquatic environments (Domingos et al., 2008, Yang et al., 2009). On the other hand, the adsorption of oxalic acid appears to destabilize nano-TiO_2 suspensions, increasing the sedimentation rate at pH 2, although no change in the sedimentation rate has been observed at pH 6.5 (Pettibone, 2008).

The adsorption of organic matter on nano-TiO_2 may also alter the adsorption of toxic compounds (Sharma, 2009). Nano-TiO_2 has been reported to show adsorption behavior towards metals such as Cu(II), Cr(III), Mn(II), Ni(II), Zn(II), Cd(II), Mo(VI) (Kaur and Gupta, 2009). When an aqueous suspension of bacteria and other microorganisms is in the presence of TiO_2 in the dark, a slight reduction in the concentration of colonies can be observed due to the possible

agglomeration of TiO_2 with the bacterial cells and subsequent sedimentation (Malato et al., 2009).

3. STUDIES OF AQUATIC ECOTOXICOLOGY OF TIO_2

NPs differ from bulk particles in terms of their heterogeneous size distribution, surface charge, composition, degree of dispersion, etc. Therefore, in a toxicology study, it is important to determine not only their exposure concentration but also other measures (Hasselov et al., 2008). At the NanoImpactNet Workshop held in 2008, a list was proposed of the six principal characteristics of nanomaterials to be discriminated in environmental studies: size, dissolution/solubility, surface area, surface charge and surface chemical composition. Information such as size distribution, crystal structure, morphology, agglomeration/dispersion, etc. may also be important (Stone et al., 2010). Nonetheless, the authors recognize that the characterization of nanomaterials may be time-consuming and costly, as well as complex, and therefore its application should depend on the objectives of the study (Stone et al., 2010). It was also agreed that the properties should be characterized in test systems and not in the "bottles" that are supplied, and that certain properties such as agglomeration and dissolution should be listed as "rates" rather than "states" in view of the dynamic nature of nanoparticulate systems.

Unfortunately, methods to measure all the properties are not available. For example, there is still no method available to measure the surface area in an aqueous dispersion of NPs. Moreover, there is still a paucity of information about the extent to which the limitations of the different methods may influence the correct interpretation of results. The bias of a technique can be reduced by using multiple techniques, although this is difficult due to time and cost constraints

(Stone et al., 2010). Hasselöv et al.'s paper (2008) presents information about the main methods available for the characterization of NPs.

The fact that TiO_2 is highly insoluble, non-reactive with other materials, thermally stable, and non-flammable enabled it to be declared innocuous to the organism (WHO, 1969). However, studies have demonstrated an apparently species specificity in the generation of lung tumors in rats that inhaled TiO_2 for long periods (Hext et al., 2005). In addition, other significant data in the literature confirm the occurrence of lung inflammation, oxidative stress and involvement of other organs after respiratory and oral exposure to nano-TiO_2 (Ferin et al., 1992, Wang et al., 2007, Warheit et al., 2007a). Recently, the International Agency for Research on Cancer (IARC) classified TiO_2 as "possibly carcinogenic for humans" (IARC, 2010).

The various possible sources of contamination of water bodies by nano-TiO_2 make it essential to assess its effects on ecosystems, i.e., its ecological, public health and economic consequences. There is still a paucity of studies about the presence of nano-TiO_2 in the environment. Natural TiO_2 NPs have been found in river water (Wigginton et al., 2007). In Switzerland, due to the climatic conditions, researchers reported nano-TiO_2 particles peeling off painted facades and being carried into surface waters, Ti concentrations of about 16 µg/L were found in urban runoff (Kaegi et al., 2008).

Nanoecotoxicology studies are relatively recent, the first publication involving an assay with fishes dated 2004 (Oberdorster, 2004). Tables 1 to 3 summarize published works about the effects of TiO_2 NPs on aquatic organisms.

With regard to the bioavailability of nano-TiO_2 to aquatic organisms, the literature is still inconclusive. In a recent paper, Johnston et al. (2010) did not observe significant absorption of nano-TiO_2 in *Oncorhynchus mykiss* exposed for 10 days to concentrations of up to 5 mg/L.

Federici et al. (2007) also did not find accumulation of nano-TiO_2 in *O. mykiss* exposed for 14 days to concentrations of up to 1 mg/L. On the other hand, some studies report that the nano-TiO_2 present in water may accumulate in *Cyprinus carpio, Danio rerio* and *Daphnia magna*, even at concentrations of 0.1 and 1 mg/L, although low factors of bioconcentration were determined (Zhang et al., 2006, Zhu et al., 2010a, b). Zhu *et al.* (2010a) report the occurrence of trophic transfer of nano-TiO_2 in *D. rerio* fed with contaminated daphnids, but discard the possibility of biomagnification. Other studies have shown that the presence of nano-TiO_2 may elevate the absorption of other contaminants in fishes, such as As and Cd (Sun et al., 2007, 2009, Zhang et al., 2007).

The results of toxicity tests have usually been expressed as lethal (LC_{50}), effective or inhibitory (EC_{50}) concentrations that cause, respectively, mortality, abnormality of inhibition to 50% of the exposed organisms. A wide variability has been found in the results reported in the literature with regard to toxicity tests. This variability may be due to the different characteristics of nano-TiO_2 and treatments applied, as well as to experimental designs. Thus, exhaustive discussion has focused on the need for the proper characterization of NPs under study, and for the standardization of nanoecotoxicological evaluation methods. The lack of information in some works makes it difficult to compare results (Warheit et al., 2008). Discussions have also focused on the lack of analytical techniques for the characterization of NPs in the media utilized for ecotoxicological assays.

Lovern and Kapler (2006) reported an LC_{50} of 5.5 mg/L in *D. magna* exposed for 48 h to filtered nano-TiO_2, but did not observe mortality or behavioral abnormalities after exposure for the same period to concentrations of up to 500 mg/L of the same nano-TiO_2, although the suspension was sonicated. Although several authors considered acute exposure to nano-TiO_2 of

low toxicity to *Daphnia* (Warheit et al., 2007b, Griffith et al., 2008, Heinlaan et al., 2008, Lee et al., 2009, Strigul et al., 2009, Wiench et al., 2009, Kim et al., 2010, Rosenkranz, 2010), prolonged exposure has presented varied results. The exposure of *D. magna* to Degussa P25 (sonicated) for 21 days showed a LC_{50} of 2.62 mg/L and alteration of the reproduction and growth rates (EC_{50} 0.46 mg/L) (Zhu et al., 2010b), while exposure for the same period to different types of BASF nano-TiO_2 (sonicated) did not cause mortality but reduced the reproductive capacity (EC_{50} 26.6 mg/L) (Wiench et al., 2009). Kim et al. (2010) did not find reproductive impairment but reported a 70% mortality rate in *D. magna* exposed for 21 days to 5 mg/L of Sigma Aldrich nano-TiO_2.

Some studies appear to suggest that nano-TiO_2 has low acute toxicity for fishes, and LC_{50} is indicated as 124,5 mg/L for *D. rerio* (Xiong et al., 2011) and >100 mg/L for *O. mykiss* (Warheit et al., 2007b). Similarly, the exposure of *D. rerio* eggs to nano-TiO_2 for 96 hours at concentrations of up to 500 mg/L did not cause alterations in the survival and hatching rates, or malformations (Zhu et al., 2008). The exposure of embryos of *Pimephales promelas* to concentrations of up to 1 mg/L for 7 days also caused no significant mortality or observable malformations (Jovanovic et al., 2011). On the other hand, some studies have shown that the prolonged exposure of fish to concentrations of 1 to 200 mg/L did not cause mortality, but observed dose-dependent elevation of the respiratory rate and swimming behavior, as well as increased production of mucus (Federici et al., 2007, Hao et al., 2009).

Evidence of adverse effects of a given contaminant at sublethal concentrations is extremely important in environmental risk assessment, since it may generate a cascade effect with consequences at the level of individuals, communities and the ecosystem. Thus, the use of biomarkers in risk assessments offers the advantage of allowing for the detection of potentially

toxic exposure well before real adverse effects occur (Nascimento et al., 2008, Prospéri and Nascimento, 2008).

Studies have shown that the toxicity of some nanomaterials such as TiO_2 may be implicated in the generation of reactive oxygen species (ROS) (Kahru and Dubourguier, 2009, Pelka et al., 2009, Sharma et al., 2009). ROS can react with the majority of biomolecules and damage lipids, proteins and nucleic acids (Valavanidis et al., 2006).

Exposure in aqueous media appears to be more severe than via the diet for *O. mykiss* (Handy et al., 2008). The prolonged exposure of fish to nano-TiO_2 induced biochemical and histopathological alterations in their gills, liver and intestines (Federici et al., 2007, Hao et al., 2009, Johnston et al., 2010, Palaniappan and Pramod, 2010). Exposure to nano-TiO_2 can trigger oxidative stress in *D. magna*, fishes and mollusks (Federici et al., 2007, Hao et al., 2009, Canesi et al., 2010a, Kim et al., 2010, Xiong et al., 2011). Lysosomal instability has also been reported in polychaetes and mollusks exposed to nano-TiO_2 (Canesi et al., 2010a, Galloway et al., 2010). The intravenous administration of high doses of nano-TiO_2 in fish has shown that it accumulated in the kidneys, with slow depuration, but no significant alterations were observed in the function of this organ (Scown et al., 2009). An experiment with *D. magna* showed that even after a period of 72 hours in clean water, the depuration of adsorbed TiO_2 was not complete (Zhu et al., 2010b).

With regard to genotoxicity in aquatic organisms, nano-TiO_2 presents controversial results. Nano-TiO_2 has presented genotoxicity in some studies (Griffith et al., 2009, Galloway et al., 2010, Jovanovic et al., 2011) but not in others (Lee et al., 2009). Griffith et al. (2009) reported that exposure to nano-TiO_2 altered the expression of 171 genes in *D. rerio* involved mainly in ribosome structure and activities, but not in the regulation of oxidative stress. Jovanovic et al. (2011) also observed upregulation of genes involved in inflammatory response (especially in

phagocytic processes), and suppression of neutrophil function in fish that received an intraperitoneal dose of nano-TiO_2. The immune system also appears to be an important target of TiO_2 NPs in bivalves (Canesi et al., 2010b).

In bioassays with aquatic organisms, the circadian cycle is usually established using fluorescent lamps. These lamps emit basically visible light, while in natural conditions these organisms are exposed to solar radiation (infrared, visible and ultraviolet light). There is ample evidence of the formation of reactive oxygen species when TiO_2 is exposed to UV radiation (Brezová et al., 2005). Several studies have reported the phototoxic effects of TiO_2 bulk or NPs), and its consequent use in the disinfection of water (Wei et al., 1994, Carp et al., 2004, Adams et al., 2006). The photocatalytic properties of nano-TiO_2 can augment its toxic effects in aquatic organisms under environmental conditions, but few studies so far have taken this into consideration. *In vitro* studies have shown that co-exposure to nano-TiO_2 and ultraviolet radiation increases cyto- and genotoxicity in fish cells (Reeves et al., 2008, Vevers and Jha, 2008). The pre- and co-illumination of nano-TiO_2 has also been shown to elevate its toxicity in daphnids (Hund and Rinke, 2006, Marcone et al., 2012).

There are still uncertainties about the characterization of exposure to nanoparticles in the testing systems of all ecotoxicity assays except those that involve the oral administration of nanoparticles. These uncertainties include how the substance is dosed and maintained in the test medium, the measurement and characterization of NPs in the test system, the understanding of the abiotic factors that influence the behavior of NPs in the test system, and a consensus about the dosimetry (Crane et al., 2008).

Today there are several guidelines for conducting ecotoxicological assays (OECD, USEPA, DIN Standards, etc.). However, their use for nanoecotoxicological assays is still under

question (Stone et al., 2010). The use of these methodologies must be evaluated for each type of nanoparticle. Testing with nano-TiO_2 presents various particularities, such as its photocatalytic properties and absorption of UV radiation, its aggregation and sedimentation behavior in water and its interaction with organic matter. Performing assays to determine lethal and effective concentrations in the proposed ranges of concentration is particularly difficult. The OECD, for example, suggests finding the LC_{50} up to the concentration of 100 mg/L, however, nano-TiO_2 forms a whitish suspension when dissolved in water, and in concentrations equal to or higher than 10 mg/L, it precipitates rapidly if no dispersion method is used. Wiench et al. (2009) found that TiO_2 does not disperse well at 10-100 mg/L and that sedimentation occurs within 24-48 hours. For uncoated TiO_2 (BASF, >99%, 70% anatase, 30% rutile, 20-30nm, 48.6m^2/g), the concentration in supernatant after 16 hours went from 100 to 83 mg/L in bidistilled water and to 33 mg/L in surface water, while agglomeration and sedimentation of coated TiO_2 were slow. Some studies have involved semi-static aquatic bioassays, changing the exposure medium every 24-48 hours (Tables 1, 2 and 3), while others have performed static assays involving mainly acute exposure.

Table 1 – Summary of papers published about the effects of nano-TiO_2 used in toxicology studies on microcrustaceans

Test species	*Product tested*	*Treatment of the product*	*Physicochemical characterization*	*Bioassay*	*Results*
D. magna (Kim et al., 2010)	Sigma Aldrich nano-TiO_2 (40 nm, 30% rutile, 70% anatase)	10% solution in water with pH 2 (without sonication) → stock solution (1 mg/L) in moderately hard synthetic water (MHW).	N4 and DLS submicron particle analyzer.	Acute assay 48h. Without feeding during the test. USEPA 1993. Chronic assay, semi-static, 21 days. Renewal of medium and daily feeding. Concentrations tested: 0, 1, 2, 5, 10 mg/L. Evaluations were made of SOD, GPX, CAT and GST activity in groups exposed for 5 days to 0, 0.5, 1, 2.5, 5, and 10 mg/L of TiO_2. GPX and GST were also tested after fractionation of the nanoparticles (<200, <400, and <800 nm)	Acute assay: mortality did not reach 50% even at 10 mg/L, so the LC_{50} could not be determined. Chronic assay: highest mortality at 5 and 10 mg/L (70 and 80 %, respectively). No reproductive impairment observed. Increase in CAT at 10 mg/L, no difference in SOD, GPx highest at 5 mg/L, GST increased at 5 and 10 mg/L. TiO_2 was found in the intestines of daphnids and glued to their antennae and external surface.
D. magna (Rosenkranz, 2010)	Degussa P25 nano-TiO_2	100 mg/L solution was prepared in culture medium for daphnids → sonication (30 min). The remaining solutions were made from serial dilutions of 1:10.	INA	Acute assay 48h. No food during the test. 100, 10, 1 and 0.1 mg/L. Chronic assay 21 days. Medium changed daily. Daily feeding. Concentrations: 0.001, 0.1 and 1 mg/L	Acute assay 48h: 10% mortality at 100mg/L. High molt frequency, dose-dependent. Chronic assay: high molt frequency only on the first day of exposure, at 1mg/L.
D. magna (Zhu et al., 2010b)	Degussa P25 nano-TiO_2 (21nm, 50m^2/g, 20% rutile, 80% anatase) Size of aggregates in culture medium: 1h - 580.5 nm; 12h – 2349.0 nm 24h – 3528.6 nm	Stock solution (1 g/L) in ultrapure water → sonication (10 min, 50 W/L, 40kHz) → new sonication (10 min, 50W/L, 40kHz) prior to dilution in culture medium for daphnids.	SEM, DLS ICP-OES (concentration of Ti in the solution and in daphnids).	Acute assay 72h semi-static. OECD 202. Medium renewed daily. No food during the test. Concentrations tested: 0.1, 0.5, 1, 5, 10, 50 and 100 mg/L. Chronic assay 21 days semi-static. OECD 211. Daily renewal of medium and daily feeding. Concentrations tested: 0.1, 0.5, 1 and 5 mg/L. Bioaccumulation and depuration test 24h of accumulation (samples were collected at 0, 2, 6, 12 and 24h) and 72h of depuration (samples were collected at 6, 12, 24, 48 and 72h). Concentrations tested: 0.1 and 1 mg/L with and without daily feeding.	Acute assay In 48h: NOEC <50 mg/L, EC_{50}> 100 mg/L, LC_{50} > 100 mg/L. In 72 h: NOEC<0.1 mg/L ; EC_{50} = 1.62 mg/L; LC_{50} = 2.02 mg/L. Chronic assay At 0.1 mg/L reproduction declined. At 0.5mg/l reproduction and growth were inhibited. Mortality was recorded in groups 1 and 5 mg/L after 8 days of exposure. EC_{50} = 0.46 mg/L, LC_{50} = 2.62 mg/L. The feeding rate decreased as the exposure concentration increased (exposure of 5h). Bioaccumulation test Group 0.1 mg/L: Concentration plateau in 12 h, BCF= 5.66x 10^4, time elapsed to accumulate 50% of the saturation level = 3.87h, time to reach 50% depuration = 26.76h. Group 1mg/L: Plateau in 24h; BCF =1.18x10^5, time elapsed to accumulate 50% of the saturation level = 3.72h, time to reach 50% depuration = 74.52h. Depuration was not complete, 20% of the saturation concentration remained in the daphnids at the end of the experiment.

					Feeding during exposure to TiO_2 increased the accumulation time and reduced the depuration time.
D. magna and *Chironomus riparius* (larvae) (Lee et al., 2009)	Sigma Aldrich nano-TiO_2 7nm (300.81 m^2/g) and 20 nm (66.604 m^2/g)	Solution (1 mg/L) in culture medium → sonication (15 min).	TEM, BET	Acute assay 96h. OECD 1984, 1998. Concentration tested: 1 mg/l.	No genotoxicity (comet assay), alteration in growth, mortality or reproduction were observed in any group.
D. magna (Strigul et al., 2009)	nano-TiO_2 prepared by hydrolysis of the titanium sulfate solution (6 nm, agglomerates 0.5 -2 mm)	Stock solution → sonication (30 min).	DLS	Acute assay 24 and 48h. OECD 202. Concentrations tested: 2.5; 8; 25; 80; 250 mg/L.	TiO_2 presented low toxicity and LC_{50} could not be calculated. Animals exposed to 80 and 250 mg/L for 24h were slower.
Daphnia magna (Wiench et al., 2009)	Bulk TiO_2 BASF nano-TiO_2: - non-coated (>99 %; 70/30 anatase/rutile; 20-30 nm; 48.6 m^2/g) - T-LITE SF (80 %, 50nm; 100m^2/g; rutile) - T-LITE SF-S - T-LITE SF-MAX It was found that 10-100 mg/L did not disperse well and sedimentation occurred in 24-48h. For non-coated TiO_2, after 16h in bidistilled water, the concentration in the supernatant went from 100 to 83mg/L and to 33 mg/L in surface water. For coated TiO_2, agglomeration and sedimentation were slow.	Stock solution (100 mg/L) in demineralized water → sonication (5min) or magnetic agitation (10 min) or both methods → dispersion in M4 or SW medium (natural surface water) → sonication and filtration (2 µm) → UV irradiation (30 min 20 W/m^2).	TEM, ultracentrifugation.	Acute assay 48h. OECD 202. Chronic assay 21 days. OECD 211. Only with T-Lite SF-S –semi-static assay (medium changed 3 times per week). Daily feeding. Concentrations tested: 0.01 to 100 mg/L.	Acute assay EC_{50}>100 mg/L in at the treatments EC_{10} non-coated nano-TiO_2 sonicated in M4 = 85.1 mg/L. In SW= 3.7 mg/L. Bulk TiO_2 sonicated in M4= 91 mg/L; in SW = 13.8 mg/L. Chronic assay There was no mortality, but reproductive effects were observed. NOEC=3 mg/L LOEC=10 mg/L EC_{50}=26.6 mg/L EC_{10} = 5.02 mg/L.
D. pulex and *Ceriodaphnids dubia* (Griffith et al., 2008)	Degussa P25 nano-TiO_2 (20 % rutile, 80 % anatase, 45.41 m^2/g; 20.5 ± 6.7 nm; ZP -25,1; polydisperse 0.197, largest particle diameter observed in suspension was 687.5nm)	Stock solution in ultrapure water (1 mg/mL) → sonication (6 W, 22.5 kHz, 6 one-half second pulses).	BET; Coulter LS 13 320; polydispersity; Zeta reader Mk 21-II; scanning micrographs.	Acute assay 48h static. American Society for testing and materials guidelines. No food given during the test.	LC_{50} >10 mg/L for both tested organisms.
D. magna and *Thamnocephalus platyurus* (Heinlaan et al., 2008)	Sigma Aldrich nano-TiO_2 (25-70 nm). Riedel-de Haen bulk TiO_2	Stock solution in ultrapure water (40 g/L) → sonication (30 min) → storage at 4°C → vortex → exposure dosage.	INA	*D. magna*: Acute assay 48h, in the dark. Standard Operational Procedures of Daphtoxkit FTM magna (1996). *T. platyurus* (larvae): Acute assay 24h, in the dark. Thamnotoxkit FTM (1995). Concentrations tested: 0.01 to 20000 mg/L	*D. magna*: LC_{50} of nano-TiO_2 ~ 20000 mg/L. (Bulk TiO_2 and other values of toxicity were not tested). *T. platyurus*: LC_{50}, LC_{20} and NOEC of nano- and bulk TiO_2 > 20.000 mg/L.
D. magna (Lovern et al., 2007)	Nano-TiO_2 30 nm (in suspension)	THF was used to ensure dispersion. The THF was eliminated by evaporation and filtration and confirmed by spectrophotometry.	TEM. Characterization according to Lovern and Klaper (2006).	Acute assay 60 min. USEPA 23. Concentration tested: 2ppm (LOEC calculated in a previous experiment).	TiO_2 did not significantly alter the heat rate, jump, movement of appendices, and curvature of the abdominal claw.

D. magna (Warheit et al., 2007b)	DuPont Haskell TiO_2: fine TiO_2 (380 nm in water, 5.8 m^2/g, 100% rutile, 99% TiO_2 and 1% aluminum) afC (140 ± 44 nm in water; 38.5 m^2/g; 79 % rutile 21 % anatase; 90% TiO_2 ; 1% amorphous silica; 7 % aluminum).	INA	DLS, BET, X-ray fluorescence, X-ray diffraction.	Acute assay 48h, static. OECD 202. Concentrations tested: 0.1, 1, 10 and 100 mg/L.	LC_{50} 48h >100mg/L for both types of TiO_2. There was 10% of immobility at concentrations of 10 and 100mg/L at the end of 48h for both compounds tested.
D. magna (Adams et al., 2006)	Sigma Aldrich nano-TiO_2 65 nm, 950 nm and 44 µm. Smaller particles (65 nm) appeared larger (on average 320 nm) and larger ones (950 nm and 44 µm) appeared smaller (320 nm and 1 µm), respectively, when in suspension.	Solution in ultrapure water (10 g/L) → agitation → exposure dosage	DLS optical microscopy.	Prolonged assay 8 days. Concentrations tested: 1, 10 and 20 ppm.	20 ppm of nano-TiO_2 was lethal for 40% of the organisms.
D. magna (Hund Rinke and Simon, 2006)	Product 1: 25 nm, mainly anatase. Product 2: 100 nm, 100% anatase.	The TiO_2 suspension was agitated and pre-illuminated in SUNTEST CPS. Particles were washed following the manufacturer's instructions → mother solution → sonication → continuous agitation and irradiation in a solar light simulation system (300-800 nm 250W, 30 min) → samples were transferred and incubated for 72 h with visible light.	INA	Acute assay 48h. ISSO 6341, OECD 202 and DIN 38412-30. Concentrations tested: 1, 1.5, 2, 2.5, 3 mg/L.	There was no concentration-effect curve, so the EC_{50} could not be determined for any group. Pre-illumination increased the toxicity of the two nano-TiO_2 products. E.g.: at 1and 2.5 mg/L of product 1, immobilization went from 0 to 20% and from 28 to 73%, respectively, when there was pre-illumination.
D. magna (Lovern and Kapler, 2006)	INA. Mean diameter of filtered TiO_2: 30nm; in sonicated solution: 100 to 500 nm	Solutions were prepared in three ways: 1) Dilution in distilled water → sonication for 30 min. 2) 20mg were placed in 200 mL THF → pulverization with nitrogen → over-night on moving plate → filtration → dilution in deionized water → evaporation of the THF → filtration. 3) Same as 2, but without THF.	TEM spectrophotometry.	Acute assay 48h. USEPA 2024. No food given during the test. Groups: 1) control, 2) THF group, 3) filtered TiO_2 (0.2, 1, 2, 5, 6, 8, and 10 ppm), and 4) sonicated and non-filtered TiO_2 (50, 200, 250, 300, 400, and 500 ppm).	Filtered TiO_2: there was no mortality at 0.2 ppm, but 1% mortality at 1ppm. LC50=5.5 ppm; LOEC= 2 ppm; NOEC= 1 ppm. Sonicated TiO_2: no group suffered mortality > 9%. NOEC, LOEC and LC_{50} not applicable. When there was no mortality, no immobility or swimming abnormalities were observed in any group.

BCF = bioconcentration factor
BET= Brunauer, Emmett, Teller method for surface area calculation
CAT = catalase activity
DLS = dynamic light scattering
EC_{10} = effective concentration for 10% of exposed organisms
EC_{50} = effective concentration for 50% of exposed organisms
GPX – glutathione peroxidase activity
GST = glutathione S-transferase activity
ICP-OES = inductively coupled plasma optical emission spectroscopy
LC_{50} = lethal concentration for 50% of exposed organisms
LOEC = lowest observed effect concentration
NOEC = no observed effect concentration
ZP = zeta potential
SEM = scanning electron microscopy
SOD = superoxide dismutase activity
TEM= transmission electron microscopy
THF = tetrahydrofuran
INA = information not available

Table 2– Summary of papers published about the effects of nano-TiO_2 used in toxicology studies on fishes

Test species	*Product tested*	*Treatment of the product*	*Physicochemical characterization*	*Bioassay*	*Results*
D. rerio adults (Xiong et al., 2011)	nano-TiO_2 from Nanjing University of Technology (anatase, purity 99%, diameter 20-70 nm, hydrodynamic diameter 251 – 630 nm, ZP -13, 1 mV) bulk TiO_2 from Tianjin Guangcheng Chemical Reagent Co. (anatase, purity 99%, diameter 128-949nm, hydrodynamic diameter 272-597, ZP -27,8mV)	test suspension in aerated single-distilled water → sonication (1,5 L,100 W, 40 kHz for 20 min).	TEM, DLS	Acute assay 96h, semi-static (solution changed every 24h). No food given during the test. Concentrations tested: 0, 10, 50, 100, 150, 200 and 300 mg/L. From biomarkers analysis, fish were exposed to 50 mg/L under light or dark conditions.	nano-TiO_2 LC50 = 124.5 mg/L SOD activity decreased in liver tissues and increased in gut tissues, in both groups (under light or dark conditions). CAT activity in liver tissue was observed to be reduced in both groups. There was elevated protein carbonyl levels. Lipid peroxides were also found in the gills and gut tissues. GSH content increased in gut tissue, and (under dark conditions) decreased in liver. MDA concentrations increased in gills and gut tissues. Morphological changes in gill cells (cell membrane damage, irregular cell outlines, pyknotic nuclei and a trend of complete disruption of gill cells). bulk TiO_2 LC50 > 300 mg/L no changes in SOD and CAT activities and in MDA content. There was an increase in GSH in gut tissue.
O. mykiss (Johnston et al., 2010)	Nano-TiO_2 (34.2 ± 1,73 nm, ZP -9), bulk TiO_2 and ionic titanium (titanium metal standard solution, Fisher Scientific).	Stock solution (250 µg/L) in ultrapure water → sonication (30min) → exposure dosage.	TEM, ICP-MS, DLS, particle sizer, CARS, multiphoton microscopy.	Prolonged assay 10 days, semi-static (change of 50% of the water every 2 days). Concentrations tested: 500 (nano-TiO_2) and 5000 µg/L (nano- and bulk TiO_2 and ionic Ti). Test exposure via diet 21 days. Concentrations tested: 0.01 and 0.1% nano-TiO_2 in food.	No significant absorption of Ti was detected in any group. The Ti concentration in the gills increased in the group exposed to ionic Ti. High levels of Ti were found in the stomach of fish fed with medium and high doses of TiO_2. TiO_2 aggregates were found on the surface of the gill epithelium after 24 and 96h of exposure and inside lamellae after 14 days of exposure.
D. rerio adult (Palaniappan et al., 2010)	Sigma Aldrich nano-TiO_2 (purity 99.7%, anatase, 20 nm, 200 ± 20 m^2/g). Particle size: 14.1 ± 1.52 nm. Nice Chemicals bulk TiO_2 (99.7% purity, anatase).	Stock solution (10 ppm) in ultrapure water → sonication (6 h) → storage at -20°C → sonication (30 min) → exposure dosage.	TEM.	Prolonged assay 14 days. Concentrations tested: 10ppm of nano-TiO_2 or 100 ppm of bulk TiO_2.	Mortality was not observed during the experiment. The biochemical constituents of the gills showed alterations. These alterations were greater in the group exposed to nano-TiO_2 than the one exposed to bulk TiO_2. Example: alterations in the amide I bands.

D. rerio (Zhu et al., 2010a)	Degussa P25 nano-TiO_2 (21 nm).	Stock solution (1 g/L in ultrapure water → sonication (10 min, 50 W/L, 40 kHz).	SEM, DLS.	Trophic transfer test. Daphnids were exposed to 0.1 or 1 mg/L of TiO_2 for 24h, after which they were collected and washed in culture medium and supplied to *D. rerio* as food. The test involved 14 days of absorption followed by 7 days of depuration (feeding with non-contaminated daphnids). The TiO_2 concentration in the daphnids was determined as follows: 4.52 ± 0.36 mg/g (in the group exposed to 0.1mg/L) and 61.09 ± 3.24 mg/g (in the group exposed to 1 mg/L). The fish were sampled on days 0, 1, 3, 5, 7, 10, 14, 15, 17, 19 and 21. Prolonged exposure test. 14 days, followed by 7 days of depuration. Semi-static (water changed daily). Concentrations tested: 0.1 and 1 mg/L.	No mortality or abnormalities were observed. Trophic transfer of TiO_2 occurred. There was no apparent biomagnification. Trophic transfer test Concentration of Ti in the fish group fed with daphnids 0.1 mg/L = 106.57 ± 14.89 mg/ kg and group fed with daphnids 1 mg/L = 522.02 ± 12.94 mg/ kg. BCF< 1. Prolonged exposure test Fish accumulated TiO_2, reaching a plateau of about 1.5 mg/kg on day 3 (group 0.1 mg/L) and of 100 mg/kg on day 10 (group 1 mg/L). BCF= 25.38 and 181.38 (at equilibrium for groups 0.1 and 1mg/L respectively). During the depuration phase, the concentration of TiO_2 in the entire body was found to decline.
D. rerio female adults (Griffith et al., 2009)	Degussa P25 nano-TiO_2 (45.41 m^2/g; ZP -25.1mV). Aggregates in powder 20.5± 6.7 nm; in suspension 220.8 to 687.5 nm.	Stock solution in ultrapure water → sonication (6s, 6W, 22kHz) → exposure dosage.	BET, SEM, scanning micrographs.	Acute assay 48h static. Concentration tested: 1000 µg/L.	Significant difference in the expression of 171 genes (microarray) - 60 up-regulated and 111 down-regulated (53 of these genes were affected by exposure to nano-copper and nano-silver). The affected genes were involved in ribosome structure and activity. No alteration was observed in genes related to regulation of oxidative stress. No histopathological differences were observed in the gills compared with the control group.
Cyprinus carpio juveniles (Hao et al., 2009)	Hongsheng Material nano-TiO_2 (50 nm, 30 ± 10 m^2/g, rutile 98%).	Solution → sonication (30 min, 100W, 40 kHz).	INA	Prolonged assay 8 days semi-static (solution changed daily). No food given during the test. Animals were collected on days 1, 2, 4 and 6 for biochemical analyses. For histopathology, the animals were exposed for 20 days. Concentrations tested: 10, 50, 100 and 200 mg/L.	No mortality occurred, but after 1 h of exposure the respiratory rate and swimming rates increased, as well as the production of mucus, in a concentration-dependent way. The biomarkers of oxidative stress varied with the concentration and exposure time. At 100 and 200 mg/L there was an increase in LPO and decrease in SOD, CAT and POD activity. The liver was more sensitive than the gills and brain. Histopathological alterations were observed mainly at the highest concentrations. The liver showed vacuolization of cytoplasm and autosomes, including necrotic cell bodies and nuclear fragments that looked like apoptotic bodies and some foci of lipidosis. The gills showed thickening, edema, fusion and hyperplasia of the lamellae and filaments.

Oncorhynchus mykiss juveniles (Scown et al., 2009)	Sigma Aldrich nano-TiO_2 (32.4nm, 46.3 m^2/g, purity >99.9 %, anatase and rutile). Particle size: 34.2 nm, 18.6 m^2/g (in powder). 400-1100 nm (in ringer and water). ZP: 0 at -0.6mV.	Solution (100 mg/L) in ringer → sonication (30 min).	BET	Intravenous administration (1.3 mg/kg). Fish and blood samples collected 6h and 90 days post-injection.	10 to 19% of injected Ti accumulated in the kidneys (up to 23 µg/g). The concentration in the kidneys did not change significantly from 6 h to 21 days post-injection, but after 90 days the concentration in the kidneys was significantly lower. The Ti level in the liver was approximately 15-fold lower. Preliminary studies showed that Ti did not accumulate in the brain, gills or spleen. No significant difference was found in blood TBARS at any time compared with the control. The histopathological analysis showed no alteration in the kidneys, but the TEM showed small aggregates apparently encapsulated around the tubules. Creatinine levels fluctuated in both the controls and the injected animals, but no effect was found in the plasma protein concentration.
C. carpio (Sun et al., 2009)	Degussa P25 nano-TiO_2 (50 m^2/g, 25 nm) Arsenite (As III) prepared from As_2O_3.	Stock solution of nanoTiO_2 (1 g/L) → sonication (10min, 50W/l, 40kHz) → exposure dosage.	INA	Chronic assay. Groups: 1) control, 2) only As III (200 µg/L ± 10.2); 3) As III + TiO_2 (10 mg/L ±1.3). Animals were placed in the aquariums 2h after the addition of As and TiO_2. Semi-static test (water changed daily). Animals were collected on days 2, 5, 10, 15, 20 and 25. Food was given once a day during the test. Speciation was evaluated of As in water, in the presence of TiO_2, with and without sunlight.	The concentration of As in the carps increased from 42% (20 days) to 185% (second day) in the presence of nano-TiO_2. The order of accumulation of As and TiO_2 in the different tissues was: viscera > gills > skin and scales > muscle. In the absence of sunlight, only a small amount of As III moved to As V (loaded, and therefore with less capacity to pass through biological membranes). With sunlight, about 75% of the As III moved to AsV in 1h.
Danio rerio adults and juveniles (Griffith et al., 2008)	Degussa P25 nano-TiO_2 (20% rutile, 80% anatase, 45.41 m^2/g; 20.5 ± 6.7 nm; ZP -25.1; polydispersion 0.197, largest particle diameter observed in suspension = 687.5 nm).	Stock solution (1 mg/mL) in ultrapure water → sonication (6W, 22.5kHz, 6 half-second pulses).	BET, Coulter LS 13 320; polydispersity; Zeta reader Mk 21-II; scanning micrographs.	Acute assay 48h static.	LC_{50}> 10 mg/L of nanoparticles
D. rerio embryos and larvae (Zhu et al., 2008)	Nanjing High Technology nano-TiO_2 (purity >99.5%, anatase, < 20 nm, mean size in suspension: 230 nm), bulk TiO_2 (purity >99%, anatase, 10000 nm).	Solution in ultrapure water → sonication (30 min).	DLS; optical microscopy; TEM	Fertilized eggs (1.5 h after fertilization) exposed to 1, 10, 50, 100 and 500 mg/L of nano- or bulk TiO_2. The solution was shaken mildly every 12h to maintain the concentration constant. Observations were made in an inverted microscope at 6, 12, 24, 36, 48, 60, 72, 84 and 96 h.	There was no mortality of embryos, no significant differences in the hatching rate, nor significant malformation in embryos and larvae.

O. mykiss juveniles (Federici et al., 2007)	Degussa P25 nano-TiO_2 (21 nm, 50 ± 15 m^2/g, 75% rutile, 25% anatase, purity 99%). Particle sizes were close to those specified by the manufacturer (24.2 ± 2.8 nm). The concentration of TiO_2 (spectrometry) in the tank reached 95-98% of the target value 10 min after dosing. The concentration in water was measured before changing the solution, to confirm that the concentration remained unchanged in 12h.	Stock solution (10 g/L) in ultrapure water → sonication (6 h, 35 kHz) → storage → sonication (30 min) → exposure dosage.	TEM, spectral scans.	Prolonged assay 14 days semi-static (80% of the water changed every 12h). Concentrations tested: 0.1; 0.5 and 1 mg/L. Food was withheld 24h prior to and during the test (except on day 10). Fish were sampled on days 7 and 14.	There was no mortality. The fish did not accumulate Ti. Changes in behavior and mucus secretion were observed at the highest concentration. The gills showed increased occurrence of edema in secondary lamellae, morphological changes in mucocyte, hyperplasia of primary lamellae, and aneurysm. Vacuolization and erosion of villosities in the intestines was observed, as well as loss of sinusoidal space, some foci of lipidosis, occasional necrotic cells and apoptotic bodies in the liver. No changes were observed in the brain. There was no clear effect of the treatment or of time on the Ti levels in the gills, liver or muscle. No hematological change was found. There was alteration of the levels of tissue Zn and Cu. A concentration-dependent reduction was found in the Na-K ATPase activity in the gills, intestines and brain at the end of the experiment (significant differences only among some groups). In general, there was an increase in TBARS at the end of the experiment in gills, intestines and brain, but not in liver. Concentration-dependent glutathione depletion occurred only in liver on day 14.
C. carpio (Zhang et al., 2007)	Degussa P25 nano-TiO_2 (50 m^2/g; 21nm).	Stock solution in ultrapure water.	Laser particle analyzer, zeta potential analyzer, ICP-OES, atomic fluorescence spectroscopy.	Chronic assay. Adsorption of Cd on TiO_2 and natural sediment particles (SP) were evaluated. Cd was added to the water (97.3 ± 6.9 µg/L) first, followed by TiO_2 (10 mg/L) or SP (10 mg/L). The animals were placed in the water 2 hours later. Food was given twice a day during the test. Fish were transferred to new solutions every day. The animals were sampled on days 2, 5, 10, 15, 20 and 25.	TiO_2 showed higher capacity to adsorb Cd than SP. SP did not have a significant influence on Cd in fish. The presence of TiO_2 elevated the accumulation of Cd. After 25 days of exposure, the concentration of Cd increased by 146 %, and was 22 µg/g. There was a positive correlation between the concentration of TiO_2 and Cd. TiO_2 and Cd accumulated mainly in the viscera and gills.
C. carpio (Sun et al., 2007)	Degussa P25 nano-TiO_2 (50 m^2/g, 25 nm, aggregates of 50- 400 nm in water.) Arsenate (As V) (prepared from Na_3AsO_4•$12H_2O$).	Stock solution of nano-TiO_2 (1g/L) → sonication (10 min, 50 W/L, 40 kHz) → exposure dosage.	TEM	Chronic assay, semi-static (water changed daily). Groups: 1) control, 2) only As V (200 µg/L ± 10.2); 3) As V + TiO_2 (10 mg/L ±1.3). Animals were placed in the aquariums 2h after the addition of As and TiO_2. Animals were collected on days 2, 5, 10, 15, 20 and 25. Food given once a day during the test.	
O . mykiss juveniles (Warheit et al., 2007b)	DuPont Haskell; Fine TiO_2 (380 nm in water, 5.8 m^2/g, 100% rutile, 99% TiO_2 and 1% aluminum). afC (140 ± 44 nm in water; 38.5 m^2/g; 79% rutile, 21% anatase, 90 % TiO_2; 1% amorphous silica; 7% aluminum).	INA	DLS, BET, X-ray fluorescence, X-ray diffraction.	Acute assay 96h static. OECD 203. Concentrations tested: 0.1, 1, 10 and 100 mg/L.	LC_{50} 96h> 100 mg/L for both types of TiO_2. There was 10% of immobility at the concentrations of 10 and 100 mg/L at end of 96h in both groups exposed to fine TiO_2.

BCF = bioconcentration factor
BET= Brunauer, Emmett, Teller method for surface area calculation
CARS = coherent anti-Stokes Raman scattering
CAT = catalase activity
DLS = dynamic light scattering
ICP-OES = inductively coupled plasma optical emission spectroscopy
ICP-MS = inductively coupled plasma mass spectroscopy
LC_{50} = lethal concentration for 50% of exposed organisms
LPO = lipid peroxidation
NOEC = no observed effect concentration
POD = peroxidase
ZP = zeta potential
SEM = scanning electron microscopy
SOD = superoxide dismutase activity
TBARS = thiobarbituric acid reactive substance assay
TEM = transmission electron microscopy
THF = tetrahydrofuran
INA = information not available

Table 3 – Summary of papers published about the effects of nano-TiO_2 used in toxicology studies on other aquatic organisms

Test species	*Product tested*	*Treatment of the product*	*Physicochemical characterization*	*Bioassay*	*Results*
polychaete *Arenicola marina* (Galloway et al., 2010)	Sigma-Aldrich nano-TiO_2 cat. no. 634662-1 (23.2 nm, equivalent spherical diameter 32.4 nm, 46.3 m^2/g, 99.9%, mixture of anatase and rutile; K 82.3 ppm, Zn 9.7 ppm, Na 6.0 ppm, Fe 3.1 ppm, Li 0.4 ppm). bulk TiO_2	Stock solution in ultrapure water → sonication (30 min) → mixed with natural treated sediment (collected at the same site where the animals were collected).	TEM, X-ray diffraction, ICP-OES	Prolonged assay 10 days. OECD/ASTM 1990. Exposure in seawater. Semi-static test (water changed every 48h). Feeding during the test. Concentrations tested: 1 to 3 g/kg of sediment.	The organic content of the sediment was 0.33 ± 0.4%. No behavioral alterations were detected. A change was observed in the feeding rate of the group exposed to 2 g/kg of nano-TiO_2 but not in the group exposed to 1 g/kg. No effect of exposure time was found. At 2 and 3 g/kg of nano-TiO_2, an impact was detected in the liposome stability (neutral red retention) and an increase in genetic impairment (comet assay). Bulk TiO_2 did not alter the rate of genetic damage compared to the control. Microscopy revealed TiO_2 aggregates of >200nm surrounding intestinal microvillosities, but no absorption by the intestinal epithelium, although TiO_2 remained in the lumen. BCF = 0.156 ± 0.075 (group 1g/kg) and 0.196 ± 0.038 (group 3 g/kg).
mollusk *Mytilus galloprovincialis* (Canesi et al., 2010a)	Degussa P25 nano-TiO_2 (purity >99.5%)	Stock suspension (100 µg/ml) in artificial seawater → sonication (15 min, 100 W, in a cold bath) → storage → sonication → exposure dosage.	TEM, BET, DLS.	Acute assay 24h. No feeding during the test. Concentrations tested: 0.05; 0.2; 1; 5 mg/l.	No mortality was found in any exposure condition. There was destabilization of the lysosomal membrane in hemocytes at 1 and 5mg/L and in the digestive glands at 0.2, 1 and 5 mg/L; as well as accumulation of lipofuscin and lysosomal neutral lipids in the digestive glands at 1 and 5 mg/L, and an increase in CAT at 1 and 5 mg/L and in GST at 0.2, 1 and 5 mg/L in the digestive glands.

