Nanotoxicologia e Ambientes Aquáticos: Estudo sobre as Interações do Nanomaterial Fulereno C_{60} e o Benzo[a]pireno no peixe *Danio rerio* (Cyprinidae, Teleostei)

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Dedico este trabalho à memória de meu pai,

Milton João de Lima Ferreira, cujo amor me

ensinou o que sei ser.

“Nós somos a maneira do Cosmo

conhecer a si mesmo.”

Carl Sagan

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Sumário

Resumo

Nanomateriais (NMs) são compostos que possuem no mínimo uma dimensão na faixa de 100 nm. Essa escala de tamanho confere aos materiais propriedades físicas e químicas diferenciadas, com aplicações em quase todas as áreas da ciência. Associada a essas propriedades está sua alta capacidade de reação com moléculas biológicas, tornando os NMs potencialmente tóxicos. A crescente produção mundial de NMs, incluindo os baseados em carbono, tem provocado preocupações quanto às suas consequências ambientais e para a saúde humana, principalmente pelo desconhecimento dos seus efeitos tóxicos nos organismos. O fulereno C_{60} é um NM de carbono cuja toxicidade tem sido um tema debatido na comunidade científica, e estudos recentes mostram que ele pode interagir com outros contaminantes ambientais, aumentando a toxicidade destes. A presente Tese, portanto, teve como objetivos: 1 – revisar o conhecimento existente sobre os efeitos dos NMs em organismos aquáticos; 2 – investigar as possíveis interações tóxicas entre o nanomaterial fulereno C_{60} e o hidrocarboneto benzo[a]pireno (BaP) usando abordagens *in vitro* e *in vivo* em um modelo de animal aquático, o peixe *Danio rerio* (Cyprinidae, Teleostei). Para isso, foram usados hepatócitos em cultura e larvas de *D. rerio* com idade de 72 horas pós-fertilização. Os resultados gerais mostram que o fulereno C_{60} pode aumentar a biodisponibilidade de contaminantes como o BaP, causando morte celular e perturbação nos mecanismos detoxificatórios. Também foi observado que o nanomaterial pode causar histopatologias graves em larvas de peixe, dano oxidativo e aumento na incidência de apoptose. Tais resultados evidenciam a importância do fulereno C_{60} como contaminante ambiental e trazem à tona a necessidade da criação de metodologias padronizadas para o estudo da Nanotoxicologia.

Palavras-chave: toxicologia, estresse oxidativo, hepatócitos, larvas.

Introdução

Assim como as diversas pressões seletivas do ambiente norteiam a evolução das espécies, a ação das espécies sobre seus ambientes também os modificam. Essa recíproca dinâmica muitas vezes alterna períodos de equilíbrio e também de extremos. Talvez o exemplo mais representativo seja o surgimento do oxigênio em nossa atmosfera, um metabólito originalmente letal para a maioria dos organismos e que direcionou a evolução nos moldes como a conhecemos hoje (Fig. 1). O modelo Darwiniano atual demonstra que o sucesso evolutivo das espécies depende basicamente de sua plasticidade genética, da capacidade de adaptação às mudanças. Isso, naturalmente, está diretamente relacionado com a velocidade em que tais mudanças ocorrem. Eventos que aceleram as mudanças de um determinado ambiente geralmente são seguidos por alterações importantes, até dramáticas, no perfil das espécies.

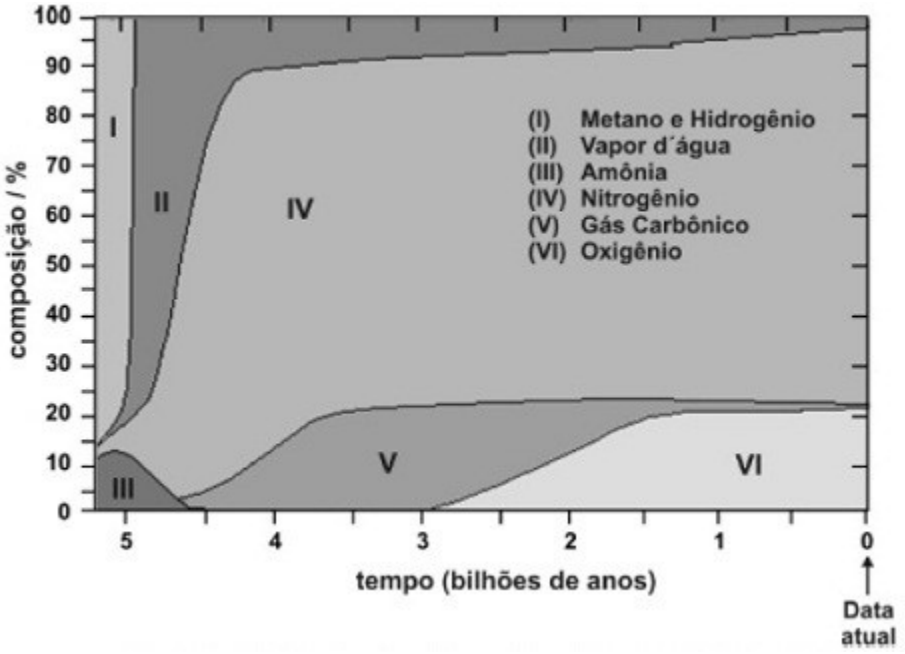

Figura 1. Composição da atmosfera e surgimento do O^2.
Fonte: adaptado de The Random House Encyclopedia, 3ª ed. 1990.

Uma das características mais marcantes da nossa jovem espécie é, sem dúvida, a capacidade de modificar os ambientes nos quais vive. Crescemos muito, e passamos a chamar essa capacidade de “impacto”, tamanho o potencial para afetar as outras espécies e a nós mesmos. Somos, portanto, uma espécie catalisadora de mudanças. E enquanto seres autoconscientes, precisamos não apenas entender o universo à nossa volta, mas também considerar a extensão das nossas ações através das inúmeras áreas da Ciência que criamos.

As descobertas de fenômenos, substâncias e processos naturais e seu uso como recurso pela humanidade historicamente representam marcos, ou “saltos” tecnológicos. Todos, evidentemente, com suas implicações ambientais proporcionais ao seu grau de importância. Na História Contemporânea, a Revolução Industrial, as descobertas da física quântica e da estrutura do DNA são exemplos importantes desses “saltos”, porque aceleraram as mudanças sociais e ambientais em um grau muito difícil de ser estimado.

Da mesma forma ocorre com o atual fenômeno tecnológico da humanidade, que é a ascensão meteórica das nanotecnologias e seu uso massivo em nível global. O desenvolvimento exponencial dos nanomateriais, por exemplo, trouxe um grande desafio às Ciências Ambientais, em especial à Toxicologia Ambiental porque os conceitos e técnicas da toxicologia clássica não podem ser extrapolados diretamente para os nano-contaminantes emergentes. A nanoescala confere aos compostos propriedades químicas e físicas totalmente novas, mesmo com tóxicos clássicos como metais, tornando os modelos de previsão obsoletos. E o surgimento de novos compostos dificultam ainda mais o entendimento dos mecanismos de interação com os organismos e ambientes.

Desse modo, em virtude do que aprendemos ao longo da História, não só é aconselhável, como torna-se obrigatório o estudo aprofundado das consequências

ambientais como parte do desenvolvimento das tecnologias.

Nanotecnologias e Nanocompostos

O físico Richard Feynman, considerado o pai da nanotecnologia, em sua famosa palestra no American Physical Society Meeting em 1959 já previa as tendências da ciência em manipular as substâncias em nível atômico (Feynman, 1992). Porém, apenas em 1974 Norio Taniguchi usou pela primeira vez o termo Nanotecnologia: *"Nano-tecnologia consiste no processamento de separação, consolidação e deformação de materiais através de um átomo ou de uma molécula"* (Taniguchi, 1974). Dentre as nanotecnologias, estão as que estudam e manipulam substâncias na nanoescala, chamadas genericamente de nanomateriais ou nanocompostos.

Nanomateriais são, por definição, substâncias que possuem no mínimo uma dimensão dentro da nanoescala (até 100 nm). Nessa faixa de tamanho, há mudanças significativas nas propriedades químicas e físicas da molécula, o que faz com que sejam amplamente exploradas (Colvin, 2003; Oberdörster et al., 2005). Embora a humanidade faça uso dessas propriedades diferenciadas desde a antiguidade, apenas há menos de duas décadas elas foram redescobertas como pivôs de uma tecnologia revolucionária (Hochella Jr., 2002; Oberdörster et al., 2007). Na atualidade, os nanomateriais fazem parte de virtualmente todas as áreas da vida humana. Desde equipamentos eletrônicos, vestuário, cosméticos, lentes, baterias, combustíveis, isoladores acústicos e térmicos, materiais semicondutores e catalisadores químicos até aplicações na medicina (instrumentos e próteses cirúrgicas, carreadores de fármacos, vetores alternativos de DNA e RNA, contrastes para diagnóstico, etc) e nas ciências ambientais (remediação de ambientes contaminados), e ainda uma gama imensa de outras aplicações (Cheng et al.,

2009; Savolainen et al., 2010).

Porém, as propriedades físico-químicas que fazem com que os nanomateriais representem de fato um “salto” tecnológico para a melhora da qualidade de vida, também podem significar sérios riscos à saúde humana e ao meio ambiente, como ocorreu com outras tecnologias. Quando estão na nanoescala, os compostos assumem propriedades mais próximas às do átomo e às leis da mecânica quântica (Tab. 1). A relação superfície/volume se eleva consideravelmente, o que significa muito mais átomos disponíveis na superfície (chegando a 50%) para reagir com moléculas do entorno, incluindo macromoléculas biológicas (Hoet et al., 2004; Lyon et al., 2006; Li et al., 2011). Os compostos na nanoescala, portanto, são intrinsecamente mais reativos do que seus equivalentes cujas partículas estão em um grau maior de agregação.

Centímetro	Gravidade, fricção, combustão
Milímetro	Gravidade, fricção, combustão, eletrostática
Micrômetro	Eletrostática, Van der Waals, browniano
Nanômetro	Eletrostática, Van der Waals, browniano, quântico
Angström	Mecânica quântica

Tabela 1. Fenômenos físicos predominantes de acordo com a escala de tamanho.
Fonte: Cartilha de Nanotecnologia – Unicamp, ABDI. 2010.

Basicamente, os termos “nanomateriais” ou “nanocompostos” englobam substâncias bastante heterogêneas, cujo único critério de agrupamento são as mudanças nas propriedades químicas e físicas que ocorrem quando o composto está na nanoescala. Quanto à sua natureza, podem ser classificadas genericamente como: compostos

inorgânicos (metais, óxidos metálicos e qualquer combinação metalóide, como os pontos quânticos); materiais baseados em carbono (fulerenos, nanotubos, grafenos, suas variações e derivados funcionalizados); mistos (dendrímeros, nanocápsulas, nanomicelas, etc) (Savolainen et al., 2010).

Contaminantes de Estudo

Fulereno C_{60}

Fulerenos são alótropos do carbono, encontrados na natureza desde eventos cósmicos como a formação de estrelas até deposição mineral em rochas. O número de átomos de carbono na molécula pode variar muito, de 20 até 540 (Kroto, 1990). Dentre os fulerenos, o mais abundante é o fulereno C_{60}, descrito pelo grupo de H. W. Kroto em 1985. Sua estrutura é a de um icosaedro regular truncado composto por 60 átomos de carbono organizados em 12 pentágonos e 20 hexágonos, apresentado trinta ligações duplas (Trpkovic et al., 2012) (Fig. 2). Esta conformação confere estabilidade química e elétrica à molécula (Kroto et al., 1991). Seu nome é uma homenagem a R. Buckminster Fuller, engenheiro famoso por construir grandes domos geodésicos com conformação icosaédrica, semelhantes à molécula proposta por Kroto. Outros sinônimos para o fulereno C_{60} são “Buckminsterfullerene” e “Buckyball”.

O fulereno C_{60} é virtualmente insolúvel em água, mas pode formar suspensões coloidais aquosas quando em agitação e incidência de luz. Isto ocorre pelo fenômeno da solvatação, onde íons ^{-}OH da água que circundam as partículas de C_{60} estabelecem um equilíbrio eletroquímico entre as forças de atração e repulsão, evitando que as partículas se agreguem em uma escala maior (Fig. 3). Contudo, a presença de outros íons na água (como sais, por exemplo) perturba este equilíbrio e faz com que se formem aglomerados,

podendo extrapolar o tamanho da nanoescala (Andrievsky et al., 1999).

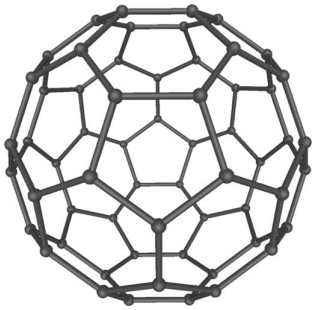

Figura 2. Modelagem gráfica computacional da molécula do fulereno C_{60}.

Fonte: Wikimedia Commons, Wikipedia. http://en.wikipedia.org/wiki/File:C60a.png (acessado em 04/2014).

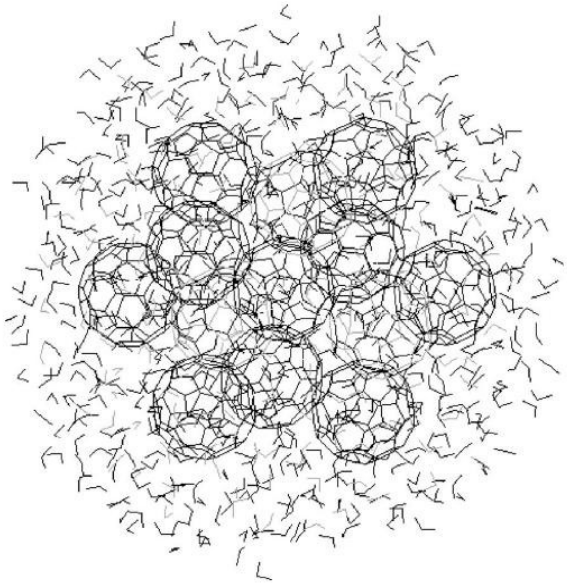

Figura 3. Modelagem gráfica computacional de um agregado com treze moléculas solvatadas de fulereno C_{60} em meio aquoso.