BCF = bioconcentration factor
BET= Brunauer, Emmett, Teller method for surface area calculation
CAT = catalase activity
DLS = dynamic light scattering
GST = glutathione S-transferase activity
TEM = transmission electron microscopy

The literature reports nano-TiO_2 aggregates of about 500 nm in water, but this number varies greatly as a function of products and treatments used. Most aquatic tests have been performed starting from the sonication of a stock solution, while few have involved only agitation or filtration of the solution (Tables 1, 2 and 3). Adams et al. (2006) employed only agitation of Sigma Aldrich nano-TiO_2 in water and observed that 65 nm sized particles formed aggregates of 320 nm, while larger particles of 950 nm and 44 μm formed aggregates of 320 nm and 1 μm, respectively. Zhu et al. (2010b) report that in a culture medium for daphnids, even with sonication, P25 formed aggregates that increased over time: 580 nm (1h), 2349 nm (12h) and 3528.6 nm (24h).

The aggregation state of NPs inevitably changes with dilution, but there is a growing discussion about the use of dispersants or sonication processes to increase the dispersion of NPs in suspension in aquatic toxicology studies, in view of their environmental applicability (Baveye and Laba, 2008, Crane et al., 2008). One argument is that the study of non-dispersive materials would be of greater relevance to what actually takes place in the environment. Moreover, sonication may cause structural changes in nanomaterials, in fact, when performed in natural waters or in the presence of any electron donor; it may result in the generation of reactive oxygen species. The sonication time required changes according to the total concentration of the nanomaterial, and once sonication or agitation has stopped, the material does not remain dispersed for very long. On the other hand, the existence of natural dispersants in the environment, such as organic matter, would validate such studies (Crane et al., 2008). However, one should not assume that aggregate materials will necessarily not be bioavailable. They may

simply change the mode of respiratory exposure on the water column to exposure via diet through sediment (Handy et al., 2008). Benthic organisms may be more exposed to NPs aggregates than to the material in the liquid phase. Similarly, the high concentration of ions in hard or marine waters will tend to cause aggregation of NPs, modifying the mode of exposure or organisms in these ecosystems (Handy et al., 2008).

A large part of acute exposure studies have been performed by withholding food from animals on the day prior to and during the bioassay. In the case of prolonged exposure, daily feeding has generally been maintained, with a few exceptions (Federici et al., 2007, Hao et al., 2009). However, it should be noted that this is also a point to be evaluated carefully and standardized, in view of the capacity of organic matter to adsorb TiO_2.

The diversity of manufactured TiO_2 NPs, the quality of the medium, the aquatic species tested, and the objectives of each research, require that exposure conditions be evaluated separately.

4. CONCLUSIONS

Evaluating the potential biological impact of nanomaterials has become increasingly important in recent years. This is particularly relevant because the rapid pace of nanotechnology development has not been accompanied by a complete investigation of its safety or by the development of suitable methodologies for this investigation.

Concern about the environmental consequences of nanotechnology has been growing and has reached public opinion. Nano-TiO_2 is a nanoproduct with applications in a variety of

areas, and is also promising for the remediation of contaminated environments. However, its potentially harmful effects should be investigated in depth to ensure its sustainable use. Because water bodies are the final destination of contaminants, the evaluation of the effects of nano-TiO_2 on aquatic organisms is extremely necessary. Several groups have started research in this area, however, their results are still not conclusive and the need remains to continue researching. In fact, the results vary considerably, probably due to differences in the experimental models and products tested. Therefore, we agree with the recommendation that nanoecotoxicology studies focus on the characterization of NPs and that the best exposure conditions for the different NPs be analyzed (considering their particular properties), in the attempt to standardize bioassays and facilitate the comparison of results. In addition, the standardization of nanoecotoxicological methodologies is useful for the construction of protocols to underpin and guide public policies.

CAPÍTULO II

ESTUDO COM PEIXES JUVENIS: EXPOSIÇÃO AGUDA

Artigo publicado: Clemente, Z. et al. Fish exposure to nano-TiO_2 under different experimental conditions: Methodological aspects for nanoecotoxicology investigations. Science of Total Environment, 463-464: 647-56, 2013.

ABSTRACT

The ecotoxicology of nano-TiO_2 has been extensively studied in recent years; however, few toxicological investigations have considered the photocatalytic properties of the substance, which can increase its toxicity to aquatic biota. The aim of this work was to evaluate the effects on fish exposed to different nano-TiO_2 concentrations and illumination conditions. The interaction of these variables was investigated by observing the survival of the organisms, together with biomarkers of biochemical and genetic alterations. Fish (*Piaractus mesopotamicus*) were exposed for 96 h to 0, 1, 10, and 100 mg/L of nano-TiO_2, under visible light, and visible light with ultraviolet (UV) light (22.47 J/cm^2/h). The following biomarkers of oxidative stress were monitored in the liver: concentrations of lipid hydroperoxide and carbonylated protein, and specific activities of superoxide dismutase, catalase, and glutathione S-transferase. Other biomarkers of physiological function were also studied: the specific activities of acid phosphatase and Na,K-ATPase were analyzed in the liver and brain, respectively, and the concentration of metallothionein was measured in the gills. In addition, micronucleus and comet assays were performed with blood as genotoxic biomarkers. Nano-TiO_2 caused no mortality under any of the conditions tested, but induced sublethal effects that were influenced by illumination condition. Under both illumination conditions tested, exposure to 100 mg/L showed an inhibition of acid phosphatase activity. Under visible light, there was an increase in metallothionein level in fish exposed to 1 mg/L of nano-TiO_2. Under UV light, protein carbonylation was reduced in groups exposed to 1 and 10 mg/L, while nucleus alterations in erythrocytes were higher in fish exposed

to 10 mg/L. As well as improving the understanding of nano-TiO_2 toxicity, the findings demonstrated the importance of considering the experimental conditions in nanoecotoxicological tests. This work provides information for the development of protocols to study substances whose toxicity is affected by illumination conditions.

1. INTRODUCTION

The development of nanotechnology is seen as an internationally important strategic issue (NAE, 2004; NNI, 2011) because it enables advances in areas such as engineering, medicine, and computer science, amongst others. A key difference between bulk and nanoscale materials is the much higher surface area of a given mass or volume of nanoparticles (NPs), compared to an equivalent weight or volume of bulk material particles. This increased surface area enhances certain properties of the materials. Titanium dioxide nanoparticles (nano-TiO_2) are one of the commonest materials used in nanotechnology (Project on Emerging Nanotechnology, 2011), with interest focusing mainly on their photocatalytic activity and the absorption of ultraviolet (UV) light at specific wavelengths (Shao and Schlossman, 1999).

TiO_2 occurs in three crystalline forms: anatase (tetragonal), rutile (tetragonal), and brookite (orthorhombic). The anatase and rutile phases possess different photocatalytic properties, with anatase showing a better combination of photoactivity and photostability, compared to rutile (Gaya and Abdullah, 2008). Nonetheless, a mixture of anatase and rutile TiO_2 is normally employed in photocatalytic processes due to its greater catalytic efficiency (Cong and Xu, 2012). There is ample evidence of the formation of reactive oxygen species (ROS) when TiO_2 is exposed to UV light (especially at 300-388 nm) and this property is used in heterogeneous photocatalysis to degrade organic and inorganic compounds (Gaya and Abdullah, 2008). This capability has applications in the production of self-cleaning surfaces, cleaning products, remediation of contaminated soil and water, deodorization of environments, and the destruction of gas-phase volatile compounds (Li et al., 2008).

Considering the wide range of possible uses of nano-TiO_2, the presence of this material in the environment is inevitable. Since aquifers are the final receptors of many pollutants, there have been several investigations of the ecotoxicity of nano-TiO_2, although the findings have been inconclusive and conflicting (Clemente et al., 2012). Nano-TiO_2 has been reported to be non-toxic to fish, although sublethal effects have been observed, mainly related to oxidative stress and inflammation (Federici et al., 2007; Hao et al., 2009; Palaniappan and Pramod, 2010; Warheit et al., 2007b). Oxidative stress occurs when there is an imbalance between the production of free radicals and the antioxidant systems in an organism (Hwang and Kim, 2007), and has been discussed to be the mechanism responsible for the toxicity of NPs including nano-TiO2. Besides that, Braydich-Stolle et al. (2009) describe that pure anatase induces membrane leakage in mouse keratinocytes, leading to necrosis. The nano-TiO_2 toxicity to daphnia has also been suggested to be related to reduction of food consumption due to agglomerates uptake (Seitz et al., 2013). Although some studies have reported no changes, others have described increases or decreases in the activities of the antioxidant enzymes catalase, superoxide dismutase, glutathione S-transferase, and peroxidase in aquatic organisms exposed to nano-TiO_2. Other interesting findings are increased levels of lipid peroxidation and protein carbonylation, indicative of oxidative damage to lipids and proteins, respectively (Federici et al., 2007; Hao et al., 2009; Xiong et al., 2011). For fish, water exposure appears to be more serious than dietary exposure (Handy et al., 2008). The exposure of fish to nano-TiO_2 can induce biochemical and histopathological alterations in the liver, gills, and intestine (Federici et al., 2007; Hao et al., 2009; Johnston et al., 2010; Palaniappan and Pramod, 2010). According to Hao et al. (2009), the liver is more sensitive than the gills and brain when fish are exposed to nano-TiO_2 in the water.

Other biomarkers have been used to a lesser extent in nanoecotoxicology, but their study can help in understanding the mechanisms involved in NPs toxicity. Metallothioneins (MTs) are proteins that act as metal chelators and free radical scavengers, so they play an antioxidant role and are involved in homeostasis and detoxification of metals (Viarengo et al., 1997). The activity of phosphatase enzymes can also be affected by metals and ROS (Aoyama et al., 2003). Since these enzymes are involved in transphosphorylation reactions, its disturbance can compromise many cellular processes (Saeed et al., 1990). Fish exposed to nano-TiO_2 have shown inhibition of Na,K-ATPase, which has an important function in the maintenance of cellular electrical potential and volume (Federici et al., 2007).

Genetic damage may also occur as a result of oxidative stress, or due to the direct interaction of contaminants with DNA (Banerjee et al., 2006; Halliwell and Gutteridge, 1992). Micronucleus and comet assays are widely used as indicators of genotoxicity in fish. The first evaluates morphological changes in the cell nucleus and the presence of micronuclei formed by chromosomes that were not incorporated into the daughter cell nucleus during mitosis, due to exposure to clastogenic substances (Schmid, 1975). The second is based on performing an electrophoresis with the cellular DNA, with damaged DNA presenting fragments that are revealed by their heterogeneous migration, forming a shape that resembles a comet (Singh et al., 1988). The genotoxic potential of nano-TiO_2 remains unclear. While some studies have observed dose-dependent genetic damage in tests conducted *in vivo* and *in vitro* (Griffith et al., 2009; Hu et al., 2010; Turkez, 2011), others have reported no genotoxicity (Landsiedel et al., 2010; Saquib et al., 2012; Singh et al, 2009).

Research in the area of nanoecotoxicology is still in its infancy, but many important issues have already been raised. These include the question of the applicability of current ecotoxicological testing protocols in studies of the toxicity of manufactured NPs (Handy et al., 2012a). In bioassays using aquatic organisms, the circadian cycle is usually established employing fluorescent lamps. These lamps mainly emit visible light, while under natural conditions the organisms are exposed to solar radiation (infrared, visible, and ultraviolet light). Although UV light is attenuated by water, in clear oceanic waters it can penetrate to a depth of 40-60 m (Ban et al., 2007; Stewart and Hopfield, 1965, cited by Acra et al., 1990), and several investigations have demonstrated the deleterious effects of environmental levels of UV light on fish. These include hematological and immunological disturbances, as well as histopathological alterations in the skin, liver, and blood cells (Salo et al., 2000; Sayed et al., 2007). The photocatalytic properties of nano-TiO_2 in the presence of UV light can enhance its toxicity to aquatic organisms under environmental conditions, but few studies have considered this effect. *In vitro* work has shown that exposure to nano-TiO_2 under UV light increases cyto- and genotoxicity in cells (Reeves et al., 2008; Vevers and Jha, 2008; Xiong et al., 2013). Pre- and co-illumination with UV light has been shown to increase the toxicity of nano-TiO_2 to daphnids and the larvae of fish and frogs (Hund-Rinke and Simon, 2006; Ma et al., 2012b; Marcone et al., 2012, Zhang et al., 2012). However, to the best of our knowledge, there have been no reports concerning the role of illumination conditions in determining the behavior of biochemical and genetic biomarkers following exposure to nano-TiO_2. The use of biomarkers in risk assessment offers the advantage of enabling early detection of potentially harmful exposure, before severe damage occurs (Nascimento et al., 2008).

The aim of this study was to evaluate the effects of nano-TiO_2 concentration and illumination conditions in acutely exposed fish, in order to establish the toxicity of nano-TiO_2 and to assist in the development of nanoecotoxicological protocols. The effect of the interaction of the two variables was assessed by observing the survival of the organisms, as well as by monitoring selected biomarkers associated with biochemical and genetic alterations. Measurements were also made of the accumulation of titanium in fish tissue. The artificial illumination conditions used were as close as possible to natural UV radiation. As test organism, it was chosen a representative native species of tropical regions and widely cultivated in pisciculture: the pacu caranha (*Piaractus mesopotamicus*).

2. MATERIALS AND METHODS

2.1 Characterization of the NPs and their stability in suspension

Evaluation of the toxicity of the TiO2 NPs was performed using titanium (IV) oxide nanopowder (Sigma Aldrich, 100% anatase, primary particle size <25 nm, 99.7% purity). A stock suspension of 1 g/L of nano-TiO2 in dechlorinated tap water was prepared by sonication for 10 min using a high frequency probe (CPX600 Ultrasonic Homogenizer, Cole Parmer, USA) operated at 150 W/L and 100% amplitude. Immediately after sonication, the required volume was used to prepare a 100 mg/L suspension under bioassay conditions (constantly aerated water in an aquarium). The hydrodynamic size, surface charge (zeta potential, ZP), and polydispersivity index (PdI) of particles in the 100 mg/L suspension were assessed by dynamic light scattering

(DLS) using a Zetasizer Nano ZS90 instrument (Malvern Instruments, UK). Colloidal stability was also evaluated from optical spectra obtained in the wavelength range 200-600 nm using a UV-Vis spectrophotometer (Model 1650PC, Shimadzu, Japan). Measurements were made at 0, 3, 5, and 24 h after preparation of the suspensions. All samples were collected in the middle of the water column. The water characteristics were: pH 7.5 ± 0.1, conductivity 1.3 ± 0.2 mS/cm, hardness 50.0 mg/L, temperature 27.0 ± 0.6 ºC, and dissolved oxygen (DO) 6.0 ± 0.6 mg/L.

2.2 Toxicity assays

The effects of nano-TiO_2 concentration, illumination condition, and interaction of these variables were evaluated using acute exposures. Toxicity was determined by observing the survival of the fish, together with changes in the biochemical and genetic biomarkers. Parallel determinations were made of the accumulation of titanium in the fish tissues.

The pacu caranha (*Piaractus mesopotamicus*) (Anexo I) is a freshwater fish widely distributed in South America (Fishbase, 2012) and has been used previously as a bioindicator in ecotoxicological studies (Sampaio et al., 2008; Silva et al., 2010). The test organisms were juvenile fish weighing 16.5 ± 3.7 g, with a total length of 9.1 ± 0.7 cm. The experiment was preceded by an acclimatization period of 15 days and followed the OECD 203 protocol (OECD, 1992), with some modifications. The fish were kept in uncovered and constantly aerated aquaria containing 9 L of water (the characteristics of the water are described in section 2.1). The animals were fasted for 24 h before the bioassay, and were not fed during the experiment.

The fish were exposed to the following concentrations of nano-TiO_2 during a 96 h period: 0 (control), 1, 10 and 100 mg/L. The suspensions in the aquaria were prepared from a stock suspension of nano-TiO_2, as described in section 2.1. All exposure suspensions were completely renewed on a daily basis. The aquaria were exposed to two different illumination conditions: visible light (visible light groups) or ultraviolet and visible light (UV light groups) (Anexo I). Each experimental condition was tested in duplicate (n=8 per group). Exposure to visible and UV light followed a photoperiod of 12/12 h (light/dark). The illumination conditions are further described in section 2.3.

After exposure, the fish were anesthetized with benzocaine (0.2 mg/mL) and then killed by medullar section. Blood was collected for micronucleus and comet assays, and samples of liver, gills, and brain were frozen at −70 °C for subsequent biochemical analyses. The axial muscle was also collected and stored at −70 °C for later analysis of titanium.

The animal manipulation procedure was approved (in June 2010) by the Commission for Ethical Use of Animals, of the State University of Campinas (CEUA/Unicamp, protocol n^o 2172-1) (Anexo II e IV).

2.3 Illumination conditions

Natural (solar) and artificial ultraviolet light were measured using an USB 2000+RAD spectroradiometer (Ocean Optics, USA). Visible light in the laboratory was measured using a digital lux meter (LD-500, ICEL, Brazil). The regions of the electromagnetic spectrum

considered were those adopted by the International Commission on Illumination (CIE, 1999): visible light (400-700 nm), UVA (400-315 nm), UVB (315-280 nm), and UVC (280-200 nm).

Natural UV light was measured during cloudless conditions at 01:00 pm on May 16, 2011 (autumn) and December 16, 2012 (spring) in Jaguariúna, Brazil (subtropical climate, 22º 42' S, 46º 59' W). In the laboratory, visible light was provided from Phillips lamps (40 W) installed in the ceiling of the room, and exposure to artificial UV light employed four lamps (Q-panel UVA340, 40 W) positioned 17 cm above the water surface.

The visible light intensity in the laboratory was 250 ± 79 lux. At the height of the aquaria, there was no detectable UV light from the fluorescent lamps installed in the ceiling. The Q-panel UVA340 lamp spectrum was from 300 to 610 nm, with an irradiance peak at 340 nm (Figure 1). The UV light flux at the water surface was 21.63 J/cm^2/h (UVA) and 0.84 J/cm^2/h (UVB). This level of irradiation was close to that provided by solar exposure at 01:00 pm in autumn (22.83 J/cm^2/h of UVA and 0.04 J/cm^2/h of UVB), and around half the solar radiation flux in spring (41.50 J/cm^2/h of UVA and 0.96 J/cm^2/h of UVB). On a sunny day in summer, UVB accounts for approximately 6% of the UV reaching the terrestrial surface, with UVA accounting for the remaining 94% (Diffey, 2002). The measured values were in agreement with this proportion, and the values for autumn were very close to those measured in temperate regions in spring (Hakkinen and Oikari, 2004; Kim et al., 2009).

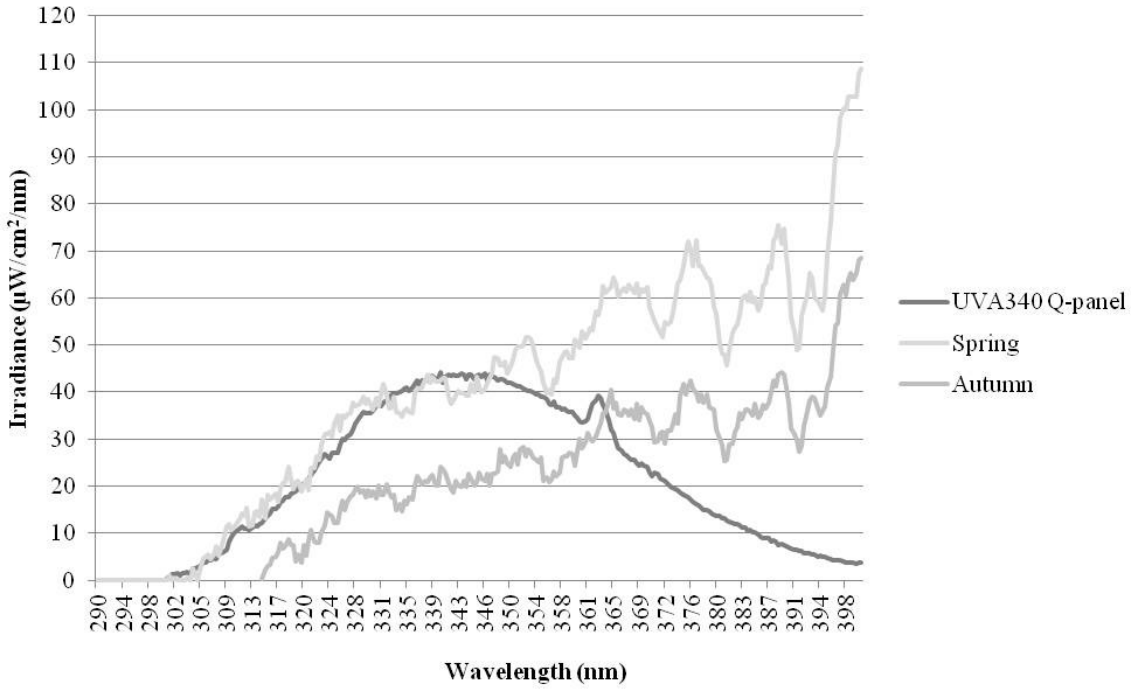

Figure 1. Electromagnetic spectra in the UV light range, obtained using an Ocean Optics USB 2000+RAD spectrometer, for UVA340 Q-panel lamps used in the bioassay (sensor-to-lamp distance: 17 cm) and solar radiation in spring and autumn in Jaguariúna (Brazil).

2.4 Biochemical analyses

Samples of liver were homogenized (1:4, w/v) in cold phosphate buffer (0.5 mol/L, pH 7), and the homogenates were centrifuged at 10,000 × *g* for 20 min at 4 °C. Aliquots of the supernatant of each sample were collected for analyses of lipid hydroperoxide (LPO – Anexo V) (Jiang et al., 1992) and carbonylated protein (PCO – Anexo VI) (Levine et al., 1994; Sedlak and Lindsay, 1968). Measurements were made of the specific activities of superoxide dismutase (SOD – Anexo VII) (Ukeda et al., 1997), catalase (CAT – Anexo VIII) (Aebi, 1984), glutathione S-transferase (GST – Anexo IX) (Keen et al., 1976), and acid phosphatase (AP – Anexo X) (Prazeres et al., 2004).

Samples of gills were homogenized (1:5, w/v) in cold buffer (pH 8.6) containing Tris-HCl (20 mmol/L), saccharose (500 mmol/L), phenylmethanesulfonyl fluoride (0.5 mmol/L), and β-mercaptoethanol (0.01%). The homogenates were centrifuged at 15,000 × *g* for 30 min at 4 °C, and the supernatant was used for determination of the metallothionein (MT) concentration (Anexo XI) (Viarengo et al., 1997).

Samples of brain were homogenized (1:4, w/v) in cold buffer (adjusted to pH 7.4 with HCl) containing sucrose (0.3 mol/L), Na_2EDTA (0.1 mmol/L), and imidazole (30 mmol/L), and then centrifuged at 10,000 x *g* for 5 min at 4 °C. The supernatant was used for assessment of Na,K-ATPase activity (Anexo XII) (Quabius et al., 1997; Sampaio et al., 2008).

The protein concentrations in the homogenates were determined using the method described by Bradford (1976), with bovine serum albumin as standard (Anexo XIII). At least 5 samples from each group were analyzed in each biochemical procedure, and all samples were analyzed in triplicate.

2.5 Genetic analyses

In the micronucleus assays, smears of blood from the fish were stained with Giemsa (Heddle, 1973; Schmid, 1975), and one thousand erythrocytes per slide were counted by optical microscopy at x1000 magnification (Laborlux K microscope, Leica, Germany). The micronucleus frequency was recorded together with any morphological alterations in the nucleus (Carrasco et al., 1990).

The comet assays were carried out as described by Singh et al. (1988). A blood sample was diluted in fetal bovine serum, stored in ice in the dark for 24 h, and then prepared for the assay (Ramsdorf et al., 2009). A 10 μL volume of this solution was mixed with 120 μL of low melting point agarose, after which a 100 μL sample was deposited on a slide coated with normal agarose. The slides were then immersed in a lysis solution (2.5 mol/L NaCl, 0.1 mol/L EDTA, 10 mmol/L Tris, 1% Triton X-100, pH 10) and kept at 20 ºC for at least 1 h. After lysis, the slides were placed in electrophoresis buffer (300 mmol/L NaOH, 1 mmol/L EDTA, pH ~13, 4 ºC) for 20 min prior to electrophoresis at 1.3 V/cm. The slides were neutralized for 15 min with 0.4 mol/L Tris buffer at pH 7.5, then dried at room temperature and stained with silver. DNA strand breaks were scored using an optical microscope at a magnification of x40. For each fish, 100 cells were visually analyzed according to the method of Collins et al. (1997).

At least 5 individuals from each group were analyzed in the micronucleus assays, and at least 3 individuals in the comet assays.

2.6 Titanium content of muscle tissue

Three pooled sets of muscle samples from each group were digested as described by Weir (2011). The samples were placed in petri dishes and dried in an oven at 100 ^{o}C. The sample weight was monitored on an hourly basis, until it remained constant. The tissues were then placed into Corning tubes and digested with analytical grade hydrogen peroxide (5 mL) and nitric acid (1 mL) at 100 ^{o}C for 4 h. The samples were subsequently removed and cooled to room temperature. After addition of nitric acid (4 mL) and hydrofluoric acid (1 mL), the samples were

heated in a sand bath (120 ^{o}C) to evaporate the acid until 1-1.5 mL of solution remained. Finally, the samples were diluted to 10 mL with ultrapure water (Milli-Q).

The analysis was performed by inductively coupled plasma optical emission spectroscopy (ICP-OES, Agilent Model 720, USA). A standard curve was prepared using a titanium ICP standard (Merck).

2.7 Statistical analysis

The biochemical and genetic variables were initially evaluated by two-way analysis of variance to investigate the influence of the factors nano-TiO_2 concentration, illumination condition (with or without UV light), and their interactions. When there was significant interaction, the responses at each concentration were compared within illumination levels, and vice versa, using bilateral t-tests for contrasts (Montgomery, 2008). When there was no evidence of interaction, the levels of the main factors were compared independently, using the same types of tests. Residual graphical analysis was used to test for normality and homoscedasticity, and a significance level of 5% was adopted for all the tests. The statistical procedures were performed using the GLM procedure of the software SAS/STAT, version 9.2.

3. RESULTS

3.1 Characterization of the NPs and their stability in suspension

The DLS results are shown in Table 1. Satisfactory measurements could only be made at the start of the experiment, with a high PdI after 3 h being indicative of the presence of a heterogeneous particle population, which compromised the quality of the size analysis. The ZP analysis could be performed throughout the period, and an average value of -31.5 ± 1.29 mV was obtained after 24 h.

Table 1. Dynamic light scattering measurements of the 100 mg/L nano-TiO_2 suspension after different time intervals: hydrodynamic size (Z-average), polydispersivity index (PdI) and zeta potential (ZP). The results are presented as means ± standard deviations.

	0 h	*3 h*	*5 h*	*24 h*
Z-average	543.9 ± 65 nm	1236 ± 15 nm	1523 ± 19 nm	1611 ± 21
PdI	0.24 ± 0.02	0.84 ± 0.07	0.93 ± 0.04	1.0 ± 0.00
ZP	- 27.8 ± 2 mV	- 32.9 ± 2 mV	- 31.4 ± 1.5 mV	- 31.9 ± 1.8 mV

The high instability of the nano-TiO_2 suspension was confirmed by UV-visible spectrophotometry (Figure 2). As time elapsed, there was a reduction in the absorbance, with the peak absorbance at 320 nm falling to 23.6, 17.9, and 4.8% of the initial value after 3, 5, and 24 h, respectively, indicating almost total precipitation of the particles. The appearance of a white precipitate was observed at the bottom of the aquaria after 24 h.

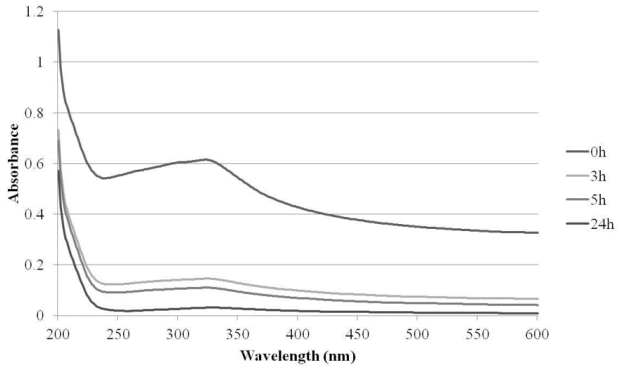

Figure 2. Colloidal stability: optical spectra obtained at different times for 100 mg/L of nano-TiO2 in an aquarium with constant aeration.

3.2 Toxicity assays

There was no mortality of fish in any group, with or without UV light, and no abnormal behavioral signa were observed. There was a concentration-dependent reduction in hepatic AP activity (Figure 3 I, $p = 0.007$). The groups exposed to 100 mg/L of nano-TiO_2 showed a significant inhibition of AP activity: the group exposed under visible light showed an inhibition of 32% ($p = 0.01$), and the group exposed under UV light showed an inhibition of 39% ($p = 0.01$), compared to the respective controls. A significant inhibition, of 27%, was also observed for the group exposed to 10 mg/L of nano-TiO_2 under visible light ($p = 0.03$).

In contrast to the groups exposed under visible light, a reduction in the PCO level (Figure 3 II) was observed for groups exposed to UV light, which revealed an effect of illumination condition ($p = 0.002$). Reductions of 75 and 62% were obtained for groups exposed to 1 mg/L ($p = 0.004$) and 10 mg/L ($p = 0.009$) of nano-TiO_2, respectively. The group exposed to 100 mg/L of nano-TiO_2 under visible light also showed a reduction in the PCO level, compared to the groups exposed to 1 mg/L ($p = 0.01$) and 10 mg/L ($p = 0.006$) of nano-TiO_2, but there was

no difference compared to the control. The significance level of the interaction of illumination condition and concentration was 0.11.

The MT concentration (Figure 3 III) showed an influence of the interaction of nano-TiO_2 concentration and illumination ($p = 0.003$), and was statistically higher for the group exposed to 1 mg/L of nano-TiO_2 and visible light, compared to the other concentrations ($p < 0.01$). It was also significantly higher compared to the group exposed to the same nano-TiO_2 concentration under UV light ($p = 0.00007$).

Micronuclei were not detected, but the extent of morphological alterations in the erythrocyte nuclei (Figure 3 IV) revealed an influence of the type of illumination ($p = 0.01$), since the alterations were more prevalent in groups exposed to UV light. Nucleus alterations were significantly higher in the group exposed to 10 mg/L of nano-TiO_2 under UV light, compared to the group exposed to the same concentration under visible light ($p = 0.03$).

No statistically significant differences between the groups were observed for the other biomarkers (Table 2), or for the muscle titanium content (Table 3).

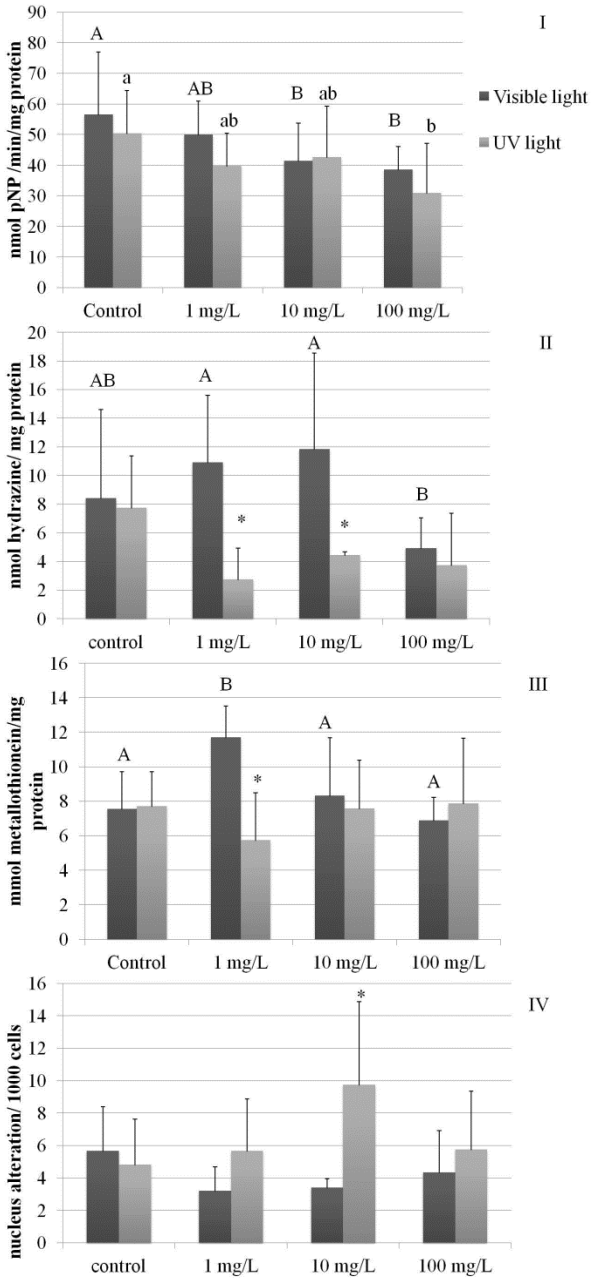

Figure 3. Biochemical and genetic biomarkers in *Piaractus mesopotamicus*: acute exposure to nano-TiO_2 (control, 1 mg/L, 10 mg/L, and 100 mg/L) under visible light or visible and ultraviolet (UV) light. Means ± standard deviations. I) Specific activity of acid phosphatase in liver; II) concentration of carbonylated proteins in liver; III) concentration of metallothionein in gills; IV) morphological alterations in the nucleus of erythrocytes (micronucleus assay). In all biomarker analyses, at least 5 samples were analyzed from each group. *$p<0.05$ between visible light and UV, for the same concentration; different uppercase letters indicate $p<0.05$ between different concentrations, using visible light; different lower case letters indicate $p<0.05$ between different concentrations, using UV light.

Table 2. Biochemical and genetic biomarkers in *Piaractus mesopotamicus* following acute exposure to nano-TiO_2 (control, 1 mg/L, 10 mg/L and 100 mg/L) under either visible light or visible and ultraviolet (UV) light. Specific activities of catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST) in the liver; lipid hydroperoxide (LPO) concentration in liver; specific activity of Na, K –ATPase in the brain; blood comet assay scores. Results are presented as means ± standard deviations.

	Control				**1 mg/L**				**10 mg/L**				**100 mg/L**			
	Visible light	*n*	*UV light*	*n*	*Visible light*	*n*	*UV light*	*n*	*Visible light*	*n*	*UV light*	*n*	*Visible light*	*n*	*UV light*	*n*
CAT (mmol of degradated H_2O_2 / min/ mg prot)	1.75 (± 0.96)	8	1.35 (± 0.47)	8	1.70 (± 1.06)	7	1.14 (± 0.23)	8	1.71 (± 0.89)	8	1.72 (± 1.20)	6	0.89 (± 0.28)	7	1.34 (± 0.63)	7
SOD (U/ mg prot)	91.44 (± 78.74)	8	85.48 (± 29.68)	8	74.71 (± 27.08)	8	58.99 (± 12.73)	7	54.80 (±7.83)	7	88.65 (± 44.06)	5	56.51 (± 24.94)	8	63.93 (± 30.81)	7
GST (µmol of conjugated CDNB/ min/ mg prot)	0.48 (± 0.18)	8	0.45 (± 0.13)	8	0.35 (± 0.09)	7	0.37 (± 0.05)	8	0.33 (± 0.04)	7	0.50 (± 0.16)	5	0.34 (± 0.04)	7	0.39 (± 0.16)	7
LPO (nmol of hydroperoxide/ mg prot)	24.74 (± 4.54)	6	23.49 (±5.39)	7	25.73 (± 8.34)	7	27.24 (± 3.43)	8	28.05 (± 7.34)	7	29.22 (± 10.18)	5	41.46 (± 27.93)	5	28.08 (± 5.71)	7
Na/K – ATPase (µmol of Pi/ mg prot/ h)	0.74 (± 0.10)	7	0.70 (± 0.13)	8	0.68 (± 0.23)	8	0.53 (± 0.17)	8	0.71 (± 0.16)	8	0.71 (± 0.23)	6	0.60 (± 0.19)	8	0.64 (± 0.09)	6
Comet assay (score of genetic damage)	0.39 (± 0.02)	3	0.60 (± 0.10)	3	0.52 (± 0.18)	5	0.47 (± 0.01)	3	0.23 (± 0.09)	3	0.33 (± 0.09)	4	0.52 (± 0.14)	3	0.47 (± 0.01)	3

Table 3. Titanium content of muscle tissue (µg of Ti/ g muscle) of *Piaractus mesopotamicus* after acute exposure to nano-TiO_2 under either visible light or visible and ultraviolet (UV) light. The results are presented as means ± standard deviations.

	Visible light		**UV light**	
	µg of Ti/ g muscle	*n*	*µg of Ti/ g muscle*	*n*
control	7.29 (± 4.11)	3	6.63 (± 3.58)	3
1 mg/L	7.92 (± 1.64)	3	13.04 (± 8.03)	3
10 mg/L	7.30 (± 1.67)	3	5.91 (± 1.04)	3
100 mg/L	6.14 (± 1.76)	3	6.34 (± 1.53)	3

4. DISCUSSION

At the start of the experiments, the measurements of nanoparticle size were in agreement with those reported previously for nano-TiO_2 (Allouni et al., 2012). However, the DLS and UV-visible spectrophotometric measurements indicated that subsequently there was substantial particle aggregation and precipitation, which resulted in a 70 % reduction in absorbance after the first 3 h. The fast aggregation and precipitation of nano-TiO_2 under aquatic test conditions is a problem that has been described in several earlier ecotoxicological studies (Clèment et al., 2013; Navarro et al., 2008). Aqueous solution pH exerts a significant effect on TiO_2 in terms of particle charge and the size of aggregates. The pH_{pzc} (when the surface of an oxide does not possess electrical charge) of anatase is 6.3 (Finnegan et al., 2007). Von der Kammer et al. (2010) showed that the degree of sedimentation of TiO_2 nanoparticles added to a medium is highly dependent on pH and the salt concentration. In solutions containing 8.4 mM of NaCl at pH >5.8, nano-TiO_2 forms polydispersed aggregates within 5 min, many of which are outside the size range measurable by DLS (French et al., 2009). These observations can be explained by Derjaguin–Landau–Verwey–Overbeek (DLVO) theory, according to which high concentrations of electrolytes or high valency anions act to diminish electrostatic energy barriers, resulting in NPs aggregation (Shih et al., 2012). The behavior of NPs in a system is dependent on the amount of added energy, which affects their dispersion, size, and surface characteristics (type and zeta potential). The ability of the particles to aggregate depends on these properties as well as particle kinetic energy, the viscosity of the water, and the presence of other materials such as small peptides or macromolecules (Shaw and Handy, 2011).

Natural surface waters have an ionic strength of between 1 µM and 1 mM. An NaCl concentration of up to 0.6% (62 mM) is recommended in pisciculture in order to avoid pathologies (French et al., 2009; Kubitza, 2007). Since the water used in the experiments had a pH of 7.5 and low hardness ($CaCO_3$ = 50 ppm) and conductivity (0.08 mS/cm), the addition of salt to the aquaria was essential, and the use of 14 mM NaCl resulted in a conductivity of 1.3 mS/cm. Due to the observed aggregation and precipitation of nano-TiO_2 in the water, it was necessary to renew the exposure media every 24 h during the bioassays in order to maintain the conditions reasonably constant. This semi-static exposure protocol has been used in other ecotoxicological studies involving nano-TiO_2 (Hao et al., 2009; Xiong et al., 2011) and has recently been recommended by Handy et al. (2012a).

The exposure to UV light was close to natural conditions. Pure water weakly absorbs UV light, and the exponential attenuation of UV (200-400 nm) is lower in distilled water than in seawater (between 10/m at 200 nm and a minimum of 0.05/m at 375 nm) (Stewart and Hopfield, 1965, cited by Acra et al., 1990). Since fish move throughout the water column, their exposure to UV is dependent on both depth and water quality. The water column in the present bioassay was 19 cm, and the exposure of the fish was comparable to natural conditions for this depth range. In a similar experiment, Salo et al. (2000) exposed fish to UVA and UVB light in an aquarium, and demonstrated that although water attenuated the UV irradiance, fish were still exposed to a substantial UV dose in the 27 cm water column. Although ecotoxicology studies involving different illumination conditions are relatively common, there are no well-defined experimental protocols to guide UV light exposure. We suggest that the illumination conditions employed here were environmentally relevant and could be used in other studies, not only with nano-TiO_2 but

also with other materials that either exhibit photocatalytic properties or whose behavior is altered when exposed to UV light, as sulfonamide antibiotics and polycyclic aromatic hydrocarbons (Huovinen et al., 2001; Jung et al., 2008).

Skin represents an important protection mechanism for fish, and research suggests that the mucus of tropical fishes contains compounds that can block both UVA and UVB radiation (Elliott, 2011). Kaweewat and Hofer (1997) demonstrated that the number of goblet cells (mucus secreting cells) in the dorsal epidermis of different fish species was significantly reduced by both artificial and solar UVB light. This reduction could result in less mucus production. At physiologically relevant doses, solar irradiation generates reactive oxygen species (ROS) that can promote lipid peroxidation in cell membranes and cause oxidative damage to DNA and proteins, leading to cell death if the mechanisms of defense and repair are inadequate (Franco et al., 2009). The micronucleus test, commonly employed to evaluate genotoxicity, revealed a general increase in the occurrence of alterations in the erythrocyte nuclei of fish exposed to UV light, compared to those kept under visible light. The illumination condition effect, as revealed by the statistical analysis, attested to the fact that UV light reached the fish. However, in the case of the control groups kept under the different illumination conditions, there were no statistically significant differences in terms of the genetic biomarkers.

For the experimental model employed, the nano-TiO_2 showed no toxicity, and exposure to UV light did not affect toxicity. These findings are in agreement with those of Griffitt et al. (2008) and Warheit et al. (2007b), who were unable to determine an LC_{50} for nano-TiO_2 using *Danio rerio* and *Oncorhynchus mykiss* under standardized experimental conditions. Xiong et al. (2011) reported an LC_{50} of 124.5 mg/L using *D. rerio*. It is likely that NPs containing weakly

soluble metal oxides, such as TiO_2, present only low toxicity (Shaw and Handy, 2011), although increased acute toxicity of nano-TiO_2 has been reported for cells and daphnids exposed to UV light (Ma et al., 2012b; Marcone et al., 2012; Reeves et al., 2008; Vevers and Jha, 2008; Xiong et al., 2013). The acute exposure of frog larvae to nano-TiO_2 under UV light caused no increase in mortality (Nations et al., 2011), but the recent work of Zhang et al. (2012) showed that the toxicity of nano-TiO_2 under UVA light increased with exposure time. When tadpoles were exposed for 14 days to nano-TiO_2 (with primary particle sizes of 5 and 10 nm), the LC_{50} was around 60 mg/L (Zhang et al., 2012). The absence of phototoxicity of nano-TiO_2 observed in the present study could have been related to the short exposure time. However, it is also possible that the larger size and complexity of the test organism resulted in lower sensitivity.

As discussed above, there was substantial aggregation of the nano-TiO_2, but the fact that aggregation occurred does not mean that the substance was not bioavailable (Handy et al., 2008). Studies *in vitro* have shown that there is an association of nano-TiO_2 with cell membranes that increases as function of nano-TiO_2 concentration. Also, these studies showed that internalization of agglomerates in endosomes and cytoplasm can occur, together with intracellular ROS generation (Allouni et al., 2012; Hussain et al., 2009; Park et al., 2008). The bioavailability of nano-TiO_2 to aquatic organisms is still unclear, with some authors finding no significant uptake (Federici et al., 2007; Johnston et al., 2010) while others have reported accumulation of Ti in fish after prolonged exposure to the element in the water or the diet (Ramsden et al., 2013; Zhu et al., 2010a). Intravenous administration to fish of high doses of nano-TiO_2 resulted in greatest accumulation in the kidneys (Scown et al., 2009), while exposure to nano-TiO_2 in the water caused accumulation of Ti primarily in the gills, as well as in liver, brain, and heart tissues (Chen

et al., 2011b). In addition to Ti found in gills and viscera, Zhang et al. (2007) also detected Ti in the muscle and skin of fish after prolonged exposure to nano-TiO_2 in the water. In the case of the experimental model employed in this study, there was no accumulation of Ti in the muscle tissue of the fish. The muscle was chosen for analysis because it is the main part of the fish consumed by humans, and is the most abundant tissue available for analysis. The fact that there was no Ti accumulation in the muscle indicates a low risk of trophic transfer to potential predators and humans. However, the risk may be greater for predators that consume the viscera as well as the muscles. It was not possible to determine the Ti contents of the liver and gills, because these organs were very small and could not be prepared in sufficient quantities. Given the conflicting reports in the literature concerning the accumulation of Ti, priority was given to biomarker analysis in the case of the gills and liver, because of the greater number of indicators. Despite the fact that no acute toxicity or bioaccumulation of Ti was detected, a number of sublethal effects were observed.

Acute exposure to nano-TiO_2 resulted in a concentration-dependent inhibition of the activity of hepatic acid phosphatase (AP). Metal species such as Hg^{2+}, Cu^{2+}, Cd^{2+}, Se^{3+}, Al^{3+}, and Pb^{3+} can inhibit AP (Blum and Schwedt, 1998), but there have been no previous reports of inhibition by Ti. The inhibition observed in the presence of the metals can be explained by their interaction with –SH groups essential for catalysis (Van Assche, 1990). Another mechanism of inhibition can be initiated by deficiency of a metal that is essential in metalloproteins or metal-protein complexes, when the essential metal is replaced by a toxic metal (Omar, 2002). It is possible that TiO_2 might influence AP synthesis, but there is no evidence for this in the literature. Reactive oxygen species such as $^1O_2$ and H_2O_2 are able to oxidize cisteine residues present at

active sites and hence inhibit the activity of tyrosine phosphatases (Aoyama et al., 2003). It is therefore possible that the observed inhibition of AP could have been due to the generation of radical species by exposure to nano-TiO_2. The inhibition of acid phosphatases can compromise several metabolic pathways as well as cell signaling.

Exposure of fish to nano-TiO_2 in water can cause histopathological changes in the gills that include membrane damage and cell disruption, epithelial hyperplasia, lamellar fusion, and morphological changes in mucocytes (Chen et al., 2011b; Federici et al., 2007; Hao et al., 2009; Xiong et al., 2011). The same studies also described increases in respiratory rate and mucus secretion, and signs of hypoxia. Johnston et al. (2010) reported the presence of nano-TiO_2 aggregates on the gill epithelium surface after exposure for 24 and 96 h, as well as inside the lamellae after 14 days of exposure. Considering these findings, with the accumulation of Ti occurring mostly in the gills, it can be hypothesized that the biochemical changes observed in this study were related to respiratory distress, rather than direct toxic effects of nano-TiO_2. The occurrence of oxidative stress in cells and organisms subjected to hypoxia is widely recognized (Blokhina et al., 2003), although future studies will be needed to confirm this hypothesis.

The gills of fish are in close contact with the surrounding medium, and together with the intestine constitute the main means of metal absorption. There was an increase in the metallothionein (MT) concentration in the gills of fish exposed to 1 mg/L of nano-TiO_2 under visible light. Bigorgne et al. (2011) also reported an increase in MT expression in *Eisenia fetida* exposed to 10 mg/L of nano-TiO_2 for 24 h, which coincided with greater absorption of Ti and increased expression of SOD. An *in silico* cross-experimental analysis of gene expression showed that nano-TiO_2 caused up-regulation of the metallothionein 2A gene (Yin et al., 2012).

Metallothioneins are involved in the regulation and detoxification of metals such as Zn and Cu. There is little known concerning the binding of MTs to metal oxides, but their adsorption onto membranes containing nano-TiO_2 was described by Cai et al. (2008). MTs also have an antioxidant role, given their ability to capture Cu^{2+} ions (and therefore reduce the production of radicals by this metal), release Zn^{2+} (which inhibits lipid peroxidation), and scavenge ROS (Viarengo et al., 1997). As discussed previously, TiO_2 can promote the generation of ROS, even in the absence of UV (Fenoglio et al., 2009). The observed increase in the MT concentration could therefore have been related to the generation of ROS by the nano-TiO_2, or to regulation of metal absorption by the organism. Moreover, there is evidence that hypoxia could activate MT gene expression (Murphy et al., 1999), which corroborates the hypothesis that the biochemical changes found in this study were associated with gill damage.

An effect on MT levels was only observed for the lower concentration of TiO_2, and co-exposure to UV light reduced the MT concentration in the 1 mg/L group to levels similar to those found for the control groups. The concept of dose-response does not necessarily apply in the case of biomarker alterations (Ji et al., 2012; Otitoju and Onwurah, 2005). The high variability of Ti accumulation in the fish, as well as the possibility of respiratory distress, could explain the lack of a concentration-response relationship and the relatively high level of variance in the toxicity data. One possibility is that high concentrations of nano-TiO_2 or ROS activate detoxification routes or antioxidant mechanisms other than those discussed in this study.

The oxidation of proteins occurs mainly by carbonylation (Yan and Forster, 2011). Work conducted *in vitro* has identified a dose-dependent induction of PCO by nano-TiO_2 (Han et al., 2012), and an increase in PCO may be accompanied by alterations in antioxidant mechanisms

(Curtis et al., 2012; Filipak Neto et al., 2008). Here, an unexpected effect of the combination of nano-TiO_2 and UV light was a reduction in PCO levels, instead of the expected induction of oxidative stress. However, in the present work there were no alterations in the activities of the antioxidant enzymes CAT, SOD and GST. Chevallet et al. (2011) reported an increase of ferritin, an acute phase protein, in macrophages exposed to nano-TiO_2. The activation of inflammatory processes due to exposure to nano-TiO_2 was described by Kobayashi et al. (2009) and Noel et al. (2012). It is known that iron and/or ferritin can accumulate during various pathological skin conditions or following chronic exposure of human skin to UV light. It is believed that the role of ferritin is to provide protection by the accommodation and "deactivation" of excess Fe ions (which can cause oxidative stress) produced by inflammatory processes or UV light (Giordani et al., 2000; Vile and Tyrrell, 1993). The activation of this protection mechanism could explain the lower levels of PCO in the groups exposed to nano-TiO_2 and UV light. This hypothesis is supported by the fact that Fe is involved in the synthesis of MT (Fleet et al., 1990) and is an essential metal in the AP structure. Induction of ferritin synthesis and the consequent decrease in Fe availability could therefore also explain the reductions in MT concentration and AP activity, discussed previously.

A chemical concentration of 100 mg/L is the maximum recommended by OECD (1992) for toxicity testing, since higher concentrations are regarded as having little environmental relevance. Kaegi et al. (2008) described the presence of 0.016 mg Ti/L in urban runoff, and also Kiser et al. (2009) described concentrations from 0.1 to 2.8 mg Ti/L in raw sewage. So, the 1 mg/L concentration used in this study was close to the highest concentration reported in the literature. In this case, few changes were observed in the biomarkers studied. However, AP

inhibition and an increase in MT were observed, which might serve as an early warning. The findings could reflect adaptation mechanisms, or be indicative of a metabolic dysfunction that could generate a cascade effect, with impacts at the cellular and tissue levels extending to the impairment of the community and the ecosystem. The results indicated that when exposure to nano-TiO_2 occurred under UV light, there was a shift in the biochemical response, with reductions in the levels of PCO and MT. Since there were no changes in the antioxidant enzymes CAT, SOD, and GST, defense mechanisms different from those discussed in this study could have been activated, which will require further investigation.

Finally, the present work showed that the effects on the biomarkers differed according to both the nano-TiO_2 concentration and the illumination conditions. Further studies will be needed in order to elucidate the causes of the observed alterations, and to determine whether there are direct effects of nano-TiO_2 on tissues. The findings help to clarify the toxic effects of nano-TiO_2 in fish, and contribute to the development of protocols for use in nanoecotoxicological studies. Although some progress has been made (Quick et al., 2011), there is still a need to understand all the factors involved in the toxicological effects of different nanoparticle formulations, both as a starting point for the development of research protocols and for better assessment of the nanotechnology risks.

5. CONCLUSIONS

Acute exposure of fish to nano-TiO_2 concentrations of up to 100 mg/L resulted in no mortality, in either the presence or the absence of ultraviolet light at environmental levels.

However, sublethal effects were observed, which were influenced by exposure to UV light. The specific activity of acid phosphatase and the concentration of metallothionein were found to be useful biomarkers for the detection of sublethal effects following acute exposure to nano-TiO_2, and should be included in investigations of nano-TiO_2 toxicity. The findings contribute to the development and implementation of protocols for use in nanoecotoxicological studies.

CAPÍTULO III

ESTUDO COM PEIXES JUVENIS: EXPOSIÇÃO PROLONGADA

Artigo aceito para publicação: Clemente, Z. et al. Biomarker evaluation in fish after prolonged exposure to nano-TiO_2: influence of illumination conditions and crystal phase. Journal of Nanoscience and Nanotechnology.

ABSTRACT

Evaluation of nano-TiO_2 ecotoxicology has been intensified over the last years, and it has been reported to have no/low toxicity to fish. However, few papers that studied the ecotoxicological properties of nano-TiO_2 considered its photocatalytic properties when exposed to ultraviolet (UV) light, as well as the effects of the different crystal phases. Despite the supposed low toxicity of nano-TiO_2 to fish, there is evidence of sublethal effects. In this study, we evaluated the effects of prolonged exposure to two different nano-TiO_2 crystal phases under different illumination conditions. Fish (*Piaractus mesopotamicus*) were exposed for 21 days to 100 mg/L of nano-TiO_2 anatase and a mixture of anatase:rutile (80%:20%) under two types of illumination: visible light and UV light at a level found in the environment (UVA and B, 22.47 $J/cm^2/h$). The following oxidative stress biomarkers were monitored in fish liver: concentrations of lipid hydroperoxide (LPO), carbonylated protein (PCO), and specific activities of superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST). Other biomarkers of physiological function as well as specific activities of acid phosphatase (AP), Na^+, K^+-ATPase and metallothionein levels (MT) were evaluated, respectively, in liver, brain and gills. Moreover, micronucleus and comet assays were performed on blood to assess genotoxicity biomarkers. Our results showed low toxicity of nano-TiO_2 to fish and lack of titanium accumulation in muscle tissue, thus substantiating literature data. However, they showed that nano-TiO_2 formulation/crystal phase and illumination influence the occurrence of sublethal effects. Pure anatase showed to cause more oxidative damage without co-exposure to UV, while the mixture

anatase:rutile showed to cause more sublethal effects when exposure occurred under UV light. These findings show that the specific activity of CAT, GST, PCO levels and comet assay results are useful as biomarkers of prolonged exposure to nano-TiO_2, since they demonstrated biochemical and genetic changes in fish exposed to TiO_2. They also show that it is particularly important to consider abiotic factors in the environment (especially illumination condition) and crystal properties when conducting nanoecotoxicological tests to assess nano-TiO_2 toxicity. Overall, our study substantiates the development and implementation of nanoecotoxicological protocols.

1. INTRODUCTION

According to the Project on Emerging Nanotechnology (2011), titanium is one of the most common material used in nano products. Titanium dioxide (TiO_2) has been commercially produced to be used as white pigment since 1900 (Grubb and Bakshi, 2010), and has been used in sunscreens for over 20 years (Labille et al., 2010). TiO_2 nanoparticles (nano-TiO_2) can be introduced into the environment by processes such as mining, TiO_2-enabled product fabrication, product use, recycling and disposal to the water environment. After being released to the environment, nano-TiO_2 can be transported to the subsurface (Labille et al., 2010), leach into the groundwater, and enter the food chain through bioaccumulation (Zhang and Guirard, 2013).

TiO_2 has three crystal phases: rutile, anatase and brookite. The rutile phase is commercially used in paints, plastics, coatings and cosmetics to provide color and opacity. The interest in anatase - TiO_2 particles in the nano range (particles with diameters between 1 and 100 nm) is due to their photocatalytic properties. TiO_2 is a semiconductor. Therefore, when it is excited by a photon in the ultraviolet range – especially at 300-388 nm (Gaya and Abdullah, 2008) – there is an electron flow from valence to conduction band. This results in charge separation, induction of a hole in the valence band and a free electron in the conduction band. These hole–electron pairs interact with H_2O or O_2 to generate reactive oxygen species (ROS), such as hydroxyl radicals and superoxide anion. The ROS-related phototoxicity of TiO_2 turns this nanomaterial into an excellent antibacterial and antiviral agent for drinking water treatment,

medical disinfection and potentially for the destruction of chemical contaminants in water, air and soil (Ma et al., 2012a).

Anatase and rutile phases have different photocatalytic properties. Compared to rutile, anatase presents a better combination of photoactivity and photostability (Gaya and Abdullah, 2008). Nonetheless, a mixture of anatase and rutile TiO_2 is usually employed in photocatalytic processes due to its higher catalytic efficiency (Cong and Xu, 2012). Also, when in the nano range, TiO_2 has a greater surface area than the bulk material, which makes the particle surface more sensitive to light and H_2O adsorption, thus improving photo efficiency (Banerjee et al., 2006).

Due to the fast growing number of commercial products that include or are made of manufactured nanoparticles, their dispersion in the environment may raise ethical, sociological and potential environmental concerns (Bigorgne et al., 2011). Literature reports LC_{50} of more than 100 mg/L for fish (Warheit et al. 2007b; Griffitt et al. 2008; Clemente et al., 2013), which indicates low toxicity to this organism. However, several authors discuss the occurrence of sublethal effects in organisms exposed to nano-TiO_2. The application of biomarkers in ecotoxicology studies has been widely studied in recent decades. Such scenarios have triggered research to establish early-warning signals, or biomarkers, which reflect adverse biological responses to anthropogenic environmental pollutants. Effects at higher hierarchical levels are always preceded by earlier changes in biological processes, allowing for the development of early-warning effect biomarker signals at later response levels. A biomarker is defined as a change in biological response (ranging from molecular, cellular and physiological responses to behavioral changes) that can be related to exposure to or toxic effects of environmental

chemicals. In an environmental context, biomarkers can be sensitive indicators that toxicants entered organisms, are distributed between tissues and are eliciting a toxic effect at critical targets (Van der Oost et al., 2003).

Many physiological parameters respond rapidly, following exposure to sublethal metal concentrations as part of a nonspecific stress response. The response is transient if the animal can compensate for the stressor or if the stressor is removed. Changes in serum enzyme activity are used as indicators of tissue injury, environmental stress, or a diseased condition. The serum enzyme activity increase rate depends on enzyme concentration in the cells, rate of leakage caused by injury, and rate of clearance of serum enzyme. Serum enzymes, such as alkaline and acid phosphatase, are considered important serum markers in the investigation of the health of concerned animal species. They are polyfunctional enzymes and responsible for removing phosphate groups from several molecule types, including nucleotides, proteins, and alkaloids, i.e. dephosphorylation, and play an important role in the skeletal mineralization of aquatic animals. In addition, phosphatase is one of the most significant enzymes involved in protein and amino acid metabolism (Heydarnejad et al., 2013).

All these mechanisms can be affected by inorganic and organic compounds. When there is an imbalance between ROS production and the organism's antioxidant defense, oxidative stress may occur, which is characterized by damage to biomolecules, such as lipids (lipid peroxidation), proteins (protein carbonylation) and DNA.

Metallothioneins (MTs) have a unique molecular structure that provides metal-binding and redox capabilities. These capabilities include maintaining metal balance that protects against intoxication by heavy metals and oxidative damage. In this sense, MTs may be seen as molecular

biomarkers that help monitor metal pollution in water ecosystems and oxidative stress (Xiang et al., 2013).

The superoxide dismutase is an antioxidant enzyme that plays a central antioxidant role. It catalyzes the reaction between superoxide radicals, producing hydrogen peroxide. In turn, H_2O_2 is captured and degraded by catalases and peroxidases enzymes. GST plays an antioxidant role and acts in the phase II of biotransformation; by conjugating GSH with xenobiotics, it facilitates xenobiotic elimination. Some changes to these enzyme activities have been linked to nano-TiO_2 exposure (Canesi et al., 2010a, Kim et al., 2010).

Water exposure to nano-TiO_2 seems to be more serious than dietary exposure to fish (Handy et al., 2008). Although some studies on nano-TiO_2 did not observe any changes to the biomarkers that are traditionally studied in ecotoxicology, as histopathology evaluation and biochemical and genetic analysis (Griffith et al., 2009; Landsiedel et al., 2010; Leed et al. 2009; Scown et al., 2009), increases or decreases in oxidative stress and inflammation biomarker levels, as well histopathological alterations have been reported in the liver, gills and intestines of fish exposed to nano-TiO_2 (Federici et al., 2007; Hao et al., 2009; Kim et al., 2010; Johnston et al., 2010; Palaniappan and Pramod, 2010; Chevallet et al., 2011; Xiong et al., 2011). While some authors report that liver is more sensitive than gills or brain (Hao et al., 2009), others state that some alterations in fish can be mainly linked to gill damage and consequent hypoxia (Boyle et al., 2013). Braydich-Stolle et al. (2009) describe that pure anatase induces membrane leakage in mouse keratinocyte, leading to necrosis. It has also been suggested that nano-TiO_2 toxicity to Daphnia is linked to reduced food consumption due to agglomerate uptake (Seitz et al., 2013).

It is still not clear how all current ecotoxicology biomarkers can be applied in nano-TiO_2 evaluation. Inhibition of Na^+, K^+-ATPase, which has an important function in maintaining cellular electrical potential and volume, was found in gills, intestines and brain of fish exposed to nano-TiO_2 by Federici et al. (2007), but no alteration was found in other studies (Boyle et al., 2013; Ramsden et al., 2013; Clemente et al., 2013). The same conflict can be seen in reports on nano-TiO_2 genotoxicity. ROS formed by exposing nano-TiO_2 to UV is highly reactive and can interact with biological molecules, causing oxidative stress (Ma et al., 2012a). Some studies showed no significant nano-TiO_2-induced DNA damage using the comet assay. However, other studies found nano-TiO_2-induced DNA damage (Prasad et al., 2013). The effect on DNA can be attributed to reduced glutathione levels with concomitant increase of lipid peroxidation and ROS generation (Chibber et al., 2013; Shukla et al., 2013).

In addition to the applicability of biomarkers, studies on the toxicity of manufactured NPs have also raised questions on the applicability of current ecotoxicological testing protocols (Handy et al., 2012a). One major issue is the exposure to ultraviolet (UV) radiation, which occurs naturally in the environment, but is not present in common bioassays. Pure water weakly absorbs UV light, and the exponential attenuation of UV (200-400 nm) in distilled water is lower than in seawater (ranging from 10/m at 200 nm to a minimum of 0.05/m at 375 nm) (Stewart and Hopfield, 1965, cited by Acra et al., 1990). Several studies have demonstrated the deleterious effects of environmental levels of UV light on fish, causing hematological, immunological and histopathological alterations (Salo et al., 2000; Sayed et al., 2007).

Although literature indicates that nano-TiO_2 has low toxicity on water organisms that were subjected to acute or prolonged exposure, ROS generated when it is exposed to ultraviolet

radiation can damage biological molecules. It is possible that laboratory studies made under light conditions without UV exposure may greatly underestimate its overall risk in the natural environment. On the other hand, some recent studies of cells, microcrustaceans and larvae have shown that by including UV light in bioassays one can change nano-TiO_2 toxicity parameters (Hund-Rinke and Simon, 2006; Reeves et al., 2008; Vevers and Jha, 2008; Marcone et al., 2012). Furthermore, it was observed that relatively low levels of ultraviolet light, consistent with those found in nature, can induce nano-TiO_2 toxicity to marine phytoplankton, the Earth's most important agent for primary production. TiO_2 was found to have no effect on phytoplankton in treatments where UV light was blocked. Under low intensity UV light, ROS increased in seawater with increasing nano-TiO_2 concentration. This may lead to increased overall oxidative stress in seawater contaminated by TiO_2 and cause decreased marine ecosystem resilience (Miller et al., 2012).

In a previous study, we showed that exposing fish *P. mesopotamicus* to nano-TiO_2 under UV light at a concentration of up to 100 mg/L for 96h did not cause mortality, but there were sublethal effects (Clemente et al., 2013). In addition, nano-TiO_2 toxicity seems to depend not only on abiotic factors, such as illumination condition, but also on its crystal phase and exposure time (Zhu et al. 2010b; Marcone et al., 2012; Clement et al., 2013). However, to our knowledge, so far no study has considered those factors conjointly when evaluating prolonged exposure of fish to nano-TiO_2. In fact, the ecotoxicological impact of the release of TiO_2 nanoparticles to the environment is still poorly documented, although they are increasingly being used in commercial goods.

The objective of this study was to evaluate the effect of nano-TiO_2 formulation/crystal phase and illumination condition in fish subjected to prolonged exposure. In order to address this matter, we investigated the interaction of both variables by observing organism survival and biomarkers associated with biochemical and genetic alterations. Several biomarkers that are widely used in ecotoxicology and are linked mainly to oxidative stress were investigated in order to evaluate nano-TiO_2 effects. We also studied titanium accumulation in fish tissue. Since the lack of knowledge of toxicity associated with nanomaterials can hinder the risk evaluation and management of these materials, this study's approach may help establishing nanoecotoxicology protocols.

2. MATERIALS AND METHODS

2.1 Characterization of NPs and of their stability in suspension

Toxicity of TiO_2 NPs was evaluated using titanium (IV) oxide nanopowder ("TA" - Sigma Aldrich, 100% anatase, primary particle size <25 nm, 99.7% purity) and Aeroxide P25 ("TM" - Degussa Evonik, 20% rutile, 80% anatase, primary particle size 25 nm, 50 m^2/g, 99% purity). These products have been widely studied and in literature they are characterized in a very similar way as by the manufacturer (Federici et al., 2007, Grassian et al., 2007, Griffith et al, 2008, Palaniappan et al., 2010).

A 1 g/L stock suspension of nano-TiO_2 in dechlorinated tap water was prepared by 10 min sonication with a high-frequency probe (CPX600 Ultrasonic Homogenizer, Cole Parmer,

USA) at 150 W/L and 100% amplitude. Immediately after sonication, the required volume was used to prepare a 100 mg/L suspension under bioassay conditions (constant aeration of the aquarium water). The hydrodynamic size, surface charge (zeta potential, ZP) and polydispersivity index (PdI) of particles in the 100 mg/L suspension were assessed through dynamic light scattering (DLS) using a Zetasizer Nano ZS90 instrument (Malvern Instruments, UK). Colloidal stability was also evaluated from optical spectra obtained in the wavelength range 200-600 nm using a UV-Vis spectrophotometer (Model 1650PC, Shimadzu, Japan). Measurements were taken at 0, 3, 5, and 24 h after preparation of the suspensions. All samples were collected at the middle level within the water column. The water had following characteristics: pH 7.5 ± 0.1, conductivity 1.3 ± 0.2 mS/cm, hardness 50.0 mg/L, temperature 27.0 ± 0.6 ºC, and dissolved oxygen (DO) 6.0 ± 0.6 mg/L.

2.2 Toxicity assay

The effects of nano-TiO_2 formulation/crystal phase, illumination condition, and the interaction of these variables were evaluated using prolonged exposures. Toxicity was determined by observing fish survival in conjunction with changes to biochemical and genetic biomarkers. In parallel, the accumulation of titanium in fish tissue was determined.

The pacu-caranha (*Piaractus mesopotamicus*) is a freshwater fish that is widely distributed in South America (Fishbase, 2012) and has already been used as bioindicator in ecotoxicological studies (Sampaio et al., 2008; Silva et al, 2010). Test organisms were juvenile fish weighing 9.4 ± 0.7 g and measuring 5.2 ± 0.4 cm in length. The experiment was preceded by

a 15-day acclimatization period and followed the OECD 204 protocol (OECD, 1984) with some modifications. Fish were kept in uncovered and constantly aerated aquaria containing 9 L of water (the characteristics of the water are described in section 2.1). The animals were fed daily throughout the experiment.

Fish were exposed for a 21-day period to three conditions: clean water (control), 100 mg/L nano-TiO_2 TA and 100 mg/L nano-TiO_2 TM. The suspensions in the aquaria were prepared from a stock suspension of nano-TiO_2, as described in section 2.1. All exposure suspensions were entirely replaced on a daily basis. The aquaria were exposed to two different illumination conditions: visible light (visible light groups) or ultraviolet and visible light (UV light groups). Each test condition was performed in duplicate (n=8 per group). Exposure to visible and UV light followed a photoperiod of 16/8 h (light/dark). Illumination conditions are further described in section 2.3.

After exposure, fish were anesthetized with benzocaine (0.2 mg/L) and then killed by medullary section. Blood was collected for micronucleus and comet assays, and liver, gills and brain samples were frozen at −70 °C for subsequent biochemical analyses. The axial muscle was also collected and stored at −70 °C for later titanium analysis.

The manipulation of animals was approved in June 2010 by the Commission for the Ethical Use of Animals of the State University of Campinas (CEUA/Unicamp, protocol n^o 2172-1, Anexo II e IV).

2.3 Illumination conditions

The employed illumination condition was the same described in our previous study (Clemente et al., 2013). Visible light was provided by Phillips lamps (40 W) that were installed on the room's ceiling. Exposure to artificial UV light employed four lamps (Q-Panel UVA340, 40 W) positioned 17 cm above the water surface. The visible light intensity in the laboratory was 250 ± 79 lux. At aquaria level there was no UV light detectable from the fluorescent lamps installed on the ceiling. The radiation of Q-Panel UVA340 lamps ranged from 300 to 610 nm, with peak emission at 340 nm. The UV light flow at the water surface was 21.63 J/cm^2/h (UVA) and 0.84 J/cm^2/h (UVB).

2.4 Biochemical analyses

Liver tissue samples were homogenized (1:4, w/v) in a cold phosphate buffer (0.5 mol/L, pH 7), and homogenates were centrifuged at 10,000 × *g* for 20 min at 4 °C. Aliquots of the supernatant of each sample were taken for lipid hydroperoxide (LPO, Anexo V) (Jiang et al., 1992) and protein carbonyl (PCO, Anexo VI) analyses (Levine et al., 1994; Sedlak and Lindsay, 1968). Specific superoxide dismutase (SOD, Anexo VII) (Ukeda et al., 1997), catalase (CAT, Anexo VIII) (Aebi, 1984), glutathione S-transferase (GST, Anexo IX) (Keen et al., 1976), and acid phosphatase (AP, Anexo X) activities (Prazeres et al., 2004) were measured.

Gill tissue samples were homogenized (1:5, w/v) in cold buffer (pH 8.6) containing Tris-HCl (20 mmol/L), saccharose (500 mmol/L), phenylmethanesulfonyl fluoride (0.5 mmol/L), and

β-mercaptoethanol (0.01%). Homogenates were centrifuged at 15,000 × *g* for 30 min at 4 °C, and the supernatant was used to determine metallothionein (MT, Anexo XI) concentration (Viarengo et al., 1997).

Brain tissue samples were homogenized (1:4, w/v) in cold buffer (adjusted to pH 7.4 with HCl) containing sucrose (0.3 mol/L), Na_2EDTA (0.1 mmol/L), and imidazole (30 mmol/L), and then centrifuged at 10,000 x *g* for 5 min at 4 °C. The supernatant was used to assess Na^+, K^+-ATPase activity (Anexo XII) (Quabius et al., 1997; Sampaio et al., 2008).

Protein concentrations in homogenates were determined using the Bradford bovine serum albumin protein assay (1976) (Anexo XIII) . At least 5 samples from each group were analyzed in each biochemical procedure, and all samples were analyzed in triplicate.

2.5 Genetic analyses

Comet and micronucleus assays have been use to determine the genotoxic and cytotoxic effect of various pollutants on fish. Micronuclei are small intracytoplasmic chromatin masses resulting from either chromosomal breakage during cell division or anaphase lagging chromosomes. On one hand comet assay is a simple, sensitive and rapid technique to detect DNA damage (single- and double-strand breaks, alkali-labile sites or DNA/DNA and DNA/protein crosslinks) in individual cells and can be very useful in genetic toxicology, especially ecogenotoxicology studies (Ahmed et al., 2013).

In the micronucleus assays, smears of blood from the fish were stained with Giemsa (Heddle, 1973; Schmid, 1975), and one thousand erythrocytes per slide were counted by optical

microscopy at x1000 magnification (Laborlux K microscope, Leica, Germany). The micronucleus frequency was recorded together with any morphological alterations in the nucleus (Carrasco et al., 1990).

Comet assays were conducted as described by Singh et al. (1988). A blood sample was diluted in fetal bovine serum, stored on ice in the dark for 24 h, and then prepared for the assay (Ramsdorf et al., 2009). 10 μL of this solution was mixed with 120 μL of low melting point agarose; then, a 100 μL sample was deposited on a slide coated with normal agarose. The slides were then immersed in a lysis solution (2.5 mol/L NaCl, 0.1 mol/L EDTA, 10 mmol/L Tris, 1% Triton X-100, pH 10) and kept at 20 ºC for at least 1 hour. After lysis, the slides were placed in an electrophoresis buffer (300 mmol/L NaOH, 1 mmol/L EDTA, pH ~13, 4 ºC) for 20 min prior to electrophoresis at 1.3 V/cm. The slides were neutralized for 15 min with a 0.4 mol/L Tris buffer at pH 7.5, then dried at room temperature and stained with silver. DNA strand breaks were scored using an optical microscope with magnification of x40. For each fish, 100 cells were visually analyzed according to the Collins et al. (1997) method. At least 4 individuals from each group were analyzed in a micronucleus assay and at least 3 individuals in a comet assay.

2.6 Titanium content in muscle tissue

At least three sets of pooled muscle samples from each group were digested as described by Weir (2011). The samples were placed on Petri dishes and dried in an oven at 100 ^{o}C. The sample weight was monitored every hour, until it remained constant. The tissues were then placed into Corning tubes and digested with analytical grade hydrogen peroxide (5 mL) and nitric

acid (1 mL) at 100 ^{o}C for 4 hours. The samples were subsequently removed and cooled to room temperature. After addition of nitric acid (4 mL) and hydrofluoric acid (1 mL), the samples were heated in a sand bath (120 ^{o}C) to evaporate the acid until 1-1.5 mL of solution remained. Finally, the samples were diluted to 10 mL with ultrapure water (Milli-Q). The analysis was performed using Inductively Coupled Plasma/Optical Emission Spectroscopy (ICP-OES, Agilent Model 720, USA). A standard curve was prepared using titanium ICP standard (Merck).

2.7 Statistical analysis

Biochemical and genetic variables were initially evaluated through two-way analysis of variance to study the influence of nano-TiO2 formulation (TA or TM), illumination condition (with or without UV light), and their interactions. When there was significant interaction, the responses at each formulation were compared within illumination levels, and vice versa, using bilateral t-tests for contrast (Montgomery, 2008). When there was no evidence of interaction, the levels of the main factors were compared independently, using the same types of test. Residual graphical analysis was used to test for normality and homoscedasticity; a significance level of 5% was adopted for all the tests. The statistical procedures were performed using the GLM procedure of SAS/STAT software, version 9.2.

3. RESULTS

3.1 Characterization of NPs and of their stability in suspension

Satisfactory DLS measurements were only taken at the beginning of the experiment. The initial mean particle size distribution (Z-average) in the 100 mg/L TA suspension was 543.9 ± 65 nm with a polydispersity index (PdI) of 0.24 ± 0.02 and a zeta potential (ZP) of 27.8 ± 2.7 mV. However, after 3 h, the suspension PdI shifted to 0.84, indicating the presence of a heterogeneous particle population, which compromised the quality of the analysis. The same occurred with 100 mg/L TM, which showed at 0h: Z-average 871.8 ± 90 nm; PdI 0.006 ± 0.01; ZP -27.6 ± 0.4 mV. After a 24-hour period, the ZP was -31.5 ± 1.29 mV and -27.8 ± 0.46 mV for TA and TM, respectively.

The high instability of the nano-TiO_2 suspension was confirmed by spectrophotometry (Figure 1). As time elapsed, there was a reduction in absorbance, indicating almost total precipitation of both formulations. For TA, the peak absorbance at 320 nm fell to 23.6 %, 17.9% and 4.8% of the initial value after 3, 5 and 24 hours, respectively. For TM, the initial absorbance at the same wavelength fell to 25.7%, 17.7% and 6.3% of the initial value after 3, 5 and 24 hours, respectively. After 24 hours, a white precipitate was observed at the bottom of the aquaria.

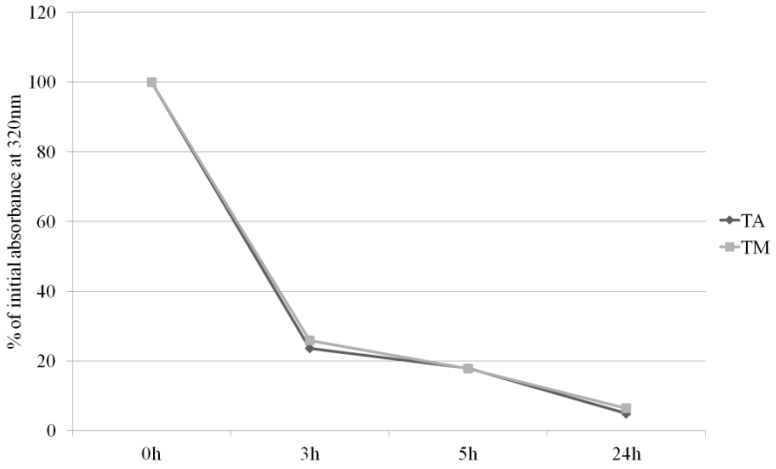

Figure 1. Colloidal stability: percentage (%) of initial absorbance at 320 nm of 100 mg/L TA and TM suspension in aquariums with constant aeration.

3.2 Toxicity test

We observed no fish mortality in any group, with or without UV light, and no abnormal behavioral symptoms. SOD ($p = 0.01$) and CAT ($p = 0.01$) activities showed to be under the influence of the illumination condition. In general, there was a reduction of enzymatic activities when fish were exposed to UV light. The contrast revealed that SOD activity (Table 1) was 39 % smaller in the control group exposed to UV light than in the control group exposed to visible light ($p = 0.02$). CAT activity (Figure 2I) of group TA with visible light was 112% higher than the control with visible light ($p = 0.02$), and 211% higher than TA with UV light ($p = 0.004$). The significance level of the interaction of illumination condition and formulation was 0.29.

PCO (Figure 2II) revealed an influence of the interaction of illumination and nano-TiO_2 formulation ($p = 0.007$). Under visible light, PCO in animals of the groups exposed to TA was 22% higher than the control ($p = 0.03$), while the opposite occurred, when fish were exposed to

UV light, being almost 55% lower than TA under visible light ($p = 0.01$) and the control under UV light ($p = 0.01$). When co-exposed to UV light, the group exposed to TM showed a 157% higher PCO than the group exposed to visible light ($p = 0.02$).

The specific activity of GST (Figure 2III) showed an effect of the formulation of nano-TiO_2, ($p = 0.0004$), being higher in groups exposed to TA ($p = 0.002$) and TM ($p = 0.0006$) under UV light compared to control under UV light. The significance level of the interaction of illumination and formulation was 0.17. Under visible light, the group exposed to TM also showed a 42% higher GST activity than the control ($p = 0.01$). GST activity in the control group exposed to UV light was 36% smaller than in the control group under visible light ($p = 0.03$).

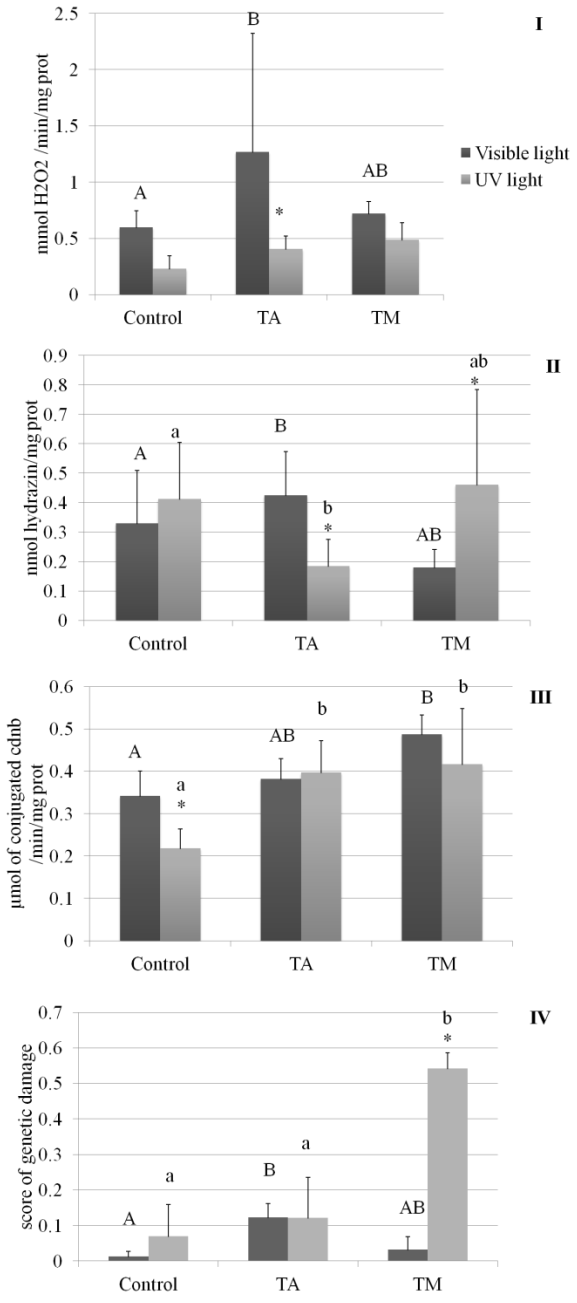

Figure 2. Biochemical and genetic biomarkers in *Piaractus mesopotamicus*: 21-day exposure to nano-TiO_2 (Control, TA and TM) under visible light or ultraviolet (UV) and visible light. Data are presented as mean ± standard deviation. I) Specific activity of catalase in liver; II) protein carbonylation in liver; III) Specific activity of glutathione S-transferase in liver; IV) Comet assay in erythrocytes. * $p<0.05$, between Visible light and UV light, in the same formulation; different uppercase letters indicate $p<0.05$ among formulations under Visible light; different lower case letters indicate $p<0.05$ among formulations under UV light.

Micronucleus ($p = 0.04$) and comet assays ($p < 0.001$) showed an influence of the interaction of illumination condition and nano-TiO_2 formulation. Micronuclei (Table 1) were not found, but the extent of morphological alterations in the erythrocyte nucleus was 93% higher in the control group under UV light than in the control under visible light ($p = 0.02$) and respectively 90% and 145% higher than TA ($p = 0.03$) and TM ($p= 0.008$) with UV light. In the comet assay (Figure 2IV), co-exposure to TM and UV increased the occurrence of genetic damage in fish erythrocytes by over 346 % compared to groups exposed to TM under visible light ($p < 0.001$), the control ($p < 0.001$) and TA ($p<0.001$) under UV light. The group exposed to TA showed a genetic damage score over 800% higher than the control ($p = 0.03$) under visible light.

No statistically significant difference between the groups was observed for other biomarkers (Table 1) or for muscle titanium content (Table 2).

Table 1. Biochemical and genetic biomarkers in *Piaractus mesopotamicus* following a 21-day exposure to nano-TiO_2 (control, TA 100 mg/L and TM 100 mg/L) under either visible light or ultraviolet (UV) light. Results are presented as mean ± standard deviation. Specific activities of superoxide dismutase (SOD), acid phosphatase (AP) and lipid hydroperoxide concentration (LPO) in the liver; metallothionein (MT) concentration in the gills, specific activity of Na, K –ATPase in the brain; micronucleus assay on erythrocytes are presented.