Fonte: Andrievski et al., 2002.

A toxicidade do fulereno C_{60} é um assunto atualmente em debate na comunidade científica, e é um dos tópicos da presente tese. Os primeiros ensaios toxicológicos com este nanomaterial indicavam um alto grau de toxicidade, inclusive em peixes (Oberdörster, 2004; Oberdörster et al., 2006; Zhu et al., 2006; Zhu et al., 2007). Outros poucos trabalhos como o de Andrievsky et al. (2005) apontavam erros metodológicos nestas primeiras

publicações e afirmavam que o fulereno C_{60} não era tóxico, mas estas observações não eram unânimes. Mais tarde, foi demonstrado que o uso de solventes orgânicos na preparação das suspensões de fulereno C_{60} (na maioria dos casos tetrahidrofurano, THF) era o fator responsável pelo menos por parte da toxicidade verificada. Compostos derivados do THF como γ-butirolactona e tetrahidro-2-furanol ficavam remanescentes nas suspensões, e a princípio não eram detectados (Henry et al., 2007). A técnica de preparo das suspensões com THF foi sistematicamente repetida em vários trabalhos, e por causa disso o entendimento dos reais efeitos do nanomaterial em organismos sofreu graves atrasos.

Atualmente há autores que postulam que a toxicidade do fulereno está associada apenas à sua fotoexcitação e os efeitos deletérios em peixes são mínimos (Henry et al., 2011). O mecanismo de fotoexcitação envolve geração de espécies reativas de oxigênio (ERO) como oxigênio singleto ($^1O_2$) e radical ânion superóxido ($O_2^{\bullet -}$) através da alternância dos estados de excitação das moléculas na partícula do C_{60} (Kato et al., 2009; Chae et al., 2011). Outros autores propõem dois mecanismos de toxicidade: um dependente de ERO (fotoexcitação) e outro por interação física com biomoléculas (Trpkovic et al., 2012). Contudo, há uma lacuna de informação com respeito aos seus efeitos durante o período de desenvolvimento dos animais (considerando apenas dados confiáveis com suspensões sem o uso de solventes orgânicos).

Além dessa problemática, há o conceito de “Cavalo de Tróia” proposto inicialmente por Limbach et al. (2007) para nanopartículas de sílica. Os autores postularam que nanomateriais podem agravar os efeitos tóxicos de outros contaminantes ambientais, “carregando” tais contaminantes para dentro de sistemas biológicos. Este tipo de carreamento se verificou também em outros trabalhos com fulereno (Baun et al., 2008;

Azevedo Costa et al., 2012; Henry et al., 2013), mas os mecanismos pelos quais isto ocorre ainda não estão claros. No ambiente aquático, já foi demonstrado que o fulereno C_{60} aumenta a biodisponibilidade de mercúrio através da adsorção do metal nas partículas do nanomaterial (Henry et al., 2013). No entanto, postula-se que os eventos que fazem com que o nanomaterial aumente a captação de outros contaminantes pelos organismos envolvam não só a adsorção, mas também a interação das nanopartículas com membranas biológicas, aumentando a permeabilidade aos tóxicos (Baun et al., 2008; Azevedo Costa et al., 2012).

Benzo[a]pireno

O benzo[a]pireno (BaP) é um hidrocarboneto aromático policíclico (HAP) formado por cinco anéis benzênicos e produzido por múltiplas fontes, como exaustão de veículos, combustões incompletas de carvão, geradores de calor e energia, processos industriais, fumaça de cigarros, entre outras (Miller e Ramos, 2001). Foi sintetizado pela primeira vez em 1933 e identificado como o principal agente carcinogênico no alcatrão (Boysen e Hecht, 2003). É um importante contaminante ambiental, amplamente distribuído em quase todos os ambientes, incluindo os aquáticos (Palanikumar et al., 2012). Sua característica lipofílica facilita a entrada em membranas biológicas e acumulação em organismos, sendo um potente agente pró-oxidante, mutagênico, carcinogênico e teratogênico (Kamaraj et al., 2007). A cadeia alimentar é considerada a principal via de exposição a humanos, contribuindo com aproximadamente 97% da ingestão de BaP por dia (Miller e Ramos, 2001).

A molécula do BaP apresenta dois sítios de maior reatividade: a chamada “região da baía”, onde se localiza o carbono alfa, e uma área eletrodensa chamada de “região K”

(Fig. 4). Ambos os sítios estão relacionados ao potencial carcinogênico do BaP pela reação com os sistemas celulares de detoxificação e formação de metabólitos. Sua metabolização é bastante complexa e pode envolver várias etapas (Fig. 5). Apesar das vias metabólicas alternativas variarem de acordo com a espécie, as vias clássicas são muito conservadas ao longo da escala filogenética. Tais vias de detoxificação por enzimas de fase I incluem oxidases de função mista, como o CYP1A e epóxido-redutases. Enzimas de fase II como epóxido-hidrolases e as de conjugação como glutationa-S-transferases (GSTs), UDP-glucoronil transferases e sulfotransferases também estão envolvidas nesse processo. O BaP é reconhecido também como um ligante do receptor aril de hidrocarbonetos (rAH), um fator de transcrição envolvido na regulação de enzimas de metabolização de xenobióticos, incluindo enzimas detoxificatórias de fase I e II. Acredita-se que essa é uma das razões pelas quais a exposição ao BaP induz ao mesmo tempo dano oxidativo (p.e., por aumento na expressão de monooxigenases) e respostas antioxidantes (p.e., por aumento na expressão de enzimas do sistema de defesa antioxidante). Porém, a maior parte da toxicidade do BaP resulta da sua detoxificação incompleta, gerando principalmente BaP-dióis, BaP-quinonas e BaP-epóxidos (Fig. 5) que induzem a formação de adutos de DNA e de proteínas e também geram ERO (Miranda et al., 2006). Por ser um contaminante ubíquo, seus efeitos associados a outros contaminantes ambientais também tem sido considerados (Miller e Ramos, 2001). Porém, até o momento, não são conhecidos trabalhos que avaliem sua co-exposição a nanomateriais.

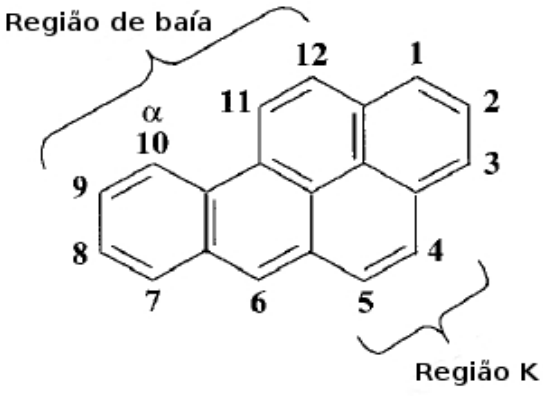

Figura 4. Fórmula estrutural da molécula do benzo[a]pireno (BaP).
Fonte: adaptado de Miller e Ramos, 2001.

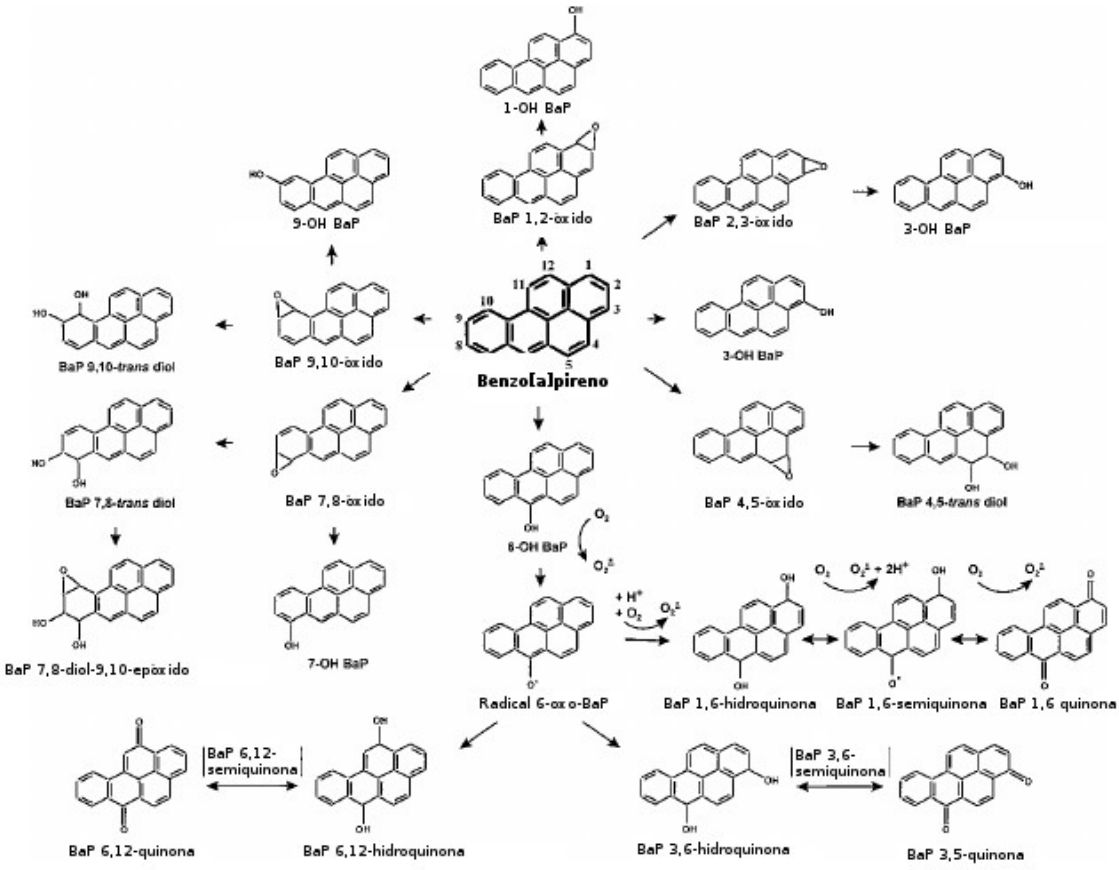

Figura 5. Produtos conhecidos da metabolização do benzo[a]pireno (BaP).
Fonte: adaptado de Miller e Ramos, 2001.

Modelo Experimental

A escolha do modelo experimental para ambientes aquáticos é muito importante em estudos de impactos ambientais. Peixes geralmente são bons modelos pois estão sujeitos não apenas à exposições pontuais, mas a todos os contaminantes presentes em um corpo de água. O peixe *Danio rerio* (Ciprinidae, Teleostei), comumente chamado “paulistinha” no Brasil e “zebrafish” pela comunidade científica em geral, é uma espécie de climas quentes endógena do sul-asiático (Índia, Bangladesh, Nepal e Paquistão) (Lawrence, 2007). É considerado um excelente modelo para estudos toxicológicos *in vitro* e *in vivo* devido à sua facilidade de manutenção, ter seu genoma sequenciado e apresentar respostas metabólicas extrapoláveis a outros organismos mesmo filogeneticamente distantes (Hermann et al., 2004; Diekmann e Nagel, 2005; Best e Alderton, 2008). Linhagens de hepatócitos de zebrafish em cultura são comumente usadas para ensaios toxicológicos (Bopp e Lettieri, 2008) incluindo nanomateriais (Azevedo Costa et al., 2012). Embriões de zebrafish também são amplamente utilizados em estudos sobre prejuízos no desenvolvimento (George et al., 2011; Hsu et al., 2013). A escolha da linhagem ZF-L de hepatócitos em cultura no primeiro trabalho deve-se ao fato de que o fígado é um órgão-alvo da exposição a BaP, devido ao seu papel essencial na metabolização de toxinas. No trabalho com larvas de zebrafish, o período escolhido para a exposição foi o de 72 horas pós-fertilização (hpf), pois trata-se de uma fase crítica no desenvolvimento do animal quando vários órgãos e estruturas como a boca e as brânquias começam a ter funcionalidade (Kimmel et al., 1995).

Os estudos com cultura de células tem suas limitações e geram discussões a respeito do seu significado estatístico, dado que há um componente de pseudo-replicagem inerente à condição de cultura celular em laboratório. Contudo, são

ferramentas estratégicas para muitas condições experimentais, além do fato de que são alternativas viáveis ao uso experimental de animais. Por outro lado, larvas de peixe podem fornecer informações essenciais sobre o impacto de contaminantes sobre determinadas populações. Tais informações também podem ser extrapoláveis a humanos, sendo uma grande fonte de aplicações para a área da saúde.

Tal paradigma sobre o uso de abordagens sistêmicas versus mecanísticas tem especial importância na Nanotoxicologia. Enquanto a aplicação ou simulação de situações mais próximas da ocorrência natural fornece respostas mais extrapoláveis em termos ecotoxicológicos, pouco revela sobre o que de fato ocorre em nível bioquímico e molecular. Esse fato assume maior relevância à medida que as variáveis aumentam, e no caso dos nanomateriais, o aparecimento da nova variável *tamanho* é o fator crítico que pode fazer com que o comportamento de um mesmo composto oscile entre benéfico/inerte/tóxico (Lyon et al., 2006; Jiang et al., 2008).

Logo, estudos *in vivo* são essenciais para fornecer dados sobre os efeitos sistêmicos dos nanomateriais, enquanto investigações *in vitro* fornecem uma visão mais aguçada da interação de suas propriedades com ambientes biológicos. O uso integrado desses dois tipos de abordagens é uma estratégia útil para estabelecer rumos e perspectivas na área emergente da Nanotoxicologia.