	Control				**TA**				**TM**			
	Visible light	*n*	*UV light*	*n*	*Visible light*	*n*	*UV light*	*n*	*Visible light*	*n*	*UV light*	*n*
SOD (U/ mg prot)	13.63 (± 41.61)	6	8.68 (±15.17)*	4	100.44 (± 25.10)	5	94.40 (± 26.73)	6	117.28 (±11.25)	4	85.13 (± 36.51)	7
AP (nmol of pNP / min/ mg prot)	41.13 (± 21.03)	6	29.41 (± 3.79)	4	42.61 (± 21.80)	6	79.84 (± 72.69)	8	41.87 (12.96)	5	35.52 (± 7.42)	7
LPO (nmol of hydroperoxide/ mg prot)	14.99 (± 3.63)	7	18.59 (± 4.63)	3	19.54 (± 5.65)	8	16.23 (±3.84)	8	17.47 (± 5.40)	5	15.29 (± 1.94)	6
MT (mmol metallothionein/ mg prot)	4.90 (± 3.77)	4	5.45 (± 0.86)	3	5.84 (± 3.82)	5	3.69 (± 1.32)	5	3.85 (± 2.47)	4	4.13 (± 2.51)	6
Na/K – ATPase (µmol of Pi/ mg prot/ h)	0.57 (± 0.37)	7	0.63 (± 0.18)	4	0.45 (± 0.14)	9	0.54 (± 0.13)	9	0.54 (± 0.26)	7	0.60 (± 0.27)	8
Micronucleus assay (morphological alteration in nucleus /1000 cells)	7.25 (± 2.76)	8	14.00 (± 10.33) [a] *	4	5.64 (± 3.29)	9	7.38 (± 2.97)[b]	8	9.00 (± 6.71)	7	5.71 (± 3.79)[b]	8

* indicates $p<0.05$, between visible light and UV light, in groups exposed to the same formulation;
different lower case letters indicate $p<0.05$ among groups exposed to UV light.

Table 2. Titanium content in muscle tissue (µg of Ti/g muscle) of *Piaractus mesopotamicus* after a 21-day exposure to nano-TiO_2 under either visible light or ultraviolet (UV) light. The results are presented as means ± standard deviations.

	Visible light		**UV light**	
	µg of Ti/g muscle	*n*	*µg of Ti/g muscle*	*n*
Control	8.17 (± 4.57)	4	13.21 (± 1.69)	3
TA	14.28 (± 13.76)	5	11.28 (± 5.24)	4
TM	12.00 (± 4.62)	3	7.80 (± 4.07)	3

4. DISCUSSION

To date, only very few studies have been published on prolonged exposure of fish to nano-TiO_2 (Federici et al., 2007, Chen et al, 2011b; Boyle et al. 2013; Ramsden et al, 2013). To the best of our knowledge, none of these studies has evaluated all biomarkers that are discussed in this paper, although one of them considered illumination when evaluating fish toxicity.

A previous study by our group evaluated the employed illumination conditions, which were deemed consistent with environmental UV radiation levels (Clemente et al., 2013). In physiologically relevant doses, sun exposure generates reactive oxygen species (ROS), which may also interact with lipids and proteins (Valavanidis et al., 2006; Hwang and Kim, 2007; Franco et al., 2009). However, our study did not observe any difference between control groups as to their lipid peroxidation (LPO) and protein oxidation (PCO) levels. On the other hand, when comparing the control group that was kept under visible light to the one under UV light, we observed a statistically significant reduction in the activity of antioxidant enzymes SOD and GST and also an increased number of nuclear morphologic alterations in red blood cells of fish (micronucleus test). SOD and GST are known oxidative stress biomarkers. While the former

eliminates superoxide anion, the latter conjugates reactive species and other electrolytes (Van der Oost et al., 2003, Valavanidis et al., 2006). Reduced SOD and GST activities might be related to an inhibition or depletion of these antioxidant mechanisms due to excessive generation of ROS. It is also well known that when water is exposed to ionizing radiation, OH• is produced (Halliwell and Gutteridge, 1992), which can react with the DNA and form multiple products (Halliwell and Gutteridge, 1992, Banerjee et al., 2006). This would explain the genotoxicity that was observed in the control group that was exposed to UV radiation.

Throughout our study, we observed intense aggregation and precipitation of nano-TiO_2. This is a complicating factor in the assessment of material toxicity that has been discussed in several studies (Adams et al., 2006; Wiench et al., 2009; Zhu et al. 2010b). The presence of ions (conductivity of 1.3 mS/cm) and a pH close to the point of zero charge of TiO_2 (pHzpc) of the water used in the bioassays explain the intense formation of aggregates (Finnegan et al., 2007; Von der Kammer et al., 2010). Some authors question the environmental relevance of using methods that ensure the complete dispersion of nano-TiO_2 in exposure media (Baveye e Laba, 2008, Crane et al., 2008). As a matter of fact, in the environment, NP aggregation and precipitation will likely occur. Some studies have, however, indicated that the presence of organic matter may increase the stability of nano-TiO_2 particles, which suggests that in the environment they probably do not aggregate as easily (Domingos et al., 2008; Yang et al., 2009; Arvidsson et al., 2011).

In order to better understand the potential fate and behavior of NPs in water systems, it is essential to understand their interaction with natural water components, such as environmental colloids and natural organic matter, under a variety of physicochemical conditions, such as pH,

ionic strength and type and concentration of cations. To date, little is known on these interactions, specifically concerning engineered nanoparticles. Sorption of contaminants onto NPs depends on their properties, such as composition, size, purity, structure and solution conditions, such as pH and ionic strength (Christian et al., 2008). Pure anatase TiO_2 NPs exhibited stronger affinity and higher sorption capacity than materials that were composed of anatase with additional amounts of rutile (Giammar et al. 2007).

Due to its nanoparticle aggregation and precipitation and since it is the most commonly used and recommended method in literature (Handy et al., 2012a), fish were exposed to semi-static conditions with 24-hour renewal of the suspensions. The suspension renewal emulates an environment with constant nanoparticle inflow. The exposure method might significantly affect nano-TiO_2 toxicity. Sentz et al. (2013) exposed Daphnia to nano-TiO_2 under two different experimental conditions (semi-static and continuous flow), and report that reproduction was more affected when they were exposed to anatase under semi-static conditions. The authors suggest that higher toxicity might be due to the formation of precipitate on the bottom of the exposure unit. The ingestion of aggregates would affect Daphnia's food intake and thus their nutritional status.

Nano-TiO_2 bioavailability to water organisms is still not clear, but the fact that aggregation occurs does not mean that the material is not bioavailable (Handy et al., 2008). Some authors report Ti accumulation in tissues (Zhang et al., 2006; Zhu et al., 2010a, b), while others did not observe a significant absorption by water organisms (Federici et al., 2007; Johnston et al., 2010). Ramsden et al. (2013) report Ti accumulation in *Danio rerio* that were exposed at 1 mg/L for 14 days with a 100% depuration after 21 days. The experimental model used in this study did

not present any Ti accumulation in fish muscle tissue. Intravenous administration of high doses of nano-TiO_2 in fish resulted in greater accumulation in the kidneys (Scown et al., 2009), while exposure to nano-TiO_2 in water caused Ti accumulation primarily in the gills, as well as in liver, brain, and heart tissues (Chen et al., 2011b). In addition to Ti found in gills and viscera, Zhang et al. (2007) also detected Ti in the muscle and skin of fish after prolonged exposure to nano-TiO_2 in the water. Our study chose to analyze muscle tissue, since it is the fish part that humans consume and is the most abundant. Absence of Ti accumulation in fish muscle tissue means that there is a low risk to human health from trophic transfer. However, the risk to other predators, which also consume fish viscera, cannot be ignored.

According to literature (Federici et al., 2007; Warheit et al., 2007b; Griffitt et al., 2008; Ramsden et al., 2013), exposure to nano-TiO_2 has not caused fish mortality under any of the tested conditions. Despite intense nano-TiO_2 precipitation and the fact that no significant Ti accumulation was observed in fish tissue, our study observed effects on biomarkers that were dependent on the nano-TiO_2 formulation used and the illumination condition.

Ma et al (2012b) showed that nano-TiO_2 toxicity under simulated solar radiation increased by two to four orders of magnitude compared to toxicity under ambient laboratory light, with a 48-h median lethal concentration (LC_{50}) of 29.8 mg/L in *D. magna* and a 96-h LC50 of 2.2 mg/L in medaka. The capacity of causing oxidative stress through the generation of ROS has been suggested as paradigm to access potential toxicity of engineered NPs (Fenoglio et al. 2009, Xiong et al., 2012). In vitro studies correlate the occurrence of oxidative stress to reduced cell viability, following exposure to nano-TiO_2. ROS is an important factor in the apoptosis process. Park et al. (2009) report GSH reduction, oxidative stress induced gene expression and

inflammation, caspase-3 activation and chromatin condensation in human bronchial epithelial cells exposed to P25.

TA caused greater alterations in studied biomarkers in the absence of UV radiation. The onset of oxidative stress became evident through the increased activity of the antioxidant enzyme CAT, protein carbonylation (PCO) and DNA damage score (comet assay) in fish exposed to TA under visible light, when compared to other groups. Our results substantiate papers published by Kim et al. (2010) and Xiong et al. (2011), which report increased CAT activity in water organisms exposed to nano-TiO_2. However, reduced CAT and SOD activity and expression were reported as well (Hao et al., 2009, Cui et al., 2010). Hussain et al (2009) report that the administration of catalase reduced proinflammatory responses in bronchial epithelial cells exposed to anatase and to anatase:rutile mixture, which indicates that the oxidative stress is related to said responses, especially to H_2O_2 generation. Fenoglio et al. (2009) have already described free-radical-generation by nano-TiO_2 even without UV radiation. In this study, the authors report that in the dark, anatase reacted with organic molecules over broken C-H bridges.

Compared to the control group, co-exposure to TA and UV radiation showed an increased GST activity, reduced PCO, and equal CAT and DNA damage. A previous study by our group (Clemente et al., 2013) has also indicated a PCO reduction, when fish were exposed for 96 hours to TA under UV radiation. According to Blumberg et al (2004), it is not always easy to establish whether biomarker alterations have a pathological cause or are a mechanism for physiological adjustment. It is logical to think that the exposure to TA under UV light should increase oxidative stress, but it was not clear in this study. Our hypothesis is that the exposure to TA without or with UV light activates different antioxidant mechanisms. The apparent lack of

oxidative stress in fish exposed to anatase under UV light compared to group under visible light may be the reflection of an intense antioxidant answer to a high generation of ROS. The increase in GST activity that was observed in this group may very well be one of those mechanisms, but other mechanisms than those studied here, can be involved too.

Prolonged exposure to TM in the absence of UV light has also increased GST activity. Other biomarkers, however, have remained at levels that were comparable to the ones found in the control group. Our results confirm the available literature, which reports increased GST activity in Daphnia and mollusks exposed to nano-TiO_2 (Canesi et al., 2010a, Kim *et al.*, 2010). In our study, we observed that GST remained elevated with co-exposure to TM and UV radiation. However, antioxidant mechanisms were not capable of containing damages to proteins and DNA, since there was an increase of PCO and of breaks in DNA strands, shown by the comet assay DNA damage score.

Nano-TiO_2 genotoxicity is still controversial. Dose-dependent DNA damages caused by exposure to nano-TiO_2 have already been described through *in vivo* and *in vitro* testing (Griffith et al., 2009; Singh et al, 2009; Hu et al., 2010; Turkez, 2011). Some papers also report elevated cytotoxicity and genotoxicity when nano-TiO_2 is irradiated (Reeves et al., 2008, Vevers and Jha, 2008, Xiong et al., 2013). However, several did not observe genotoxicity (Landsiedel et al., 2010; Leed et al. 2009; Saquib et al., 2012; Shuwe et al., 2007). Based on the evidence presented in some studies, the International Agency for Research on Cancer (IARC) classified TiO_2 as "possibly carcinogenic to humans" (group 2B) (IARC, 2010).

We chose to test the 100 mg/L concentration, because in a previous acute exposure study (Clemente et al, 2013), it did not cause any mortality and only minor sublethal effects. A

concentration of 100 mg/L is the maximum the OECD (1984) recommends for toxicity tests; higher concentrations are reckoned to have little environmental relevance. Gottschalk et al. (2009) calculated the predicted environmental concentrations (PEC) for nano-TiO_2 in the US and Europe. Simulations ranged from 21 µg/L for surface waters to 4 µg/L in wastewater treatment effluents. In Arizona, Kiser et al. (2009) report Ti concentrations ranging from 185 µg/L to 2800 µg/L in municipal wastewater treatment influents/intakes. In average, removal efficiency for particles smaller than 700 nm was 42%. The release of synthetic nano-TiO_2 from urban applications to the water environment was reported by Kaegi et al. (2008), who showed that nano-TiO_2 leached from nano-TiO_2-containing paint and entered receiving waters, where concentrations reached 16 µg/L. With continued use of TiO_2-containing commercial products, it is to be anticipated that environmental levels of TiO_2 will steadily increase and eventually be discharged to water systems (Ma et al., 2012b). Therefore, it is very unlikely that the concentration we tested here is found in the environment, but it can be used as basis for future lab studies and to establish regulatory policies.

Studies have shown that the anatase:rutile combination has higher photoactivity than other sources. Evonik's P25 nano-TiO_2 is the most commonly used product in photocatalytic processes (Nogueira and Jardim, 1998, Malato et al., 2009). For *Daphnia magna*, Clément et al. (2013) report a $EC50_{72h}$ for anatase nanoparticles and a anatase:rutile mixture 30 to 70 times smaller than for rutile. Marcone et al. (2012) observed that, under UVA, toxicity of P25 to *Daphnia similis* is greater than of nano-TiO_2 anatase-S they had produced. They discussed the possibility that different outcomes were due to the products' different photoactivation and consequent formation of free radicals. Our results also indicate that the anatase:rutile combination

generated more sublethal effects in fish exposed to environmental UV levels than pure anatase nano-TiO_2 did.

It still has not become clear why the different crystal phases of TiO_2 present different photocatalytic properties. Some authors suggest that anatase has a higher photoactivity than rutile, because it has an indirect band gap, and the band gap and Fermi level are higher, and present different O_2 adsorption rates (Banerjee et al., 2006; Sun and Xu, 2010). The synergistic effect between anatase and rutile particles was thought to be related to greater absorption of UV light by rutile (Coatingsys, 2009), but recently Cong and Xu (2012) proposed it to be due to O_2 transfer from anatase phase to rutile that explores the masked photoactivity of rutile particles.

The association between cell membranes and nano-TiO_2 cytotoxicity seems to depend on crystal phase and size (Allouni et al., 2012; Xiong et al., 2013). Xiong et al. (2013) suggest that the dependence of nano-TiO_2 cytotoxicity on size is related to the generation of ROS and adsorption of biomolecules by particles, which are inversely correlated to NP size. Allouni et al. (2012) report that the percentage of cells associated to nano-TiO_2 was significantly higher in particles that contained the anatase:rutile mixture than in formulations containing only one of the crystal phases. Johnston et al. (2010) reported the presence of nano-TiO_2 aggregates on the surface of gill epithelium after acute exposure, as well as in gill lamellae after 14 days of exposure. Studies indicate that the sublethal effects that have been observed in fish may be related to gill damage and consequent hypoxia (Federici et al., 2007; Hao et al., 2009; Chen et al., 2011a, b; Xiong et al., 2011). All that considered, we suggest that the difference by which organisms respond to the tested formulations is related to the different photocatalytic properties, and thus to ROS generation, as well as to the different adsorption and absorption of nano-TiO_2

formulations by this organism, especially in gills. In this study, we were not able to quantify titanium in gills, since there was little material available. The quantity was sufficient, however, to determine the concentration of metallothionein, a biomarker of metal exposure and oxidative stress. Some papers indicate MT induction in organisms exposed to nano-TiO_2 (Bigorne et al., 2011; Clemente et al., 2013), but this study did not present MT level alterations in gills, which could substantiate this hypothesis.

In order to correctly assess nanotechnology risks, we first have to know all factors that are involved in the different NP formulations and in toxicity generation. Our study adds to the knowledge of TiO_2 nanoparticles toxicity. It clearly shows that the effects of nano-TiO_2 on fish depend on formulation/crystal phase and on illumination condition. Aligned with literature (Chen et al., 2011b; Wang et al., 2011; Ramsden et al., 2013), our results indicate that prolonged rather than acute exposure intensifies biomarker alterations. Therefore, studies on nano-TiO_2 toxicity in water organisms based on prolonged and chronic exposure should have greater relevance to estimates of environmental risk. Responses to biomarkers commonly used in ecotoxicology may vary for different reasons. However, we can still not determine if these findings are indicative of pathologic conditions or homeostatic mechanisms. Further research is needed, but our results add to the knowledge required to study the factors that may influence responses in organisms exposed to nano-TiO_2.

5. CONCLUSIONS

Prolonged exposure of fish to nano-TiO_2 concentrations of up to 100 mg/L under visible light or co-exposed to UV light at environmental levels resulted in no fish mortality . However, changes in biomarkers mainly linked to oxidative stress were observed. Biomarker changes depend on formulation/ crystal phase and illumination condition. In the case of nano-TiO_2 anatase, oxidative stress could be observed when exposure occurred under visible light; it was highlighted by an increased catalase activity, protein carbonylation and genetic damage. On the other side, groups exposed to the anatase:rutile mixture showed protein carbonylation, genetic damages and a higher glutathione S-transferase activity when co-exposed to UV light. Difference in the photocalytic properties and ROS generation of both nano-TiO_2 formulations may be related to different biomarker responses, as well as different nano-TiO_2 adsorption or absorption by the organism, especially in gills.

The parameters tested catalase and glutathione S-transferase activities, and protein carbonylation levels, and the comet assay showed that they could be useful as prolonged nano-TiO_2 exposure biomarkers . On the other hand, metallothionein, lipid peroxidation and specific activities of acid phosphatase and Na^+/K^+-ATPase activities did not respond to any condition tested.

The inclusion of abiotic factors (specially environmental UV levels), as well as the investigation of different formulations and the intensification of prolonged and chronic exposure in nano-TiO_2 toxicity studies are essential to obtain a more accurate and detailed risk assessment of this nanotechnology.

CAPÍTULO IV

ESTUDO COM MICROCRUSTÁCEOS

Artigo aceito para publicação: Clemente, Z. et al. Minimal levels of ultraviolet light enhance the toxicity of TiO_2 nanoparticles to two representative organisms of aquatic systems. Journal of Nanoparticle Research.

ABSTRACT

A number of studies have been published concerning the potential ecotoxicological risks of titanium dioxide nanoparticles (nano-TiO_2), but the results still remain inconclusive. The characteristics of the diverse types of nano-TiO_2 must be considered in order to establish experimental models to study their toxicity. TiO_2 has important photocatalytic properties, and its photoactivation occurs in the ultraviolet (UV) range. The aim of this study was to investigate the toxicity of nano-TiO_2 to indicators organisms of freshwater and saline aquatic systems, under different illumination conditions (visible light, with or without UV light). *Daphnia similis* and *Artemia salina* were co-exposed to a sublethal dose of UV light and different concentrations of nano-TiO_2 in the form of anatase (TA) or an anatase/rutile mixture (TM). Both products were considered pratically non-toxic under visible light to *D. similis* and *A. salina*. Under this condition, the $EC50_{48h}$ value for *D. similis* was >1000 mg/L (both products) and for *A. salina* were 480.67 mg/L (TA) and 284.81 mg/L (TM). Exposure to nano-TiO_2 under visible and UV light enhanced the toxicity of both products. In the case of *D. similis*, TM was more toxic than TA, showing values of $EC50_{48h}$ = 60.16 and 750.55 mg/L, respectively. *A. salina* was more sensitive than *D. similis*, with $EC50_{48h}$ = 4 mg/L for both products. Measurements were made of the growth rates of exposed organisms, together with biomarkers of oxidative stress and metabolism (glutathione S-transferase, glutathione peroxidase, catalase, superoxide dismutase and acid phosphatase). The results showed that the effects of nano-TiO_2 depended on the organism, exposure time, crystal phase and illumination conditions, and emphasized the need for a full characterization of nanoparticles and their behavior when studying nanotoxicity.

1. INTRODUCTION

Rapid industrial development, increased urban population densities, and inadequate or absent waste treatment systems have resulted in a wide range of contaminants reaching water bodies. As a result, there has been a renewed focus on water pollution problems and possible treatment methods. Photocatalysis is a technique that can be used to degrade pollutants, forming harmless substances such as CO_2 and H_2O (Macwan et al., 2011). TiO_2 is a crystalline semiconductor that possesses the important property of being able to be photoactivated at wavelengths in the range 300-338 nm (Nogueira and Jardim, 1998; Gaya and Abdullah, 2008). Its resistance to corrosion and decomposition, combined with its low cost and the possibility of using solar ultraviolet (UV) radiation, makes TiO_2 especially attractive for use in heterogeneous photocatalysis (Malato et al., 2009). The naturally-occurring crystalline phases of TiO_2 are anatase, rutile, and brookite, of which anatase and rutile are the most common. The rutile form is thermodynamically stable at all normal temperatures and pressures (USEPA, 2010). However, the anatase form shows the highest photocatalytic activity, since it possesses a more negative conduction band potential (higher potential energy of photogenerated electrons), it has a high specific surface area, and is photochemically stable as well as relatively inexpensive (Gaya and Abdullah, 2008; USEPA, 2010). TiO_2 anatase is recognized for its strong photoinduced redox potential and its effectiveness in the purification and disinfection of both air and water, and is used for the remediation of contaminated environments (Macwan et al., 2011). Nonetheless, there is evidence of synergism between the crystal phases, and that an anatase/rutile blend is more

photoactive, compared to the pure phases. The nano-TiO_2 material P25® (produced by Evonik Degussa) is the mixture most commonly employed in photocatalityc processes (Nogueira and Jardim, 1998; Malato et al., 2009).

A range of TiO_2 nanoparticles (NPs) are now in production (Xiaobo, 2009), varying in terms of particle size, surface area, purity (affected by doping, coating, or quality control issues), surface characteristics, crystalline shape, and chemical reactivity, amongst other properties. Since it is already widely used, and also shows promise in new emerging applications, nano-TiO_2 has been the subject of a number of ecotoxicological investigations. However, questions have been raised concerning both the suitability of existing nanoecotoxicological protocols and the need for standardization (Handy et al., 2012a). Furthermore, a general lack of appropriate characterization of these materials hinders comparison of existing studies, which have often shown conflicting results due to differences in experimental protocols as well as the materials used.

The photoactivation of nano-TiO_2 generates reactive oxygen species (ROS) able to degrade organic and inorganic compounds (Chatterjee and Dasgupta, 2005; Fujishima and Zhang, 2006). The ROS production has also been proposed to be the principal cause of the material toxicity towards different organisms. An imbalance between the production of ROS and the antioxidant systems of the organism can lead to a condition known as oxidative stress, resulting in damage to biomolecules including proteins, lipids, and DNA (Hwang and Kim, 2007). Ma et al. (2012a) reported that the immobilization of *D. magna* exposed to nano-TiO_2 was related to the production of ROS. Another mechanism of toxicity could be by physical action; it has been suggested that the toxicity of nano-TiO_2 to *Daphnia* may result from reduced food consumption due to the ingestion of agglomerates of the nanomaterial (Seitz et al., 2013). Use of nuclear

microscopy indicated that Ti was only located in the digestive tract of *Daphnia* exposed to nano-TiO_2 (Keller et al., 2010). However, in work using confocal microscopy, Li et al. (2011) found that Ti was present in the digestive tract, tissues, brood chamber, and appendages of *Ceriodaphnia dúbia*. The authors suggested that nano-TiO_2 was unable to penetrate the gut wall, and proposed an exposure route involving direct contact, ingestion, and internal contact with tissues and embryos. In another study, Braydich-Stolle et al. (2009) reported that pure anatase induced rupture of the keratinocyte membrane in rats, leading to necrosis.

A wide range of EC50 values (the concentration that causes toxic effects in 50% of the exposed population) have been reported for microcrustaceans exposed to nano-TiO_2 (Clemente et al., 2012). Most studies describe the substance as being non-toxic to *Daphnia* (Griffith et al., 2008; Heinlaan et al., 2008; Lee et al., 2009; Kim et al., 2010; Rosenkranz, 2010), although $EC50_{48h}$ values of 26.6 mg/L (Wiench et al., 2009) and 42 mg/L (Li et al., 2011) have also been reported. Lovern and Kapler (2006) obtained an $EC50_{48h}$ of 5.5 mg/L for *D. magna* exposed to filtered nano-TiO_2, but observed no mortality or behavioral abnormalities for exposures during the same period to concentrations of up to 500 mg/L of the same nano-TiO_2, but with sonication of the suspension. Extension of the exposure period from 48 to 72 h appeared to substantially increase the toxicity of the nano-TiO_2, with $EC50_{72h}$ values of 1.30, 3.15, and 3.44 mg/L for anatase sizes of 15, 25, and 32 nm, respectively (Clément et al., 2013), and a value of 1.62 mg/L for P25 (Zhu et al., 2010b).

There are few studies that have evaluated the toxicity of nano-TiO_2 in *Artemia*, an important indicator organism in inland saltwater ecosystems. Barelds (2010) exposed *Artemia* nauplii to concentrations up to 10 mg/L of nano-TiO_2, and were not able to establish an $EC50_{24h}$;

however, greater toxicity (12.6% mortality) was observed in the organisms exposed to the lowest concentration (0.01 mg/L), compared to groups exposed to 1 and 10 mg/L.

In bioassays employing aquatic organisms, the circadian cycle is usually established using fluorescent lamps. These mainly emit visible light, while under natural conditions the organisms are exposed to solar radiation (infrared, visible, and ultraviolet light). The photocatalytic properties of nano-TiO_2 can increase its toxicity to aquatic organisms under natural conditions, and examples of this can be found in the literature (Ma et al., 2012b; Marcone et al., 2012; Clemente et al., 2013; Xiong et al., 2013). The effect of UV radiation on aquatic organisms varies according to species and the UV wavelength employed. There have been reported ED50 values for UVB light as 0.035 mW/cm^2 (48 h exposure) for *D. magna* (Kim et al., 2009), 0.0028 mW/cm^2 (24 h exposure) for *Brachionus* sp. (Kim et al., 2011), and 580 J/m^2 (120 h exposure to UVA and UVB, corresponding to 13 mW/cm^2) for *Artemia franciscana* (Dattilo et al., 2005). However, there appears to be no information in the literature concerning UV irradiation ED50 values for *D. similis* and *A. salina*.

The determination of concentrations that cause no adverse effect on physiological parameters in a longer exposure time is extremely relevant to propose maximum allowable limits for water bodies. The results that have been reported for sublethal effects in aquatic organisms exposed to nano-TiO_2 differ widely and are sometimes contradictory. Nonetheless, such studies are important, since they can provide evidence of adverse effects for use in environmental risk assessments. The use of biomarkers to evaluate risk has the advantage of enabling early detection of potentially toxic exposure (Nascimento et al., 2008). Although some studies have not found any changes, others have described increases or decreases in the activities of the antioxidant

enzymes catalase, superoxide dismutase, glutathione S-transferase, and peroxidase, in aquatic organisms exposed to nano-TiO_2 (Federici et al., 2007; Hao et al., 2009; Scown et al., 2009; Kim et al., 2010). The effects of nano-TiO_2 have not been evaluated for other equally important enzymes such as the phosphatases, which are involved in a variety of transphosphorylation reactions and can be affected by metals and ROS (Aoyama et al., 2003).

The objective of this study was to evaluate the acute and long-term toxicity of different formulations of nano-TiO_2 to microcrustaceans exposed under varying illumination conditions, by observing their mobility, biochemical analysis, and growth rate assessments. Ecotoxicological tests were conducted using organisms that were representative of the same trophic level in different aquatic ecosystems: freshwater (*Daphnia similis*) and saltwater (*Artemia salina*).

2. MATERIALS AND METHODS

2.1 Characterization of the NPs and their stability in suspension

Evaluation of nano-TiO_2 toxicity in microcrustaceans was performed using titanium (IV) oxide nanopowder ("TA" - Sigma Aldrich, 100% anatase, primary particle size <25 nm, 99.7% purity) and Aeroxide P25 ("TM" - Degussa Evonik, 20% rutile, 80% anatase, primary particle size 25 nm, 50 m^2/g, 99% purity). These commercial products have been extensively studied, and their measured characteristics have been reported to be very close to the manufacturers'

specifications (Federici et al., 2007; Grassian et al., 2007; Griffith et al., 2008; Palaniappan et al., 2010).

Stock suspensions (1 g/L) of each nano-TiO_2 in Milli-Q water were prepared by sonication for 10 min using a high frequency probe (CPX600 Ultrasonic Homogenizer, Cole Parmer, USA) operated at 20% amplitude (120 W/L). Immediately after the sonication, suitable volumes were removed in order to prepare suspensions at concentrations of 1, 10, and 100 mg/L under the same bioassay conditions (dilution in the *Daphnia* or *Artemia* culture media).

The hydrodynamic size, surface charge (zeta potential, ZP), and polydispersion index (PdI) of the particles in the 100 mg/L suspensions were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS90 instrument (Malvern Instruments, UK). The colloidal stability of the 1, 10, and 100 mg/L suspensions was evaluated by means of optical spectra obtained in the wavelength range 200-600 nm using a UV-Vis spectrophotometer (Model 1650PC, Shimadzu, Japan). The measurements were made 0, 3, 6, and 24 h after preparation of the suspensions. All samples were collected from the center of the water column. For each suspension, the precipitation rate was calculated from the angular coefficient of the linear regression curve obtained using the logarithm of the absorbances at 325 nm.

2.2 Test organisms and culture media

Daphnia similis neonates were separated from a colony maintained in the laboratory (Anexo XV), using a sieve with 0.5 mm orifices. The culture and exposure media were prepared using tapwater that had been previously filtered for 48 h using a filter containing activated

carbon. The characteristics of the water used for the *Daphnia* were: pH 7.9 ± 1; conductivity 133 ± 1 µS/cm; total hardness 2º dGH; temperature 20 ± 1 ºC; dissolved oxygen (DO) 6 ± 0.5 mg/L.

Nauplii of *Artemia salina* were obtained after 48-h incubation of commercial cysts of *Artemia* (Maramar®, Brazil) in 3% saline solution (Red Sea Salt®) prepared with distilled water. For the growth tests, saccharose (3%) was added to the exposure medium. The characteristics of the water used for the *Artemia* were: pH 8 ± 1; conductivity 40 ± 1 mS/cm; temperature 20 ± 1 ºC; dissolved oxygen 6 ± 0.5 mg/L.

The bioassays were performed using small glass Petri dishes (5 cm diameter) containing 10 ml of exposure medium. All the tests were performed in quadruplicate, with five individuals for each replicate (n = 20 per group).

2.3 Illumination conditions

Measurements of natural (solar) and artificial ultraviolet radiation were performed using a spectroradiometer (Model USB 2000+RAD, Ocean Optics, USA) and a radiometer (Model VLX-3W, Cole Parmer, USA) with different sensors for UVA (365 ± 2 nm), UVB (312 ± 2 nm), and UVC (254 ± 2 nm). The intensity of visible light in the laboratory was measured using a digital lux meter (Model LD-500, ICEL, Brazil). The regions of the electromagnetic spectrum considered were those adopted by the International Commission on Illumination (CIE, 1999): visible light (400-700 nm), UVA (400-315 nm), UVB (315-280 nm), and UVC (280-200 nm).

In the laboratory, visible light was provided from standard 40 W fluorescent lamps (Phillips) installed in the ceiling of the room, and UV light exposure was provided using a

reflector containing two lamps UVA-340 Q-Panel 40 W. The intensity of visible light in the laboratory was 250 ± 79 lux. At the height at which the tests were conducted, no UV radiation was detected from the fluorescent lamps. The spectrum of the UVA-340 lamps was from 300 to 610 nm, with an irradiance peak at 340 nm. During the tests, the UV radiation flux was regulated by adjusting the distances from the lamps. Attenuation of the UVA and UVB radiation, as a function of distance from the lamps, is illustrated in Figure 1.

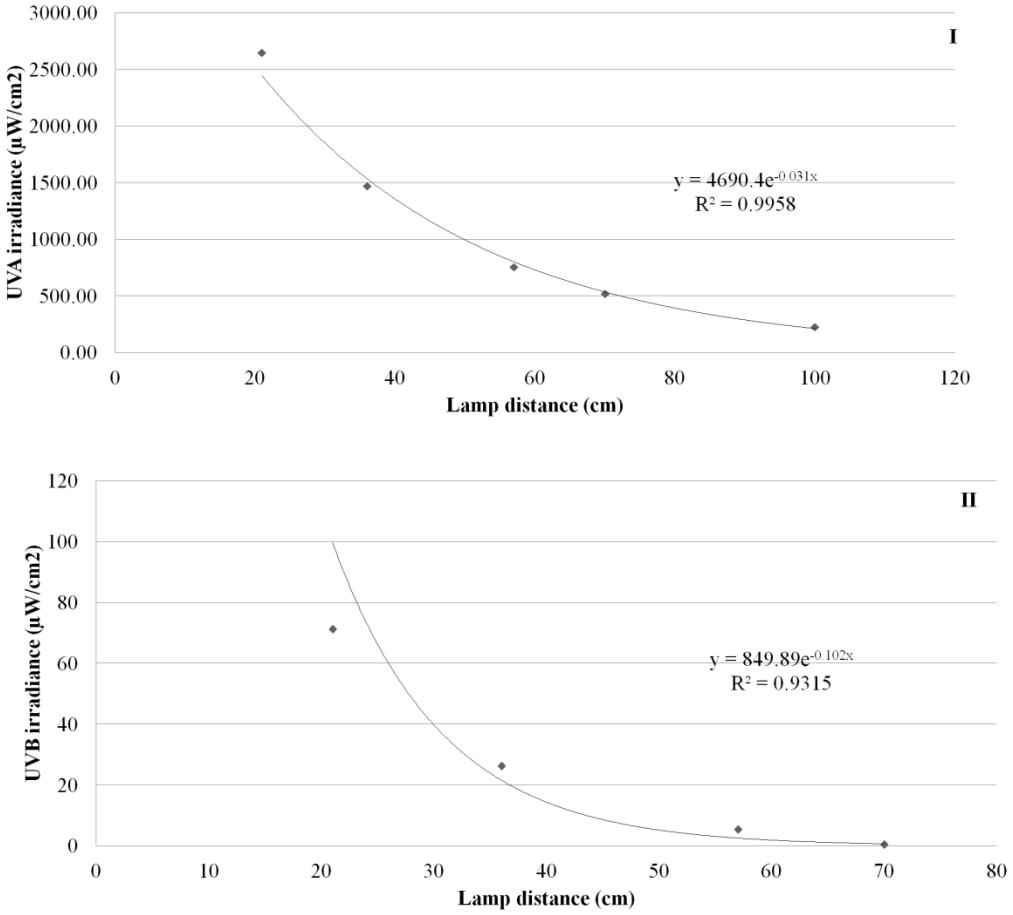

Figure 1. Ultraviolet irradiance fluxes obtained at different distances from two Q-Panel UVA-340 lamps installed under a reflector. The measurements employed an Ocean Optics USB 2000+RAD spectroradiometer. I: UVA irradiance (315-400 nm); II: UVB irradiance (280-315 nm).

2.4 Acute toxicity test – ultraviolet radiation

The UV exposure dose was regulated by adjusting the distance between the UV lamps and the Petri dishes containing the organisms in their respective culture media. Pre-tests were performed to determine the distances to be used in the final test. The effect of UV radiation on *Daphnia* and *Artemia* mobility was determined for a 48 h interval, using distances between 10 and 110 cm from the UV lamps (Anexo I). A control group was kept in the same room, but without exposure to the UV radiation.

2.5 Acute toxicity test – nano-TiO_2

The toxicity of each nano-TiO_2 formulation to *Daphnia* and *Artemia* was evaluated during a 48 h period, using two illumination conditions: visible light (visible light groups) or ultraviolet and visible light (UV light groups). During exposure to the UV radiation, the organisms were kept sufficiently distant from the lamps such that the UV exposure itself did not result in any immobility. The *Daphnia* were kept at a distance of 150 cm from the lamps, corresponding to a total UV dose of 0.046 mW/cm^2 (0.17 $J/h/cm^2$), as measured using the spectroradiometer. The *Artemia* were kept at a distance of 65 cm from the lamps, corresponding to a total UV dose of 0.6 mW/cm^2 (2.3 $J/h/cm^2$). These UV radiation doses were equivalent to 5% of the UV $ED50_{48h}$ calculated for each species.

The exposure media were prepared as described in Section 2.1, by diluting the stock suspension in the appropriate culture medium for each test organism. The organisms were not fed during the tests, and all the exposure media were renewed after 24 h.

Pre-tests were performed to establish the concentrations of nano-TiO_2 to be used in the final tests. Under visible light, the *Daphnia* were exposed to 0 (control), 100, and 1000 mg/L of both nano-TiO_2 formulations. Under UV light, five nano-TiO_2 concentrations were tested, together with a control group. The *Daphnia* were exposed to 6.25, 12.5, 25, 50, and 100 mg/L of TM, and to 62.5, 125, 250, 500, and 1000 mg/L of TA. Under visible light, the *Artemia* were exposed to 250, 500, and 1000 mg/L of TA, and to 166, 500, and 1000 mg/L of TM. Under UV light, the *Artemia* were exposed to 5, 10, 20, 40, and 80 mg/L of TA, and to 1, 2, 5, 10, and 20 mg/L of TM.

At the end of the exposure time, the total of individuals showing mobility in each recipient was registered. The data were used to determine the $EC50_{24h}$ and $EC50_{48h}$ values.

2.6 Growth test

In order to identify the occurrence of any sublethal effects on the microcrustaceans exposed to nano-TiO_2, the rates of growth of the *Daphnia* and *Artemia* exposed to TA and TM were evaluated using the same illumination conditions described for the acute toxicity tests (section 2.5). *D. similis* neonates and nauplii of *A. salina* were exposed for 96 h to concentrations that were shown not to cause any imobility in pilot experiments. The *Daphnia* were exposed to 0 (control), 1 and 10 mg/L of TA and TM, under visible light or UV and visible light. The *Artemia*

were exposed to 0 (control), 0.06, and 0.6 mg/L of TA and TM, under visible light or UV and visible light. The concentrations used were equivalent to around 1.5 and 15% of the lowest nano-TiO_2 $EC50_{48h}$ found for each organism.

The bioassays were performed using uncovered 24-well polystyrene plates, with the organisms (n = 10 per group) kept individually in wells containing 2 mL of solution (Anexo I). The suspensions were totally renewed on a daily basis, and the organisms were fed once daily (after renewal of the exposure media) with dehydrated *Chlorella pyrenoidosa* (1 drop of 2 g/L suspension per well).

The organisms were photographed at the beginning of the test and after every 24 h, using an Optika 4083B3 camera coupled to a stereomicroscope (Optika, Italy). Six-fold magnification was used, and measurement of the size of the organisms was performed using Optika View (v. 7.1.1.5) software that had been previously calibrated using a graduated slide. The measurements of the *D. similis* were made from the front of the eye to the base of the tail spine (Figure 2I), and those of the *A. salina* from the front of the eye to the end of the tail (Figure 2II). The growth rates were evaluated by calculating the angular coefficients of the linear regression curves for the growth of individual organisms after 96 h.

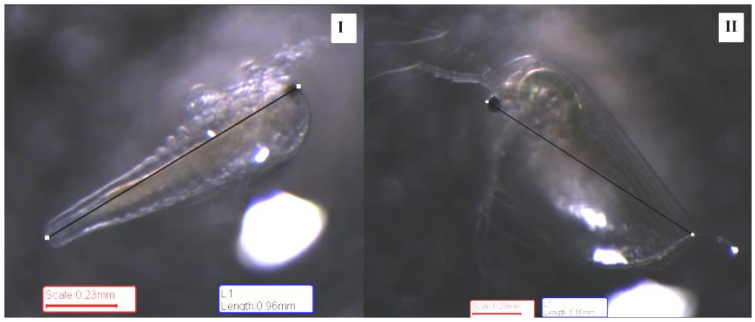

Figure 2. Size measurements of the microcrustaceans. I: *Artemia salina* after 96 h of exposure (control group); II: *Daphnia similis* after 96 h of exposure (control group).

2.7 Biochemical analyses

Biochemical analysis were only performed for *D. similis*, because the number of individuals required to obtain sufficient sample for analysis was smaller than the number required in the case of *A. salina*, due to the much smaller size of the latter organisms. The *Daphnia* were exposed for 24 h to different concentrations of TA and TM, under the same illumination conditions described for the acute test. The exposure was halted after 24 h because it was difficult to renew the suspensions due to the large number of individuals in each receptacle, and to be able to evaluate the occurrence of biochemical alterations prior to the immobility that had already been identified after 48 h. In addition to control groups, the following concentrations were tested: 7.5, 75, and 750 mg/L of TA, and 0.6, 6, and 60 mg/L of TM. For each formulation, the concentrations tested corresponded to 1, 10, and 100% of the $EC50_{48h}$ under UV light. Around 100 adult organisms (at least 3 replicates) were exposed to the test conditions in Petri dishes (10 cm diameter, 1 cm water column height) containing 50 mL of solution. At the end of the exposure period, the organisms were collected using a sieve (21.2 mm mesh), washed with distilled water, weighed, and stored at -80 ºC prior to the biochemical analyses.

The samples were homogenized in 50 mM phosphate buffer (pH 7), using a 1:10 mass/volume ratio, and centrifuged at 10,000 x *g* for 10 min at 4 ^{o}C. The supernatant was used to determine the activities of glutathione S-transferase (GST), glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD), and acid phosphatase (AP), together with the protein concentration. At least three pools of organisms were analyzed for each group, and all measurements were made in triplicate. The analyses were performed using a Sunrise microplate

absorbance reader (Tecan, Austria), with the exception of the CAT analysis, which was performed using cuvettes in a UV-Vis spectrophotometer (Model 1650PC, Shimadzu, Japan).

For measurement of the GST activity, 50 µL of supernatant was added to 100 µL of reactant solution (consisting of 3 mM 1-chloro-2,4-dinitrobenzene plus 3 mM reduced glutathione), and the absorbance at 340 nm was monitored for 2 min (Keen et al., 1976) (Anexo IX).

The GPx activity was measured by adding 50 µL of supernatant to 200 µL of reactant solution (consisting of 1.5 mM azide, 1.5 U/mL glutathione reductase, 1.5 mM reduced glutathione, 0.153 mg/mL β-NADPH, and 0.3 mM EDTA, in 50 mM phosphate buffer at pH 7) and 50 µL of 0.002% H_2O_2. The absorbance of the solution was monitored for 2 min at 340 nm (Sigma, 2000) (Anexo XIV).

The CAT activity was measured by adding 10 µL of supernatant to 990 µL of reactant solution (0.03 M hydrogen peroxide in 50 mM phosphate buffer at pH 7). The absorbance of the solution was monitored for 1 min at 240 nm (Aebi, 1983) (Anexo VII).

For the determination of SOD activity, 10 µL of supernatant was added to 10 µL of 3 mM EDTA, 10 µL of 15% BSA, 150 µL of 3 mM xanthine in 50 mM sodium carbonate buffer (pH 9.8), 110 µL of 0.75 mM nitro blue tetrazolium (NBT), and 10 µL of 4.5 U/mL microbial xanthine oxidase (Ukeda et al., 1997) (Anexo VI).

The AP activity was measured by adding 10 µL of supernatant to 15 µL of 0.1 M sodium acetate buffer (pH 5) and 125 µL of a 5 mM solution of p-nitrophenyl phosphate (pNPP). The mixture was incubated for 30 min at 37 ºC, and the reaction was halted by adding 150 µL of 1 M NaOH prior to measuring the absorbance at 405 nm (Prazeres et al., 2004) (Anexo X).

The data obtained from the different biochemical assays were normalized according to the total protein contents of the samples, quantified using the Bradford method (Bradford 1976). A standard curve was constructed using bovine serum albumin (BSA) at concentrations ranging from 0.1 to 1.5 mg/mL. 250 μL of Bradford reagent (Sigma) was placed in a microplate, together with 5 μL of supernatant that had been diluted 1:50 (v/v) in phosphate buffer (50 mM, pH 7). The absorbance at 595 nm was measured after an incubation period of 10 min (Anexo XIII).

2.8 Statistical analysis

The 24-h and 48-h ED50 values (the dose required to affect 50% of the exposed population) of UV exposure and the 24-h and 48-h EC50 (the concentration required to affect 50% of the exposed population) of nano-TiO_2 exposure, together with the corresponding 95% confidence intervals, were calculated using probit analysis (Statgraphics Plus v. 5.1 software). The EC50 values were considered to be statistically different when there was no overlap of the 95% confidence intervals. The growth rates and the biochemical data were compared using two-way ANOVA. The factors considered for each exposure were the illumination conditions (with and without UV), the concentrations of TA and TM, and the interaction between these parameters. The Holm-Sidak post-test was used to compare the groups, using a significance level of 5%. The normality of the data was evaluated using the Shapiro-Wilk test. These analyses employed Sigma Plot (v. 11.0) software.

3. RESULTS

3.1 Characterization of the NPs and their stability in suspension

The initial absorbances of the suspensions of TM were generally higher than for TA (at the same concentration). The absorbances of the 1 mg/L suspensions were very close to those of the media without nano-TiO_2 (blanks), so it was not possible to correctly evaluate either this or lower concentrations. The nano-TiO_2 suspensions presented an absorbance peak at 325 nm, and this wavelength was used to determine the precipitation of the suspensions over the course of time (Figure 3). The nano-TiO_2 suspensions showed substantial instability, and precipitation was greater at higher concentrations (Figure 4). After 24 h, the precipitation rates of the highest concentration suspensions of TM exceeded those of the TA suspensions. The 100 mg/L suspension of TM precipitated at a rate that was between 1.2 and 2.5 times higher than that of TA, depending on the medium used. The opposite was observed for 10 mg/L suspensions, where the precipitation rate of TA was between 1.3 and 2 times greater than that of TM. At the highest concentrations evaluated, the precipitation rates of each nano-TiO_2 formulation were similar in the *Artemia* media, but the precipitation rates in the *Daphnia* media were slower.

The 100 mg/L suspensions of TM showed rapid reductions of absorbance for all three media evaluated, with values equivalent to around 24, 9, and 3% of the initial absorbances after 3, 6, and 24 h, respectively (Figure 3I). Reductions in the absorbances of the 10 mg/L suspension of TM were more gradual. The absorbance of the 10 mg/L suspensions diminished to 84, 63, and 30% of the initial value after 3, 6, and 24 h, respectively (Figure 3II).

The absorbance of the TA suspensions in the *Artemia* media diminished more abruptly, compared to the *Daphnia* media. In the first three hours, the absorbance of the 100 mg/L suspensions of TA reduced to 10, 20, and 43% of the initial value for the media containing *Artemia*, *Artemia* with saccharose, and *Daphnia*, respectively (Figure 3III). After 24 h, 3, 7, and 24%, respectively, of the initial absorbances were measured in these same media. The suspensions containing 10 mg/L of TA precipitated more progressively (Figure 3IV). After 3 h, the absorbances of the 10 mg/L suspensions of TA diminished to 34, 49, and 70% of the initial values, for the media containing *Artemia*, *Artemia* with saccharose, and *Daphnia*, respectively. After 24 h, around 20% of the initial absorbance was measured.

Due to the instrumental detection limit, the DLS measurements were only conducted for the 100 mg/L suspensions (Table 1). The intense formation of aggregates and rapid precipitation of nano-TiO_2 affected the quality of the readings, as evidenced by the high values of the polydispersion index (PdI) obtained for all the suspensions. Hence, although single particle population peaks at around 700 nm were detected for the suspensions of TA in the three media, Z-average values above 1 µm were obtained in almost all cases. The presence of ions in the media, pH values close to the pHzpc of nano-TiO_2, and low zeta potential values probably contributed to the instability of the suspensions. Intense aggregation and precipitation of nano-TiO_2 in the exposure media is in agreement with earlier findings (Ma et al., 2012b; Pagnout et al., 2012).

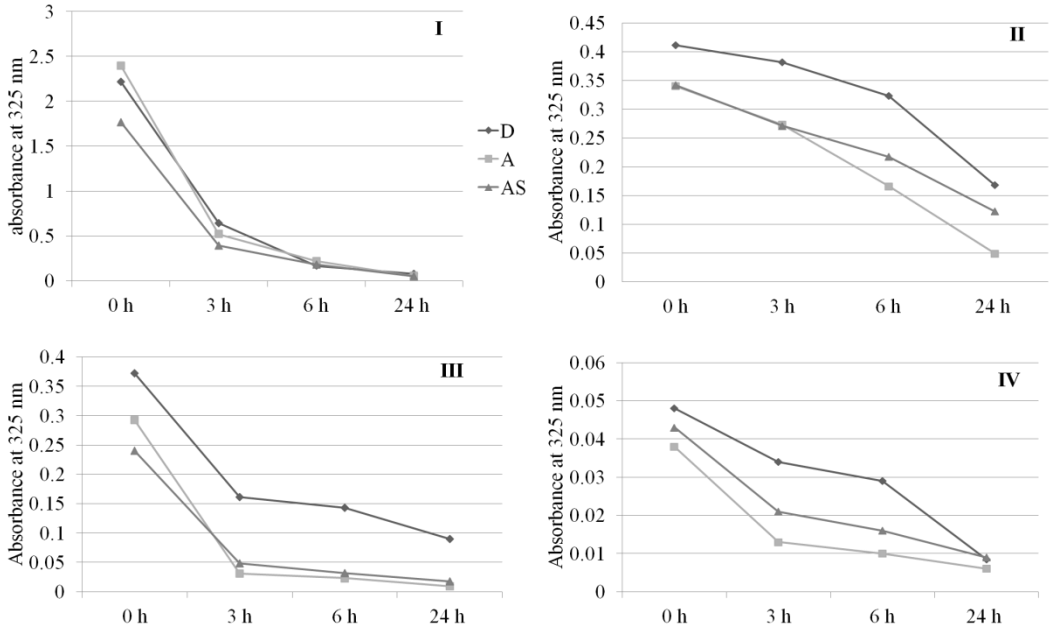

Figure 3. Colloidal stability of the nano-TiO_2. Absorbances at 325 nm of the suspensions of nano-TiO_2 in the exposure media for *Daphnia* (D), *Artemia* (A), and *Artemia* with saccharose (AS), according to time. I) 100 mg/L TM; II) 10 mg/L TM; III) 100 mg/L TA; IV) 10 mg/L TA.

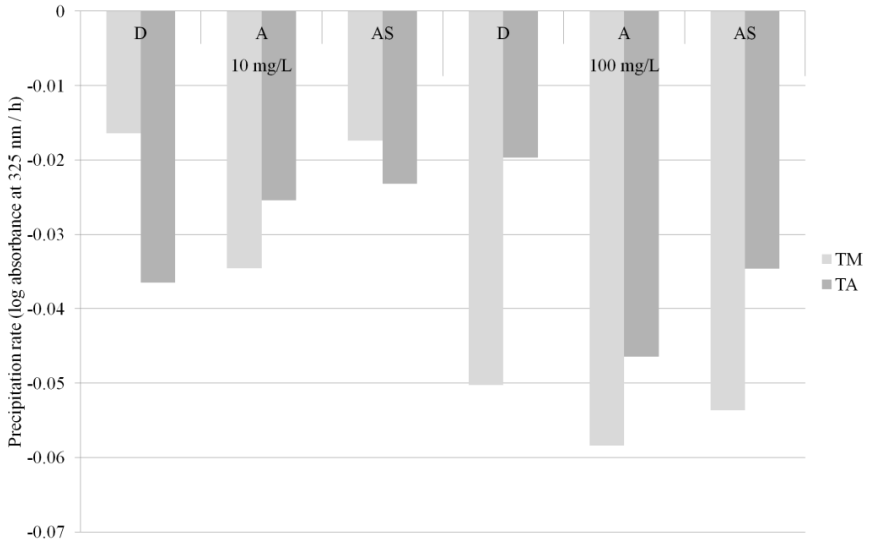

Figure 4. Precipitation rates (log of values of absorbances at 325 nm / h) of the nano-TiO_2 suspensions 10 and 100 mg/L (TM and TA) in the exposure media for *Daphnia* (D), *Artemia* (A), and *Artemia* with saccharose (AS).

Table 1**.** DLS measurements of 100 mg/L suspensions of TA and TM in the media used for *Daphnia* (D), *Artemia* (A), and *Artemia* with saccharose (AS). Average size of the particles in suspension (Z-average), polydispersion index (PdI), size of the main particle population (peak), and zeta potential (ZP). Results are presented as the mean (± standard deviation) of three readings.

			0 h	**3 h**	**6 h**	**24 h**
A	**TM**	Z-average (nm)	1407.3 (± 222.6)	1790.33 (± 163.5)	1692.6 (± 96.4)	3711.23 (± 4879.8)
		PdI	0.06 (± 0.05)	0.42 (0.01)	0.44 (± 0.1)	0.7 (± 0.2)
		peak (nm)	1466.0 (± 257.2)	1150.6 (± 111.2)	1097.9 (±148.5)	351.6 (± 207.4)
		ZP (mV)	2.4 (± 0.9)	3.0 (± 0.9)	2.2 (± 1.0)	- 7.4 (± 5.8)
	TA	Z-average (nm)	1601.6 (± 58.7)	2383.0 (± 108.5)	2444.6 (± 967.8)	1715.0 (± 980.0)
		PdI	0.7 (± 0.1)	1 (± 0.0)	0.9 (± 0.08)	0.9 (± 0.1)
		peak (nm)	706.8 (± 45.9)	507.3 (± 64.0)	637.6 (± 267.2)	642.5 (± 207.5)
		ZP (mV)	-1.9 (± 0.7)	-4.3 (± 1.2)	-5.6 (± 3.0)	-8.2 (± 5.5)
A S	**TM**	Z-average (nm)	1640 (± 585.6)	3602.3 (± 556.1)	3267.0 (± 614.5)	4657.5 (± 1529.4)
		PdI	0.08 (± 0.1)	0.5 (± 0.09)	0.6 (± 0.09)	1.0 (± 0.0)
		peak (nm)	1689.0 (± 616.3)	1399.3 (± 445.8)	1034.6 (± 191.0)	236.3 (± 225.8)
		ZP (mV)	-1.5 (± 0.8)	-1.9 (± 1.5)	-1.1 (± 1.8)	-4.0 (± 1.6)
	TA	Z-average (nm)	2108.6 (± 103.6)	2786.6 (± 397.9)	1945.6 (± 235.1)	2483.5 (± 1587.4)
		PdI	0.8 (± 0.1)	0.8 (± 0.2)	0.4 (± 0.0)	0.8 (± 0.2)
		peak (nm)	743.4 (± 61.2)	721.1 (± 302.6)	1237.6 (± 78.6)	733.8 (± 691.7)
		ZP (mV)	-2.8 (± 0.5)	-3.2 (± 0.7)	-3.2 (± 1.8)	-8.67 (± 3.5)
D	**TM**	Z-average (nm)	779.7 (± 8.8)	1403.0 (± 91.5)	1189.0 (± 28.5)	1373.3 (± 360.2)
		PdI	0.1 (± 0.03)	0.3 (± 0.06)	0.4 (± 0.03)	0.9 (± 0.06)
		peak (nm)	825.2 (± 3.5)	1164.3 (± 84.0)	780.8 (± 19.3)	360.7 (± 92.8)
		ZP (mV)	-20.1 (± 0.7)	-17.2 (± 0.2)	-21.5 (± 1.0)	-13.3 (± 2.0)
	TA	Z-average (nm)	1023.3 (± 36.5)	764.43 (± 24.4)	826.1 (± 20.8)	578.2 (± 52.9)
		PdI	0.4 (± 0.05)	0.3 (± 0.06)	0.3 (± 0.1)	0.4 (± 0.1)
		peak (nm)	721.5 (± 33.0)	613.0 (± 48.8)	623.6 (± 66.0)	400.1 (± 46.0)
		ZP (mV)	-19.2 (± 0.3)	-20.4 (± 3.1)	-18.9 (± 0.3)	-21.5 (± 0.7)

3.2 Acute toxicity tests

In all the acute toxicity tests, survival in the control groups exceeded 90% after 48 h exposure, confirming that the experimental conditions were satisfactory (OECD 202, 1984). The distances of the UV lamps and the corresponding UV doses required to immobilize 50% of the exposed *D. similis* are presented in Table 2.

Table 2. Distances of the UV lamps and doses of ultraviolet radiation (UVA and UVB) required for immobilization of 50% (EC50) of the *Daphnia similis* after 24-h and 48-h exposures. The 95% confidence intervals of the ED50 are given in parentheses.

	Lamp distance (cm)	*UV irradiation (mW/cm^2) - radiometer*	*UV irradiation (mW/cm^2) - spectroradiometer*
24-h	33.49 (28.70-37.95)	0.53 (0.48 – 0.59)	1.71 (1.49 – 1.98)
48-h	50.91 (45.21-56.08)	0.35 (0.32-0.40)	0.99 (0.85 – 1.19)

The toxicity of the UV radiation to *A. salina* was tested to distances of up 10 cm (a dose of 3.5 mW/cm^2, equivalent to 12.7 J/h/cm^2), and no immobility was observed for exposure periods of up to 48 h. However, at a lamp distance of 10 cm, the rapid evaporation of water hindered the bioassays. Evaluation of the toxicity of the nano-TiO_2 under UV radiation therefore employed a lamp distance of 65 cm, corresponding to 0.6 mW/cm^2 (2.3 J/h/cm^2), which was 20 times lower than the $ED50_{48h}$ (13 mW/cm^2, equivalent to 47 J/h/cm^2) for nauplii of *Artemia franciscana*, reported by Dattilo et al. (2005). In these tests, the radiation doses were calculated using the spectroradiometer measurements.

The 24-h and 48-h EC50 values of TA and TM for *D. similis* and *A. salina* are provided in Table 3. TA and TM showed no toxicity to *D. similis* after 24 h, under any of the conditions

tested, or after exposure for 48 h under visible light. In the groups exposed to 1000 mg/L for 48 h under visible light, the immobility rates were 5 and 25% for TA and TM, respectively. It was therefore not possible to determine the corresponding EC50 values. Under UV light, determination of the $EC50_{48h}$ values for the two nano-TiO_2 formulations showed that TM was significantly more toxic to *D. similis*, compared to TA.

A. salina showed greater sensitivity to acute exposure, compared to *D. similis*, under all the conditions tested. Exposure to nano-TiO_2 under UV clearly increased the toxicity of both TA and TM. The most severe condition was exposure to the formulations for 48 h under UV radiation. A statistically significant difference between the EC50 values of TA and TM was only observed for the 48 h exposure under visible light, when TM was also more toxic than TA.

Table 3. Nano-TiO_2 toxicity to *Daphnia similis* and *Artemia salina* (24-h and 48-h exposures), under visible light or visible and UV light. The EC50 values are given, together with the corresponding 95% confidence intervals. Different lower case letters indicate statistically significant differences between the EC50 values for the same organism.

		EC50 24-h (mg/L)		**EC50 48-h (mg/L)**	
		Visible light	*UV light*	*Visible light*	*UV light*
Daphnia similis	TA (anatase)	>1000.00	>1000.00	>1000.00	750.55 (599.56 – 1008.92)a
	TM (anatase/rutile)	>1000.00	>1000.00	>1000.00	60.16 (48.30 - 77.94)b
Artemia salina	TA (anatase)	949.07 (783.16 – 1269.15)a	14.40 (10.64 – 19.13)b	480.67 (382.18 – 604.24)c	4.05 (2.35 – 5.62)d
	TM (anatase/rutile)	945.75 (758.36 – 1317.90)a	16.68 (13.87 – 21.14)b	284.81 (213.01 – 374.83) e	4.03 (2.98 – 5.40)d

3.3 Growth tests

Daphnia similis

After 96 h of exposure, the mobility in the control groups exceeded 90%, under both illumination condition, and the size of the organisms increased by 14.4%. There was no statistically significant difference between the growth rates of the control groups with or without exposure to UV light (Figures 5I and 5II).

However, exposure to TM revealed an influence of concentration ($p<0.001$) on the growth rate, which was generally higher in the groups exposed to 10 mg/L of TM. The rate of growth in the group exposed to 10 mg/L under UV light was 41% greater than that of the UV control group ($p=0.006$), and 45% greater than that of the group exposed to 1 mg/L under UV light ($p=0.003$). Comparisons among the remaining groups revealed no statistically significant differences. Exposure to TA showed no effect on the growth rate at any concentration or under any illumination condition.

Artemia salina

In all groups, the mobility after 96 h of exposure exceeded 83%, with the control group showing a size increase of 119 ± 12%. The growth rate in the control group was 20% higher under UV light than in the absence of UV light ($p=0.004$) (Figure 5III). When the organisms were exposed to TM, the only statistically significant effect ($p<0.001$) concerned the illumination condition, with increased growth rates of the organisms exposed to UV light, for both the control group ($p<0.001$) and the group exposed to 0.06 mg/L of TM ($p=0.019$). In the case of exposure to

TA (Figure 5IV), the statistical analysis revealed that the effect of concentration depended on the illumination condition, demonstrating that there was an interaction between the two factors (p=0.045). The post-test did not identify any significant differences among the remaining groups.

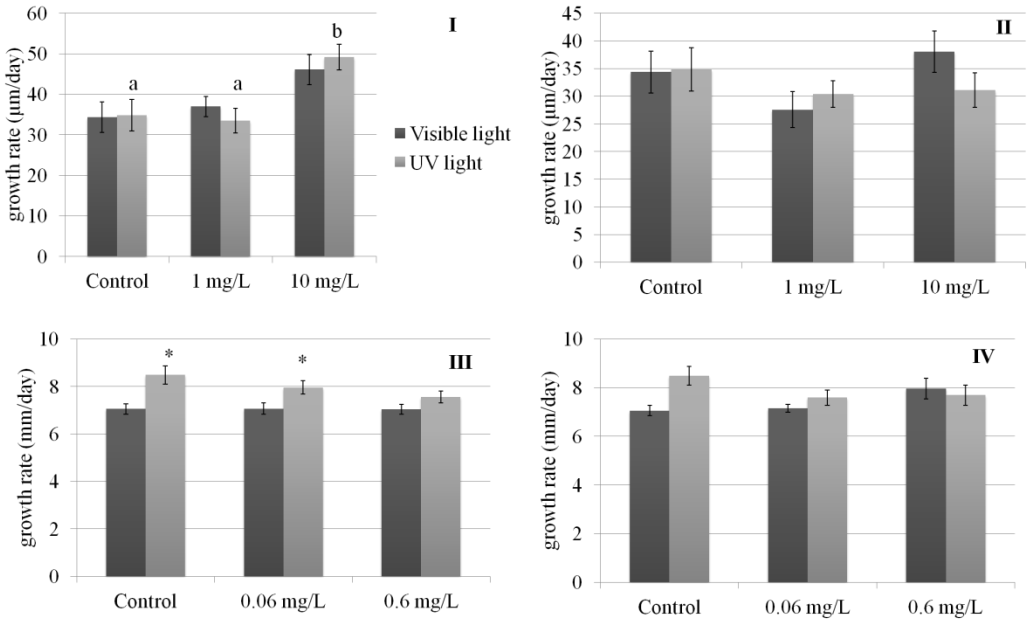

Figure 5. Growth rates of microcrustaceans exposed for 96 h to nano-TiO_2 under visible light or visible and UV light. I: Exposure of *Daphnia similis* to TM; II: Exposure of *Daphnia similis* to TA; III: Exposure of *Artemia salina* to TM; IV: Exposure of *Artemia salina* to TA. Mean ± standard error. An asterisk (*) indicates p<0.05 between groups exposed with and without UV, for the same concentration. Different lower case letters indicate p<0.05 between groups exposed under UV light.

3.4 Biochemical analyses in D. similis

When the organisms were exposed to TA, the specific activity of CAT (Figure 6I) showed an effect of both illumination condition (p=0.006) and concentration (p<0.001), but there was no interaction between the two factors (p=0.13). There was no difference between the

controls, but the CAT activity for the group exposed to 75 mg/L under UV light was 42% greater than for the same group under visible light ($p=0.002$). Considering the groups that were not exposed to UV light, the CAT activity diminished by 31% for the group exposed to 750 mg/L, compared to the control group ($p<0.001$). The groups exposed to UV light showed a reduction of 22 and 34 % in CAT activity when exposed to 7 ($p=0.009$) and 750 mg/L ($p<0.001$), respect to control group. The specific activity of GST (Figure 6II) showed no effect of nano-TiO_2, but there was an effect of illumination condition ($p=0.018$), with the activity for the control group under UV light being 22% higher than for the control group under visible light ($p=0.005$). The effect of the TA concentration on the specific activity of AP (Figure 6III) depended on illumination condition, demonstrating that there was interaction between the two factors ($p=0.017$). The AP activity differed for the controls with and without UV light ($p=0.001$). For the groups exposed under UV light, the AP activity was 20-24% lower for those treated with 7, 75, and 750 mg/L of TA, compared to the control group ($p\leq0.001$). No differences among the different treatment groups were observed for the specific activities of SOD and GPx (Table 4).

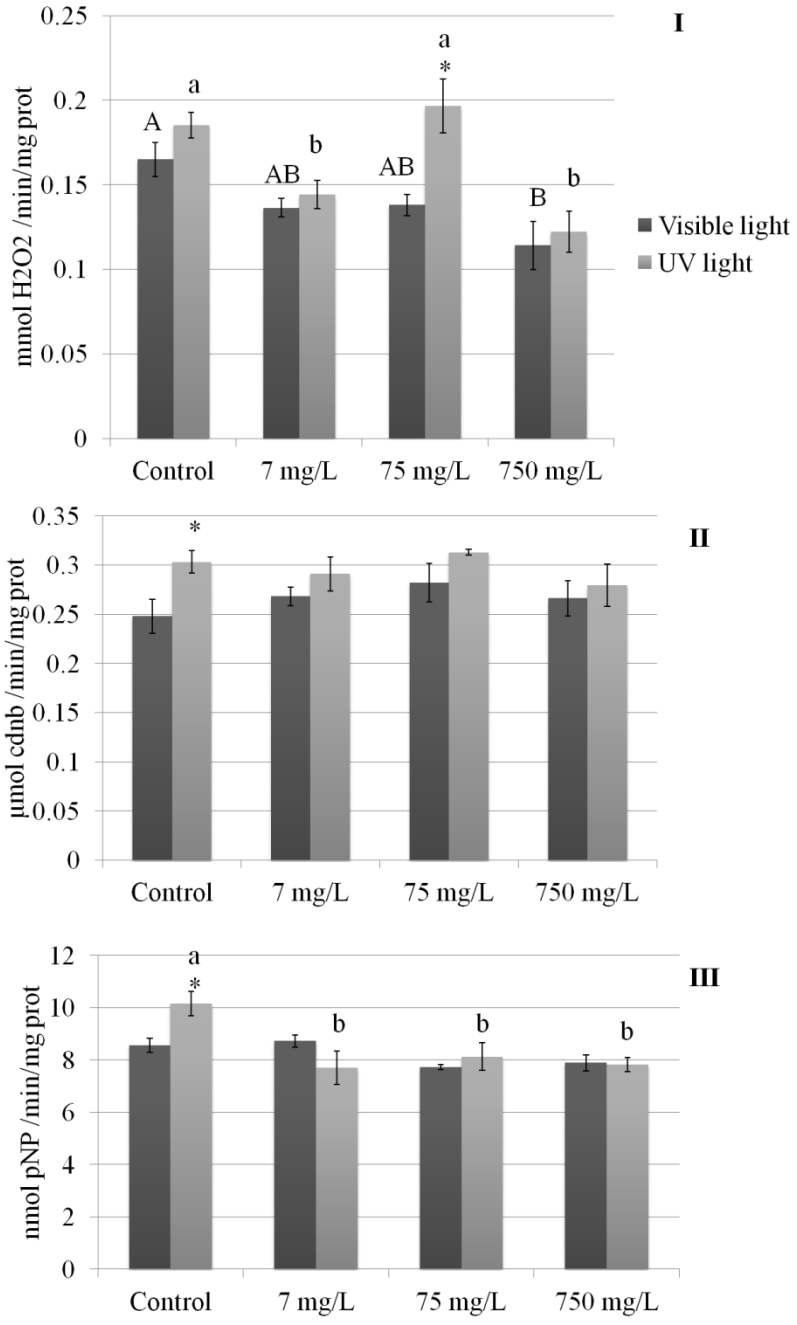

Figure 6. Biochemical analyses in *Daphnia similis* exposed for 24 h to nano-TiO_2 (control, 7, 75, and 750 mg/L of TA), under visible light or visible and UV light. Specific activities of (I) catalase (CAT), (II) glutathione S-transferase (GST), and (III) acid phosphatase (AP). In all analyses, at least three samples were analyzed for each group. Mean ± standard error. An asterisk (*) indicates $p<0.05$ between groups with and without UV, for the same concentration. Different upper case letters indicate $p<0.05$ between different concentrations under Visible light. Different lower case letters indicate $p<0.05$ between different concentrations under UV light.

When the organisms were exposed to TM, the specific activity of SOD (Figure 7) was the only biomarker that showed a response, with evidence of interaction between the TM concentration and illumination condition (p=0.03). The SOD activity for the group exposed to 0.6 mg/L under UV light was around 42% smaller, compared to the group exposed to the same concentration without UV (p=0.013). Among the groups exposed to TM under visible light, those exposed to 6 mg/L (p=0.001) and 60 mg/L (p=0.003) presented SOD activities that were 57 and 51% lower, respectively, compared to the 0.6 mg/L group; however, there was no difference relative to the control. Exposure to TM had no statistically significant effect on the remaining biochemical biomarkers (Table 5). The main results are summarized in Table 6.

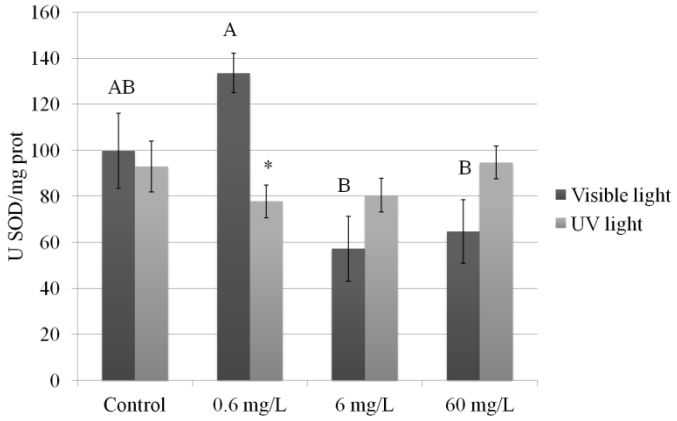

Figure 7. Specific activity of superoxide dismutase (SOD) in *Daphnia similis* exposed for 24 h to TM under visible light or visible and UV light. Mean ± standard error. An asterisk (*) indicates $p<0.05$ between groups exposed with and without UV, for the same concentration. Different upper case letters indicate $p<0.05$ between groups exposed to different concentrations under Visible light.

Table 4. Biochemical analyses in *Daphnia similis* exposed during 24 h to nano-TiO_2 (control, 7, 75, and 750 mg/L of TA under visible light or visible and UV light. Specific activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx). Results are presented as means ± standard errors.

	Control				**7 mg/L**				**75 mg/L**				**750 mg/L**			
	Visible light	*n*	*UV light*	*n*	*Visible light*	*n*	*UV light*	*n*	*Visible light*	*n*	*UV light*	*n*	*Visible light*	*n*	*UV light*	*n*
SOD (U/mg prot)	99.73 (± 16.27)	8	92.87 (± 11.05)	8	95.69 (± 14.57)	4	151.41 (± 31.66)	4	72.91 (± 3.50)	4	89.44 (± 27.18)	4	64.99 (± 11.21)	4	91.28 (± 19.49)	3
GPx (nmol β-NADPH consumed/min/mg prot)	0.84 (± 0.11)	8	0.83 (± 0.03)	8	0.82 (± 0.40)	3	1.20 (± 0.50)	3	1.07 (± 0.25)	4	0.83 (± 0.25)	4	0.71 (± 0.10)	4	0.74 (± 0.15)	3

Table 5. Biochemical analyses in *Daphnia similis* exposed for 24 h to nano-TiO_2 (control, and 0.6, 6, and 60 mg/L of TM), under visible light or visible and UV light. Specific activities of catalase (CAT), glutathione S-transferase (GST), glutathione peroxidase (GPx), and acid phosphatase (AP). Results are presented as means ± standard errors.

	Control				**0.6 mg/L**				**6 mg/L**				**60 mg/L**			
	Visible light	*n*	*UV light*	*n*	*Visible light*	*n*	*UV light*	*n*	*Visible light*	*n*	*UV light*	*n*	*Visible light*	*n*	*UV light*	*n*
CAT (mmol H_2O_2 degraded/min/mg prot)	0.16 (± 0.01)	8	0.18 (± 0.008)	8	0.17 (± 0.01)	4	0.16 (± 0.02)	4	0.19 (± 0.02)	4	0.17 (± 0.003)	4	0.16 (± 0.01)	4	0.14 (± 0.01)	4
GST (µmol CDNB conjugated/min/mg prot)	0.24 (± 0.01)	8	0.30 (± 0.01)	8	0.24 (± 0.02)	4	0.21 (± 0.01)	4	0.23 (± 0.03)	4	0.25 (± 0.02)	4	0.25 (± 0.01)	4	0.22 (± 0.02)	4
GPx (nmol β-NADPH consumed/min/mg prot)	0.84 (± 0.11)	8	0.83 (± 0.03)	8	0.67 (± 0.28)	3	0.80 (± 0.16)	3	0.80 (± 0.07)	4	0.72 (± 0.16)	4	0.83 (± 0.29)	4	0.69 (± 0.13)	4
AP (nmol pNP formed/min/mg prot)	8.55 (± 0.26)	8	10.15 (± 0.46)	8	7.81 (± 0.11)	4	8.61 (± 0.25)	4	9.72 (± 0.28)	4	8.88 (± 0.85)	4	8.62 (± 0.49)	4	8.97 (± 0.58)	4

Table 6. Summary of results obtained for *D. similis* and *A. salina* exposed to TA (anatase) and TM (anatase/rutile mixture): acute toxicity (EC50 48-h), prolonged toxicity (growth during 96 h), and biochemical analysis (24-h exposure).

Bioindicator	**Nano-TiO_2**	**Illumination condition**	**Acute toxicity**	**Prolonged toxicity**		**Biochemical analysis**	
				Causative factor[1]	**Comparison among groups[2]**	**Causative factor[1]**	**Comparison among groups[2]**
D. similis	TA	Visible light	>1000 mg/L	No effect	No effect	Illumination condition and concentration (CAT) Illumination condition (GST) Interaction (AP)	↓ CAT at 750 mg/L
	TA	UV light	750.55 mg/L		No effect		↓ CAT at 7 and 750 mg/L ↑ CAT at 75 mg/L ↓ AP at 7, 75, and 750 mg/L
	TM	Visible light	>1000 mg/L	Concentration	No effect	Interaction (SOD)	No effect
	TM	UV light	60.16 mg/L		↑ at 10 mg/L		↓ SOD at 0.6 mg/L
A. salina	TA	Visible light	480.67 mg/L	Interaction	No effect	-	-
	TA	UV light	4.05 mg/L		No effect		-
	TM	Visible light	284.81 mg/L	Illumination condition	No effect	-	-
	TM	UV light	4.03 mg/L		↑ in control and at 0.06 mg/L		-

[1] Indicates $p<0.05$ using two-way ANOVA to identify the effects of illumination condition, concentration, and interaction between these two factors.
[2] Indicates $p<0.05$ for comparisons among groups using the Holm-Sidak post-test.

4. DISCUSSION

The UV light levels employed in the bioassays with *Daphnia* and *Artemia* corresponded to doses that were around 250 and 19 times lower, respectively, than the solar irradiation measured in spring and autumn in a subtropical region (Clemente et al., 2013). More than 10% of the UVB radiation incident on the surface of clean seawater can penetrate to a depth of 15 m (Calkins, 1974; cited by Acra et al., 1990). Considering that during the bioassays, the water column measured only 1 cm, and that in the environment *Daphnia* and *Artemia* can move freely in the water column, it is reasonable to suppose that despite attenuation of the UV radiation by the water, the organisms were exposed to a substantial dose of UV light, compatible to that obtained at greater depth in aquatic ecosystems (Stewart and Hopfield, 1965; cited by Acra et al., 1990). During the bioassays with nano-TiO_2, UVA was the major component of UV light and UVB levels were virtually zero, but according to Ma et al. (2012a), an absence of UVB has no significant impact on the production of ROS during photocatalysis in the presence of nano-TiO_2. UVA radiation, on the other hand, appears to be fundamental for the process.

The results are in agreement with previous reports indicating that typical light conditions do not induce any acute toxicity in *Daphnia* (Hund-Rinke and Simon, 2006; Lovern and Klaper, 2006; Ma et al., 2012b), and that the inclusion of ultraviolet radiation in bioassays alters the nano-TiO_2 toxicity (Reeves et al., 2008; Vevers and Jha, 2008; Marcone et al., 2012; Tong et al., 2013). Minimal levels of UV radiation acted to increase the nano-TiO_2 toxicity, enabling the establishment of EC50 values that differed according to the formulation and the bioassay conditions. The results indicated that the $EC50_{24h}$ and $EC50_{48h}$ values exceeded 100 mg/L for *D.*

similis and *A. salina*, under common conditions of illumination, hence classifying the nano-TiO_2 as being practically non-toxic to these organisms (USEPA, 1985). Under the UV light exposure conditions employed, for *D. similis* the $EC50_{48h}$ of the anatase/rutile mixture was around 12 times lower than that of the formulation containing pure anatase. The anatase/rutile mixture could therefore be classified as slightly toxic to *Daphnia*, while anatase was practically non-toxic (USEPA, 1985). In the bioassays with *Artemia*, for both formulations tested, the $EC50_{24h}$ values obtained under standard illumination conditions diminished around 60-fold when exposure to UV radiation was included. In the case of the 48-h exposures, the $EC50_{48h}$ of the formulation containing the anatase/rutile mixture diminished around 70-fold when UV radiation was included, while that of pure anatase diminished 120-fold. Under UV radiation, the $EC50_{48h}$ values of both formulations for *Artemia* were similar, and classified the nano-TiO_2 as being moderately toxic to *A. salina*.

As discussed previously, the anatase/rutile mixture is more photoactive than the pure crystal phases. Clément et al. (2013) reported that for *Daphnia magna*, the $EC50_{72h}$ values of NPs of anatase and an anatase/rutile mixture were between 30 and 70 times lower than that of rutile. In other work, a nano-TiO_2 (P25®) $EC50_{48h}$ value of 29.8 mg/L was found for *D. magna* exposed under UV radiation (Ma et al., 2012b). Marcone et al. (2012) observed that under UVA, the toxicity of P25® to *D. similis* was greater than that of an anatase-S nano-TiO_2 produced by them, and discussed the possibility that this could be related to differences in the photoactivation of the two products, and consequently also in the rates of ROS generation.

The reasons for the different photocatalytic properties of the different TiO_2crystal phases remain unclear. Hypotheses that have been raised include differences between the crystal phases

in terms of the band gap, Fermi level, adsorption of O_2, and absorption of UV radiation (Banerjee et al., 2006; Coatingsys, 2009; Sun and Xu, 2010; Cong and Xu, 2012). The association with the cellular membranes and cytotoxicity of nano-TiO_2 seems to be dependent on both size and crystal phase (Allouni et al., 2012; Xiong et al., 2013). Allouni et al. (2012) reported that the percentage of cells associated with nano-TiO_2 was significantly higher for particles containing the anatase/rutile mixture than for formulations containing only one of the crystal phases. Based on the available evidence, we suggest that the different responses induced in the organisms tested could have been related to the different photocatalytic properties (and, therefore, generation of ROS), and/or different rates of adsorption or absorption of the nano-TiO_2 formulations by the organisms. Nevertheless, the USEPA (2010) has warned that sources of rutile (whether mineral or synthetic) can contain metallic impurities, such as oxides of iron and vanadium, and the presence of this type of contamination could help to explain the greater toxicity of the anatase/rutile mixture.

Clèment et al. (2013) reported that *D. magna* presented greater acute sensitivity to TiO_2, compared to other organisms tested, and that NP size was more critical than its concentration. $EC50_{72h}$ values of 1.30, 3.15, and 3.44 mg/L were obtained for anatase NP sizes of 15, 25, and 32 nm, respectively, and the anatase NPs were more toxic than rutile particles. The EC50 values for rotifers (in saline medium) were 5.37 and 10.43 mg/L for anatase sizes of 15 and 32 nm, respectively (Clèment et al., 2013). In the present work, *A. salina* showed to be more sensitive than *D. similis*. In general, *Artemia* is more resistant to various contaminants, compared to *Daphnia*, although exceptions have been described for substances such as arsenic and iron sulfate, whose EC50 values are lower for *Artemia* (Calleja et al., 1994). The lower nano-TiO_2

EC50 values under UV light obtained for *A. salina*, compared to *D. similis*, could have been related to the greater UV dose employed for the first species, but the different behavior of the NPs in the media utilized for each species could also have played a role in influencing the responses.

Azevedo et al. (2004) found that low concentrations of NaCl (2 g/L) did not affect the degradation of phenol using photocatalysis in the presence of P25®, but that high concentrations (50 g/L) reduced the efficiency of the process by 81%. In tests employing *Escherichia coli* (Pagnout et al., 2012), the addition of electrolytes (NaCl, $CaCl_2$, and Na_2SO_4) progressively reduced the toxicity of P25 at pH values below the pHzpc, while the toxicity increased for pH values above the pHzpc. Values of 6.3 have been described for the pHzpc of anatase and P25® (Finnegan et al., 2007; Kosmulski, 2009). Such findings could explain the greater sensitivity shown by *A. salina*, given that the bioassay was performed at pH 8.7 under conditions of high salinity (30 g/L). The pH, electrolyte concentration, and valency of the ions determine the strength of electrostatic forces, influencing the balance of attraction and repulsion between the nanoparticles, as well as between the nanoparticles and the cells exposed to them.

Reactive species of oxygen (ROS) are capable of causing damage to biomolecules, and an increase in their production could explain the greater toxicity of nano-TiO_2 under UV light. The 24-h exposure of *Daphnia* to concentrations at and below the $EC50_{48h}$, under UV light, resulted in different responses of the biochemical biomarkers studied. Exposure to anatase caused inhibition of the CAT and AP activities. The inhibition of CAT activity was observed in the groups exposed to 750 mg/L, with and without UV, as well as in the group exposed to 7 mg/L under UV light. In the presence of UV light, AP activity was inhibited at all the anatase

concentrations tested. Exposure to TM only affected the SOD activity, which was inhibited at the lowest concentration tested (0.6 mg/L) under UV light; nonetheless, there was no statistically significant difference, compared to the control group.

The literature still remains inconclusive concerning the effects of nano-TiO_2 on biochemical biomarkers. Zhu et al. (2011) exposed marine molluscs to anatase (10 nm) for 96 h, using concentrations of up to 10 mg/L, and observed sublethal effects but no mortality. At 1 mg/L (but not at 10 mg/L) there was an increase in the activity of SOD, while at 1 and 10 mg/L there were reductions in GSH content. Work with other aquatic organisms exposed to nano-TiO_2 has also provided evidence of reductions in the activity and expression of CAT and SOD (Hao et al., 2009; Cui et al., 2010). Inhibition of the CAT and SOD activities by a variety of contaminants, and consequent oxidative stress, has been described in several studies (Butler and Hoey, 1986; Sanchez-Moreno et al., 1989; Sun and Oberley, 1989; Kumagai et al., 1995; Bagnyukova et al., 2005). However, it was not possible to find any report concerning the direct inhibition of these enzymes by nano-TiO_2. High concentrations of H_2O_2 can reversibly inhibit and irreversibly inactivate the CAT activity (Lardinois et al., 1996). Modifications in SOD resulting in enzymatic inhibition can also be caused by H_2O_2 (Gottfredsen et al., 2013). In the same way, reactive species of oxygen, such as $*O_2$ and H_2O_2, are able to inhibit the activity of phosphatases by oxidizing the cisteine residues present at the active sites (Aoyama et al., 2003). Hussain et al. (2009) found that the administration of catalase reduced pro-inflammatory responses in bronchial epithelial cells exposed to anatase and an anatase/rutile mixture, indicating that such responses were related to oxidative stress, especially the generation of H_2O_2. The occurrence of oxidative stress could be related to the direct production of ROS by the nano-TiO_2,

or be a result of hypoxia caused by adherence of the nano-TiO_2 to the organisms, resulting in respiratory difficulties. Exposure to UV light enhances the generation of ROS by nano-TiO_2, and in the present work, this acted to increase the toxicity of the materials to *Daphnia* and *Artemia*, in terms of both immobility and sublethal effects.

The test involving the growth of *Daphnia* has shown a good correlation with tests of chronic toxicity over periods of 14 to 21 days (Hanazato, 1998). Sublethal concentrations of anatase nano-TiO_2 did not induce any changes in the growth rate of the *Daphnia*, while at the highest concentration the anatase/rutile mixture increased the growth rate, especially under UV light. The exposure of *Artemia* to sublethal concentrations of nano-TiO_2 resulted in increased growth rates when the organisms were exposed under UV light. Furthermore, the effect of anatase concentration depended on the illumination condition, with a tendency for an increased growth rate when the anatase concentration was increased, but slower growth when the organisms were exposed under UV light. Further investigations will be necessary in order to fully understand these findings, but a possible explanation could be related to bactericidal effects of the UV radiation and the nano-TiO_2, and consequently a better state of health and improved growth of the organisms.