Hipóteses e Objetivos

Em virtude das lacunas de conhecimento sobre os efeitos dos nanomateriais em ambientes aquáticos; das evidências de efeitos do tipo “Cavalo de Tróia” dos nanomateriais, e da discussão sobre a real toxicidade do fulereno C_{60}, as hipóteses da presente Tese se apresentam como:

1. O nanomaterial fulereno C_{60} possui a capacidade de aumentar a biodisponibilidade e toxicidade de outros contaminantes como o benzo[a]pireno (BaP) no ambiente aquático.

2. O nanomaterial fulereno C_{60} pode induzir efeitos tóxicos em animais aquáticos mesmo na ausência de fotoexcitação das partículas.

Sendo assim, os objetivos foram:

1) Objetivo Geral

- Investigar as possíveis interações tóxicas entre o nanomaterial fulereno C_{60} e o hidrocarboneto BaP usando abordagens *in vitro* e *in vivo* em um modelo de animal aquático, o peixe *Danio rerio* (Cyprinidae, Teleostei).

2) Objetivos Específicos

- Revisar a literatura científica disponível acerca dos efeitos dos nanomateriais em organismos aquáticos, a fim de estabelecer um panorama do "estado-da-arte" da nanotoxicologia em ambientes aquáticos.

- Verificar os efeitos da co-exposição aos contaminantes acima mencionados sobre o estado redox de hepatócitos em cultura e larvas de *D. rerio* utilizando marcadores de estresse oxidativo.

- Verificar os efeitos do fulereno C_{60} sobre a bioacumulação de BaP em cultura de hepatócitos de *D. rerio.*

- Verificar o potencial de inibição enzimática do fulereno C_{60} em uma enzima chave na detoxificação do BaP, a glutationa-S-transferase pi (GST-π), utilizando ferramentas de modelagem computacional.

- Verificar os efeitos da co-exposição aos contaminantes acima mencionados em larvas

de *D. rerio* considerando a ocorrência de alterações histopatológicas em diferentes órgãos.

Parte I

Capítulo de Livro: Ecotoxicological Risks of Nanomaterials

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4. Ecotoxicological risks of nanomaterials

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Abstract. The state of the art in the toxicological effects of nanomaterials (NMs) is analyzed with a main focus in the responses of aquatic organisms. Several examples are included, examining the toxicity of both organic and inorganic nanoparticles particularly in oxidative stress responses. Data from the literature is included in order to compare nanomaterials and bulk material toxicity in daphnids. Effects of nanomaterials on immune responses are also discussed. Overall, literature reviewed indicates that some species are more sensitive to NMs, and the complex interaction that NMs establish with abiotic factors such as light (UV and visible) radiation.

1. General aspects of nanotoxicology

Because of their small size (at least one dimension in the range 1-100 nm), nanomaterials (NMs) have many physicochemical properties that differs of those that

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are not at this scale. Not all of these properties are beneficial, since some studies have been demonstrated that NMs have adverse effects on environmental and human health. Current research have showed that physicochemical properties of NMs such as size, shape, surface area, solubility, chemical composition, dispersion factor are very important to determine the biological response in live systems. In fact, several studies have demonstrated accumulation of NMs in several organs, but *in vitro*, data present clear limitations in extrapolating to the whole organism. However, such data present the benefit of accuracy by assessing the biological responses in highly controlled conditions. This kind of assay has been used to determine toxicology mechanisms AS degradability and formation of radical oxygen species (ROS). For this purpose, to study nanotoxicology *in vitro* is necessary to make some considerations, as relevant particle properties, handling and delivery [1]. Considering size, for example, once NMs of smaller size can enter the cells mitochondria through various pathways inducing oxidative stress and cell death. The surface area also needs to be taken into account since the relatively larger surface areas of NMs can induce greater production of ROS that can damage DNA [2]. Similarly, the exposure route is also important, once slightly or completely soluble NMs might release toxic or nontoxic ions that undergo chemical reactions to form ROS [3]. In addition to these physicochemical properties of NMs, the relevant exposure concentration and time, the cell line, assay toxicology, control experiments and positive/negative reference materials are extremely important to develop an *in vitro* approach [1]. Within a cell, these NMs can be metabolized and/or altered and of most important toxicological concern is that some NMs are transported across cell membranes, especially into mitochondria where the NMs induce mitochondrial perturbation, which include the initiation of apoptosis and decrease ATP production. These perturbations can lead to cytotoxicity and increase generation of ROS, depletion of the antioxidant glutathione (GSH) and reduction of mitochondrial membrane potential in rat liver cells line [4].

Toxicological effects of NMs in aquatic environments have been little studiedspecies. The study of Kahru and Dubourguier [5] summarized several toxicological data of several kinds of NMs in algae, ciliate daphnids and fish species. They found that algae and daphnids were the most sensitive species for synthetic NMs, with the exception of the organic NM fullerene, where the most sensitive species were ciliates. A related ecotoxicological issue is the study of the comparative toxicity of NMs and the bulk material, an essential issue if considered that up to date there is a lack of regulatory normative for NMs in the environment. Some authors presented data that indicate few differences in the toxicity of NMs and the respective bulk formulation, with

exception of CuO [5] and others have also observed that lung cells showed higher cytotoxicity when exposed to CuO nanoparticles than to $CuCl_2$ [6].

Some studies using the microcrustacea *Daphnia magna* as test organism showed that nanosized silver induced lower survival rate (43.3%) than bulk silver (86.7%) at the same concentration (0.1 mg/L) after 96 h exposure [7]. Other authors found in *D. magna* a higher toxicity of nanosized ZnO after 48 h exposure (LC_{50}: 3.2 ± 1.3 mg/L) than in its bulk form (LC_{50}: 8.8 ± 1.4 mg/L) [8]. Other toxicity data available for this species include LC_{50} (72 h) for nanosized TiO_2 (2.02 mg/L) [9] and LC_{50} (96 h) for the organic NM fullerene (~2.0 mg/L) [10]. Taking into account the toxicity classification proposed by U.S. Environmental Protection Agency for inter-chemical comparison, LC_{50} values entering in the range of > 1 to ≤ 10 mg/L are considered moderately toxic [11], as seems to be for the toxicological data cited above.

2. Toxic effects of nanomaterial on the immune system

It is important to consider that some of the interactions of NMs may result in other forms of damage beyond those described above. Immune reactivity and the formation of foreign bodies such as granulomas have been also reported [12]. Indeed, an important finding of immuno-modulatory effects of nanoparticles was first recorded in the late '90s by the group of researchers of Dr. Erlanger at Columbia University-USA. Their findings showed that the C_{60} has antigenic properties when functionalized with serum albumin or derivative of lysine, thereby acting as a hapten which can induce, in rats, antibodies after intraperitoneal injection [13]. Latter it was reported that monoclonal antibodies specific for C_{60} also recognized and specifically linked to carbon nanoparticles, and raised the question of lack of knowledge about potential immune responses when functionalized nanoparticles come into contact with the body acting as sensitizing agents [14].

The immune disorders that can be generated through NMs are still unknown. Although the reticuloendothelial system, which is composed of phagocytic cells in the liver, lymph nodes and spleen, can remove or isolate NMs, self-protein interactions with particles may change their antigenicity and initiate autoimmune responses. Nanoparticle-protein complexes also have more mobility and may facilitate the uptake of the antigen. This can lead to boost of primary and secondary immune responses by changing the function of antigen presentation to cells of the innate immune system, and also through its impact on the function of these cells leading to an exaggerated immune response against common environmental allergens [15]. Finally, it is possible that the immune system directly recognizes the nanomaterial, as exemplified

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3.2. Synthesis and characterization of carbon nanomaterials

For carbon nanotubes several methods posses some common features as, for example, the fact that the catalysts are usually transition metals like nickel, cobalt, iron and copper. Nanotubes syntheses also produce several by-off products, such as amorphous carbon, fullerenes and metal catalyst residues which can be at least in part responsible of some toxic effects [16]. A minimum set of characteristics/properties about NMs should include the elemental composition of the particles as well as surface morphology, degree of crystallinity and imaging by TEM (transmission electron microscopy). In ecotoxicological studies also it is needed measure key abiotic factors that may influence characterization of NMs such as media pH, Ca^{2+} concentration, the presence of natural organic matter and ionic strength [17]. With regard to the characterization of nanomaterials, among the techniques used besides TEM, it can be mentioned: thermo gravimetric analysis, Raman spectroscopy and scanning electron microscopy, among others. Raman spectroscopy, as reported by Jorio and coauthors [18], is a technique to characterize carbonaceous materials by identifying the types of chemical unions, providing information regarding thedisorder of the crystal lattice. Featuring micro-focus features investigations are getting accurate to identify the different crystalline and amorphous forms that make up the samples.

The technique of thermo gravimetric analysis (TGA) measures the quantity of the various materials present in the sample; the mass is loosed with the increase of temperature. This makes the proper technique to indirectly identify the concentrations of the structures in the sample because of each carbon structure burn at different temperatures. However, the temperature can be affected by the density, the surface area of the material, the presence of metals and the heating rate [19]. The transmission electron microscopy (TEM) was the first technique used for the characterization of nanomaterials. It uses an electron beam that when strikes the samples surface some types of signals can be generated. However, the main problem of this technique is the small sample size, usually of the order of 10^{-12} – 10^{-13}g, providing information specific to a small amount of the NM that is being characterized [17]. Other technique, the scanning electron microscopy (SEM), presents a great focus depth that allows the analysis of uneven surfaces with a huge magnification. Thus, the observed image is the projection of the thickness of the material, different to that observed on a surface.

3.3. Functionalization of carbon nanomaterials

Several processes, including encapsulation have been seen as a form of potential uses of NMs in biological and chemical applications. Sometimes, the

introduction of functional groups on the surface of NMs (functionalization) affords greater solubility and sometimes reduced toxicity [20]. As an example it can be cited the fullerenol, a derivative polyhydroxylated of fullerene that when functionalized with hydroxyl group is recognized as an efficient antioxidant showing hepatoprotective and nephroprotective effect in cell rats [21]. Thus, in studies involving aquatic ecotoxicity is need to take into account that many NMs, like unmodified fullerenes, have poor aqueous solubility, producing large aggregates of tens to hundreds of nanometers. The dispersion of NMs is achieved through the use of solvent, stirring or sonication to promote the formation of smaller clusters. In view of this, a major problem when faced with experiments of toxicity in aquatic organisms is that NMs are generally insoluble in water and capable of aggregation and/or agglomeration making it difficult to quantify the actual NM amount experienced by test organisms. These transformations are favored by increased ionic strength and calcium concentration, like those found in sea water, beyond turbulent waters and a variety of chemical found in this environment that may act as dispersants and/or stabilizers [22]. For all that, studies *in vitro* can demonstrate in a controlled manner how the NM behaves within the cells without interferences, thus enabling an interpretation of the initial responses that could happen in the whole organism.

3.4. Toxic effects of carbon nanomaterials: Nanotubes and fullerenes

Fullerene cytotoxicity seems to depend on the type of cells used and how C_{60} test suspensions are prepared [23]. In oysters, it was observed a rapid accumulation of C_{60} particles in digestive gland cells, where fullerene aggregates tended to be localized and concentrated into lysosomes [24], inducing a significant increase in lysosomal lipofuscin in a observed concentration-dependent effect. In other study it was clearly demonstrated the ability of different fullerene preparations, including nanocrystalline C60/70 (THF/nC60/70) and polyhydroxylated C60/70 [C60/70(OH)n], to modulate the cytotoxicity of the main pro-inflammatory cytokine TNF [25]. Following these authors, the mechanisms underlying the observed effects possibly involved the interaction of fullerene preparations with TNF-induced oxidative stress and subsequent mitochondrial depolarization in target cells.

It is important to state the conflicting results that exist in terms of the pro or antioxidant properties of fullerene. In aquatic organisms it has been reported in the fish *Carassius auratus* a lowering of lipid peroxidation levels in brain and gills after fullerene exposure although with a concomitant higher lipid damage in liver [26]. Other studies have also shown in rats an antioxidant behavior of fullerene after exposure *in vivo* in rats [27]. At least in part some of

the differences must be related to the photo-excitability of fullerene with UV and visible light. Fullerene photo-activation induces the generation of several ROS, including singlet oxygen and hydroxyl radicals, among others [28]. In brain carp, higher lipid peroxides content was reported after fullerene exposure under fluorescent light [29]. Other studies have reported lower mortality in embryo of zebrafish *Danio rerio* after exposure to fullerene in the dark. The same study showed lower embryo mortality if a co-exposure of fullerene with a GSH precursor (N-acetylcysteine) was performed, indicating that oxidative stress is involved in fullerene toxicity [30]. In bacteria isolated from the mucus secretion of the common carp *Cyprinus carpio*, it was observed higher ROS concentration after exposure to fullerene under light in some isolated bacteria, but not in other. A different responsiveness was also registered in terms of growth: some bacteria showed the growth inhibited after fullerene exposure whereas other did not presented this response [31]. In fact, polychaete species confronted to fullerene in the sediment without light showed absence of toxicity and augmented antioxidant response [32].

Another important point concerning fullerene toxicology refers to the methodologies employed for preparing the suspensions. Early ecotoxicological studies reported reduction of the antioxidant GSH in fish using fullerene prepared with an organic solvent, tetrahydrofuran (THF) [33], but later some concerns about THF toxicity were raised. Employing *Daphnia magna* as test organism, it was reported a clear higher toxicity of fullerene suspensions prepared with THF than in suspensions obtained after stirring fullerene in MilliQ water [34] and this methodology was subsequently employed in other ecotoxicological studies [35]. More recent studies have determined that the higher toxicity of fullerene-THF mixture was related to a THF metabolite, γ-butyrolactone, that presents toxicity and can be metabolized to the neurotransmitter gamma amino butyric acid (GABA). Zebrafish exposed to mixtures of fullerene-THF or water-THF presented augmented expression of antioxidant genes [36], a result that cautions to the pro-oxidant effects of fullerene if the assays were conducted using THF. More recently, studies employing a cell line and *Daphnia magna* reported that fullerene-THF suspensions are no toxic if side products are previously eliminated by additional washing steps, a methodological procedure that should aid for the evaluation of the intrinsic toxicological effects of fullerene [37].

Carbon nanotubes (CNTs) are known as oxidative stress inducers in cells. In general, mitochondrial damage seems to be a recurrent toxicity route for NMs, including CNTs [22, 38-41]. Such damages may appear with evidences of apoptosis and/or necrosis in many cell lineages: human skin fibroblasts, human T-limphocytes, rat macrophages and others. Apparently, the main apoptosis mechanism triggered by fullerene and CNTs involves activation of

caspases, probably by release of cytocrome c due to excessive mitochondrial ROS generation [25]. There are also evidences that CNTs may mimic or interfere with the cellular microtubule system and thereby disrupt the mitotic spindle apparatus, leading to aberrant cell division [42]. Inflammatory responses were also observed when human epidermal keratinocytes and human skin fibroblast was exposed to CNTs [43]. The mechanism is likely due to the production of reactive oxygen species, leading to the activation of the NF-jB. Engineered carbon nanotubes can activate the complement system, the biochemical cascade that removes pathogens, which consists of two pathways: the classical complement pathway is activated by antigen–antibody complexes, whereas the alternative pathway is antibody independent. Interestingly, both single-walled carbon nanotubes (SWNTs) and double-walled carbon nanotubes (DWNTs) stimulated the classical pathway [44], but only DWNTs triggered the alternative pathway. The mechanism of this selective complement activation remains unknown.

4. Toxicology of inorganic nanomaterials

4.1. Introduction

In the last times have being increasing the use of metal NMs such as titanium oxide (TiO_2), zinc oxide (ZnO), gold (Au) and others. These inorganic NMs have been several applications in medical and cosmetic industry [45]. Data to be summarized suggest that inorganic NMs can entry in several cells compartments and accumulate in organs and tissues affecting the reproduction, inducing reactive oxygen concentration (ROS) generation, altering antioxidant defense system and causing oxidative damages in different organisms. However, few data are available about NMs effects in aquatic organisms and the possible interaction of these NMs with other compounds that could synergize its toxicity.

4.2. Titanium dioxide nanoparticles

Nanoparticles of TiO_2 ($npTiO_2$) are usually considered non-toxic and has being used in many products and applications [46]. However, the possible effect of these nanomaterials in aquatic organisms is still limited. In the species *Arenicola marina* after $npTiO_2$ exposure was observed DNA damage [47]. In zebrafish (*Danio rerio*) was showed that $npTiO_2$ are accumulated in organs as liver, gills, heart and brain [48]. Besides information about distribution and accumulation to known biochemical effects are important to understand the toxicity of this nanomaterial.

Several biomarkers can be used to evaluate $npTiO_2$ effect, including antioxidant responses as superoxide dismutase (SOD) and catalase (CAT) activity, reduced glutathione levels (GSH), lipid peroxidation and others. In fact, in zebrafish it was observed after exposure to $npTiO_2$ an increase in SOD activity, GSH levels and lipid peroxidation in gut, while a decrease in the SOD and CAT activity in the liver. In the case of gills was observed an increase in hydroxyl radical ($HO^{\cdot}$) generation concomitant to oxidative damage in terms of lipid peroxidation [49]. These results suggest that $npTiO_2$ can induce toxicity in tissues of zebrafish. Besides, $npTiO_2$ can impair reproductive capacity, a clear environmental risk at the ecosystemic and population levels of organization [50].

In cells of goldfish (*Carassius auratus)* exposure to $npTiO_2$ and UVA induced an increases in $HO^{\cdot}$ generation and a consequent DNA damage, a result consistent with the photo-active nature of $npTiO_2$ [50]. $NpTiO_2$ seem mediate reactive oxygen species (ROS) production and to modulate the antioxidant system as seen, for example, in *Daphia magna* where $npTiO_2$ exposure augmented CAT, glutathione-S-transferase (GST) and glutathione peroxidase (GPx) activity [51]. So, in some aquatic species the $npTiO_2$ can induce oxidative stress generating a great problem to organisms. $NpTiO_2$ are also suggested to strongly diminish the lisossomal membrane stabilization in digestive gland of *Mytilus galloprovincialis* and significant increase in lysosomal lipofuscin, the end-products of lipid peroxidation [22]. Furthermore, the cytotoxic properties of $npTiO_2$ appear to correlate with their phase composition, were anatase titanium dioxide was 100 times more toxic than an equivalent sample of rutile titanium dioxide, and the generation of reactive oxygen species under UV illumination correlated well with the observed biological responses [52]. Additionally, the crystal structure of titanium dioxide also dictates the mode of cell death: anatase $npTiO_2$, regardless of size, were reported to induce necrosis, whereas rutile $npTiO_2$ triggered apoptosis through the formation of reactive oxygen species [52].

4.3. Gold nanoparticles

Gold in its bulk is considered a noble metal, non-toxic with applications in therapeutic and medical areas, so it was believed that gold nanoparticules (npAu) will be non-toxic. NpAu are also used in biosensors where they markedly enhance sensitivity and specificity of detection because of their unique physical, chemical, mechanical, magnetic and optical properties [53]. However, npAu has shown to induce oxidative damage in lung fibroblast cells of mammals [54]. In the blue mussel *Mytilus edulis* it was observed a clear difference in the distribution of npAu among organs, where digestive gland

showed to accumulate more npAu than gills and mantle (62.05; 0.52 and 0.02 µg/g tissue, respectively) [55]. In the same study was observed that npAu diminished the reduced/oxidized glutathione ratio in the digestive gland indicating that more accumulation can interfere in the redox state in terms of GSH. NpAu seem to have effects on hepatocytes of the fish *Oncorhynchus mykis* and showed to increase significantly the production of ROS [56]. The degree of recognition and internalization of nanomaterials by macrophages likely influences their biodistribution. There was reported that gold nanoparticles (40 nm) injected into mice were taken up primarily by macrophages resident in the liver and secondarily by macrophages in other organs [57].

4.4. Silver nanoparticles

The largest number of applications of engineered inorganic nanomaterials involves silver nanoparticules (npAg) in products ranging from textiles, air and water filters, food packaging, medical device and wall paintings [58]. The cause of their multiple applications and uses lays in its remarkable biocides properties [59]. AgNPs have a broad antimicrobial spectrum and have also been reported to be very effective against viruses, fungi and algae [60]. It has been shown that npAg are incorporated into the cell wall of pathogenic bacteria, resulting in membrane disruption and cell death. The antimicrobial mechanism of npAg is related to cell wall damage due to the presence of reactive oxygen species (ROS) originated on the surface of nanoparticles, and the release of silver toxic ions that interact with cellular components [61]. Once inside the cell, npAg can block DNA transcription, disrupt bacterial respiration and ATP production, and react with proteins -SH groups, which can lead to an inactivation of functional enzymes [62]. *In vitro* studies carried out in human and rat liver cells suggests that npAg increases ROS production and decreases reduced glutathione (GSH) levels by inhibition of enzymes that synthesize them, causing damage in the DNA, lipid peroxidation (LPO), protein oxidation, and cell apoptosis [63]. At the molecular level, Chae et al. [64] observed changes in the expression of genes related to stress biomarkers (cytochromes, metallothionein, glutathione-S-transferase) in liver of the medaka fish. Finally, effects such as mortality, retarded growth, morphological abnormalities, circulatory disturbances, neurotoxicity, oxidative stress and behavior changes have been observed in recent studies carried out on fish embryos exposed to npAg [65].

4.5. Selenium nanoparticles

Selenium (Se) is an essential micronutrient to most organisms playing important role in antioxidant defenses once this element is indispensable to

many enzymes activity as glutathione peroxidase. However, Se nanoparticules (npSe) can be more toxic in medaka fish (*Oryzias latipes*) in comparison with sodium selenite, in fact, npSe showed major accumulation capacity in the liver than gills and muscle. This hyper-accumulation can induced ROS generation indicated by increase in GSH levels and decrease in SOD activity [66].

5. Concluding remarks

The chapter summarized the toxicological data available for aquatic organisms. Several points must be considered in order to clearly evaluate the potential environmental risks of NMs, including the methodology to prepare NMs suspensions, the size of the assayed nanoparticles, the presence of abiotic factors like light irradiation, among others. Although the toxic database of NMs for aquatic organisms is still limited some reviews [5] have stressed that sensitivity of some organisms like algae and daphnids to NMs. More studies and technologies are needed in the near future to analyze NMs presence in the environmental in order to better evaluate its potential risks.

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Parte II

Artigo: Co-exposure of the organic nanomaterial fullerene C_{60} with benzo[a]pyrene in *Danio rerio* (zebrafish) hepatocytes: Evidence of toxicological interactions

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Co-exposure of the organic nanomaterial fullerene C_{60} with benzo[a]pyrene in *Danio rerio* (zebrafish) hepatocytes: Evidence of toxicological interactions

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ABSTRACT

Compounds from the nanotechnology industry, such as carbon-based nanomaterials, are strong candidates to contaminate aquatic environments because their production and disposal have exponentially grown in a few years. Previous evidence shows that fullerene C_{60}, a carbon nanomaterial, can facilitate the intake of metals or PAHs both *in vivo* and *in vitro*, potentially amplifying the deleterious effects of these toxicants in organisms. The present work aimed to investigate the effects of fullerene C_{60} in a *Danio rerio* (zebrafish) hepatocyte cell lineage exposed to benzo[a]pyrene (BaP) in terms of cell viability, oxidative stress parameters and BaP intracellular accumulation. Additionally, a computational docking was performed to investigate the interaction of the fullerene C_{60} molecule with the detoxificatory and antioxidant enzyme πGST. Fullerene C_{60} provoked a significant ($p < 0.05$) loss in cellular viability when co-exposed with BaP at 0.01, 0.1 and 1.0 μg/L, and induced an increase ($p < 0.05$) in BaP accumulation in the cells after 3 and 4 h of exposure. The levels of reactive oxygen species (ROS) in the cells exposed to BaP were diminished ($p < 0.05$) by the fullerene addition, and the increase of the GST activity observed in the BaP-only treated cells was reduced to the basal levels by co-exposure to fullerene. However, despite the potential of the fullerene molecule to inhibit π GST activity, demonstrated by the computational docking, the nanomaterial did not significantly ($p > 0.05$) alter the enzyme activity when added to GST purified extracts from the zebrafish hepatocyte cells. These results show that fullerene C_{60} can increase the intake of BaP into the cells, decreasing cell viability and impairing the detoxificatory response by phase II enzymes, such as GST, and this latter effect should be occurring at the transcriptional level.

1. Introduction

The fate of products and effluents from the nanotechnology industry has been a growing matter of concern because their production and disposal have exponentially risen in the last few years (Kahru and Dubourguier, 2010). The current data about the actual risks to humans and to the environment are not conclusive, and this is mainly due to the lack of information concerning their mechanisms of toxicity, actual concentrations and chemical behavior in the environment (Christian et al., 2008; Aschberger et al., 2011). However, the novel chemical and physical properties arising from the nanoscale greatly enhance the reactivity of the nanoparticles with biomolecules, making the nanomaterials potentially toxic and capable of harming the environment (Kahru and Dubourguier, 2010). On the other hand, it must also be considered that some works show low toxicity levels of carbon nanomaterials (such as

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fullerenes) in fish, at least with respect to oxidative stress parameters (Fraser et al., 2011; Henry et al., 2011).

Despite the debate concerning the actual toxicity level of the nanomaterials, especially in the aquatic environment, there is a consensus that nanomaterials may potentially affect biological systems not only *per se*, but also through interaction with other compounds (Christian et al., 2008; Henry et al., 2011). Considering their high reactivity, a question arises about what can happen when nanomaterials are in the presence of other toxic molecules. One of the first attempts to investigate this issue was conducted by Limbach and Wick (2007), who measured the oxidative stress in human lung epithelial cells induced by nano-silica doped with a number of metals. This study found higher damage in the treatments with cobalt- and manganese-doped silica nanoparticles than in metals or silica alone. Because nano-silica facilitated the uptake of the metals by the cells, this mechanism was so-called the "Trojan horse" effect. This type of delivery mechanism displayed by nanomaterials has been investigated in a few additional nanotoxicological studies, mainly with metallic nanoparticles. For example, Fan et al. (2011) showed that nano-TiO_2 enhanced copper bioaccumulation and toxicity in the crustacean *Daphnia magna*, even at low nanomaterial concentrations. It was also found that nano-TiO_2 enhanced arsenate toxicity in *Ceriodaphnia dubia* (Wang et al., 2011) and, when doped with the lanthanide Ce(IV), it caused deformation in the cell morphology of a human hepatocyte cell line (Mao et al., 2010).

Studies investigating co-exposure with carbon-based nanocompounds, such as nanotubes and fullerenes, are less common. Fullerene C_{60} is a worldwide produced nanomaterial with a unique cage-like molecular structure made solely of carbon. Although highly hydrophobic, due to its electronic configuration it can form strong C_{60}—H_2O bonds when in colloidal water suspensions (Andrievsky et al., 2002; Khokhryakov et al., 2006), resulting in stable nano-aggregates that can promote deleterious effects in biological systems (Murdock et al., 2008; Ehrenberg et al., 2009).