The literature reports contradictory results concerning the impact of nano-TiO_2 on the growth of microcrustaceans. Several authors have described a negative impact of exposure to nano-TiO_2 on the reproduction and growth of *Daphnia* (Zhu et al., 2010b; Fouqueray et al., 2012; Campos et al., 2013), while other work has found an absence of any effect (Lee et al., 2009). Dabrunz et al. (2011) observed a reduction in the moulting frequency of *D. magna* exposed to 2 mg/L of nano-TiO_2, while Rosenkranz (2010) found a dose-dependent increase in the moulting

frequency of *D. magna* exposed to P25, with organisms exposed to 1 µg/L of P25® being significantly larger than those of a control group. Li et al. (2011) found that exposure of *Ceriodaphnia dubia* to 20 mg/L of P25 caused an increase in the organisms size, relative to the control, while exposure to 50 and 100 mg/L had negative effects on growth and reproduction. The authors did not discuss the greater growth of the organisms exposed to 20 mg/L, but suggested that the observed reductions might not have been related to oxidative stress, but rather to an interruption in the assimilation and consumption of energy. It was demonstrated that at concentrations of 10-100 mg/L of nano-TiO_2, a fine layer of the material adhered to the algae supplied as food. The formation of aggregates of algae and nano-TiO_2 was also observed by Campos et al. (2013). This interaction caused precipitation and interfered in the bioavailability of both the algae and the nano-TiO_2, consequently affecting the energy supply of the organisms.

Since the 0.6 mg/L concentrations of the formulations did not cause any changes in the growth rates, mortality, or behavior of the test organisms, in either the presence or absence of UV light, this could be considered to be the 'no observed effects concentration' (NOEC) for *A. salina*. Taking the growth tests into consideration, the NOEC values for *D. similis* would be 10 mg/L for anatase (with and without UV light), and 1 and 10 mg/L for the anatase/rutile mixture, with and without UV radiation, respectively. However, the biochemical biomarkers indicated effects at concentrations different to those evaluated in the growth tests. Hence, in the case of *Daphnia*, the NOEC values may be indicated as <0.6 mg/L for anatase/rutile mixture in the presence of UV light, and 75 mg/L for anatase under visible light. These values are above the predicted environmental concentrations (PEC) of nano-TiO_2 calculated for the United States and Europe (21 ng/L for surface waters, and 4 µg/L for sewage treatment effluents) (Gottschalk et al.,

2009). Nonetheless, concentrations of Ti from 185 to 2800 µg/L have been detected in municipal sewage treatment wastewater lagoons in Arizona (Kiser et al., 2009), and given the continuing growth in the production and use of materials containing nano-TiO_2, it is possible that the presence of these substances in aquatic ecosystems could increase over time. Projected production volumes indicate that the quantity of nano-TiO_2 could increase exponentially (Robichaud et al., 2009). The changes observed in the biomarkers studied here should therefore serve as an alert.

Intense aggregation and precipitation of the nano-TiO_2 was observed during the bioassays, in agreement with the literature (Sharma, 2009). The characteristics of the medium, such as pH, ionic strength, and the presence of organic matter, should be taken into consideration in nanoecotoxicological tests, as well as the characteristics of the nano-TiO_2 employed. Different precipitation rates were observed, depending on the medium and the concentration and type of nano-TiO_2. Hence, although the discussion presented here has been based on nominal initial concentrations, the differences observed between the groups, in terms of the parameters evaluated, should be considered with care, since they relate to a dynamic system whose behavior still remains to be fully understood (Keller et al., 2010).

Finally, the correct evaluation of the risk of nanotechnology requires prior understanding of all the factors involved in the behavior of different NP formulations, as well as in the generation of toxicity. The present work adds knowledge concerning the toxicity of nanoparticles of TiO_2 in two organisms that play crucial roles in freshwater and saltwater ecosystems. It was clearly evident that interpretation of the effects of nano-TiO_2 in aquatic organisms depends on the type of bioassay, the nature of the formulation (especially crystal phase), and the illumination

conditions employed. The results emphasize the need to adapt ecotoxicological evaluation protocols to enable them to be used in nanoecotoxicological studies, especially in the case of nano-TiO_2, for which the behavior of the particles in the exposure medium needs to be taken into consideration, together with the photocatalytic properties of the material.

5. CONCLUSIONS

Determination of the nano-TiO_2 toxicity using bioassays depends on the organism, culture medium, and exposure time employed. It also depends on the crystal phase and the illumination conditions. Exposure to UV radiation at minimal environmental levels increases the nano-TiO_2 toxicity. *Artemia salina* showed greater acute sensitivity to nano-TiO_2, compared to *Daphnia similis*, in either the presence or absence of UV light. Under UV light, the anatase/rutile mixture was more toxic to *D. magna*, compared to pure anatase. For *A. salina*, the two crystal phases only showed different effects when the exposure was performed in the absence of UV light, with the mixture being more toxic than pure anatase. The acute exposure of *Daphnia* to concentrations at and below the $EC50_{48h}$ of nano-TiO_2, under UV irradiation, inhibited the specific activities of catalase, superoxide dismutase, and acid phosphatase, indicating the occurrence of oxidative stress. At sublethal concentrations, the nano-TiO_2 did not show any negative impacts on the growth of *Daphnia* and *Artemia*. The results indicated that the nano-TiO_2 could potentially have negative impacts in freshwater and saltwater ecosystems. The findings contribute to the discussion of nanoecotoxicological protocols and their implementation.

CAPÍTULO V

ESTUDO COM EMBRIÕES DE PEIXE

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ABSTRACT

The popularity of TiO_2 nanoparticles (nano-TiO_2) lies in their wide range of nanotechnological applications, together with low toxicity. Meanwhile, recent studies have shown that the photocatalytic properties of this material can result in alterations in their behavior in the environment, causing effects that have not yet been fully elucidated. The objective of this study was to evaluate the toxicity of two formulations of nano-TiO_2 under different illumination conditions, using an experimental model coherent with the principle of the three Rs of alternative animal experimentation (reduction, refinement, and replacement). Embryos of the fish *Danio rerio* were exposed for 96 h to different concentrations of nano-TiO_2 in the form of anatase (TA) or an anatase/rutile mixture (TM), under either visible light or a combination of visible and ultraviolet light (UV). The acute toxicity and sublethal parameters evaluated included survival rates, malformation, hatching, equilibrium and overall length of the larvae, together with biochemical biomarkers (specific activities of catalase (CAT), glutathione S-transferase (GST) and acid phosphatase (AP)). Both TA and TM caused accelerated hatching of the larvae. Under UV irradiation, there was greater mortality of the larvae of the groups exposed to TM, compared to those exposed to TA. Exposure to TM under UV irradiation altered the equilibrium of the larvae. Alterations in the activities of CAT and GST were indicative of oxidative stress, although no clear dose-response relationship was observed. The effects of nano-TiO_2 appeared to depend on both the type of formulation and the illumination condition. The findings contribute to elucidation of the factors involved in the toxicity of these nanoparticles, as well as to the establishment of protocols for risk assessments of nanotechnology.

1. INTRODUCTION

Titanium dioxide nanoparticles (nano-TiO_2) are used industrially in the areas of cosmetics and pharmaceuticals, amongst others, as well as in environmental applications, and are increasingly encountered in daily life. The use of these materials can often be beneficial, while at the same time questions remain concerning the environmental risks of nanotechnology. Around 70 million organic and inorganic substances have been recorded (CAS, 2013), and a full understanding of their possible adverse effects on human health and the environment represents a considerable challenge. The field of ecotoxicology therefore requires tools to enable it to keep pace with rapid chemical and technological development.

Toxicity tests have traditionally been performed using rodents, in accordance with the norms of the OECD (Organization for Economic Cooperation and Development) and the USEPA (United States Environmental Protection Agency). The same agencies describe the use of fish as model organisms in aquatic toxicology. However, scientific research using animals has been much discussed in terms of bioethics, and there have been renewed efforts to identify alternative techniques that do not require the use of animals. Various networks and research centers have been established to develop and validate new methods that comply with the internationally recognized principle of the three Rs of alternative research, which aims to reduce the numbers of animals used in experiments (Reduction), improve techniques in order to avoid unnecessary pain and suffering (Refinement), and substitute tests using animals by alternative methods (Replacement) (RENAMA, 2012; ECVAM, 2013).

Tests employing fish embryos (the Fish Embryo Test, FET) are used in ecotoxicology because the results obtained have a strong correlation with the outcomes of acute toxicity tests using adult fish (Knobel et al., 2012), and it is likely that the embryos do not have the same perception of pain as the adults, due to the immaturity of the nervous system (Lammer, 2009). Furthermore, in relation to the three Rs, the fact that fish embryos are incapable of independent feeding means that these techniques can be considered to be coherent with the principles of Refinement and Replacement (Esch et al., 2012).

In 2008, the OECD implemented a study designed to validate the FET test (García-Franco, 2011), using zebrafish (*Danio rerio*). The zebrafish model is used for in vivo toxicity tests and is effective in identifying the mechanisms underlying toxicity. The main advantages of its use are the large numbers of eggs laid, rapid development, and the transparency of the eggs (Braunbeck and Lammer, 2006). The organism also shows 70% genetic similarity with humans (Howe et al., 2013). In addition to evaluating toxicity and the occurrence of malformations, the FET test can be used to detect sublethal effects. The FET model therefore fits between traditional studies employing cell cultures and those that use mammalian models (Lin et al., 2013). To date, there have been few studies that have applied the FET method in nanoecotoxicological assessments. However, its use is promising, especially considering the wide variety of nanomaterials that have emerged over a very short space of time, the small volumes involved in the tests, and the low levels of waste generation.

There are a number of issues that remain to be addressed concerning the appropriate use of the FET test. The bioavailability of nano-TiO_2 to aquatic organisms remains unclear. Although a number of studies have found no significant accumulation (Federici et al., 2007; Johnston et al.,

2010), others have reported the accumulation of Ti in aquatic organisms exposed to the element present in either the water or the diet (Zhu et al., 2010a; Ramsden et al., 2013). Chen et al. (2011b) found that adult *D. rerio* exposed to nano-TiO_2 accumulated Ti in the gills, liver, heart, and brain, indicating that the nanoparticles (NPs) could traverse biological barriers, including the blood-brain barrier. There is evidence that nano-TiO_2 can adhere to the chorion of the embryo, where it can be absorbed and then distributed uniformly throughout the tissues of the fish, without any tissue-specificity or photodependence (Bar-Ilan et al., 2011). The accumulation of Ti has also been observed in tissues of the larvae of *D. rerio*, especially in the intestine (associated with the microvilli), the gill lamellae, the liver, and the skeletal muscle (Bar-Ilan et al., 2013).

Other bioassays, such as tests using hydra, a freshwater microinvertebrate, can be used to detect the teratogenic potential of chemical substances (Environment Canada, 2010). Studies with hydra and other aquatic organisms such as microcrustaceans and fish have indicated that the toxicity of nano-TiO_2 is negligible (Blaise et al., 2008; Griffith et al., 2008; Yeo and Kang, 2010; Xiong et al., 2011; Clemente et al., 2013). Similar findings have been reported for fish embryos (Zhu et al., 2008; Paterson et al., 2011; Ma et al., 2012b).

Nevertheless, a number of questions have been raised concerning the bioassays used to assess the environmental risk of nanotechnology. Nanomaterials possess unique characteristics and properties that must be taken into account in the development of protocols for ecotoxicological investigations. TiO_2 is a semiconductor with the important property of being able to be photoactivated, which makes it especially attractive for use in processes based on heterogeneous photocatalysis to degrade a variety of organic and inorganic compounds (Nogueira and Jardim, 1998; Gaya and Abdullah, 2008). Exposure of TiO_2 to ultraviolet radiation (UV) in

the wavelength range 300-388 nm results in the production of reactive oxygen species (ROS) that can cause damage to biomolecules. Oxidative stress is the main reason for the toxicity attributed to nano-TiO_2. The photocatalytic properties of TiO_2 are enhanced when the compound is present in the form of nanoparticles, which can increase its toxicity to aquatic organisms under environmental conditions with exposure to solar UV radiation (Ma et al., 2012a; Marcone et al., 2012; Clemente et al., 2013; Xiong et al., 2013).

TiO_2 occurs in different crystal phases, being the photocatalytic properties and toxicity of anatase higher than rutile (Malato et al., 2009; Allouni et al., 2012; Xiong et al., 2012). Evidence has been found for synergism between the phases, with anatase/rutile mixtures being more photoactive than the pure phases. The nano-TiO_2 known as P25®, manufactured by Evonik Degussa, is the mixture that is most commonly used in photocatalytic processes (Nogueira and Jardim, 1998; Malato et al., 2009). Earlier work in our research group indicated that exposure to nano-TiO_2 can have sublethal effects in fish and microcrustaceans, depending on the crystal phase, concentration, and illumination conditions. Nonetheless, there remain a number of uncertainties, as a result of which further work is needed to fully evaluate the toxicity (especially sublethal effects) of nano-TiO_2 in the fish embryos.

Wang et al. (2011) observed that chronic exposure to nano-TiO_2 significantly diminished the reproduction rate of adult *D. rerio*, with a 29.5% reduction in the quantity of eggs produced. Effects on reproduction, as well as on the development and survival of new generations, could lead to serious impacts on ecosystems. There is therefore a need for careful scrutiny of any effects of nano-TiO_2 on embryos and larvae exposed to the substance. To this end, biomarkers can be used as effective early warning systems, and are often more useful than

direct measurements of a chemical agent in the organism. Biomarkers can provide a sensitive indication of the entry of a toxic agent into an organism, followed by its dispersal in the tissues and the induction of toxic effects in critical target regions (Van der Oost et al., 2003).

Since oxidative stress is probably the main cause of the toxicity of nano-TiO_2, the investigation of biomarkers associated with this effect should be used in the case of organisms exposed to the material. While some studies have reported no adverse effects, others have described changes in the activities of the antioxidant enzymes catalase, superoxide dismutase, glutathione S-transferase, and peroxidase in aquatic organisms exposed to nano-TiO_2 (Federici et al., 2007; Hao et al., 2009; Scown et al., 2009; Kim et al., 2010). On the other hand, equally important enzymes such as phosphatases, which are involved in a variety of transphosphorylation reactions, and can be affected by metals and ROS (Aoyama et al., 2003), have received little attention in terms of the effects of nano-TiO_2. It has been reported that exposure to nano-TiO_2 can affect the growth and size of microcrustaceans and fish (Zhu et al., 2010b; Chen et al., 2011b; Fouqueray et al., 2012; Campos et al., 2013). However, the published data are often contradictory, and comparison of results is hindered by insufficient information as well as the absence of standardized nanoecotoxicological protocols.

Although there have been many studies concerning the effects of UV radiation on aquatic organisms, no standardized ecotoxicological protocols exist for the evaluation of photosensitive compounds. The lamps and UV irradiation intensities employed have varied widely, so that it is difficult to compare results. Charron et al. (2000) reported a 75% survival rate of *D. rerio* embryos exposed to 0.15 W/m^2 of UVB for 24 and 30 h. Dong et al. (2007) obtained LD50 values of 2.32 and 855.3 J/cm^2 of UVB and UVA, respectively, for embryos exposed

during the mid-gastrula period. The exposure of tadpoles to 4 mW/cm^2 of UVA for 14 days increased the toxicity of nano-TiO_2 (Zhang et al., 2012). Increased toxicity of nano-TiO_2 was also observed in the larvae of medaka (*Oryzias latipes*) exposed daily for 4 h to a UV dose of 6.12 $W/cm^2/h$ (equivalent to a total of 97 W/cm^2 over 4 days) (Ma et al., 2012b).

In summary, there is a need to re-evaluate the safety of nano-TiO_2, as well as to standardize nanoecotoxicological protocols, including tests designed to assess the effects of UV irradiation. The objective of the present work was to investigate the toxicity of different nano-TiO_2 formulations to *D. rerio* embryos exposed under different illumination conditions, and to establish suitable experimental protocols. The parameters evaluated reflected acute toxicity and sublethal effects, and included survival rates, malformation, hatching, overall length of the larvae, and biochemical biomarkers.

2. MATERIALS AND METHODS

2.1 Characterization of the NPs and their stability in suspension

Toxicity evaluation of the nano-TiO_2 to *D. rerio* embryos employed titanium (IV) oxide nanopowders, either 100% anatase, with primary particle size <25 nm and purity of 99.7% (Sigma Aldrich), or Aeroxide P25 (Degussa Evonik), composed of 20% rutile and 80% anatase, with primary particle size of 25 nm, surface area of 50 m^2/g, and purity of 99%. These materials, denoted TA and TM, respectively, have been extensively studied and their measured

characteristics have been found to be very close to those specified by the manufacturers (Federici et al., 2007; Grassian et al., 2007; Griffith et al., 2008; Palaniappan et al., 2010).

Stock suspensions of 1 g/L of each nano-TiO_2 in Milli-Q water were prepared by sonication for 10 min using a high frequency probe (CPX600 Ultrasonic Homogenizer, Cole Parmer, USA) operated at an amplitude of 20% (120 W/L). Immediately after sonication, aliquots were removed for the preparation of suspensions containing 1, 10, and 100 mg/L of nano-TiO_2 under the same conditions used for the bioassays (with dilution in the embryo exposure medium).

The hydrodynamic diameter, surface charge (zeta potential, ZP), and polydispersion index (PdI) of the particles in the 100 mg/L suspensions were measured by the dynamic light scattering (DLS) technique, using a Zetasizer Nano ZS90 (Malvern Instruments, UK). The colloidal stabilities of the 1, 10, and 100 mg/L suspensions were determined from spectra obtained in the wavelength range 200-600 nm using a UV-Vis spectrophotometer (Model 1650PC, Shimadzu, Japan). These measurements were made 0, 3, 6, and 24 h after preparation of the suspensions, with all samples being collected from the center of the water column.

The characteristics of the water used for the embryo exposures were: pH 7.5 ± 0.5, conductivity 600 ± 10 µS/cm, hardness 5º dGH, temperature 26.0 ± 1 ºC, and dissolved oxygen content (DO) 6.0 ± 0.6 mg/L.

2.2 Toxicity assessment

The *D. rerio* husbandry is described in Anexo XVI. Embryos of *D. rerio* (1 h post-fertilization, with at least 20 organisms in each group) were exposed for 96 h to different

concentrations of TA and TM (0, 1, 10, and 100 mg/L), under two illumination conditions: visible light (visible light groups) or a combination of visible and UV light (UV light groups), as described in Section 2.3. The exposure was performed using 24-well polystyrene plates, with the embryos kept individually in 2 mL volumes of the suspension (Anexo I). After every 24 h, the embryos were transferred to plates containing fresh suspensions, prepared as described above (Section 2.1). The embryos and larvae were examined every 24 h using a stereomicroscope (Model SMZ 2 LED, Optika). At the end of the exposure period, the live larvae were photographed at a magnitude of x2 for measurement of their overall lengths using Optika View Version 7.1.1.5 software, previously calibrated using a slide with a millimeter scale.

The TA and TM exposure bioassays were performed separately, due to the large quantity of eggs required. The animal handling procedures were approved by Embrapa Environment's Ethics Commission for Animal Use (CEUA-EMA, protocol number 004/2012) (Anexo III).

2.3 Illumination conditions

The measurements of natural (solar) and artificial UV were made using a spectroradiometer (USB2000+RAD, Ocean Optics, USA). The regions of the electromagnetic spectrum selected were those adopted by the International Commission on Illumination (CIE, 1999): visible (400-700 nm), UVA (400-315 nm), UVB (315-280 nm), and UVC (280-200 nm).

In the laboratory, visible light was provided from standard fluorescent lamps (Phillips, 40 W) installed in the ceiling of the room. UV irradiation was provided using a reflector

containing two 40 W UVA340 Q-Panel® lamps, positioned 80 cm above the surface of the water. Exposure to UV and visible light followed a 16 h (light) / 8 h (dark) cycle.

The intensity of visible light in the laboratory was 250 ± 79 lux. At the height at which the tests were conducted, no UV derived from the fluorescent lamps was detected. The emission spectrum of the Q-Panel® lamps was 300-610 nm, with an irradiance peak at 340 nm, and the UV exposure dose during the tests was 1.45 J/cm^2/h (97% UVA and 0.06% UVB). This dose was equivalent to one tenth of the LD50 of UVA and UVB described by Dong et al. (2007) for *D. rerio* embryos, and close to the dose used by Ma et al. (2012b) in experiments with larvae of *O. latipes*.

2.4 Biochemical analyses

In a second experiment, 1 h post-fertilization *D. rerio* embryos were exposed for 96 h to 0, 1, and 10 mg/L of nano-TiO_2. The same parameters were evaluated, and the illumination conditions were the same as those used in the toxicity tests. The exposures were performed (in triplicate) using glass Petri dishes (5 cm diameter) containing 10 mL of suspension (providing a 1 cm water column). Each replicate contained 10 organisms, and the suspensions were renewed on a daily basis. At the end of the experiment, the live larvae were washed in phosphate buffer (0.5 M, pH 7) and stored at -80 ºC in Eppendorf tubes, using a ratio of 8 larvae to 0.5 mL of buffer.

The samples were homogenized using an Ultra-Turrax (IKA, China), then centrifuged for 10 min at 10000 x *g* and 4 ^{o}C. The supernatant was used for the biochemical analyses, comprising the specific activities of catalase (CAT), glutathione S-transferase (GST), and acid

phosphatase (AP), as well as the protein concentration. At least 3 pools of larvae were analyzed for each group, and all readings were made in triplicate. The analyses employed a microplate absorbance reader (Sunrise, Tecan, Austria), with the exception of the CAT analysis, for which a UV-Vis spectrophotometer (Model 1650PC, Shimadzu, Japan) fitted with a cuvette was used.

The specific activity of CAT was determined according to the method described by Aebi (1984). 100 µL of the supernatant were added to 900 µL of reactant solution (0.03 M hydrogen peroxide in 50 mM phosphate buffer at pH 7), after which the absorbance at 240 nm was monitored for 1 min (Anexo VIII).

The GST activity was measured using the method of Keen et al. (1976). 50 µL of sample was added to 50 µL of reactant solution (GSH 3 mM, CDNB 3 mM) and the absorbance at 340 nm was monitored for 4 min (Anexo IX).

For determination of the AP activity, 10 µL of supernatant were added to 15 µL of 0.1 M sodium acetate buffer (pH 5) and 125 µL of 5 mM p-nitrophenyl phosphate (pNPP) solution. The mixture was incubated for 40 min at 37 ºC, after which the reaction was halted by adding 150 µL of 1 M NaOH, followed by measuring the absorbance at 405 nm (Prazeres et al., 2004) (Anexo X).

2.5 Statistical analysis

The hatching rates on day 2 (TA) or day 3 (TM), together with the changes in equilibrium on day 4, were analyzed using the chi-square test (Statgraphics Plus v. 5.1 software). For each type of exposure (TA and TM), two-way ANOVA was used to analyze the overall

length of the larvae, as well as the biochemical data, considering the factors: illumination condition (with and without UV), concentration (0, 1, 10, and 100 mg/L), and the interaction between them. Comparison of the groups employed the Holm-Sidak post-test, and the normality of the data was determined using the Shapiro-Wilk test. These analyses employed Sigma Plot v. 11.0 software, and a significance level of 5% was adopted throughout.

3. RESULTS

3.1 Characterization of the NPs and their stability in suspension

The TiO_2 nanoparticles tended to aggregate and precipitate over the course of time. However, this behavior varied for the different products and suspension concentrations. For the same concentration and wavelength, the absorbances of the TM suspensions were greater, compared to those of TA. For both suspensions, the absorbance peak occurred at around 325 nm, so this wavelength was therefore used for the comparisons of suspension stability (Figure 1).

The 100 mg/L suspension of TM showed a drastic reduction in absorbance during the first few hours, with decreases to 18, 7, and 2% of the initial value after 3, 6, and 24 h, respectively. The absorbance of the 10 mg/L suspension decreased more gradually, to 68, 41, and 11% of the initial value after the same periods. The 1 mg/L suspension was more stable, since 75% of the initial absorbance remained after 24 h.

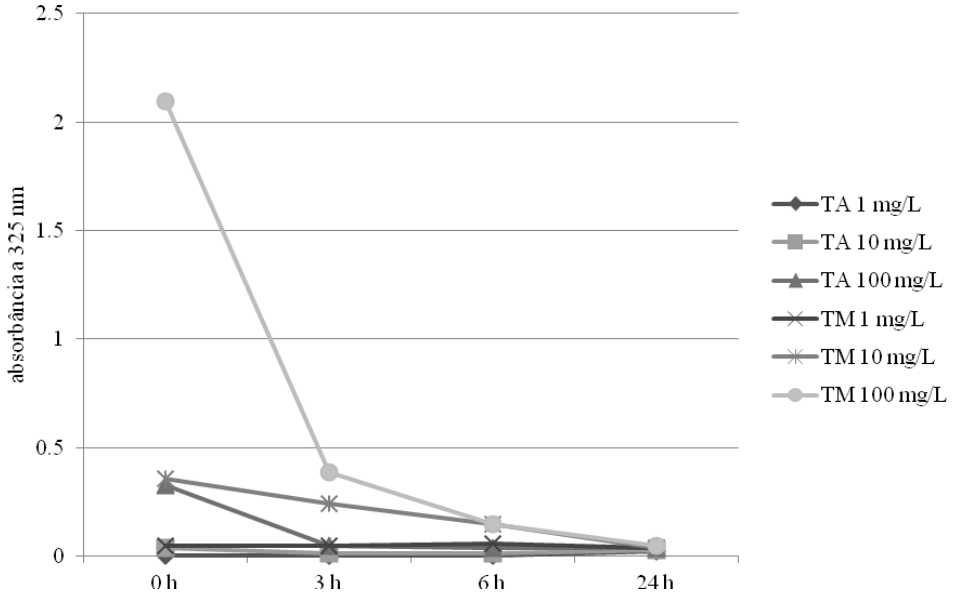

Figure 1. Colloidal stability of the nano-TiO_2. Absorbances at 325 nm of the suspensions of TA and TM (1, 10, and 100 mg/L) in the embryo exposure medium, according to time.

Similar behavior was shown by the suspensions of TA. The absorbance of the 100 mg/L suspension diminished to 15% of the initial value after 3 h, but then only decreased to 11% after 24 h. The 10 mg/L suspension showed absorbances equivalent to 39 and 30% of the initial value after 3 and 6 h, respectively. The absorbance of the 1 mg/L suspension was close to that of the blank (water without TiO_2), so it was not possible to measure the precipitation rate.

Due to the detection limit of the instrument, the DLS measurements were only performed for the 100 mg/L suspensions (Table 1). The intense aggregate formation and rapid precipitation of the nano-TiO_2 affected the quality of the readings, as evidenced by the high PdI values obtained for all the suspensions. Although unimodal particle size peaks at 400-700 nm were observed for the TA suspensions, PdI values above 0.4 and Z-averages above 800 nm were obtained for almost all the measurements. In the case of TM, all the Z-average readings exceeded

1 µm. The presence of ions in the media, pH close to the pHzpc of nano-TiO_2, and low zeta potentials probably contributed to the high instability of the suspensions. The intense aggregation and precipitation of nano-TiO_2 was in agreement with previous findings (Ma et al., 2012b; Pagnout et al., 2012).

Table 1. DLS measurements of the 100 mg/L suspensions of TA and TM in the embryo exposure medium. Average size of the particles in suspension (Z-average), polydispersion index (PdI), size of the main particle population (peak), and zeta potential (ZP). Results are presented as the mean (± standard deviation) of 3 readings.

Nano-TiO_2		**0 h**	**3 h**	**6 h**	**24 h**
TM	Z-average (nm)	1224.6 (± 166.1)	1903.3 (±12.6)	2132.0 (± 259.9)	4075.6 (± 1476.5)
	PdI	0.2 (± 0.02)	0.4 (0.07)	0.8 (± 0.1)	1.0 (0.0)
	Peak (nm)	1252.0 (± 116.2)	1109.3 (± 95.7)	662.0 (± 233.2)	470.8 (± 403.8)
	ZP (mV)	-13.2 (± 0.1)	-15.2 (± 0.6)	-19.0 (± 0.9)	-21.0 (± 2.9)
TA	Z-average (nm)	1192.3 (± 35.9)	857.3 (± 29.7)	966.2 (± 66.0)	812.9 (± 20.7)
	PdI	0.52 (± 0.03)	0.4 (± 0.05)	0.5 (± 0.1)	0.7 (± 0.04)
	Peak (nm)	731.7 (± 41.7)	636.5 (± 23.9)	619.8 (± 15.8)	412.5 (± 32.8)
	ZP (mV)	-18.5 (± 0.3)	-20.4 (± 1.1)	-20.8 (± 0.9)	-20.9 (± 1.0)

3.2 Toxicity evaluation

As also reported in other studies (Chen et al., 2011a), the nano-TiO_2 adhered to the chorion, forming an external white layer that hindered a clear view of the embryo, as a result of

which it was not possible to properly evaluate the occurrence of malformations during the embryonic period (Figure 2).

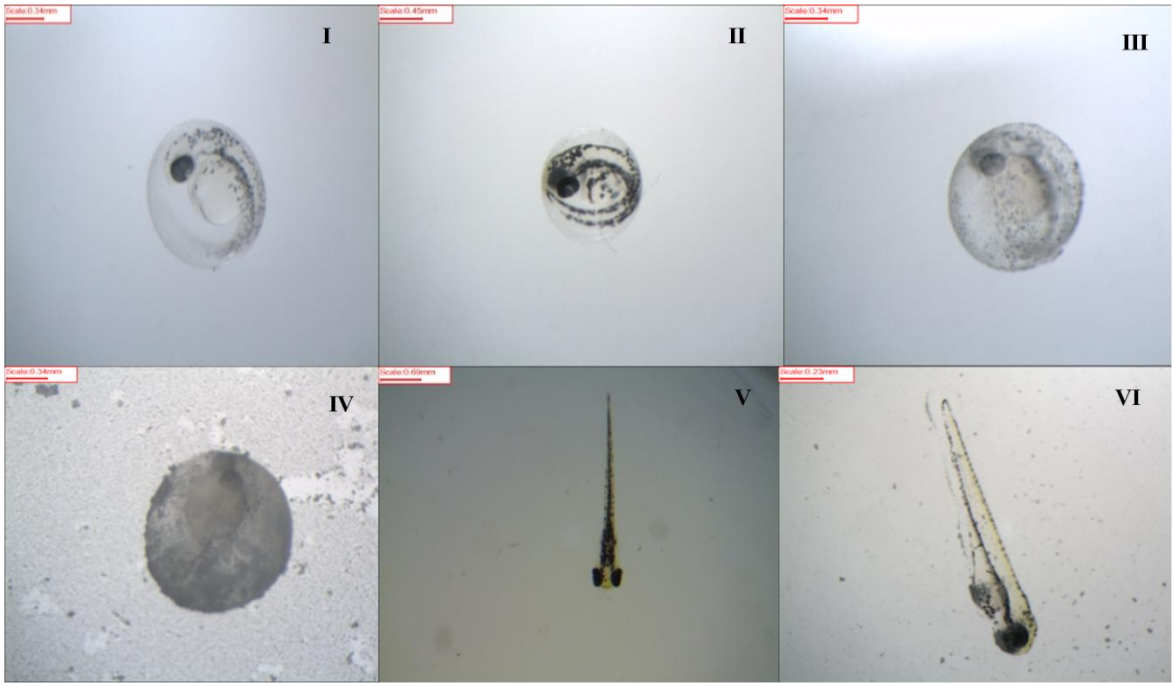

Figure 2. Embryos and larvae of *D. rerio.* I) 24 h control, II) 48 h control, III) 24 h exposure to 10 mg/L nano-TiO_2, IV) 24 h exposure to 100 mg/L nano-TiO_2, V) 72 h control, larva without change in equilibrium, VI) larva with change in equilibrium.

During the first 72 h of exposure to TA, there was no mortality in any of the groups. However, on Day 4 there was 5% mortality of the larvae in the group exposed to 100 mg/L of TA under UV light, while no mortality was observed in the other groups (Figure 3I). Hatching of the larvae began on Day 2, but the hatching rate (Figure 4I) varied between the groups ($p = 0.0015$), and was higher for the groups exposed under visible light to 10 mg/L (47%) and 100 mg/L (36%). The hatching rate was lower for the groups exposed under UV light, compared to the corresponding groups exposed without UV. By Day 4, all individuals had ecloded, after which

changes in equilibrium were observed for the larvae from all groups (including 12% of individuals in the control group), although there was no relationship to the type of exposure ($p = 0.2$) (Figures 2V, 2VI and 5I).

Statistical analysis revealed that for the total larva length variable, there was interaction ($p < 0.001$) between the illumination condition and the nano-TiO_2 concentration (Figure 6I). For all the concentrations of TA tested, the larvae exposed under UV light for 96 h were significantly shorter than those in the corresponding groups exposed under visible light ($p \leq 0.01$), with reductions of 2-6% in their length. There were no differences between the control groups. Comparison of the groups exposed under visible light revealed no significant differences, while for the groups exposed under UV, there was only a significant difference in the case of the larvae exposed to 100 mg/L, which were around 5% shorter than individuals in the control, 1, and 10 mg/L groups ($p < 0.001$).

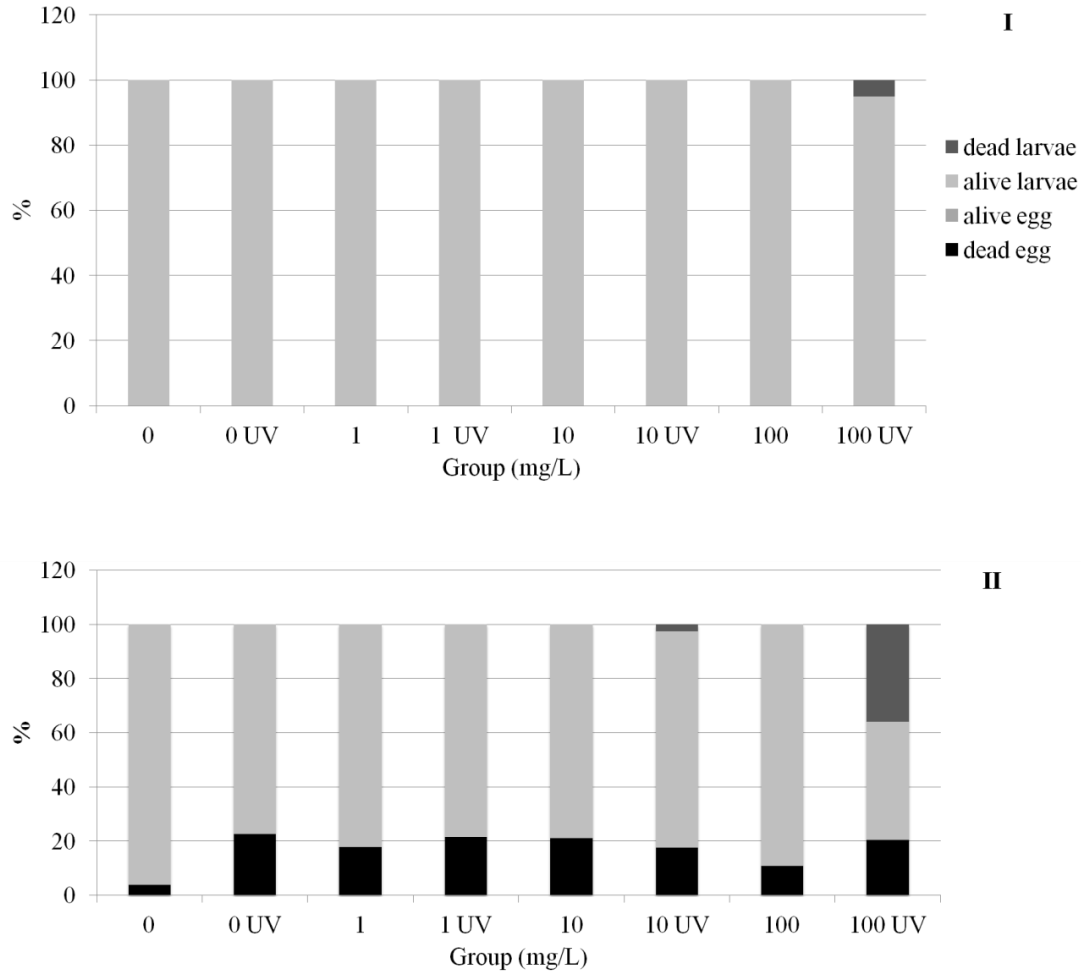

Figure 3. Test of toxicity in embryos (FET). Exposure to 0 (control), 1, 10 and 100 mg/L of nano-TiO_2, with or without exposure to UV radiation. Percentages (%) of eggs and larvae, alive and dead, after 4 days exposure to TA (I) and TM (II).

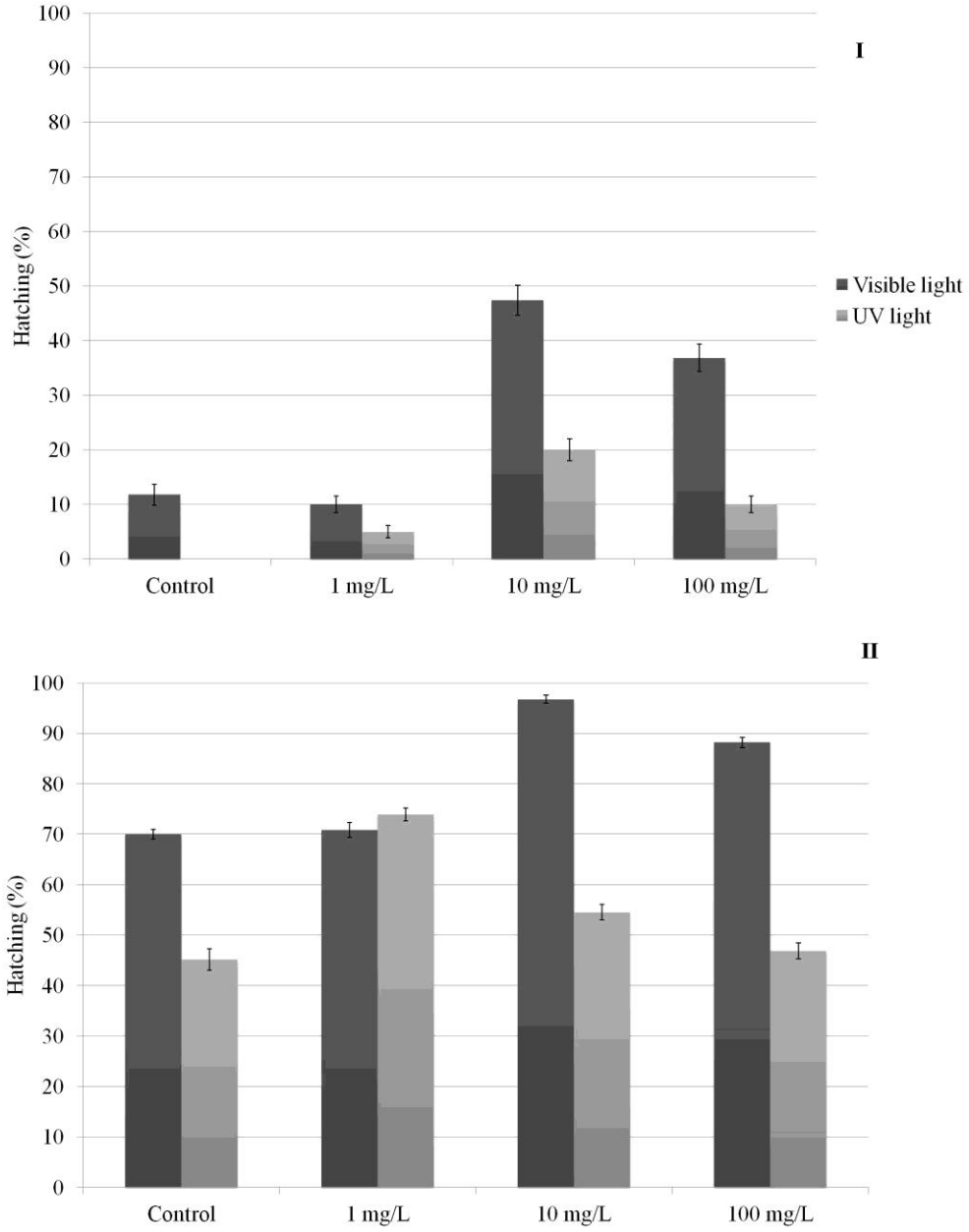

Figure 4. Hatching rates of *D. rerio* embryos exposed to 0 (control), 1, 10 and 100 mg/L of nano-TiO_2 under either visible light or a combination of visible and UV light. I) Eclosion rate of embryos exposed to TA, on Day 2. II) Eclosion rate of embryos exposed to TM, on Day 3. Percentage ± standard deviation.

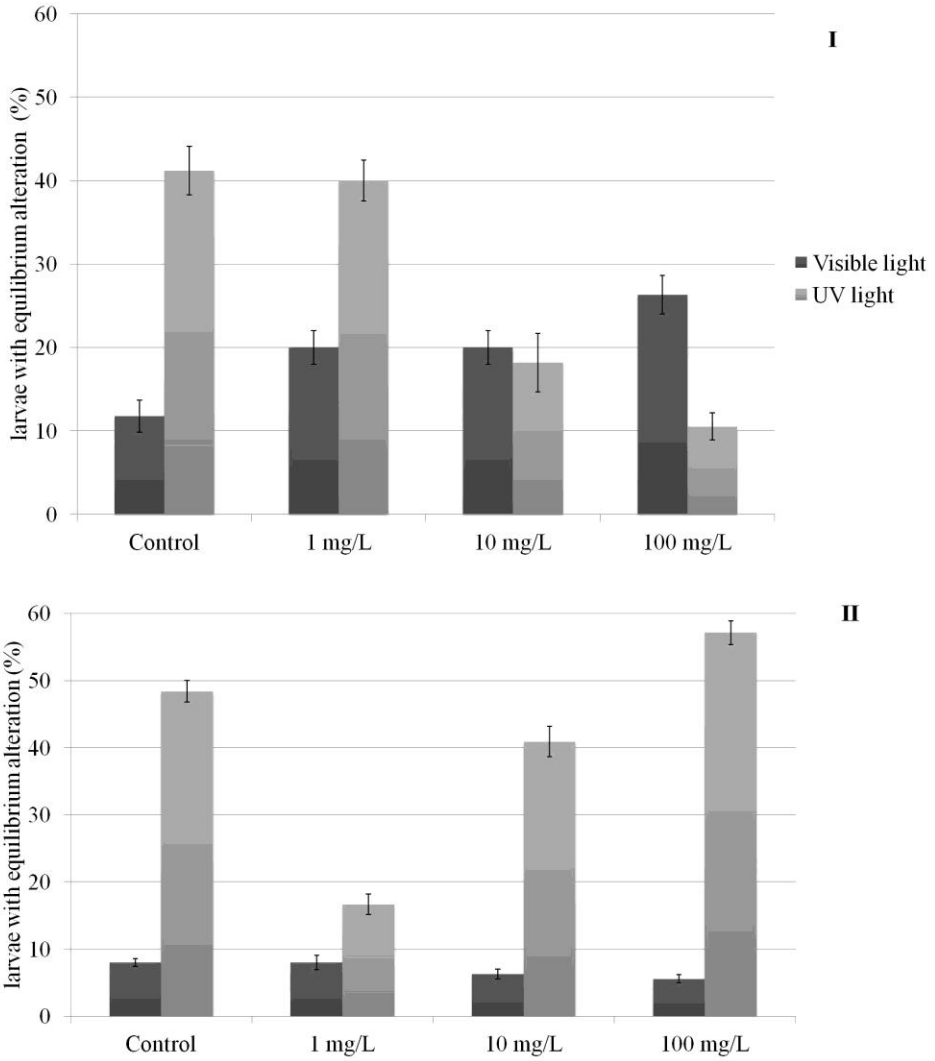

Figure 5. Equilibrium changes in *D. rerio* larvae on Day 4 of exposure to 0 (control), 1, 10 and 100 mg/L of nano-TiO_2 under either visible light or a combination of visible and UV light. Exposure to TA (I) and TM (II). Percentage ± standard deviation.