C_{60} has been widely investigated in terms of the chemical and physical interactions with a range of molecules and devices looking for applications as nano-probes, nano-sensors and nano-electrodes (Nakashima et al., 1998; Cho et al., 2005; Goyal et al., 2005) and in medicine (Partha et al., 2008; Pinteala et al., 2009; Ganji et al., 2010; Tarabukina et al., 2010; Adini et al., 2011; Santos et al., 2011). Despite being poorly studied, the uptake rate and toxicity of other environmental contaminants seem to be somehow affected when co-exposed to fullerene. Baun et al. (2008) indicated that co-exposure with fullerene C_{60} enhanced the toxicity of phenanthrene to the microcrustacean *Daphnia magna* and to the algae *Pseudokirchneriella subcapitata*. This was due, at least in part, to the high adsorption of phenanthrene molecules onto C_{60} nano-aggregates, which facilitated phenanthrene uptake. Similarly, Costa et al. (2012) observed that arsenic (As^{III}) uptake was higher in zebrafish hepatocytes co-exposed to fullerene (1 mg/L).

Among the polycyclic aromatic hydrocarbons (PAHs), benzo[a]pyrene (BaP) is one of the most important due to its ubiquitous presence in most environments. It is produced mainly during the incomplete combustion of organic matter and in cigarette smoke (Rose and Levi, 2004). It is also a carcinogen and mutagen toxicant and reactive oxygen species (ROS) generator (Sasco et al., 2004; Naspinski et al., 2008). Its detoxification process includes metabolization by phase I enzymes that can produce electrophilic epoxides that can readily bind to DNA (Walker et al., 2001). BaP contamination can be harmful through the generation of oxidative stress (Palanikumar et al., 2012), the inhibition of retinoid synthesis (Alsop et al., 2007) and the formation of DNA adducts (Kurelec et al., 1991). The exposure of cultured cells to BaP can also cause changes in gene expression (Castorena-Torres et al., 2008), oxidative impairment (Winzer et al., 2001) and an increase of the carcinogenic risk by interaction with 17β-estradiol (Chang et al., 2007), among many other deleterious effects.

In order to investigate the influence of fullerene C_{60} upon the toxicity of an important environmental contaminant, such as BaP, the present work aimed to assess the oxidative stress parameters, cell viability and bioaccumulation of BaP in ZF-L cells, an established culture of hepatocytes from the zebrafish *Danio rerio* (Cyprinidae). This cell lineage was chosen because *Danio rerio* is a highly suitable biological model widely used in toxicology, including in studies with nanomaterials (Fako and Furgeson, 2009; Costa et al., 2012). Additionally, an *in silico* study was performed by computational docking to verify the hypothesis of the interaction of the fullerene C_{60} molecule with the antioxidant and phase II detoxificatory enzyme glutathione-S-transferase (GST).

2. Materials and methods

2.1. Preparation of the chemicals

2.1.1. Preparation and characterization of C_{60} suspension

In order to produce a homogeneous suspension of C_{60} nanoparticles, 200 mg of fullerene C_{60} in powder form (99% purity, SES Research, USA) was added to 1 l of ultra-pure Milli-Q water and stirred for two months under artificial light. After this period, the suspension was centrifuged at 25,000 × *g* and 15 °C for 1 h to remove the bigger aggregates and was then sequentially filtered by 0.45 and 0.20 μm nylon membranes. This methodology was based on the work of Lyon et al. (2006) where no organic solvent was employed because these solvents can release residual degradation products that affect the toxicity of the nanomaterial (Henry et al., 2007). The concentration of the suspension was determined by measurement of the total organic carbon content in a total organic carbon analyzer (TOC-V CPH, Shimadzu Corp., Japan). The characterization of the C_{60} suspension was performed by transmission electron microscopy (TEM) in a JEOL JSM 1200 EX II transmission electron microscope operating at 100 kV. For the TEM, aliquots of the C_{60} suspension (10 μl) were disposed onto 300 mesh TEM grids (SPI) that were coated with Formvar. The analysis was performed after 24 h to allow sample evaporation, according to previous studies (Britto et al., 2012; Costa et al., 2012; Ferreira et al., 2012). As previously reported for C_{60} suspensions prepared using the water-stirring method without the addition of organic solvents (Lyon et al., 2006; Britto et al., 2012; Costa et al., 2012; Ferreira et al., 2012), the ubiquitous presence of fullerene nano-aggregates in the nanometer range were seen in the C_{60} suspension analyzed by TEM (Fig. 1).

2.1.2. Preparation of BaP solutions

BaP solutions ranging from 0.01 to 10.00 μg/mL were obtained by dissolving benzo[a]pyrene (Fluka, purity ≥ 96%) in dimethyl sulfoxide (DMSO) (Synth, Brazil). The final concentration of DMSO in contact with the cells was 1% since Filgueira et al. (2007) showed that this DMSO concentration was not deleterious for an erythroleukemic cell line. In addition, the DMSO control group showed no effects in the analyzed variables (see Section 3).

2.2. Maintenance of the hepatocytes

Zebrafish hepatocytes (ZF-L lineage) purchased from the American Type Culture Collection (ATTC) were maintained in culture flasks with 10 mL of RPMI 1640 (Gibco) medium supplemented with 10% fetal bovine serum and a 1% antibiotic/antimycotic cocktail (streptomycin, amphotericin and penicillin) at 28 °C. For the exposure assays, cells were initially removed from the flasks with 0.125% trypsin, washed with phosphate buffered saline (PBS) and transferred to 24-well culture plates (0.5 mL per well, 10^6 cells/mL)

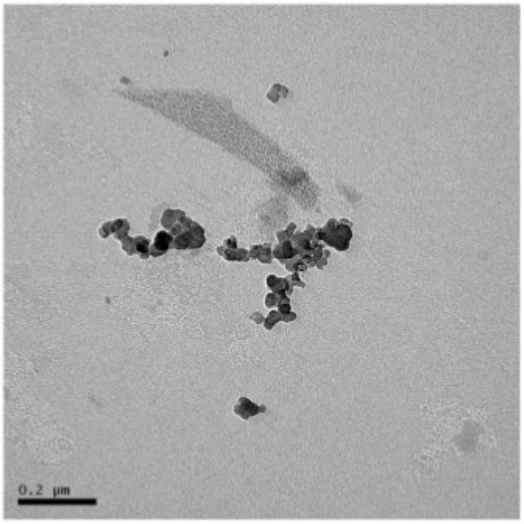

Fig. 1. Transmission electron microscopy (TEM) image of fullerene C_{60} from the suspension obtained by the solvent-free method.

to settle down and adhere. After 24 h, the cells were carefully washed with PBS and exposed to the treatments.

2.3. Experimental design and procedure

All exposures were performed with at least 10^6 cells/mL in a final volume of 400 µl per well (toxicants or vehicles plus RPMI medium), with four wells per treatment at 28 °C over 4 h. After this period, the cells were washed with PBS to remove the toxicants, and the estimation of the number of cells was performed, as well as the cell viability assay (see next section). Initially, some assays were conducted with a range of concentrations of both C_{60} (0.1, 1.0 and 10.0 mg/L) or BaP alone (from 0.01 to 10.0 µg/L) in order to determine the optimal concentrations for which the cell viability was not altered. Because none of the fullerene concentrations altered the cell viability (see Section 3) and considering previous exposure studies (Costa et al., 2012), the concentration of 1.00 mg/L of C_{60} was chosen for the further co-exposures with BaP. BaP concentrations of 0.01, 0.10 and 1.0 µg/L were chosen for the subsequent exposures because they did not impair hepatocyte viability. Control groups included a Milli Q water control (the solvent of the fullerene suspensions) and a DMSO control (BaP solvent) at a final concentration of 1%.

2.4. Estimation of number of cells and viability assays

Four control wells (800 µl of cell suspension) from the 24-well plate were pooled and diluted with RPMI medium to obtain aliquots of 100%, 75%, 50% and 25% of the original cell suspension. After that, the cells were counted in an optical light microscope, and 200 µl of the dilutions were read in duplicate at 630 nm with an ELISA microplate reader (Biotek Elx 800). The absorbance values were then fitted to the respective number of cells previously counted in the microscope, and a standard curve was made to estimate the number of cells of the treatments after reading at 630 nm (Costa et al., 2012).

The technique of intracellular reduction of 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide (MTT) to formazan by mitochondrial dehydrogenase activity was employed for the cell viability measurement. Aliquots of 20 µl of cell suspensions were added in 96-well plates and incubated for 30 min in the dark at 28 °C with 20 µl of a 12 mM MTT solution. Following the incubation, the plate was centrifuged for 7 min at 1100 rpm, the supernatant was discarded and 200 µl of DMSO was added to dissolve the blue formazan crystals. Finally, the samples were read at 490 nm in an ELISA microplate reader. The absorbance values were considered as a measure of dehydrogenase functionality and, therefore, an indirect cell viability parameter (Costa et al., 2012).

2.5. Determination of the ROS concentration

Following the exposure, the hepatocytes were centrifuged at 600 × g for 5 min at 10 °C, the supernatant was discarded and the cells were re-suspended in a solution with 40 µM of the fluorescent probe 2,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Invitrogen) in PBS. Immediately, the cell suspension was transferred to a white 96-well microplate (160 µl per well in triplicate) and read in a microplate reader fluorimeter (Victor 2, Perkin Elmer) at wavelengths of 485 and 520 nm for the excitation and emission, respectively. The ROS concentration was expressed in terms of fluorescence area resulting from the integration of the fluorescence values between 0 and 70 min after fitting to a second order polynomial. The ROS area was fitted to the estimated cells number in each treatment (Costa et al., 2012).

2.6. Glutathione-S-transferase (GST) activity assay

The activity of the phase II enzyme GST was determined through the monitoring of a conjugate formed by 1 mM of reduced glutathione (GSH) and 1 mM of 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma) in the presence of 100 µl of cell extract in PBS at 340 nm (Habig and Jakoby, 1981). The results were expressed as nanomoles of GSH-CDNB conjugate/min/mg protein at 25 °C and pH 7.40. The total protein content was assessed through a commercial kit (Doles, Brazil) based on the pirogalol method.

2.7. Quantification of the BaP concentration in the BaP working solutions

The PAH analyses were conducted using a gas chromatograph coupled with a mass spectrometer (Perkin Elmer® Clarus 500-GC-MS) and equipped with an Elite-5MS silica capillary column (Perkin Elmer® 5% phenyl-95% methylpolysiloxane; 30 m × 0.25 mm, 0.25 µm film thickness). The injector was kept at 280 °C in splitless mode. The temperature program started at 40 °C, increased at a rate of 10 °C min^{-1} to 60 °C, then increased at 5 °C min^{-1} to 290 °C, was maintained at 290 °C for 5 min and then increased at 10 °C min^{-1} to 300 °C and was held constant for 10 min. Helium was used as the carrier gas (1.5 mL min^{-1}). The MS operating conditions were: interface at 290 °C, ion source at 200 °C and electron energy of 70 eV. The data were acquired under selected ion monitoring (SIM) mode. Compound identification was based on the individual mass spectra and the GC retention time in comparison to literature, library data, and authentic standards. Standards were injected and analyzed under the same conditions as the samples. The limit of detection (LOD) of BaP was in the range of 1.75 ng mL^{-1}, and the limit of quantification (LOQ) was 5 ng mL^{-1}. The procedure was checked for recovery efficiencies by analyzing uncontaminated samples spiked with BaP standards. The average recoveries ($n = 5$) ranged from 88% to 101%. PAH surrogate standards (p-therphenyl-d14) were added to all samples to monitor the procedures of sample extraction, recovery and analysis. The average recoveries of the surrogate standards added samples varied from 91% to 117%. One laboratory blank and one duplicate were run with every 10 samples. The coefficient of variation of the BaP concentrations in the

duplicates was less than 15%. Still, to evaluate the precision of the analysis, two replicates of the samples were analyzed. The relative standard deviation (RSD) of the replicates varied between 2 and 5%. Regular analyses of the reference material from the International Atomic Energy Agency Analytical Quality Control Services (Organic Contaminants in Marine Sediment-IAEA-417) and semi-annual participation in the intercomparison exercises promoted by the Canadian Association for Laboratory Accreditation (CALA) have shown satisfactory quality control. The measured concentrations confirmed that the nominal concentration (1000 ng mL^{-1}) was within 1018 ± 30.0 ng mL^{-1}.

2.8. Estimation of BaP intracellular accumulation

The BaP (or its metabolites) intracellular accumulation (1.0 μg/L) over time (1, 2, 3 and 4 h of incubation) with and without co-exposure to C_{60} (1.0 mg/L) was assessed following the protocol described by Filgueira et al. (2007). The readings were performed after washing the cells with PBS, and aliquots of 160 μL were put in a white 96-well plate to read in a fluorimeter at the wavelengths of 340 and 450 nm for excitation and emission, respectively.

2.9. In silico assay of the interaction of fullerene C_{60} molecule with π GST

Due to the results obtained in the GST activity assay (see Section 3), a mathematical simulation (computational docking) of the interaction between the molecules of C_{60} and GST was performed to investigate the potential affinity of the fullerene C_{60} for GST enzyme, which could interfere with the enzymatic activity. For this simulation, the class pi mitochondrial GST (π GST) was chosen as the model for the C_{60} docking. This isoform was selected due to the high number of mitochondria present in hepatocytes, the availability of computational data from a mouse liver π GST, which possess a good analogy with the zebrafish π GST, and the recent evidence of the role of π GST in BaP detoxification in zebrafish (Garner and Di Giulio, 2012). The docking simulations of the fullerene with mouse liver π GST complexed with S-(P-nitrobenzyl) glutathione (PDB code 1GLQ) were performed using AutoDock Vina 1.1.1 [1] followed by redocking with AutoDock 4.0.1. Before the simulations, the inhibitor S-(P-nitrobenzyl) glutathione was removed from the structure, and the enzyme was geometrically optimized using the Universal Force Field (UFF) implemented in the Avogadro 0.9 software. The fullerene molecule was constructed in Avogadro, and its geometry was optimized using UFF. The enzyme was kept in its catalytic (dimeric) form. AutoDock Tools was used to create the inputs in the .pdbqt format for the simulations in AutoDock Vina. A second docking was made using AutoDock to confirm the data obtained by AutoDock Vina. The entire system was considered for the simulations. The grid box was centralized at the coordinates $x = 63.504$, $y = 18.195$ and $z = 5.743$, with dimensions of 60, 60 and 60 Å using a spacing of 1 Å and the exhaustiveness set to 50. All other parameters were used as defaults. The conformation with the lowest binding free energy was accepted as the best affinity model. The conformations and interactions were analyzed using the software Accelrys Discovery Studio Visualizer 2.5 and PyMOL. A redocking was conducted using the S-(P-nitrobenzyl) glutathione to validate the method. In this case, the molecule was successfully positioned at a similar position to the crystallographic conformation, with an RMSD less than 1.