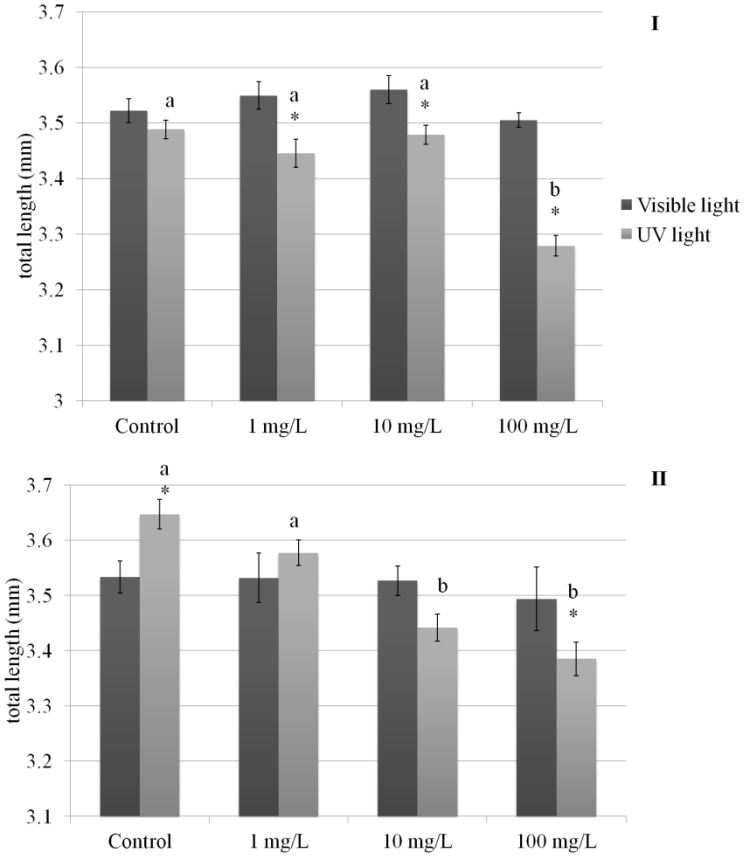

Figure 6. Total length of *D. rerio* larvae exposed for 4 days to 0, 1, 10 and 100 mg/L of nano-TiO_2 under either visible light or a combination of visible and UV light. Exposure to TA (I) and TM (II). Means ± standard errors. Two-way ANOVA, followed by the Holm-Sidak post-test: *$p < 0.05$ between illumination conditions with and without UV, for the same concentration; different lower case letters indicate $p < 0.05$ between different concentrations, under UV.

Exposure to TM resulted in the coagulation of eggs in all groups (Figure 3II). After 96 h, the total number of dead eggs corresponded to 3.8% in the control group under visible light, and 22.5% in the control group under UV. All the live eggs had ecloded by Day 4 of exposure, but on Day 3, when hatching had begun in all groups, differences were observed between the groups in terms of the hatching rate ($p = 0.0001$) (Figure 4II), which was 38% higher in the group exposed to 10 mg/L under visible light, compared to the control group under visible light. In the group exposed to 100 mg/L under UV, the hatching rate was 33% lower than for the control group

under visible light, but similar to that of the control group under UV light. The remaining groups showed hatching rates that were similar to those of the controls. At the end of the exposure period, there was no mortality of larvae in the control groups (Figure 3II), while the groups exposed to 10 and 100 mg/L under UV light showed 3 and 36% mortality, respectively. Summing the numbers of dead eggs and larvae, the 100 mg/L under UV group showed 56.4% mortality after 4 days of exposure. In the remaining groups, total mortality was around 20%, similar to that of the control group under UV light.

After hatching, there were changes in the equilibrium of larvae in all groups exposed to TM (Figure 5II), and statistical analysis revealed a dependence on the type of exposure ($p < 0.001$). Around 8% of individuals in the control group under visible light showed changes in equilibrium, and similar rates were observed for the remaining groups exposed under the same illumination condition. For the control group exposed to UV light, the percentage of individuals showing equilibrium changes increased to 48%. Exposure to 1, 10, and 100 mg/L of TM under UV light resulted in equilibrium changes in 16, 41, and 57% of individuals, respectively. Statistical analysis revealed that there was an effect of the interaction between the TM concentration and the illumination condition on the size of the larvae ($p = 0.002$) (Figure 6II). Comparison between the groups exposed under visible light revealed no significant differences, while for the treatments under UV light the differences were significant. For the two illumination conditions, there were differences between the control groups ($p = 0.008$) and the 100 mg/L groups ($p = 0.03$). The total lengths of the larvae in the groups exposed to 10 and 100 mg/L under visible light were around 6 and 7% shorter, respectively, than those of individuals in the control

group exposed under UV light ($p < 0.001$), and 2 and 4% shorter than in the 1 mg/L under UV light group ($p \leq 0.004$).

3.3 Biochemical analyses

The groups exposed under UV light generally showed enzymatic activities that were lower than those for the groups exposed under visible light (Figure 7). The control groups with and without UV light differed in terms of the activities of CAT ($p < 0.001$) and AP ($p = 0.004$), but no difference was found for the activity of GST ($p = 0.1$). For the control groups, exposure to UV reduced the activities of CAT and AP by 59.7 and 35.9%, respectively.

For CAT (Figure 7I), there was an effect of the interaction of the two factors ($p = 0.01$), since the influence of the TA concentration altered according to the illumination condition. The CAT activity was reduced by 48% in the group exposed to 10 mg/L of TA under UV, compared to the corresponding group exposed under visible light ($p < 0.001$). Under visible light, the CAT activity of the group exposed to 1 mg/L of TA was lower compared to the control group ($p = 0.045$) and the 10 mg/L group ($p < 0.001$).

GST activity (Figure 7II) was affected by both the type of light ($p = 0.006$) and the nano-TiO_2 concentration ($p < 0.001$), but there was no interaction between the two factors ($p = 0.135$). Exposure to 10 mg/L of TA without UV light resulted in increased GST activity, compared to the control ($p = 0.002$) and 1 mg/L ($p < 0.001$) groups without UV, while co-exposure of the 10 mg/L group to UV reduced the activity by 22% ($p = 0.002$). For the groups

exposed to TA, there was an effect of illumination condition on the activity of AP ($p = 0.04$), but no difference between the groups was found using the Holm-Sidak post-test (Figure 7III).

The groups exposed to TM showed an effect of illumination condition on the activities of CAT ($p < 0.001$), GST ($p = 0.001$), and AP ($p < 0.001$). Compared to the activity under visible light, the CAT activity (Figure 7IV) was reduced by 55% in the groups exposed to 1 ($p < 0.001$) and 10 mg/L ($p < 0.001$) of TM under UV light. The activity of GST (Figure 7V) was reduced by 30% in the group exposed to 1 mg/L of TM ($p = 0.001$). Application of the Holm-Sidak post-test found no differences between the groups in the case of AP (Figure 7VI).

Table 2 provides a summary of the main results.

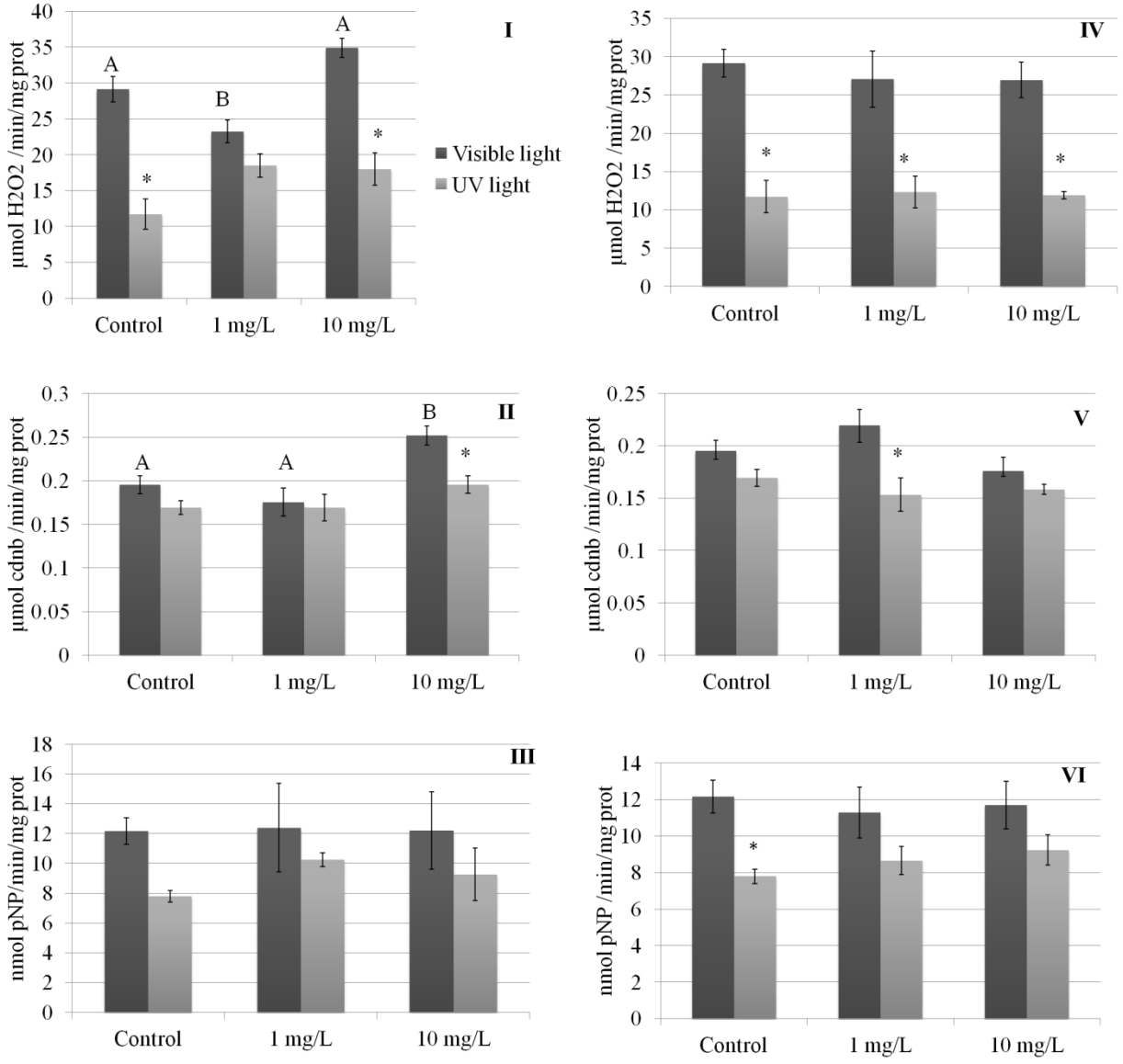

Figure 7. Biochemical analyses (mean ± standard error) of *D. rerio* larvae exposed for 4 days to 0, 1 and 10 mg/L of nano-TiO_2 under either visible light or a combination of visible and UV light. (I) Specific activity of catalase (CAT) in larvae exposed to TA; (II) specific activity of glutathione S-transferase (GST) in larvae exposed to TA; (III) specific activity of acid phosphatase (AP) in larvae exposed to TA; (IV) specific activity of CAT in larvae exposed to TM; (V) specific activity of GST in larvae exposed to TM; (VI) specific activity of AP in larvae exposed to TM. In all analyses, at least 3 samples were analyzed for each group. Two-way ANOVA, followed by the Holm-Sidak post-test: *$p < 0.05$ between illumination conditions with and without UV, for the same concentration; different upper case letters indicate $p < 0.05$ between different concentrations, under visible light.

Table 2. Summary of the results obtained for *D. rerio* embryos and larvae exposed to TA (anatase) and TM (anatase/rutile mixture) nano-TiO_2 for 4 days: acute toxicity, eclosion rate, total larva length, and specific activities of catalase (CAT), glutathione S-transferase (GST), and acid phosphatase (AP).

Nano-TiO_2	Illumination	Acute toxicity	Eclosion	Equilibrium change	Larva size		CAT		GST		AP	
					Influencing factor[1]	Comparison between groups[2]	Influencing factor[1]	Comparison between groups[2]	Influencing factor[1]	Comparison between groups[2]	Influencing factor[1]	Comparison between groups[2]
TA	Visible light	No effect	↑ (10, 100 mg/L) in relation to control	No effect		No effect		↓ (1 mg/L)		↑ at 10 mg/L		No effect
TA	UV light	Mortality of larvae: 5% (100 mg/L)	↑ (1, 10, 100 mg/L) in relation to control ↓ In relation to visible light		Interaction	↓ (100 mg/L) in relation to control ↓ (1, 10, 100 mg/L) in relation to visible light	Interaction	↓ (0, 100 mg/L) in relation to visible light	Concentration and illumination	↓ at 10 mg/L in relation to without UV	Illumination	No effect
TM	Visible light	No effect	↑ (10, 100 mg/L) in relation to control	No effect		No effect		No effect		No effect		No effect
TM	UV light	Mortality of larvae: 3% (10 mg/L) 36% (100 mg/L) Mortality of eggs and larvae: 56.4% (100 mg/L)	↓(0, 10, 100 mg/L) in relation to visible light	↑ (UV radiation) in relation to visible light ↓ (1, 10 mg/L) and ↑ (100 mg/L) in relation to control	Interaction	↓ (10, 100 mg/L) in relation to control	Illumination	↓ (0, 1, 10 mg/L) in relation to visible light	Illumination	↓ (1 mg/L) in relation to visible light	Illumination	↓ (0 mg/L) in relation to visible light

[1] Indicates $p < 0.05$ using two-way ANOVA to identify the effects of the factors: illumination condition, concentration, and interactions.
[2] Indicates $p < 0.05$ for comparison between the groups, using the Holm-Sidak post-test.

4. DISCUSSION

Previous work has indicated that dissolved metals derived from metal oxides can retard the hatching of embryos, probably by the inhibition of hatching enzyme 1 (Lin et al., 2013; Massarsky et al., 2013). This seems to be the case for nanoparticles containing Ag, Zn, Cu, and Ni, but not for TiO_2. The present results indicated that exposure to nano-TiO_2 accelerated hatching of the embryos, corroborating the findings of Paterson et al. (2011), who reported the premature hatching of *O. latipes* embryos exposed to P25 nano-TiO_2 at concentrations of 0.03-14 µg/L. Compared to the corresponding controls, for both formulations, exposure both with and without UV increased the hatching rate at the beginning of the larval stage. This could have been related to blockage of the pores of the chorion with the nano-TiO_2, hence hindering respiration and the excretion of metabolites. The pores of the chorion measure between 300 nm and 1 µm, and it has been observed that a variety of NPs can adsorb onto its external surface (Rawson et al., 2000; Fent et al., 2010; Lin et al., 2013). An increased respiration rate can facilitate the release of enzymes related to hatching and rupture of the chorion (Leung and Bulkey, 1979). The observed changes in the activities of the enzymes CAT and GST were indicative of oxidative stress, which could have been associated with hypoxia (Blokhina et al., 2003). It is also possible that hatching could have been stimulated by damage to the chorion caused by an excess of ROS.

The chorion protects the embryo from exposure to external agents, including nano-TiO_2 (Bar-Ilan et al., 2011), and its loss seems to make the organism more susceptible. In the present work, no significant malformation or mortality was observed during the

embryonic period, although there were adverse effects on the larvae, which depended on the formulation type and concentration, and the illumination condition.

Under standard illumination conditions, exposure to TA or TM did not affect the survival of the organisms, and there were no differences in either the sizes of the larvae at the end of the 96 h exposure period or in acid phosphatase activity (an important metabolic biomarker). The results concurred with earlier work that found no evidence for changes in survival rates or malformations in fish embryos exposed to nano-TiO_2 (Zhu et al., 2008; Paterson et al., 2011; Ma et al., 2012b).

Nevertheless, exposure to nano-TiO_2 under UV irradiation caused mortality of larvae in the groups exposed to 100 mg/L of TA or TM, and to 10 mg/L of TM. The mortality rates remained below 36%, so it was not possible to establish LC50 values. The maximum concentration tested (100 mg/L) was as recommended in the OECD (1992) protocol, on the basis that concentrations higher than this would have no environmental relevance. Exposure for 23 days, under the same irradiation conditions, enabled calculation of an LC50 value of 1 µg/L for embryos of *D. rerio*, associated with an increase in the DNA adduct 8-hydroxy-2'-deoxyguanosine (8-OHdG), which is an indicator of oxidative stress (Bar-Ilan et al., 2013). In the same work, it was also found that hydroxyl radicals were produced in illuminated suspensions, but not in the absence of illumination, and that toxicity was not due to products of reactions with the plastic of the plates used in the bioassays, but rather to the association of the nano-TiO_2 with the embryos, leading to photosensitivity (Bar-Ilan et al., 2011, 2013). Bar-Ilan et al. (2011) reported an $LC50_{120h}$ value of 300 mg/L for *D. rerio* embryos exposed under UV, and Ma et al. (2012b) obtained an $LC50_{96h}$ value of 2.2 mg/L for P25 nano-TiO_2 using embryos of *O. latipes*, which were

also exposed under UV light. Differences in the irradiation intensity, as well as in the experimental protocols and the species employed, can therefore help to explain the variability in the results.

The larvae in the groups exposed to nano-TiO_2 under UV light were smaller, compared to those in the groups exposed without UV and the control group. This was probably due to earlier hatching, resulting in poorer development and greater susceptibility. Similar findings were reported by Paterson et al. (2011). Furthermore, hatching in a medium containing a high concentration of ROS could affect the development and survival of the larvae.

The present results are broadly in line with the findings of earlier work. Using *D. rerio*, Chen et al. (2011b) observed an inhibition of growth that was both concentration and time-dependent, as well as a decrease in the proportional size of the liver. Other reported phenomena in embryos exposed to nano-TiO_2 (P25) under UV light include retarded growth, cranio-facial and tail malformation, and pericardial edema (Bar-Ilan et al., 2011, 2013; Paterson et al., 2011).

The levels of UV radiation employed in the bioassays with the embryos corresponded to a dose that was around 17 and 29 times lower than that delivered by solar UV irradiation in a subtropical region in autumn and spring, respectively (Clemente et al., 2013). Despite the attenuation of UV radiation by water, in clear oceanic waters UV can penetrate to a depth of 40-60 m (Ban et al., 2007; Stewart and Hopfield, 1965, cited by Acra et al., 1990), and several studies have reported that environmental levels of UV radiation can cause adverse effects in fish (Kaweewat and Hofer, 1997; Elliott, 2011). It is known that ROS generated in the presence of solar radiation can promote lipid peroxidation

in cell membranes, and cause oxidative damage to DNA and proteins, leading to cell death when the mechanisms of defense and repair are insufficient (Franco et al., 2009). In the present work, exposure to UV radiation was responsible for delayed hatching and reductions in the activities of CAT, GST, and AP. It is known that high concentrations of ROS can inhibit the activity of CAT (Lardinois et al., 1996) and AP (Aoyama et al., 2003). Reductions in the activities of GST and AP are indicative of lower metabolic rates in these organisms.

Exposure to TA affected the activities of GST and CAT, and in the latter case, the concentration effect was dependent on the illumination condition. The CAT activity was reduced in the groups exposed to 1 mg/L without UV light, and to 10 mg/L with UV, while the GST activity was increased in the group exposed to 10 mg/L without UV. Similar findings have been reported in studies with other aquatic organisms exposed to nano-TiO_2, albeit without considering the influence of UV irradiation. While some investigations have reported increases in the activity of the antioxidant enzyme CAT (Kim et al., 2010; Xiong et al., 2011), others have found reductions in the activity of the same enzyme (Hao et al., 2009; Cui et al., 2010). The present results are in accordance with the literature that has described increases in the activity of GST in daphnia and mollusks exposed to nano-TiO_2 (Canesi et al., 2010a; Kim et al., 2010). GST plays an antioxidant role and acts in Phase II biotransformation, assisting in the elimination of xenobiotics. Zhu et al. (2011) demonstrated that despite an absence of acute toxicity, marine mollusks (*Haliotis diversicolor supertexta*) exhibited oxidative stress after exposure to nano-TiO_2, as shown by changes in the activity of the antioxidant enzyme superoxide dismutase, as well as in the levels of reduced glutathione and lipoperoxidation.

In order to ensure the safe use of nano-TiO_2, more in-depth investigation is needed of other parameters, including those that reflect behavioral and neurophysiological changes. Here, equilibrium changes were observed in the larvae exposed to UV light. Although co-exposure to 1 and 10 mg/L of the anatase/rutile mixture reduced the occurrence of these alterations, co-exposure to 100 mg/L increased it to a level similar to that observed for the control under UV irradiation. It is possible that the oxidative stress caused by exposure to UV light could be related to damage to the fins, swim bladder, or nervous system, and that low concentrations of nano-TiO_2 might act to block UV. However, at higher concentrations of nano-TiO_2, there appeared to be greater damage, which could have been due to enhanced production of ROS. Exposure to the same product has been associated with changes in parameters related to swimming (such as maximum speed and average activity time) in *D. rerio* larvae (Chen et al., 2011a). It has also been suggested that the behavioral endpoints may be more sensitive for detection of the toxicity of nano-TiO_2, compared to other markers such as survival rates and hatching (Chen et al., 2011a).

Although the changes found here were indicative of the occurrence of oxidative stress in the organisms exposed to nano-TiO_2, a clear response was not observed in all the test groups, and it was not possible to directly attribute greater ROS production to exposure to UV radiation. The high instability of the suspensions means that the correlations between concentrations and responses should be treated with caution, since the findings are discussed in terms of nominal initial concentrations. In addition, although oxidative stress seems to be an important cause of toxicity of nano-TiO_2, there are also other factors that may be associated with the adverse effects induced by this material. Chen et al. (2011a) observed that behavioral changes in *D. rerio* larvae caused by exposure to nano-TiO_2 (P25)

were not affected by co-exposure to antioxidants. Full development of the digestive tract and the ingestion of food by the larvae occur at around the fifth day post-hatching (Lawrence, 2007); it is therefore unlikely that the toxicity observed in these organisms was due to the ingestion of nano-TiO_2. It has been suggested that the toxicity could be due to dyspnea and hypoxia resulting from the adsorption of nano-TiO_2 onto the surfaces of the respiratory organs, and subsequent damage to the tissues (Federici et al., 2007; Hao et al., 2009; Chen et al., 2011b; Xiong et al., 2011; Boyle et al., 2013). Recent work indicates that nano-TiO_2 is a potential respiratory inhibitor in fish, related to reduction in levels of glycogen and protein in the tissues (Vutukuru et al., 2013).

The size of the particles, as well as the zeta potential values, aggregate formation, and precipitation, were similar for the formulations studied. Meanwhile, different effects were observed in the organisms exposed to either pure anatase or the anatase/rutile mixture. On one hand, the instability of the suspensions hindered accurate comparison of the effects caused by a given concentration, while on the other hand it is known that the physico-chemical properties of anatase and rutile differ, especially in terms of photoactivity (Gaya and Abdullah, 2008), leading to differences in toxicity. Various hypotheses have been raised to try to explain the reasons for the different photocatalytic properties of the two crystal phases, including differences in the band gap, Fermi level, adsorption of O_2, and absorption of UV radiation (Banerjee et al., 2006; Coatingsys, 2009; Sun and Xu, 2010; Cong and Xu, 2012). In addition, the crystal phase and particle size influence the association of the particles with the cell membranes, hence affecting cytotoxicity (Allouni et al., 2012; Xiong et al., 2013). Considering the evidence as a whole, it appears that the different responses of the organisms to the formulations tested may be related to differences

in both the photocatalytic properties (and consequently the generation of ROS) and the rates of adsorption/absorption of the different nano-TiO_2 formulations by the organism. It is also important to consider the origin of the material, since rutile derived from either mineral or synthetic sources can contain metallic impurities, such as oxides of iron and vanadium, which could also contribute to the different observed effects (USEPA, 2010).

Despite the widely-reported intense aggregation and precipitation of nano-TiO_2, adverse effects were observed in the exposed organisms. There is no reason to suppose that the sedimentation process should not occur in nature, or that this might reduce the environmental risk caused by the transfer of nano-TiO_2 to water bodies (Bar-Ilan et al., 2013). An additional consideration is that fish embryos may remain in the sediment of still waters, while after hatching the larvae can move throughout the entire water column, so that they may be exposed to both precipitated and resuspended material. The presence of organic matter in the medium can also affect the dispersal of nano-TiO_2 and its toxicity (Tong et al., 2013).

In summary, the present work contributes to the evaluation of the risks of nanotechnology, using a promising experimental model to generate information concerning the ecotoxicology of nano-TiO_2. Although the data suggested that nano-TiO_2 presented low acute toxicity to fish embryos, there were sublethal effects that may have been due to adaptive mechanisms, as well as changes that were indicative of possible risks to the environment. The effects of nano-TiO_2 are varied and need to be explored in greater detail, considering the diverse factors that influence its toxicity. Exposure to UV radiation is without doubt a factor that should be included in assessments of the toxicity of nano-TiO_2. The different crystal forms and formulations of nano-TiO_2 should be evaluated

individually, and further information is needed concerning the ways in which particle aggregation and precipitation could affect the outcomes of bioassays. It is then necessary to develop protocols to ensure the stability and reproducibility of the experimental systems.

5. CONCLUSIONS

Nano-TiO_2 presented low acute toxicity to the embryos and larvae of *Danio rerio*, although exposure under UV irradiation increased mortality rates. It was not possible to establish $LC50_{96h}$ values. However, while exposure to pure anatase at a concentration of 100 mg/L resulted in 5% mortality of the larvae at the end of a 96 h exposure period, the rate for the anatase/rutile mixture was much higher, at 36%. Sublethal effects were observed, with premature hatching of the larvae exposed to nano-TiO_2, and impaired development of the organisms exposed to nano-TiO_2 under UV. Changes were detected in the equilibrium of larvae co-exposed to the anatase/rutile mixture and UV radiation. Effects on biochemical biomarkers (catalase, glutathione S-transferase, and acid phosphatase) were dependent on both the type of formulation and illumination condition, and were indicative of the existence of oxidative stress. The use of the FET test to determine the effects of nano-TiO_2 was coherent with the three Rs of humane animal research. The evaluation procedure enabled use of a range of morphological, functional, and biochemical parameters, with savings in terms of cost and time, as well as in the space required and the amount of waste generated. The findings suggest that modifications of standard experimental conditions, especially considering illumination conditions, should be implemented in future nanoecotoxicological investigations.

CONCLUSÕES GERAIS

O nano-TiO_2 apresentou baixa ou nenhuma toxicidade aguda em todos os bioensaios. Entretanto, diversos efeitos subletais foram constatados em nível bioquímico, genético, fisiológico e comportamental, dependendo do bioensaio e das condições de exposição empregadas. Assim, ao contrário do que os estudos iniciais vinham apontando, de que o nano-TiO_2 não apresentaria risco ambiental, nosso estudo indica que em condições mais próximas às ambientais, este material pode representar um risco, que precisa ser caracterizado em detalhe. Os efeitos observados podem representar mecanismos adaptativos ou danos transitórios ou permanentes que podem acarretar efeitos em cascata sobre um indivíduo, uma comunidade e, por fim, um ecossistema.

Apesar dos resultados confirmarem que a toxicidade do nano-TiO_2 está relacionada à ocorrência de estresse oxidativo, há evidências de que esse mecanismo pode ser decorrente de lesões em órgãos respiratórios e consequente hipóxia, mais do que à sua propriedade de gerar espécies reativas de oxigênio. Ainda, outros mecanimos, como inflamação e alteração na ingestão de alimentos, podem estar envolvidos.

Neste estudo ficou claro que a toxicidade do nano-TiO_2 está relacionada à forma cristal e à condição de iluminação. Com exceção da exposição aguda de peixes juvenis, os bioensaios evidenciaram que a mistura de anatase e rutilo apresenta maiores efeitos adversos a biota aquática do que anatase puro. Sob exposição à radiação ultravioleta, alguns efeitos são pronunciados.

Alguns dos biomarcadores avaliados mostraram-se mais úteis do que outros para identificação de efeitos adversos decorrentes da exposição ao nano-TiO_2. Em especial, a atividade de fosfatase ácida, glutationa S-transferase, catalase, e os níveis de metalotioneína, proteínas carboniladas e de dano genético apresentaram resposta às condições de estudo. Em larvas de peixe, foram particularmente interessantes a avaliação do tamanho corporal, bem como da taxa de eclosão e da presença de alteração de equilíbrio. As atividades de Na^+/K^+-ATPase, superóxido dismutase, glutationa peroxidase e os níveis de lipoperoxidação não apresentaram respostas evidentes frente às condições de estudo. Entretanto, ficou claro que a abordagem de um conjunto de biomarcadores apresenta maior utilidade do que uma abordagem isolada. Isso porque as respostas dos biomarcadores estudados variaram consideravelmente em cada bioensaio, dependendo da formulação de nano-TiO_2 e da condição de iluminação empregada. Ainda, em poucos casos ficou evidente uma relação concentração-resposta. Esses achados podem estar relacionados à instabilidade das suspensões de nano-TiO_2, visto que a intensa agregação e precipitação que ocorre durante os bioensaios tornam de difícil controle as características da exposição, e portanto, a discussão levantada tendo como base as concentrações nominais podem não corresponder à real concentração de exposição. Desta forma, a principal dificuldade encontrada no estudo da ecotoxicidade do nano-TiO_2 é a instabilidade da suspensão. Alguns estudos vêm sendo desenvolvidos para tornar as condições de exposição relativamente constantes e reduzir os erros e variabilidade dos resultados relacionados a essa questão.

Estes achados contribuem assim para a discussão e estabelecimento de protocolos nanoecotoxicológicos. Evidenciou-se que adaptações nas condições de iluminação durante os bioensaios fazem-se necessárias na avaliação de materiais com propriedades

fotocatalíticas, de forma a incluir níveis de radiação ultravioleta compatíveis com o meio. Os bioensaios com microcrustáceos e larvas de peixes apresentaram maior sensibilidade, praticidade e economia de recursos com relação aos bioensaios com peixes juvenis. O tamanho reduzido e a transparência desses organismos pode explicar a maior sensibilidade dos mesmos ao nano-TiO_2 sob radiação UV. Dessa forma, a inclusão de espécies aquáticas que apresentem transparência pode ser recomendada para avaliação do nano-TiO_2. Porém, cada bioensaio permitiu a avaliação de parâmetros diferentes, enriquecendo o estudo. Assim, a exemplo do que já é preconizado no estudo de outros compostos, a investigação nanoecotoxicológica deve priorizar o estudo de possíveis efeitos adversos através de bioensaios com organismos representativos de diferentes ecossistemas e níveis tróficos. Ainda, a inclusão de espécies como *P. mesopotamicus* e *D. similis* pode ser recomendada para avaliação de efeitos nanoecotoxicológicos em espécies nativas. Por fim, os esforços devem visar a uma ampla avaliação e obtenção de uma resposta robusta à sociedade dos riscos envolvidos nas nanociências e nanotecnologias.

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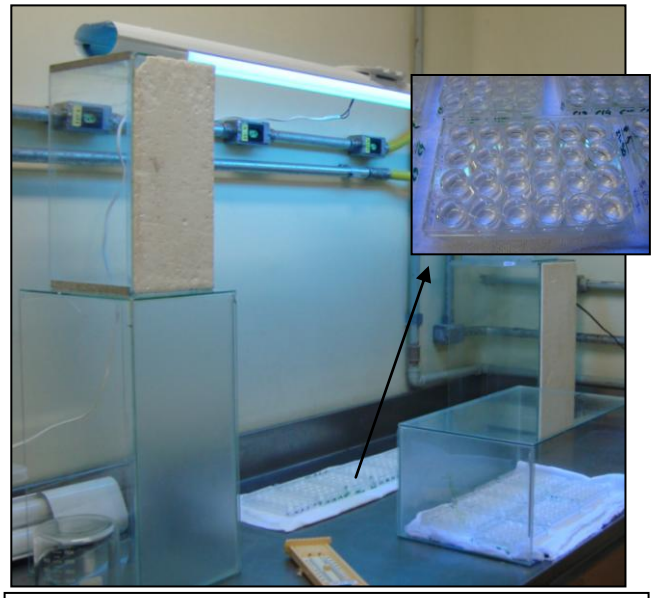
ANEXO I – Organismos e condições experimentais

I) Exemplar de *Piaractus mesopotamicus*

II) Condições experimentais em bioensaios com *P. mesopotamicus*, exposição a nano-TiO_2 sem (A) e com (B) radiação UV.

III) Condições experimentais em bioensaio com Daphnia, exposição à diferentes distâncias das lâmpadas de radiação UV.



IV) Condições experimentais em bioensaio de crescimento em Daphnia e toxicidade em embriões de *Danio rerio*. Em detalhe, placa de poliestireno.

ANEXO II – Certificado de aprovação CEUA Unicamp

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

C E R T I F I C A D O

Certificamos que o Protocolo nº **2172-1**, sobre **"Avaliação dos efeitos tóxicos de NANO-TIO2 em peixes"**, sob a responsabilidade de **Prof. Dr. Leonardo Fernandes Fraceto / Zaira Clemente**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética no Uso de Animais – CEUA/Unicamp em **28 de junho de 2010**.

C E R T I F I C A T E

We certify that the protocol nº **2172-1**, entitled **"Evaluation of NANO-TIO2 toxic effects in fish"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **June 28, 2010**.

Campinas, 28 de junho de 2010.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva

ANEXO III – Certificado de aprovação CEUA Embrapa Meio Ambiente

COMISSÃO DE ÉTICA NO USO DE ANIMAIS – CEUA
EMBRAPA MEIO AMBIENTE

PARECER DO PROTOCOLO **Nº 004/2012**

Em atenção ao **protocolo nº 004/2012** registrado na CEUA Embrapa Meio Ambiente na data de 06/09/2012, como membro da CEUA Embrapa Meio Ambiente, venho emitir o **parecer favorável** ao projeto entitulado "Avaliação dos efeitos tóxicos do nano-TiO2 em embriões de *Danio rerio*", o qual as atividades estão sob a responsabilidade da Dra. Vera Lucia Castro, devido a metodologia proposta estar em consonância com demais pesquisas realizadas, segundo constatado nos artigos científicos referenciados pela pesquisadora responsável.

Jaguariúna, 20 de setembro de 2012.

ELISABETH FRANCISCONI FAY
Parecerista/Membro CEUA

CLAUDIO MARTIN JONSSON
Coordenador da CEUA

ANEXO IV – DECLARAÇÃO CEUA UNICAMP

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha dissertação tese de Doutorado intitulada "Avaliação ecotoxicológica do dióxido de titânio nanoparticulado, sob diferentes condições de iluminação",

( ) não se enquadra no § 3º do Artigo 1º da Informação CCPG 01/08, referente a bioética e biossegurança.

Tem autorização da(s) seguinte(s) Comissão(ões):

( ) CIBio – Comissão Interna de Biossegurança , projeto No. _____, Instituição: _____.

(X) CEUA – Comissão de Ética no Uso de Animais ,
Projeto No 2172-1, Instituição: Universidade Estadual de Campinas.
Projeto No 004/2012, Instituição: Embrapa Meio Ambiente.

( ) CEP - Comissão de Ética em Pesquisa, protocolo No. _____, Instituição: _____

** Caso a Comissão seja externa ao IB/UNICAMP, anexar o comprovante de autorização dada ao trabalho. Se a autorização não tiver sido dada diretamente ao trabalho de tese ou dissertação, deverá ser anexado também um comprovante do vínculo do trabalho do aluno com o que constar no documento de autorização apresentado.*

Aluno: Zaira Clemente

Orientador: Prof Dr Leonardo Fernandes Fraceto

Para uso da Comissão ou Comitê pertinente:
(X) Deferido ( ) Indeferido

Carimbo e assinatura

Prof. Dr. ALEXANDRE LEITE RODRIGUES DE OLIVEIRA
Presidente da Comissão de Ética no Uso de Animais CEUA/UNICAMP

ANEXO V - CONCENTRAÇÃO DE HIDROPERÓXIDO LIPÍDICO

1. Princípio do método

Este método tem por princípio a rápida oxidação do Fe^{+2} mediada por peróxidos sob condições ácidas, e posterior formação do complexo Fe^{+3}-laranja de xilenol (fonte de absorção de luz) na presença do estabilizador BHT. Este método pode ser usado para detectar hidroperóxidos lipídicos a nível nanomolar (Jiang et al., 1991).

2. Preparo das soluções

2.1 Solução reação (BHT 4 mM, H_2SO_4 25 mM, sulfato ferroso amoniacal 250 μM, alaranjado de xilenol 100 μM em metanol 90%)

para 100 mL: 99,75 mL metanol 90%
0,0076 g xilenol orange
0,25 mL ácido sulfúrico (H_2SO_4)
0,0882 g Butil hidroxitolueno (BHT)
0,0098 g sulfato ferroso de amônio (adicionar por último!)

Adicionar reagentes na ordem descrita
Homogeneizar em agitador magnético
Manter a temperatura ambiente.
Proteger da luz.
Solução apresenta uma coloração dourada, quando preparada adequadamente. Após acréscimo de H_2O_2, torna-se púrpura.

2.2 Curva padrão de peróxido de hidrogênio

Para fazer a curva, inicialmente diluir H_2O_2 30% (990 μL de água + 10μL H_2O_2), e medir sua absorbância a 240 nm. Calcular a concentração da solução pura através da equação de Beer-Lambert:
$A=\varepsilon . d . c$

Onde:
A = absorbância a 240nm
ε = coeficiente de extinção molar do H_2O_2 a 240 nm (40 $M^{-1}.cm^{-1}$).
c= concentração da solução diluída
d= caminho óptico (na cubeta, d=1 cm).

A seguir, calcular uma curva padrão, de modo a obter as seguintes concentrações de H_2O_2, em volumes finais de 1ml:

100 µM 50 µM 25 µM 12,5 µM 6,25 µM 3,12 µM 1,56 µM 0 (branco)

3. Preparo das amostras

Amostras de fígado de pacu caranha (*Piaractus mesopotamicus*) são homogeneizadas em tampão fosfato 0,05 M pH 7, na proporção 1:4 (peso : volume). A seguir as amostras são centrifugadas (10.000 *x*g, 20 min, 4 °C) e o sobrenadante é diluído em metanol (proporção 1:2, volume:volume). As amostras são sonicadas durante 2 min e novamente centifugadas (10.000 *x*g, 4^{o} C, 10 min). O sobrenadante é utilizado para análise.

4. Procedimento

Em tubos eppendorf (realizar tudo em triplicata), colocar:
100 µL de amostra ou H_2O_2 da curva padrão
900 µL solução reação

Homogenizar em vortex
Incubar por 30 min, a temperatura ambiente.
Transferir para cubetas de 1 ml e ler absorbância a 560nm.

5. Cálculo da concentração de hidroperóxido

A concentração de hidroperóxido pode ser calculada a partir da curva padrão ou do coeficiente de extinção molar de hidroperóxidos proposto por Jiang et al. (1991):

$$[\text{hidroperóxidos}] = A \cdot \text{diluição} \cdot \varepsilon^{-1} \cdot d^{-1} \cdot [\text{proteína}]$$

Onde:
[hidroperóxidos]= mmol de hidroperóxidos por mg de proteínas.
A = valor de abs a 560 nm após descontar o branco.
Diluição (da amostra) = 30 µL de amostra para 300 µL de volume final, ou seja, 10x
ε = coeficiente de extinção molar aproximado para H_2O_2, hidroperóxido de cumeno ou hidróxido de butila (560 nm)
$\varepsilon = 4{,}3 \cdot 10^4\ M^{-1}.cm^{-1}$
d = caminho óptico (para cubeta = 1 cm)
[proteína] = concentração de proteínas totais em mg/mL

Obs: metanol precipita proteínas, portanto o cálculo da concentração de hidroperóxido deve basear-se na leitura da concentração de proteínas nas amostras diluídas em metanol.

ANEXO VI - CONCENTRAÇÃO DE PROTEINAS CARBONILADAS

1. Príncípio do método

Proteínas podem ser oxidadas/carboniladas durante processo de estresse oxidativo (carbonilação é um dos tipos de dano oxidativo que pode ocorrer). A 2,4-dinitrofenil-hidrazina (DNPH) reage com proteínas carboniladas (apenas do tipo aldeído e cetona) formando dinitrofenil hidrazonas que podem ser detectadas a 358-370 nm. Carbonilas com grupos funcionais como ácidos carboxílicos, amidas e ésteres não reagem como o DNPH. (Levine et al., 1994; Quinlan e Gutteridge, 2000).