2.10. Verification of the effect of C_{60} on the activity of GST in purified extracts

Based on the results from the docking assay, and in order to investigate whether the modulation of GST activity observed in the treatments BaP + C_{60} was induced by the direct interaction of the nanomaterial with the enzyme (see Section 3), an *in vitro* assay was run in which the GST activity was measured in GST purified extracts previously exposed to C_{60}. The purified extracts of GST from ZF-L cells were obtained through a commercial kit (MagneGST®, Promega), and the procedure was followed according to manufacturer's instructions. The method is based on the binding of glutathione-conjugated magnetic particles with GST enzymes present in the samples, which allows for the separation of these enzymes from the rest of the cellular extract. Once the purified extracts were obtained, an exposure assay was performed in which the GST extracts were mixed with 10 mg/L fullerene C_{60} over 4 h at 28 °C in the absence of light. After the exposure, a GST activity assay was performed identically to the method described in Section 2.6.

2.11. Statistical analysis

Data from all assays were analyzed by means of ANOVA (Zar, 1984) after the verification of normality and homogeneity of variances; if even one of the assumptions was violated, mathematical transformations were applied. *Post hoc* comparisons among the treatments were performed through the Newmann–Keuls method, and a significance level of 0.05 was adopted for all steps of the analysis.

3. Results

Because the cell viability was not significantly ($p > 0.05$) reduced by any of the three tested C_{60} aggregates (Fig. 2a), and based on previous evidence of oxidative balance disturbance in fish, both *in vivo* (Oberdörster, 2004) and in ZF-L cultured cells (Costa et al., 2012), a concentration of 1.0 mg/L was adopted for the subsequent co-exposures with BaP. BaP, however, was capable of reducing cell viability ($p < 0.05$) at 10.0 μg/L (Fig. 2b), thus the concentrations of 0.01, 0.1 and 1.0 μg/L were chosen for co-exposure to C_{60}. At those BaP concentrations, fullerene C_{60} significantly ($p < 0.05$) lowered the cell viability during co-exposure experiments (Fig. 3).

The exposure to 1.00 μg/L of BaP resulted in an augmented intracellular accumulation of BaP (or its metabolites) in ZF-L cells only when co-exposed to fullerene C_{60} (Fig. 4a). The longer the incubation time was, the higher the accumulation values ($p < 0.05$). Fig. 4b shows the fluorescence units in the blank samples (without cells), demonstrating that C_{60} did not interfere ($p > 0.05$) with the readings at the wavelengths used for the BaP accumulation measurements.

Fig. 5 shows the levels of intracellular ROS of the exposed ZF-L cells. The BaP-only treatments did not significantly ($p > 0.05$) increase the ROS generation when compared to the respective controls. On the contrary, the co-exposure with C_{60} decreased ($p < 0.05$) the basal ROS level.

The activity of the phase II enzyme GST increased ($p < 0.05$) after exposure to 0.10 and 1.00 μg/l of BaP (Fig. 6). However, the co-exposure to C_{60} reversed the GST activity to its basal levels despite the presence of BaP.

Fig. 7 shows a 3D representation from the docking simulation of the C_{60} in the π GST molecule. The results showed that the fullerene C_{60}, in its more stable conformation (Gibbs free energy: −11.5 kcal/mol), was situated at a region of the enzyme postulated as the binding site of HEPES, near the C-terminal region between the elements β2 and α1. This region, due to the presence of the amino acids Arg18, Ala22, Trp28 and Phe192, produces a hydrophobic surface that favors fullerene binding stabilization through Van der Waals forces (Fig. 7b). Moreover, the data revealed that fullerene acts *via* three cation-π type interactions with the residual Lys188, and such interactions seem to be the main force contributing to the affinity of the nanomaterial with the HEPES binding site of π GST.

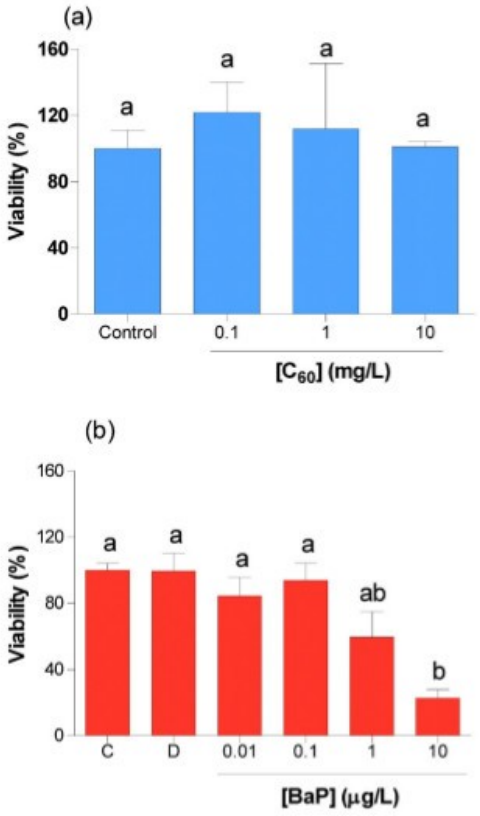

Fig. 2. Cell viability measurement after 4 h of exposure employing the method of reduction of MTT by mitochondrial dehydrogenases. C: Milli Q water control. D: dimethyl sulfoxide (DMSO) control. (a) Percentage of viable cells exposed to fullerene C_{60} (0.1, 1.0 or 10.0 mg/L). (b) Percentage of viable cells exposed to BaP (0.01, 0.1, 1.0, or 10.0 µg/L). $N = 4–16$ independent experiments.

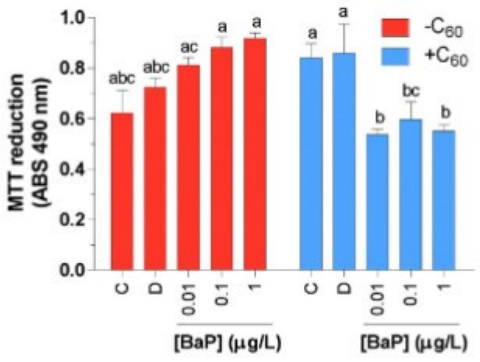

Fig. 3. Absorbance values of MTT reduction in cells treated with BaP (0.01, 0.10 or 1.0 µg/L) with or without fullerene C_{60} (1.0 mg/L). C: Milli Q water control. D: dimethyl sulfoxide (DMSO). Same letters indicate the absence of statistically significant ($p > 0.05$) differences. $N = 4–8$ independent experiments.

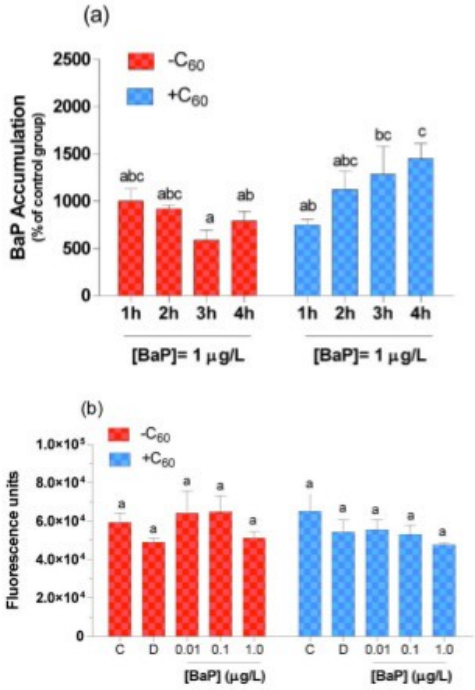

Fig. 4. Intracellular accumulation of BaP in ZF-L cells exposed to BaP with or without fullerene C_{60} (1.0 mg/L). C: Milli Q water control. D: dimethyl sulfoxide (DMSO) control. (a) Accumulation kinetics of BaP (1.00 µg/L) throughout 4 h of exposure; data are expressed as percentages of the control group. (b) Fluorescence units from the readings in samples without cells after 4 h of incubation to BaP (0.01, 0.10 or 1.00 µg/L). Same letters indicate the absence of statistically significant ($p > 0.05$) differences. $N = 3–4$ independent experiments.

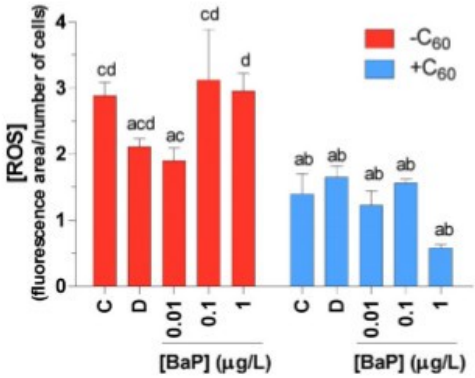

Fig. 5. Reactive oxygen species (ROS) concentration after 4 h of exposure to BaP (0.01, 0.10 or 1.00 µg/L) with or without fullerene C_{60} (1.0 mg/L). C: Milli Q water control. D: dimethyl sulfoxide (DMSO) control. Data are expressed as relative fluorescence area adjusted to the number of viable cells of each treatment. Same letters indicate the absence of statistically significant ($p > 0.05$) differences. $N = 3–4$ independent experiments.

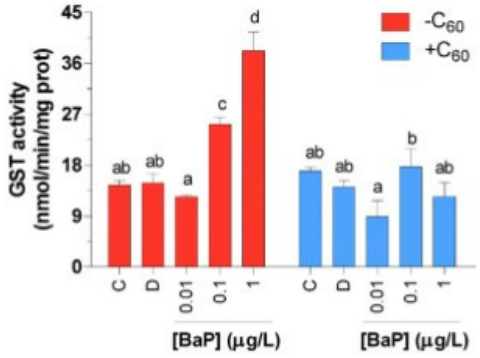

Fig. 6. Specific activity of glutathione-S-transferase (GST) in ZF-L cells exposed for 4 h to BaP (0.01, 0.10 or 1.00 µg/L) with or without fullerene C_{60} (1.0 mg/L). C: Milli Q water control. D: dimethyl sulfoxide (DMSO) control. Same letters indicate the absence of statistically significant ($p > 0.05$) differences. $N = 3–8$ independent experiments.

The exposure of the ZF-L purified extracts to 10 mg/L of C_{60} for 4 h had no effect on the GST activity ($p > 0.05$). The Control groups produced 12.95 ± 4.38 nanomoles of GSH-CDNB conjugate/min/mg protein, whereas the C_{60} groups produced 14.13 ± 4.22 nanomoles of GSH-CDNB conjugate/min/mg protein.

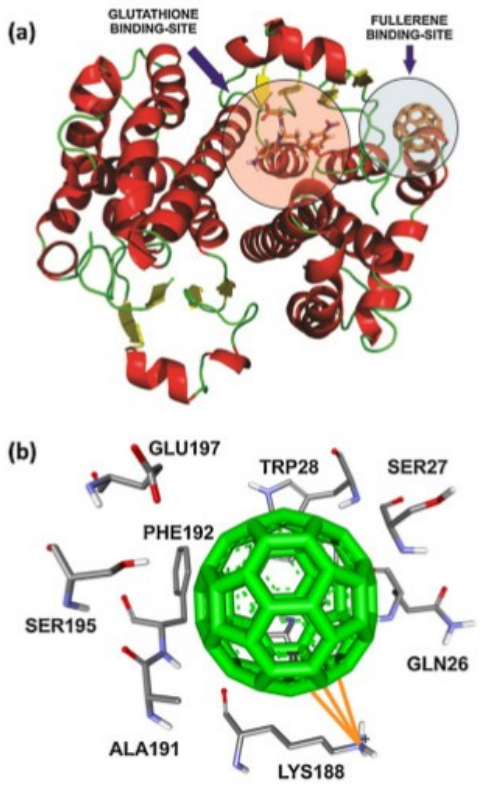

Fig. 7. (a) Scheme of pi glutathione-S-transferase (GST) isoform, showing the binding site of glutathione and the HEPES allosteric site where fullerene C_{60} showed the highest affinity. (b) Amino acid residues close to fullerene C_{60}. The model shows the interaction with lysine residue 188.

4. Discussion

Fullerene toxicity is a controversial issue. Kahru and Dubourguier (2010) compiled fullerene toxicological data for fourteen organisms and classified this nanomaterial as very toxic, taking into account the lowest median $L(E)C_{50}$ values for all test organisms. However, some studies indicate the absence of fullerene toxicity (i.e., Xia et al., 2010), whereas others considered that ROS generation by aqueous fullerene suspension is minimal (i.e., Henry et al., 2011). Recently, Trpkovic et al. (2012) stated that fullerene toxicity can be elicited by ROS-dependent (when photo-excited) and ROS-independent mechanisms, where the latter is considered to be through cell membrane damage and/or induction of autophagy. An ROS-independent pathway should be considered responsible for the cytotoxicity observed in the present study because fullerene and BaP exposures were performed in incubators in the dark at 28 °C.

Yang et al. (2010) raised the possibility of aqueous fullerene suspensions acting similarly to dissolved organic matter (DOM), changing the bioavailability of toxic molecules (such as PAH). This concept was related to the 'Trojan horse' paradigm first postulated by Limbach and Wick (2007). In addition, Henry et al. (2011) highlighted the potential environmental risk of fullerene due to its capacity to act as a carrier for other contaminants. However, the "Trojan horse' concept needs to be better studied. The original paper of Limbach and Wick (2007) compared the levels of intracellular ROS between silica nanoparticles containing metals and the corresponding oxides. Other authors, such as Baun et al. (2008), considered the "Trojan horse' effect under the view of the augmented accumulation of a toxic molecule (as phenanthrene) when co-exposed with a nanomaterial, such as fullerene, and the toxicological consequences of this co-exposure. The same concept was considered by Sun et al. (2009), in terms of arsenic accumulation in carp gills after co-exposure with titanium dioxide nanoparticles, and by Costa et al. (2012) studying arsenic accumulation in zebrafish hepatocytes after co-exposure to fullerene. Following the postulation of Baun et al. (2008), the present work demonstrated the deleterious effects and higher accumulation of BaP (or its metabolites) when co-exposed with fullerene C_{60} and the consequences in terms of cytotoxicity, intracellular ROS and detoxification capacity.

The effects of mixtures of pollutants in the environment are usually hard to predict due to many factors. This task is even more difficult when nanomaterials are under study in virtue of their inherent properties, which can amplify or alleviate the toxic effects of other compounds. To the best of our knowledge, information about the influence of the physical–chemical characteristics of toxic molecules on nanomaterial interactions is currently lacking. Fullerene C_{60} has induced loss in cell viability when co-exposed with BaP, which did not occur with cells treated with BaP only (Fig. 3). This result is probably due to the increase of the BaP intracellular accumulation caused by fullerene C_{60} (Fig. 6). Once a higher BaP concentration is inside the cells, the increasing damage may lead to the observed loss in the mitochondrial dehydrogenase functionality, as measured by the MTT assay. This finding is in accordance with the work of Baun et al. (2008), as mentioned above. Al-Subiai et al. (2012) registered higher genotoxicity in mussel haemocytes when fluoroanthene and fullerene were co-exposed. However, this is not always true. Yang et al. (2010) reported lower histological damage induced by fluoroanthene when co-exposed with fullerene under UV radiation, and Baun et al. (2008) observed that fullerene did not influence the toxicity of atrazine and methyl parathion to the algae *P. subcapitata* and the crustacean *D. magna*.

The presence of fullerene C_{60} reduced the intracellular concentration of ROS (Fig. 4), resulting in an antioxidant effect. This

may be due to the low number of viable cells in BaP + C_{60} treatments or to the ability to react with radicals, which is attributed to the C_{60} molecule (Andrievsky et al., 2009; Xia et al., 2010). This property is postulated as a non-stoichiometric reaction, in which a self-neutralization could occur when the molecule is in a hydrated state, and it could give the observed scavenging characteristics to the nanomaterial (Andrievsky et al., 2009). Previous studies from our group employing cell suspension from carp *Cyprinus carpio* brains registered a reduction of intracellular ROS after 2 h of exposure to 1 mg/L of fullerene, also showing an antioxidant behavior of an aqueous suspension of this nanomaterial (Acosta et al., 2012).

The activity of the total GST was raised in the BaP-only treatments, which is a classical effect of this PAH and is associated to the generation of ROS (Vieira et al., 2008; Palanikumar et al., 2012). Interestingly, co-exposure to C_{60} hinders this increase, keeping the enzyme activity at the basal levels (Fig. 6), a result that can be deleterious for cell viability (as observed) because of the lowering of the detoxifying capacity. Moreover, the computational docking showed that the C_{60} molecule can potentially affect the GST activity because of its affinity for a hydrophobic region of π GST, which is postulated as an allosteric site of HEPES. Such interaction may alter the C terminal region of the enzyme, producing conformational changes that can modify the xenobiotic binding site (Ji et al., 1997). From a toxicological point of view, this evidence is relevant because it demonstrates that fullerene C_{60} can induce deleterious effects by impairing important detoxificatory responses, such as the phase II mechanisms.

However, the nanomaterial did not affect the enzyme activity in the GST purified extracts of ZF-L cells, even at a concentration of 10 mg/L. A possible explanation is that, although the molecule of fullerene has the potential to inhibit π GST activity, it could not bind to the allosteric site of HEPES due to the nanoparticle size, which is a consequence of the aggregation state of fullerene (an aspect not considered in the docking analysis). The lack of effects in the purified extracts in terms of the inhibition of GST activity contrasts with the cell assays, where a clear inhibition of this enzyme was observed, suggesting that the deleterious effects of fullerene may be occurring at the transcriptional level. Schlenk et al. (2008) stated that GST enzymes are more abundant in the liver, being the π-class homolog the predominant form in cyprinids. In this way, although 1-chloro-2,4-dinitrobenzene (CDNB) is a substrate for several GST isoforms (Schlenk et al., 2008), it is expected that the measured activity should reflect the catalytic activity of the π isoform when measured in zebrafish hepatocytes.

Mashino et al. (2001) proved in a previous study that fullerene functionalized with carboxylic groups inhibited glutathione reductase, another enzyme that has glutathione as co-substrate. Thus, both the agglomeration of fullerene molecules in the aqueous suspension and the fact that the nanomaterial was in a non-functionalized form should explain the lack of inhibitory potency in the assays with purified extracts and suggests indirect toxicity mechanism(s). At the present, the hypothesis of the role of the fullerene as a down-regulator of GST transcription is being analyzed at our laboratory.

5. Conclusions

Altogether, the results show that fullerene elicited toxic effects in ZF-L cells by increasing the intake of BaP, decreasing cell viability and impairing the detoxificatory response by the phase II enzyme GST. This latter effect probably occurs at the transcriptional level. The potential affinity of fullerene to π GST needs further investigation, since this isoform is postulated as the predominant GST class in cyprinids.

Conflict of interest statement

The authors declare that there are no actual or potential conflicts of interest in the present work.

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Parte III

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Fullerene C_{60} and benzo[a]pyrene (BaP) elicit oxidative stress and histopathological injuries in *Danio rerio* (zebrafish) larvae

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ABSTRACT

The harmful effects of co-exposure of the carbon nanomaterial fullerene C_{60} with other environmental toxicants are largely unknown, although evidences can be found in literature. Also, fullerene toxicity is currently under discussion in scientific community, mainly due to the lack of standard procedures to produce the working suspensions in assays. The present work investigated the effects of the co-exposure of aqueous-stirred suspensions of fullerene C_{60} with a classical environmental contaminant, benzo[a]pyrene (BaP) in the final larval stages of the zebrafish *Danio rerio* (Cyprinidae, Teleostei). The parameters verified included oxidative stress markers, apoptosis and histopathologies in larvae. The results revealed that fullerene elicited several deleterious effects in zebrafish larvae as alone or co-exposed to BaP. Such damages were verified in terms of high levels of protein oxidation (carbonyl groups), apoptosis and histopathological alterations in multiple larvae organs. Additionally, co-exposure to BaP and fullerene induced more severe damages to morphology of larvae organs, indicating that a interaction between the toxicants had occurred.

Keywords: histopathology, apoptosis, protein oxidation, carbon nanomaterials, fish.

Introduction

The concerns with respect to the environmental consequences of the astonishing rising of the nanotechnologies has been expressed since before the proposal of the scientific branch of nanotoxicology by.(Donaldson (2004). The difficulties to detect and characterize nanomaterials in the environment, which include lack of specific methodologies and analytic techniques fitted to nanoparticles, mean that these data are scarce until today, a situation that difficult the estimation of environmental risks generated by nanotechnologies (Handy et al., 2008; David, 2013). Nevertheless, there are evidences supporting such concerns, as the work of Kaegi et al. (2008) which had detected nano-TiO2 from exterior facades paints in urban storm water runoff. Authors sampled the discharge water just before a natural surface water body, demonstrating that the contamination of the environments by nanomaterials is incontestably occurring.

Carbon nanomaterials such as fullerene C_{60} are highly hydrophobic, but they can form stable colloidal particles in water due to hydration (Andrievsky et al., 2002; Scharff et al., 2004). The C_{60} molecule can be easily bound to a number of functional groups for literally hundreds of applications (Mirkov et al., 2004; Sun and Xu, 2006; Sigwalt et al., 2011; Hendrickson et al., 2012). Also, its pristine form has affinity with biomolecules such as proteins and nucleic acids due to the tendency to form π- π bonds (Nakamura and Isobe, 2003; Benyamini et al., 2006). However, the presence of ions disturbs the delicate balance between attraction and repulsion strengths, allowing the particles to aggregate and settle down (Deguchi et al., 2007).

Therefore, toxicity of fullerene C_{60}, as well as any other nanomaterial, is strongly dependent on many parameters like aggregation state, particle surface chemistry, lattice structure and incidence of light (Petersen and Henry, 2012). In aquatic scenarios, environmental components such as organic matter modulate such parameters (Mashayekhi et al., 2012), making difficult to preview the effects of the nanomaterial in organisms as fish, for example. It has been proposed that fullerene C_{60} can elicit toxicity by means of photo-activation, with consequent generation of reactive oxygen

species (ROS), or physical interaction with cellular components (Trpkovic et al., 2012). Although the issues around its own toxic effects there are substantial evidences that fullerene C_{60} can modulate the toxicity of other environmental contaminants such as phenanthrene (Baun et al., 2008) and mercury (Henry et al., 2013). Also, our research group have found that C_{60} enhanced the uptake of arsenium (Azevedo Costa et al., 2012) and benzo[a]pyrene (BaP) (Ferreira et al., 2014) in zebrafish hepatocytes, inducing an increase in toxic effects of these toxicants. These findings are in according to the evidences firstly demonstrated by Limbach et al. (2007) with nano-silica and manganese, and other studies with nano-TiO2 and copper (Fan et al., 2011) and cerium (Mao et al., 2010). The term 'Trojan horse' was coined by Limbach et al. (2007) to describe a situation where nanomaterials favor the entry of other toxic molecules to cells.

The polycyclic aromatic hydrocarbon (PAH) BaP is a classical and ubiquitous environmental toxicant, produced by multiple sources including incomplete combustion, vehicle exhaustion and cigarette smoke (Miller and Ramos, 2001). Its harmful effects as a mutagen, carcinogen and teratogen agent has been demonstrated to be related to the production of metabolites such as BaP-diols, BaP-quinones and BaP-epoxides by CYP1A complex, inducing the formation of DNA and protein adducts and generation of ROS (Boysen and Hecht, 2003; Miranda et al., 2006). The effects of BaP in fish has been well-established at the biochemical level as a disruptor of redox/antioxidant mechanisms (Palanikumar et al., 2012) as well as an inducer of pathologies, injuries in development and reproduction (Alsop et al., 2007; Incardona et al., 2011).

The ciprinid *Danio rerio* (zebrafish) is considered as an excellent model for toxicological studies with a number of toxicants such as PAHs (Amanuma et al., 2002) and nanomaterials (George et al., 2011), including developmental nanotoxicology studies (Zhu et al., 2007; Usenko et al., 2008). Considering such scenario, the present work aimed to investigate the effects of the co-exposure of fullerene C_{60} and BaP in a critical period of zebrafish development (72 to 96 hpf) in which the animal is vulnerable to environmental toxicants. Toxicity was assessed in terms of

histopathological alterations, induction of apoptosis and oxidative stress markers such as ROS concentration and protein carbonylation.

Methods

Experimental Procedures

All procedures in the present work including maintenance, handling, exposure and euthanasia of the animals were carried out according to standard experimental procedures for fish (Brand et al., 2002) and were approved by the institutional Ethical Committee for Animal Use (CEUA) of the Universidade Federal do Rio Grande-FURG, process number 23116.002165/2013-47.

Zebrafish larvae were obtained from a system for fish maintenance equipped with a recirculating water flow and UV-C lamp, temperature set at 28±1º C, pH 7.5 and light-dark cycle of 14-10 h. For fish mating, adult female and male individuals (proportion 2:1, respectively) were chosen and placed in separated aquariums overnight with an apparatus to collect the eggs. At the first hours of light in the morning, eggs were collected, washed and placed in beakers with temperature of 28±1 ºC. The eggs were cleaned daily and after hatching, larvae were maintained until 72 hpf (day 3), when the exposures were carried out.

For the exposures, larvae 72 hpf were then placed in 24-well culture plates (five larvae per well) in a total volume of 1 ml, as follows: 800 µl of water from the zebrafish system filtered by a 0.22 µm pore size filter; 100 µl of the concentrations of BaP, ultra-pure water (control) or DMSO (BaP diluent); 100 µl of ultra-pure water (treatments without fullerene) or suspension of fullerene C_{60}. Each well corresponded to one treatment, being at least five larvae per treatment in each exposure. The treatment groups were: Ctr(ultra-pure water), C0 (DMSO 0.1%), C1 (BaP 0.01 µg l^{-1} nd C2 (BaP 0.1 µg l^{-1}) with or without the addition of fullerene C_{60} (final concentration: 1 mg l^{-1}). The above concentrations of BaP and fullerene were elected based on the previous findings with

zebrafish hepatocytes performed by our research group (Ferreira et al. 2014). The exposures were performed in the dark, and after 24 h, larvae were washed three times with filtered water from the zebrafish system and prepared for the subsequent assays.

Production and Characterization of Fullerene C_{60} Suspensions

Suspensions of fulleren C_{60} were produced without addition of any organic solvents and was based in the methodology of Lyon et al. (2006): 200 mg of fullerene C_{60} powder (99% purity, SES Research, USA) was placed in clean erlenmeyers free of metal residuals with 1 L of ultrapure water and allow to stirr uninterruptedly during 60 days under artificial light in order to produce a stable colloidal suspension. When finished this period, the supernatant was collected after centrifugation at 25,000 × g and 15 ºC for 1 h and then filtered in sequence by filters of 0.45 and 0.22 μm of pore size. Fullerene actual concentration in the resulting suspension was measured through determination of the total organic carbon content in a carbon analyser TOC-V CPH (Shimadzu, Japan).