2. Preparo das soluções

2.1 HCl 2M
para 100 mL: 16,56 mL HCl 37%
83,44 mL água destilada

2.2 Solução reagente (DNPH – 2,4 dinitrophenyl hydrazine 10 mM, HCl 2M)
para 20 mL: 0,039 g DNPH
20 ml HCl 2 M

2.3 Ácido tricloroacético (TCA) 28%
para 100 mL: 28 g TCA
100 mL água destilada

2.4 Solução etanol- acetato de etila (1:1)
para 100 mL: 50 mL etanol absoluto
50 mL acetato de etila

2.5 Cloreto de Guanidina 6 M
para 100 mL: 57,3 g
100 mL água destilada

3. Preparo da amostra

Amostras de fígado de pacu caranha (*Piaractus mesopotamicus*) são homogeneizadas em tampão fosfato 0,05 M pH 7, na proporção 1:4 (peso : volume). A seguir as amostras são centrifugadas (10.000 *x*g, 20 min, 4 °C) e o sobrenadante é diluído com o mesmo tampão para realização da análise (proporção 1:10, volume: volume).
Obs: [proteínas] adequada = 1000-2000 µg/mL

4. Procedimento

1. Separar duas alíquotas de 200 µL por amostra (uma para o teste e outra para o branco);
2. Adicionar 500 µL de HCl 2M aos brancos;
3. Adicionar 500 µL de solução reagente aos tubos teste;
4. Deixar tubos a 30-37°C por 90 min;
5. Remover os tubos e colocá-los em gelo;
6. Adicionar 700 µL de TCA 28%;
7. Homogeneizar em *vortex* por 3 min;
8. Centrifugar a 9000 *xg* por 10 min e descartar sobrenadante;
9. Ressuspender o *pellet* em 1 mL de solução etanol – acetato de etila;
10. Misturar em *vortex* por 2 min;
11. Centrifugar a 9000 *x*g por 10 min e descartar sobrenadante;
12. Repetir o procedimento 10-12 mais duas vezes;
13. Ressuspender o *pellet* em 1 mL de cloreto de guanidina 6M;
14. Misturar em *vortex* por 1 min;
15. Centrifugar a 9000 *x*g por 3 min para eliminar eventual "material não solúvel";
16. Transferir 150 µL para microplaca e ler absorbância a 370 nm;
17. Medir a concentração de proteina nas amostras (tubos teste) com cloreto de guanidina.

5. Cálculo da concentração de proteínas carboniladas

$$[\text{proteínas carboniladas}] = A \cdot \text{diluição} \cdot \varepsilon^{-1} \cdot d^{-1} \cdot [\text{proteína}]^{-1}$$

[proteínas carboniladas] = mmol de hidrazinas por mg de proteína.
A = absorbância a 370 nm, descontando os brancos individuais dos testes.
diluição (da amostra) = 100 µL de amostra diluida 1:5 em 500 µL solução final pra leitura, ou seja, 25x.
ε = coeficiente de extinção molar das hidrazonas = $2{,}1.10^4\ M^{-1}.cm^{-1}$
d= caminho óptico (para volume final de 150 µL, d = 0,45 cm).
[proteína] = concentração de proteínas totais em mg/mL

ANEXO VII - ATIVIDADE DE SUPERÓXIDO DISMUTASE

1. Princípio do método

A superóxido dismutase (SOD) é uma enzima antioxidante responsável pelo combate aos ânions superóxido. O método baseia-se na seguinte reação:

$$\text{Xantina} + O_2 + H_2O \xrightarrow{XO} \text{ác. úrico} + O_2^* + H^+$$

$$\text{NBT (oxidado)} + O_2^* \rightarrow \text{NBT (reduzido)} + O_2$$

$$O_2^* + 2H^+ \xrightarrow{SOD} 02 + H_2O_2$$

Na presença de xantina e O_2, a xantina oxidase (XO) produz o ânion superóxido (O_2 *). O NBT ganha elétrons (sofre redução) na presença de O_2*. A reação de produção de NBT reduzido (azul de formazan - composto colorido que absorve em 560nm) é inibida na presença de SOD pelo fato de requerer O_2*. Quanto maior a atividade de SOD, maior a inibição na formação de NBT reduzido, e portanto menor a formação do composto colorido (Ukeda et al., 1997).

2. Preparo de soluções

2.1 Tampão carbonato de sódio (T.C.S.) 50 mM, pH 9,8 a temperatura ambiente

Para 100 mL: Solução A (Bicarbonato de sódio 0,1M)
0,288 g de $NaHCO_3$
3,43 mL de água destilada

Solução B (Carbonato de sódio 0,1M)
0,232 g de Na_2CO_3 anidro
2,19 mL de água destilada.

Misturar as soluções A e B e completar para 100 mL com água destilada. Verificar o pH.

2.2 EDTA 3 mM (Dissodium salt dihidrate)
para 5 mL: 0,0055 g EDTA sal dissódico
5 mL água destilada

2.3 Solução estoque Xantina Oxidase (XO) 22,5 U/mL

0,01 g de XO (XO microbial source, X2252 Sigma, 9 U/mg)
4 mL de água destilada (diluir o frasco inteiro)

2.4 Solução XO 4,5 U/mL

0,2 mL de solução estoque XO (22,5 U/mL)
0,8 mL de água destilada.

2.5 BSA 15 %
para 5 mL: 0,75 g BSA
5 mL água destilada

2.6 Xantina 3 mM (Xanthine X0626 Sigma®)
para 10mL: 0,0045g
10 mL T.C.S.
Sonicar durante 5 min. Estável por 1 semana.

2.7 NBT (nitro blue tetrazolium) 0,75 mM (Sigma®)
para 10 mL: 0,0061g
10 mL água destilada
Proteger da luz. Estável por 1-2 semanas.

3. Preparo da amostra

Amostras de fígado de *Piaractus mesopotamicus* (pacu caranha) são homogeneizadas em tampão fosfato 0,05 M pH 7, na proporção 1:4 (peso : volume). A seguir as amostras são centrifugadas (10.000 × *g*, 20 min, 4 °C) e o sobrenadante é diluído com o mesmo tampão para realização da análise (proporção 1:10, volume: volume).

Pools de *Daphnia similis* são homogeneizados em tampão fosfato 0,05 M pH 7 na proporção 1:10 (peso : volume). A seguir as amostras são centrifugadas (10.000 *x*g, 10 min, 4 °C) e o sobrenadante é utilizado para realização da análise.

4. Procedimento
Colocar em microplaca, na ordem:

150 µL xantina 3 mM
110 µL NBT 0,75 mM
10 µL BSA 15%
10 µL EDTA 3 mM
10 µL amostra ou tampão fosfato ou sod comercial
10 µL XO 4,5 U/ml

A XO deve ser adicionada por último, para iniciar a reação.
Ler a 560 nm durante 10 min, a 25^{o}C. Agitar a microplaca durante 20 seg antes do início e durante 20 seg entre cada leitura (21 leituras, com intervalo de 30 seg = 10 min).

5. **Cálculo da atividade enzimática**

A XO deve produzir uma atividade da ordem de 0,025/min para a reação não inibida para aproximadamente 5 min (Sigma, 1999) ou 0,25 em 20 min (Ukeda et al., 1997).
O decréscimo da atividade causada pela SOD para a reação não inibida deve ser da ordem de 40 – 60% (Sigma, 1999).

Calcular a % de inibição da reação ocasionada pela SOD (em 10 min):

$$\% \text{ Inibição} = \frac{\text{Abs/min reação não inibida - Abs/min reação inibida}}{\text{Abs/min reação não inibida – Abs/min branco}} \times 100$$

Para cálculo da atividade em Unidades:
Unidades/mL extrato = % inibição / (50%) . (volume da amostra contendo SOD)
1 Unidade corresponde à inibição de 50% da taxa de formação de formazano.
Para cálculo da atividade específica (U/mg prot), multiplicar pelo fator de diluição e dividir pela concentração de proteína na amostra.

ANEXO VIII - ATIVIDADE DE CATALASE

1. Princípio do método

A catalase (CAT) é uma enzima antioxidante que cataliza a seguinte reação:

$$H_2O_2 \xrightarrow{\text{catalase}} 2\ H_2O + O_2$$

O método consiste em mensurar a atividade da catalase através do consumo de peróxido de hidrogênio exógeno, gerando oxigênio e água. A decomposição de H_2O_2 é acompanhada pela diminuição da absorbância a 240 nm (Aebi, 1983).

2. Preparo das soluções

2.1 Solução Reação de H_2O_2 0,03 M

Para 100 ml: 0,34 mL de H_2O_2 (30%)
99,66 mL de tampão fosfato 0,05 M pH 7
A solução deve ser protegida da luz.

3. Preparo da amostra

Amostras de fígado de pacu caranha (*Piaractus mesopotamicus*) são homogeneizadas em tampão fosfato 0,05 M pH 7, na proporção 1:4 (peso : volume). A seguir as amostras são centrifugadas (10.000 × *g*, 20 min, 4 °C) e o sobrenadante é diluído com o mesmo tampão para realização da análise (proporção 1:20, volume: volume).

Pools de *Daphnia similis* são homogeneizados em tampão fosfato 0,05 M pH 7 na proporção 1:10 (peso : volume). A seguir as amostras são centrifugadas (10.000 *x*g, 10 min, 4 °C) e o sobrenadante é utilizado para realização da análise.

Pools de larvas de *Danio rerio* são homogeneizados em tampão fosfato 0,05 M pH 7 na proporção de 8 larvas em 500 µL. A seguir as amostras são centrifugadas (10.000 *x*g, 10 min, 4 °C) e o sobrenadante é utilizado para realização da análise (100 µL de amostra + 900 µL de solução reação).

4. Procedimento

Utilizar cubetas de quartzo (pois permite passagem de radiação ultravioleta).

1) Na cubeta, colocar: 990 µL de solução reação de H_2O_2 0,03M
10 µL de amostra

2) Agitar a solução e imediatamente monitorar a queda da absorbância por 1’30’’ a 240 nm.

Obs: a absorbância inicial (no tempo 0 min.) deve ser de aproximadamente 0,5.
A diferença de absorbância em 15 seg ($\Delta A240 / \Delta At = 15\ s^{-1}$) deve estar entre 0,02 e 0,1.

5. Cálculo da atividade enzimática

Atividade específica das CAT = $\Delta abs.min^{-1}$. diluição . ε^{-1} . d^{-1} . $[proteínas]^{-1}$

Onde:
Atividade específica das catalases= mmol de H_2O_2 degradado por minuto por mg de proteínas.
diluição = 10 µL de amostra em 1000 µL de volume final, ou seja, 100x
ε = coeficiente de extinção molar do H_2O_2 (λ 240nm) = 40 $M^{-1}.cm^{-1}$
d= caminho óptico (na cubeta) = 1 cm
[proteínas] = concentração de proteinas totais em mg/mL.

ANEXO IX - ATIVIDADE DE GLUTATIONA S-TRANSFERASE

1. Princípio do método

A glutationa S-transferase (GST) representa uma importante família de isoenzimas pertencentes à fase II do metabolismo. São assim denominadas pelo seu papel como catalisadoras da conjugação de vários compostos eletrofílicos ou oriundos da fase I, com o tripeptídeo glutationa reduzida (GSH). Além disso, ligando-se covalentemente a compostos eletrofílicos, as GSTs atuam como enzimas antioxidantes. O princípio do método baseia-se no fato de que as GSTs catalisam a reação de conjugação do substrato CDNB (1-cloro- 2,4-dinitrobenzeno) com a GSH (glutationa reduzida), formando um tioéter que pode ser monitorado pelo aumento de absorbância a 340 nm (Keen et al., 1976).

2. Preparo das soluções

2.1 Solução mãe de glutationa reduzida (GSH) 20 mM

Para 15 mL: 0,092 g de GSH 99-100%
15 mL de tampão fosfato 0,05 M pH 6,5

2.2 Solução mãe 1-cloro-2,4- dinitrobenzeno (CDNB) 60 mM

Para 5 mL: 0,0607 g de CDNB
5 mL de etanol absoluto PA

2.3 Solução Reação (GSH 3 mM CDNB 3 mM)

Para 100 mL: 80 mL de tampão fosfato 0,05 M pH 6,5
15 mL de GSH 20 mM
5 mL de CDNB 60 mM

3. Preparo das amostras

Amostras de fígado de pacu caranha (*Piaractus mesopotamicus*) são homogeneizadas em tampão fosfato 0,05 M pH 7, na proporção 1:4 (peso : volume). A seguir as amostras são centrifugadas (10.000 *x*g, 20 min, 4 °C) e o sobrenadante é diluído com o mesmo tampão para realização da análise (proporção 1:80, volume: volume).

Pools de *Daphnia similis* são homogeneizados em tampão fosfato 0,05 M pH 7 na proporção 1:10 (peso : volume). A seguir as amostras são centrifugadas (10.000 *x*g, 10 min, 4 °C) e o sobrenadante é utilizado para realização da análise.

Pools de larvas de *Danio rerio* são homogeneizados em tampão fosfato 0,05 M pH 7 na proporção de 8 larvas em 500 µL. A seguir as amostras são centrifugadas (10.000 *x*g, 10 min, 4 °C) e o sobrenadante é utilizado para realização da análise (50 µL de amostra + 50 µL de solução reação).

4. Procedimento

1) Em microplaca, colocar, na ordem:
50 µL de amostra
100 µL de solução reação (GSH 3Mm, CDNB 3mM).
Obs: para Branco, colocar 50 µL tampão + 100 µL solução reação.

2) Agitar microplaca e medir imediatamente absorbância a 340 nm durante 2 min.

5. Cálculo da atividade enzimática

Atividade específica GST: ΔAbs. Min^{-1} . diluição . ε^{-1} . d^{-1} . [proteínas]$^{-1}$

Onde:
Atividade específica GST = µmol de GSH-CDNB formado por minuto por mg proteínas.
ΔAbs. Min^{-1}= variação de absorbância em 1 min
Diluição (da amostra)= 50 µL em 150 µL de volume final, ou seja 3x
d =caminho óptico percorrido pelo feixe de luz (para volume total de 150 µL no micropoço d=0,45)
ε = coeficiente de extinção molar (λ 340nm) para o CDNB em pH 6,5 = 9,6 $mM^{-1}.cm^{-1}$
[proteínas] = concentração de proteínas em mg/mL

ANEXO X - ATIVIDADE DE FOSFATASE ÁCIDA

1. Princípio do método

A fosfatase ácida (FA) é uma enzima que catalisa reações de transfosforilação, e está envolvida em diversos processos celulares. O princípio do método é o seguinte:

(a) 4-nitrofenil fosfato + H_2O $\xrightarrow[\text{pH 4.9}]{\text{fosfatase ácida}}$ 4-nitrofenol + Pi

(b) 4-nitrofenol $\xrightarrow[\text{dissociação e rearranjo}]{\text{pH 11}}$ íon 4-nitrofenolate

A hidrólise do 4-nitrofenilfosfato pela fosfatase ácida em pH 4,9 libera 4-nitrofenol. A reação é parada elevando-se o pH a 11 através da adição de NaOH. Nesse pH é produzido o íon 4-nitrofenolate, fortemente colorido e cuja absorbância pode ser medida a 405 nm. A hidrólise do substrato não pode ser monitorada continuamente porque a diferença de absorbância entre o ester fosfato e seu parente fenol ocorre apenas em solução alcalina, enquanto a fosfatase ácida trabalha em pH ácido.

2. Preparo das soluções

2.1 Solução NaOH 1M
Para 100 mL: 4 g NaOH
100 mL água destilada

2.2 Tampão acetato de sódio 0,1 M pH 5
Para 100 mL: 0,57 ml ácido acetico (100%/ densidade 1,05)
6,4 mL NaOH 1 M
completar para 100 mL com água destilada

Misturar com agitador magnético
Medir e ajustar pH se necessário

2.3 Solução estoque p-nitrofenil fosfato (pNPP) 0,1 M

Para 5 ml: 0,185 g pNPP (disodium 4-nitrophenyl phosphate)
5 mL de água destilada

Proteger da luz e manter refrigerado.

2.4 Solução reação pNPP 5 mM
Para 10 ml: 0,5 mL solução estoque pNPP 0,1M
9,5 mL água destilada

Proteger da luz e manter refrigerado. A temperatura ambiente é estável por apenas 1h.

3. Preparo das amostras

Amostras de fígado de pacu caranha (*Piaractus mesopotamicus*) são homogeneizadas em tampão fosfato 0,05 M pH 7, na proporção 1:4 (peso : volume). A seguir as amostras são centrifugadas (10.000 × *g*, 20 min, 4 °C) e o sobrenadante é diluído com o mesmo tampão para realização da análise (proporção 1:10, volume: volume).

Pools de *Daphnia similis* são homogeneizados em tampão fosfato 0,05 M pH 7 na proporção 1:10 (peso : volume). A seguir as amostras são centrifugadas (10.000 × *g*, 10 min, 4 °C) e o sobrenadante é utilizado para realização da análise.

Pools de larvas de *Danio rerio* são homogeneizados em tampão fosfato 0,05 M pH 7 na proporção de 8 larvas em 500 µL. A seguir as amostras são centrifugadas (10.000 × *g*, 10 min, 4 °C) e o sobrenadante é utilizado para realização da análise.

4. Procedimento em microplaca

1) Em microplaca, colocar:
15 µL tampão acetato de sódio 0,1 M pH 5
10 µL de amostra
125 µL de solução reação pNPP
Obs: para Branco, substituir 10 µL de amostra por 10 µL de tampão de preparo da amostra. Incubar juntamente com as amostras, e depois adicionar NaOH.

2) Agitar microplaca e incubar a 37^{o}C durante 30 min.
4) Paralisar a reação adicionando 150 µL de NaOH 1M.
5) Agitar a microplaca e medir a absorbância a 405 nm.

Obs: O ideal é que a absorbância da reação, descontando o branco, esteja entre 0,2 e 1.

2) Cálculo da atividade enzimática

Atividade específica FA = $A \cdot \varepsilon^{-1} \cdot d^{-1} \cdot$ diluição $\cdot T^{-1} \cdot [\text{proteína}]^{-1}$

Atividade específica FA = mmol de pNP formado por minuto por mg de proteína.
A = absorbância a 405 nm, descontando o branco
diluição (da amostra)= 10 µl de amostra dil 1:10 em 300 µL mistura de reação, ou seja, 300x.
T= tempo de incubação, ou seja, 30 min.
ε = coeficiente de extinção molar do pNP = 18.300 $M^{-1}.cm^{-1}$
d= caminho óptico (para volume final de 300 µL, d = 0,9 cm).
[proteína] = concentração de proteínas na amostra (mg/mL).

ANEXO XI - CONCENTRAÇÃO DE METALOTIONEÍNA

1. Princípio do método

O método consiste em obter um extrato etanol/clorofórmio da fração citosólica para isolar uma fração de metalotioneína parcialmente purificada. A concentração de metalotioneína nas amostras é então quantificada espectrofotometricamente através da análise de grupos de tiol (-SH) dos resíduos de cisteína (metalotioneina tem 20-30% de cisteina), usando DTNB (Viarengo et al., 1997).

2. Preparo das soluções

2.1 Tampão Tris-sacarose (20 mM Tris-HCl, 500 mM sacarose, pH 8,6)
para 150 mL: 0,472 g Tris-HCl
25,65 g sacarose
100 mL água destilada
Ajustar pH e completar para 150 mL com água destilada.

2.2 Tampão Sódio-Fosfato 0,2 M pH 8
para 100 mL: Solução A
3,5 g $Na_2HPO_4.12H_2O$
50 mL água destilada

Solução B
1,37 g $NaH_2PO_4.H_2O$
50 mL água destilada

Misturar solução A e B, em volumes iguais e ajustar o pH.

2.3 Solução NaCl 250 mM
para 10 mL: 0,146 g NaCl
10 mL água destilada

2.4 Solução EDTA 4 mM HCl 1N
para 10 mL: 0,82 mL HCl 37%
9,18 mL água destilada
0,0148 g EDTA

2.5 Tampão de homogeneização (Tris-HCl 20 mM, Sacarose 500 mM, PMSF 0,5 mM, β-mercaptoetanol 0,01%)
para 100 mL: 100 mL tampão Tris-Sacarose
0,0087 g phenylmethylsulphonylfluoride(PMSF)
10 μL β- mercaptoetanol

2.6 Solução de Ellman (NaCl 2M; DTNB 0,43 mM em tampão Na-fosfato 0,2 M, pH 8)
para 100 mL: 100 mL tampão Na-fosfato 0,2M pH 8
11,68 g NaCl
0,017 g DTNB (5,5'-dithio-bis(2-nitrobenzoic acid)

2.7 Solução 1
para 25 mL: 23,24 mL etanol absoluto
1,75 mL clorofórmio Manter a -20^{o}C

2.8 Solução 2
para 100 mL: 97,87 mL etanol absoluto
2,17 mL HCl 37% Manter a -20^{o}C

2.9 Solução 3
para 100 mL: 87 mL etanol absoluto
12 mL tampão tris-sacarose
1 mL clorofórmio Manter a -20^{o}C

2.10 Curva padrão de GSH

Preparar solução estoque 1000 μM e a partir dela fazer uma diluição seriada

GSH 1000 μM
para 10 mL: 0,003 g GSH
10 mL água destilada

[GSH]	solução GSH anterior (μL)	solução edta-HCl (μL)
1000 μM		
500 μM	500	500
250 μM	500	500
125 μM	500	500
62,5 μM	500	500
31,25 μM	500	500
15,62 μM	500	500
7,8 μM	500	500
3,9 μM	500	500

3. Procedimento

1. Homogenizar brânquias em tampão de homogenização (1:5, peso:volume)
2. Centrifugar a 15000 *x*g por 30 min a 4^{o}C
3. Em novo tubo:
   300 μL sobrenadante (obs guardar 20-40 μL para proteina)
   340 μL solução 1
4. Centrifugar a 6000 *x*g por 10 min a 4^{o}C

5. Em novo tubo:
   490 µL do sobrenadante
   1500 µL solução 2
6. Agitar e incubar a -20^{o}C por 1h
7. Centrifugar a 6000 *x*g por 10 min a 4^{o}C
8. Descartar sobrenadante (metalotioneína encontra-se no precipitado) e ressuspender o precipitado com:
   1000 µL solução 3
9. Agitar e centrifugar a 6000 *x*g por 10 min a 4^{o}C
10. Descartar o sobrenadante e ressuspender o precipitado com:
    50 µL NaCl 250 mM
    50 µL edta-HCl
11. Agitar e adicionar 1 mL de solução de Ellman
12. Agitar e centrifugar a 3000 *x*g por 5 min
13. Transferir 150 µL do sobrenadante a microplacas e ler absorbância a 412 nm.

Para curva, colocar em tubos:
100 µL amostra
1000 µL solução de Ellman
Transferir 150 µL a microplacas

4. Cálculo da concentração de metalotioneína

Plotar a absorbância a 412 nm (descontado o branco) da curva de GSH versus a concentração de GSH em cada padrão e determinar a equação da curva. Determinar a concentração de grupos SH nas amostras aplicando a equação da curva.

$[MT] = [SH] \cdot 0{,}29 \cdot [proteína]^{-1}$

Onde:
[MT] = mmol metalotioneína por mg de proteína.
[SH] = concentração de grupamentos SH (mmol SH/mL), obtido através da aplicação da equação da curva padrão de GSH (1µM MT-SH = 1 µM GSH = 0,055 M MT). 29% de MT é composto por SH.
[proteína] = concentração de proteína nas amostras (mg/mL).

ANEXO XII – ATIVIDADE DE NA^+/K^+ - ATPASE

1. Princípio do método

A Na/K-ATPase é uma enzima de membrana importante na osmoregulação e manutenção do potencial de membrana celular.
O método baseia-se na incubação da enzima com o substrato (ATP), e mensuração do Pi liberado por hidrólise. A atividade de Na^+/K^+-ATPase é distinguida das demais atividades hidrolíticas de ATP, através da diferença de Pi liberado nos meios sem e com inibidor seletivo da enzima (ouabaína) (Quabius et al., 1997; Sampaio et al., 2008).

2. Preparo das soluções

2.1 Tampão SEI (Sacarose 0,3 M, Na_2EDTA 0,1 mM, Imidazol 0,03 M, pH 7,4)
Para 200 mL: 20,54 g Sacarose
0,0074 g Na_2EDTA
0,408 g Imidazol
Completar para 200 mL com água destilada

Separar cerca de 20 mL da solução acima e medir o pH do restante (180 mL), sendo que, com agitação fica em torno de 8,5-9,0. Corrigir o pH para 7,4 com ácido clorídrico concentrado.
Após o preparo do tampão adicionar 70 μL de β-mercaptoethanol em 200 mL de tampão SEI (para manter a atividade da enzima), guardar na geladeira (validade estimada de 1 mês se estiver na geladeira).

2.2 Tampão de Incubação (NaCl 250 mM, $MgCl_2$ 10 mM, Na_2EDTA 1 mM, Imidazol 10 mM, $Na^{+2}ATP$ 3mM)
1,461 g NaCl
0,2033 g $MgCl_2$
0.037 g Na_2EDTA
0,0681 g Imidazol
0.165 g $Na^{+2}ATP$
Completar para 100 mL com água destilada

2.3 Tampão de incubação com ouabaína 2,5 mM
Adicionar 0,09 g de ouabaína a 50 mL de tampão de incubação
Proteger da luz.

2.4 Tampão de incubação com KCl 13 mM
Adicionar 0,048 g de KCl a 50 mL de tampão de incubação.
Proteger da luz.

2.5 Reagente de cor (H_2SO_4 0.66 mM, Molibdato de amônia: 9.2 mM, $FeSO_4 . 7H_2O$ 0.33 mM)

3,66 mL H_2SO_4
1,14 g Molibdato de amônia
9,2 g $FeSO_4 . 7 H_2O$

Obs.: acrescentar $FeSO_4 . 7 H_2O$ somente na hora do uso.

Misturar TCA 8,6% a igual volume de reagente de cor. Ex: 20 mL TCA + 20 mL reagente de cor.
Realizar a análise imediatamente ou após 15 ou 30 minutos da adição da mistura acima.

2.6 Padrão de fosfato 1,62 mM

0,00813 g $Ca_5 (PO_4)_3OH$
10 mL água destilada
30 µL HCl (para dissolver fosfato de cálcio)

3. Preparo das amostras

Amostras de cérebro de *P. mesopotamiccus* (pacu caranha) são homogeneizadas em tampão SEI (1:5, peso:volume) e centrifugadas (10.000 *x*g, 5 minutos, 4°C). O sobrenadante é utilizado para realização da análise enzimática, sem diluição adicional.

4.Procedimento

Cada amostra deve ser incubada (em triplicata) separadamente com tampão de incubação com KCl e tampão com ouabaína. Para isso, deve-se colocar, em microplaca:

Em 3 pocinhos:
5 µL amostra ou padrão
100 µL tampão de incubação com KCl

Em outros 3 pocinhos:
5 µL amostra ou padrão
100 µL tampão de incubação com ouabaína.

Incubar durante 30 min a 25°C, no escuro.
Adicionar 200 µL de reagente de cor com TCA e sulfato ferroso, para parar a reação.
Ler a 595 nm.

5. Cálculo da atividade enzimática

Atividade Na/K- ATPase = ΔAbs amostra. ΔAbs padrão^{-1} . [Pi] . [proteína]$^{-1}$. T

Onde:
Atividade específica de Na/K- ATPase em µmol Pi liberado/mg de proteína/hora.
ΔAbs = Abs leitura do tampão com KCl – Abs da leitura do tampão com ouabaína

[Pi] = concentração de fosfato no padrão (1620 µmol/L)
[Proteína] = concentração de proteína na amostra (mg/L).
T = tempo de incubação (h)

ANEXO XIII - CONCENTRAÇÃO DE PROTEÍNA

1. Princípio do método

O método baseia-se na reação de proteínas com o corate Brilliant Blue G. A faixa de concentração deve ser de 0,1 a 1,4 mg/mL de proteína, usando albumina de soro bovino (BSA) como proteína padrão (Bradford, 1976).

2. Preparo das soluções

Preparar uma curva padrão de albumina de soro bovino (BSA) realizando diversas diluições de uma solução mãe a 2 mg/mL, da seguinte forma:

[] BSA (mg/mL)	µL de BSA mãe (2 mg/mL)	µL de água destilada
Branco	0	200
0,1	10	190
0,25	25	175
0,5	50	150
0,75	75	125
1	100	100
1,25	125	75
1,5	150	50

3. Preparo da amostra

As amostras a serem analizadas são homogeneizadas e diluídas em tampão de forma a que a concentração de proteína fique dentro dos limites da curva padrão com BSA.

Amostras de fígado de pacu caranha (*Piaractus mesopotamicus*) são homogeneizadas em tampão fosfato 0,05 M pH 7, na proporção 1:4 (peso : volume). A seguir as amostras são centrifugadas (10.000 *x*g, 20 min, 4 °C) e o sobrenadante é diluído com o mesmo tampão para realização da análise (proporção 1:100, volume: volume).

Pools de *Daphnia similis* são homogeneizados em tampão fosfato 0,05 M pH 7 na proporção 1:10 (peso : volume). A seguir as amostras são centrifugadas (10.000 *x*g, 10 min, 4 °C) e o sobrenadante é utilizado para realização da análise.

Pools de larvas de *Danio rerio* são homogeneizados em tampão fosfato 0,05 M pH 7 na proporção de 8 larvas em 500 µL. A seguir as amostras são centrifugadas (10.000 *x*g, 10 min, 4 °C) e o sobrenadante é utilizado para realização da análise.

4. Procedimento

Em microplaca, colocar:
5 µL de amostra ou BSA
250 µL de reagente de Bradford Sigma® (puro).

Agitar microplaca durante 30 seg
Incubar durante 5 a 45 min a temperatura ambiente
Ler absorbância a 595 nm.

Obs: O complexo proteina-corante é estável por 60 min.

5. Cálculo da concentração de proteína

Plotar a absorbância (descontado o branco) versus a concentração de proteína em cada padrão. Determinar a concentração de proteína nas amostras comparando a absorbância (descontado o branco) com a curva padrão.

ANEXO XIV - ATIVIDADE DE GLUTATIONA PEROXIDASE

1. Princípio do método

A glutationa peroxidase (GPx) é uma enzima antioxidante que remove o excesso de H_2O_2 formado pelo estresse oxidativo a partir de ânions superoxido. A atividade de GPx é estimada pelo decréscimo da absorbância a 340 nm devido à oxidação de β-NADPH (β-nicotinamida adenina dinucleotideo fosfato). Ou seja quanto maior a atividade da GPx no extrato, maior velocidade de formação de GSSG (glutationa oxidada) e maior a velocidade de oxidação de β-NADPH para β-NADP. Para analise de GPx dependente de Se usa-se H_2O_2 como substrato. Para análise de GPx independente de Se usa-se hidroperóxido de cumene.

$$2\ GSH + H_2O_2 \xrightarrow{\text{Glutationa peroxidase}} GSSG + 2\ H_2O$$

$$GSSG + \beta\text{-NADPH} \xrightarrow{\text{Glutationa redutase}} \beta\text{-NADP} + 2\ GSH$$

2. Preparo das soluções

2.1 Tampão fosfato 50 mM (pH 7) com EDTA 0,4 mM

a) 0,272 g de KH_2PO_4 diluídos para 40 mL de água destilada
b) 0,534 g de $Na_2HPO_4 . 2\ H_2O$ diluídos para 60 mL de água destilada

Misturar 40 mL de "a" com 60 mL de "b" para fazer 100 mL de tampão. Verficar pH (se necessario ajustar com NaOH 1M).
Adicionar 14,88 mg de EDTA dissódico para cada 100 mL do tampão.

2.2 Solução Azida 2 mM em tampão fosfato 50 mM pH 7 com EDTA

Para 50 mL: 0,013 g azida de sódio
diluir em 50 mL do tampão fosfato 50mM pH 7 com EDTA.

2.3 Solução tampão fosfato 10 mM com 1,0 mM Dithiothreitol (DTT) pH 7,0

Preparar 1 L de tampão diluindo 1:5 o tampão fosfato 0,05 M, pH 7.
Adicionar 0,015 g de DL- Dithiothreitol em 1 L de tampão fosfato 10 mM.

2.4 ß-NADPH (beta nicotinamida adenina dinuleotideo fosfato - forma reduzida) 12,5 mg/mL

Dissolver o conteúdo de um frasco (25 mg) em 2 mL de água destilada

2.5 Solução de glutationa redutase (GR) 6 U/mL

0,033 mL de Glutationa redutase Sigma 450 U/mL (frasco com 500 U tem 1,11 mL; frasco com 2,5KU/2500 U tem 5,55 mL).
Diluir em 2,5 mL de água destilada gelada.

2.6 Glutationa reduzida (GSH) 200 mM

0,03 g GSH
Diluir em 0,5 mL de água destilada

2.7 Solução de peróxido de hidrogênio 0,002%

14 µL peróxido de hidrogênio 30 %
Diluir em 200 mL de água destilada

2.8 Solução reação (azida 1,5 mM, GR 1,5 U/mL, GSH 1,5 mM, βNADPH 0,153 mg/mL)

Para 10 mL: 7,5 mL solução azida 2 mM
2,5 mL GR 6 U/mL
75 µL GSH 200 mM
122 µL ß-NADPH 12,5 mg/L

3. Preparo das amostras

Pools de *Daphnia similis* são homogeneizados em tampão fosfato 0,05 M pH 7 na proporção 1:10 (peso : volume). A seguir as amostras são centrifugadas (10.000 × *g*, 10 min, 4 °C) e o sobrenadante é utilizado para realização da análise.

4. Procedimento

Em microplaca de 96 poços, colocar, na ordem:
50 µL de amostra
200 µL de solução reação
50 µL de solução de peróxido de hidrogênio 0,002%

Obs: para o Branco, substituir amostra por 50 µL de tampão com DTT.
Agitar a microplaca e medir imediatamente absorbância a 340 nm durante 2 min (14 leituras, com intervalo de 10 seg, agitar 3 seg antes da leitura).

5. Cálculo da atividade enzimática

Atividade específica GPx: $\Delta Abs. Min^{-1} . \varepsilon^{-1} . d^{-1} . [proteínas]^{-1}$

Onde:
Atividade específica GPx = µmol de βNADPH consumido por minuto por mg proteínas.
ΔAbs. Min^{-1} = variação de absorbância em 1 min
d = caminho óptico percorrido pelo feixe de luz (para volume total de 300 µL no micropoço d = 0,9)
ε = coeficiente de extinção molar β-NADPH (λ 340 nm) = 6,22. $mM^{-1}.cm^{-1}$
[proteínas] = concentração de proteínas em $mg.mL^{-1}$

ANEXO XV - MANUTENÇÃO DE *DAPHNIA SIMILIS*

As daphnias são mantidas em aquários de 10 L, contendo água previamente aerada e recirculada através de filtro com carvão ativado, sendo adicionados 200 µL de Vitamina B12 (1 mg/mL) e 1 mL de Selenito de Sódio (20 µg/mL) ao meio. A troca dos meios de cultivo e limpeza dos aquários é realizada semanalmente.

As daphnias são mantidas a 20 ± 1^{o}C e iluminação constante (~1500 lux). A alimentação é fornecida duas vezes ao dia, administrando-se 10 mL de uma suspensão de *Chlorella pyrenoidosa* (2 mg/mL).

ANEXO XVI - MANUTENÇÃO DE PEIXES *DANIO RERIO* E OBTENÇÃO DOS EMBRIÕES PARA REALIZAÇÃO DE TESTE DE TOXICIDADE.

1. Objetivo

Este método consiste na exposição de embriões de peixe (*Danio rerio*) a várias concentrações do agente químico ou diferentes condições experimentais, por um período de 2 a 4 dias, nas condições prescritas. Tal procedimento permite determinar a concentração letal média (CL50) do agente tóxico e ocorrência de malformações.

2. Manutenção dos pais

Machos e fêmeas de *Danio rerio* com idade entre 4 e 12 meses devem ser mantidos juntos em aquários com capacidade para 20 L de água. Os animais devem ser mantidos em água declorinizada limpa, a 27 ± 1 ^{o}C, contendo até 0,3% de cloreto de sódio, submetidos a um ciclo claro/ escuro de 16/8h. A alimentação com ração Tetramin Tropical Flakes® deve ser fornecida duas vezes ao dia, em quantidade que seja consumida pelos animais em até 5 minutos, para que não haja acúmulo de detritos no aquário (Lawrence, 2007). Recomenda-se a suplementação com náuplios de artêmias, uma vez ao dia.

3. Água reconstituída

Para manutenção dos embriões e larvas, deve ser preparada uma solução de água reconstituída, com no mínimo 24h de antecedência ao teste, conforme descrito a seguir (USEPA, 2002).
Água moderadamente dura (preparada com água destilada ou água Milli Q):

$NaHCO_3$	96 mg/L
$MgSO_4$	60 mg/L
KCl	4 mg/L
$CaSO_4.2\ H_2O$	60 mg/L

Os três primeiros sais devem ser adicionados à água e a solução deve ser aerada durante 24 horas. O $CaSO_4.2H_2O$ deve ser adicionado em um pouco de água separadamente e agitado até que o sal esteja completamente dissolvido, após o que pode ser adicionado à solução com os demais sais.
A solução de água reconstituída deve ser mantida coberta, sob forte aeração. As condições para seu uso com os ovos são: temperatura 26 ± 1^{o}C, condutividade 290 ± 30 µS/cm, oxigênio dissolvido >80% , pH 8.

4. Obtenção dos ovos para realização do teste de toxicidade

Cerca de três dias (por exemplo, na sexta-feira) antes do dia em que se deseja obter os ovos (por exemplo na segunda-feira), machos e fêmeas de *D. rerio* devem ser separados (Figura I indica como diferenciar machos e fêmeas) e mantidos em aquários diferentes. No dia

anterior ao que se deseja obter os ovos, os peixes devem ser transferidos para "aquários de acasalamento" com capacidade para 10L de água, mantidos separados por uma divisória de vidro com pequenos furos. Manter a proporção de 2 machos:1 fêmea. Os aquários devem estar equipados com filtro contendo carvão ativado e aeração constante, de forma a suprir os dois compartimentos do aquário, e o fundo deve estar recoberto com duas camadas de bolinhas de gude (Figura II). Recomenda-se que os animais sejam alimentados antes de serem transferidos para o aquário de acasalamento, para que não haja excesso de detritos quando da obtenção dos ovos.

No dia em que se deseja obter os ovos, antes que as luzes do laboratório se acendam, o filtro deve ser desligado e a divisória do aquário deve ser retirada. O acasalamento e a postura ocorrem quando as luzes se acendem. Aguardar cerca de 30 min para colher os ovos.

Para colher os ovos, retirar delicadamente os peixes e bolinhas de gude do aquário. Passar a água através de um pequeno coador plástico do tipo usado para coar leite/chá (Figura III).

Utilizando pipetas plásticas do tipo Pasteur, transferir os ovos para a água reconstituída preparada para manutenção dos ovos. Remover os ovos mortos/coagulados (Figura IV). Caso haja muita sujeira na água (restos de ração, fezes), transferir os ovos novamente para água reconstituída limpa.

Para evitar contaminação dos ovos, pode-se lavá-los com água contendo hipoclorito de sódio (preparada diluindo 0,1 ml de água sanitária 5,25% em 170 mL de água). Para a lavagem, colocar os ovos na solução com água sanitária diluída, durante 1 a 2 minutos, e depois passá-los duas vezes em água limpa.

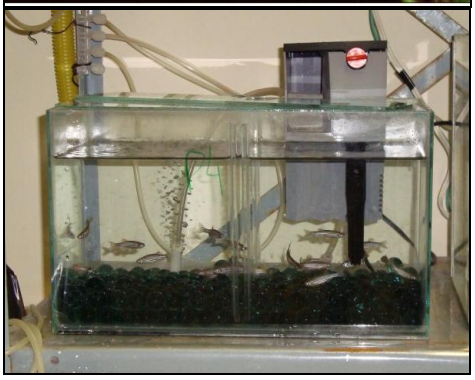
O ideal é que os ovos sejam transferidos para as condições de teste em até 1 hora –pós-fertilização (acendimento das luzes). O teste é realizado segundo o método proposto pela OECD (2006).

Os ovos devem ser inspecionados através de estereomicroscópio ou microscópio invertido antes de transferi-los para as condições de teste. Ovos não fertilizados não demonstrando clivagem ou ovos apresentando irregularidades durante a clivagem (por exemplo assimetria, formação de vesículas) ou injúrias no córion devem ser descartados.

Os embriões devem ser inspecionados a cada 24 horas, através de estereomicroscópio. Deve ser feito registro fotográfico dos embriões, anotando-se o aumento empregado. O teste pode ter duração de 2 a 4 dias. Alterações nos *endpoints* e tempos de observação são possíveis. As observações dos *endpoints* devem ser registradas em arquivo excel conforme modelo apresentado ao final deste anexo. Na tabela Excel, o número 1 deve ser registrado na célula correspondente ao *endpoint* constatado para cada indivíduo.

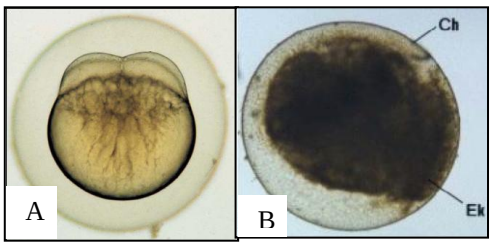
Ao fim do experimento, calcula-se a porcentagem de indivíduos que apresentaram determinados *endpoints* e a sobrevivência de ovos/larvas em cada tempo de observação. Os dados são utilizados para cálculo da CL50, através da análise de probito.

I) *D. rerio* adultos. A fêmea (indivíduo acima) pode se diferenciada do macho (indivíduo abaixo) pelo seu abdômen globoso e a ausência de coloração avermelhada/amarelada ao longo das listras prateadas. Fonte: Braunbeck e Lammer, 2006.



II) Aquário para acasalamento

III) Técnica para colheita dos ovos. Inverter um coador plástico e despejar sobre ele a água contendo os ovos, de modo lento. Verter os ovos em um recipiente com água reconstituída.



IV) Ovos recém-coletados. A) ovo vivo, em clivagem (aproximadamente 1h); B) ovo morto/ coagulado. Ch = córion, Ek = ovo coagulado. Fonte: Braunbeck e Lammer, 2006.

Modelo de tabela de registro de observação dos embriões

Dados Brutos																																							
		Dia 1									Dia 2										Dia 3										Dia 4							
Réplica	[concentração]	Ovo coagulado	Ovo vivo	Alteração em Olho	Alteração em pigmentação	Ausência de separação da cauda	Alteração em absorção de saco vitelino	Edema	Atraso no desenvolvimento		Ovo coagulado	Ovo vivo	Larva viva	Larva morta	Alteração em pigmentação	Batimento cardíaco	Atraso no desenvolvimento	Ausência de otholito	Edema	Deformidade de Cauda	Ovo coagulado	Ovo vivo	Larva viva	Larva morta	Alteração em pigmentação	Alteração em Absorção Saco vitelino	Deformidade de Cauda	Alteração de Equilíbrio	Edema	Atraso no desenvolvimento	Ovo coagulado	Ovo vivo	Larva viva	Larva morta	Absorção Saco Vitelino	Deformidade de Cauda	Alteração de equilíbrio	Edema cardíaco