The characterization of the C_{60} suspension was done by transmission electron microscopy (TEM) in a JEOL JSM 1200 EX II transmission electron microscope operating at 100 kV. Samples were allowed to evaporation after 24 h and analysis was performed according to previous studies (Britto et al., 2012; Azevedo Costa et al., 2012; Ferreira et al., 2012; Ferreira et al., 2014).

Determination of the Content of Protein Oxidative Modifications

This assay was done through a commercial immunohistochemistry kit (OxyIHC^{TM} Oxidative Stress Detection Kit, Millipore) according to the manufacturer's instructions. The method is based on the reaction of protein carbonyl groups resulting from oxidative damage with 2,4-dinitrophenylhydrazine (DNPH). Specific antibody anti-DNP-derivatized proteins was used for detection of the carbonyl content, followed by a biotin-conjugated secondary antibody, streptavidin-conjugated HRP and 3,3'-diaminobenzidine (DAB) staining for development of the color (non-fluorescent dye). Euthanized larvae were not fixed but placed directly in ethanol 70% and immediately processed as described above for histology, except by the hematoxilin-eosin staining

step and using slides previously covered with Silane (Sigma). Samples were observed and photographed at visible light in a microscope Olympus BX 51 equipped with a high resolution digital camera Olympus DP 72. Protein oxidatively modified in larvae was qualitatively scored and expressed as values of the Bernet Index (Bernet et al., 1999) adapted to protein damage. Details regarding such index are described in **Methods – Evaluation of Histopathological Modifications.**

Determination of Reactive Oxygen Species (ROS)

The methodology for determination of ROS was adapted from Hermann et al. (2004). Immediately after the exposure, larvae were incubated during 2 h with the fluorochrome dichlorofluorescein diacetate (H_2DCF-DA, Invitrogen) 1 μM in DMSO 0.1% at 28 ºC, washed and anesthetized with 50 mg l^{-1} of tricaine methanesulphonate (Sigma) during approximately 1 min. The animals were then placed in microscope slides to observation and acquisition of the images in an inverted epifluorescence microscope Olympus IX 81 equipped with a high resolution digital camera Olympus DP 72 (wavelengths: range of blue light for excitation and range of green light for emission). After the acquisition of the images, larvae were euthanized with 200 mg l^{-1} of tricaine methanesulphonate. The analysis of the images was performed with the open source software ImageJ (Schneider et al., 2012) in which the fluorescence of each larvae was fitted to its respective area. Since the vitellus possesses a highly fluorescent background, the yolk sac was not considered for the analysis. Data represent fluorescence units expressed as percentages of the control group.

Quantification of Apoptosis in Larvae

A commercial immunohistochemistry kit (Click-iT® TUNEL Alexa Fluor® Imaging Assay, Invitrogen) was employed for detection of apoptosis and it was follow the manufacturer's instructions. The assay is based on the method of TUNEL in which an insertion of a derivatized dUTP is done at the 3'-OH ends of fragmented DNA, a well-established evidence of apoptosis. The dUTP is copper-catalyzed bound (click reaction) to the fluorophore Alexa Fluor® to detect the fluorescence in DNA damaged sites. A DNAse positive control was also ran in some slices of

control larvae in order to produce strand breaks. Samples slices with 2 μm of thickness were placed in slides also previously covered with Silane and prepared as described above for histology, except by the hematoxilin-eosin staining step. Larvae were observed and photographed in an epifluorescence microscope Olympus BX 51 equipped with a high resolution digital camera Olympus DP 72 in wavelengths of blue light for excitation and green light for emission. Quantification of apoptosis was performed by averaging the occurrence of fluorescent spots in the slices of each larvae. Analysis was performed also taking into account the different organs of the animals.

Evaluation of Histopathological Modifications

In order to observe the alterations in larvae tissue morphology, it was performed the standard procedures for histology. As the exposure period finished, larvae were euthanized with 200 mg l^{-1} of tricaine methanesulphonate and then placed in paraformaldehyde 4% during 30 min. After the subsequent histological procedures, samples were cut in series of 6 μm thickness slices through an automatic micrometer (Leica RM 2255), placed in slides and stained with haematoxilin-eosin method (Carson and Hladik, 2009). In order to visualize all the possible organs, it was choose sagital cuts. The slides were observed in a light microscope Olympus BX 51 equipped with a high resolution digital camera Olympus DP 72. The injuries in organs were qualitatively analyzed and are described in **Results**. In addition, the lesions were scored according to an index developed for histopathological analysis of fish organs, herein referred as Bernet Index (Bernet et al., 1999). This score takes into account the type of reaction observed, the severity degree of the lesion and its level of importance to the organ. The organ index is the sum of the values of all modification observed, and the larvae index is the sum of the organs index.

Statistical analysis

Statistical analysis was done by means of ANOVA (Zar, 1984). Normality and homogeneity of variances was verified and when at least one of the assumptions was violated, mathematical transformations were applied. Post-hoc comparisons among treatments were performed through the

Newmann-Keuls method, and a significance level of 0.05 was adopted for all steps of the analysis.

Results

Characterization of Fullerene C_{60} Suspension

As described in **Methods**, the characterization of the C_{60} suspension was performed by TEM. As can be seen in Figure 1, the particles were at the nanoscale (1-100 nm), satisfying the basic criterion for assessing the toxicity of nanomaterials in biological models (Colvin, 2003). Also, the suspension showed crystalline aggregates with some rounded shapes, which were very similar to those reported in other works that employed the same preparation methodology (Lyon et al., 2006).

Oxidative Stress Parameters and Apoptosis

Figure 2b shows the ROS concentration data in zebrafish larvae resulting from three independent experiments. In the treatments without C_{60}, the two concentrations of BaP induced fluorescence values significantly ($p<0.05$) higher than the control, with BaP C2(-) C_{60} (0.1 $\mu g\ l^{-1}$) also higher ($p<0.05$) than C0(-) C_{60} treatment (DMSO, control for BaP). However, the addition of C_{60} apparently enhanced ROS production in the controls Ctl(+) C_{60} and C0(+) C_{60} (water and DMSO, respectively) but not in BaP-treated larvae.

Oxidatively modified protein content is showed in Figure 2a. C2(-) C_{60} treatment (BaP 0.01 $\mu g\ l^{-1}$) presented a significant ($p<0.05$) augment in damage compared with other treatments without fullerene. However, fullerene raised ($p<0.05$) the protein carbonylation despite the presence or absence of BaP.

Incidence of apoptosis can be seem in Figure 2c. BaP treatments without C_{60} were significantly ($p<0.05$) higher than controls. Again, the presence of fullerene provoked an increase ($p<0.05$) in the incidence of apoptotic cells in all (+)C_{60} treatments. Liver, eyes and intestine apparently are the most affected organs.

Figure 3 depicts representative images of larvae of ROS assay and carbonylated proteins. It can be seem differences in ROS (fluorescence) among control (-)C_{60} and control (+)C_{60} and

treatments. Higher staining also is showed in immunohistochemistry images of treatments with fullerene, indicating occurrence of protein damaged.

Estimation and Characterization of Histopathological Modifications in Larvae

Table 1 shows the summary of morphological modifications found in organs by treatment. All observed organs presented multiple alterations triggered by C_{60} exposure, while BaP (-) C_{60} treatments were found to induce only liver injury (possible evidence of hepatic steatosis). BaP(+) C_{60} treatments induced all the modifications present in other treatments and additional morphological alterations: disorganization of the cells conformation in liver and intestine (with absence of the nucleus in enterocytes), absence of lens in the eyes and increased steatosis in liver.

Figure 4 provides graphical information regarding the observed histopathologies, focusing on the different organs. The estimation was done by means of a lesion index referred here as Bernet Index (Bernet et al., 1999) (see Materials and Methods). Index values of the larvae were clearly higher in (+) C_{60} treatments. High values of liver, eyes an intestine reflect the severe injuries found in these organs.

The morphological alterations in larvae tissues can be visualized in Figures 5, 6 and 7. Several lesions and deformities in multiple organs are indicated in figures.

Discussion

Currently, the discussion concerning the toxicity of fullerene C_{60} in fish tends to conclude that this nanomaterial has little deleterious effects, at least with respect to generation of ROS (Henry et al., 2011). This conclusion is mainly due to the fact that many of the toxicological studies with C_{60} and fish were carried out with methodologies that employed THF, leading to false positive outcomes (Andrievsky et al., 2005; Henry et al., 2007). In addition, some studies which employed solvent-free methods to produce the suspensions and the exposure was carried out in the dark indicated absence/minimal toxicity (Shinohara et al., 2009; Fraser et al., 2011). Although, it is a matter of concern the ability of fullerene to modulate the toxicity of other contaminants in the

environment, apparently by enhancing the bioavailability of such compounds (Baun et al., 2008; Azevedo Costa et al., 2012; Henry et al., 2013; Ferreira et al., 2014). In the present work, it was demonstrated that aqueous-stirred fullerene suspensions elicited severe deleterious effects and enhanced some histopathological alterations of BaP in the final stages of development of zebrafish larvae.

The zebrafish larvae at 72 hpf has already developed most of its morphogenesis. Almost all organs are functional, and metabolic rate slows considerably (Kimmel et al., 1995). Although this stage is after the critical development periods, fish still vulnerable to environmental toxicants. The mouth initiates to protrude and open, the gills starts its functionality and digestory system becomes more elongated, meaning additional ways of exposure to contaminants. Once toxicants reach its targets, maturation of the organs can be affected and the growing process compromised, leading to a low probability of survival and reproduction (DiGiulio and Hilton, 2008; Roberts, 2012).

The apparent lack of effect in ROS generation observed in C_{60} when co-exposed to BaP (Fig. 2b) is not supported by the protein damage data. Carbonyl groups content was exacerbated in BaP (+) C_{60} treatments (Fig 2a), indicating that oxidative damage occurred at notable levels (as well as in all fullerene treatments). However, it can be postulated that if the excess of ROS reacted with proteins, the ROS levels would not appear to rise, as seemed. In this way, protein damage could be a fingerprint of a previous ROS peak provoked by fullerene.

Apoptosis triggered by carbon nanomaterials is commonly found in literature (Orlova et al., 2013; Wang et al., 2014). *In vitro* investigations indicate the prevalence of the intrinsic apoptotic pathway. A carboxylic- C_{60} derivative was found to induce apoptosis via JNK pathway, inhibition of PARP cleavage and release of cytochrome c (Lao et al., 2009). Carbon nanotubes also show the involvement of mitochondria and ROS production, with participation of TNF-α and Bax (Cheng et al., 2011), or NF-κB and AP-1 factor (Ravichandran et al., 2010). In the present work, apoptosis data correlated with protein oxidation. Exposure to BaP treatments and all the (+) C_{60} treatments enhanced the incidence of apoptosis (Fig 2c). This supports the idea that a ROS overproduction

could be occurred. This ROS peak would damaged seriously proteins and important cellular components, triggering apoptosis events. In this way, such oxidative environment was likely to trigger intrinsic mitochondrial apoptosis rather than extrinsic mechanisms.

Larvae exposed to the treatments with the toxicants presented severe histological alterations in multiple organs. The mechanisms underlying the observed deleterious effects of fullerene-only treatments are not completely clear. It has been postulated that C_{60} could trigger membrane damage and autophagy without ROS production (Trpkovic et al., 2012). Autophagy is a common outcome in cells exposed to nanomaterials as a cellular response to foreign bodies that can result in cell death (Zabirnyk et al., 2007), involving disturbance of cellular signaling pathways (Ling Wu et al., 2014; Roy et al., 2014). Carbon nanomaterials fit this profile because of its tendency to cause inflammatory responses (Liu et al., 2013). This can be seem in the intestine of larvae of (+) C_{60} treatments, were catarrhal enteritis was evident (Table 1; Fig. 6D; Fig. 7F). Therefore, the observed histopathologies in organs can be related to the interaction of nanoparticles with cellular components such as membranes which ultimately could promote autophagy. Nevertheless, there is also an important oxidative stress component in the toxicity observed, since proteins were oxidatively damaged.

Liver is a vital organ for the development and growth in teleosts, being responsible by most of the glycogen and/or lipid stocks, detoxificatory machinery and also haematopoiesis (Hinton et al., 2008; Roberts, 2012; Jovanovic et al., 2013). Although the liver is known as a target organ for BaP (Michurina et al., 2000), all the fullerene treatments also induced hepatic injuries (Table 1; Fig. 4B; Figs. 7A, 7C and 7E). The presence of large vacuoles was evident in livers of BaP-treated as well as in all fullerene-treated larvae, indicating the possible occurrence of steatosis. If true, this indicates that the nanomaterial disturbed the fatty acids metabolism, acting as a classical hepatotoxin such as BaP. Since fullerene induced high levels of protein oxidation (Fig. 2A; Figs. 3B, 3C), a possible toxicity mechanism could be the reduced synthesis or loss in functionality of apoproteins, which is one of the reasons for fatty degeneration (DiGiulio and Hilton, 2008).

It also should be stressed that larvae of (+) C_{60} treatments presented poor content or absence of vitellus (Table 1), indicating that some kind of disturb in uptake or absorption of nutrients is occurring. Overall consequences of fullerene exposure to the animals can be summarized as follows: 1. larvae probably are blind due to severe retinal disorganization of the eyes; 2. the gills may have their functionality impaired due to aplasia; 3. cranium deformity and stretching of the brain can compromise fish behavior and survival; 4. larvae probably will have energetic metabolism and immune system impaired due to liver injuries; 5. the absorption function of the intestine is impaired due to abnormal striated border, inflammation and tissue disorganization; 6. locomotion capacity may be reduced due to kyphosis, deformed swim bladder and muscular flaccidity.

The effects of the interaction of BaP and C_{60} were not clearly evident with respect to oxidative stress parameters or apoptosis, probably due to the exacerbated deleterious effects of fullerene. Although, observation of morphological alterations can bring to light some evidences. BaP (+) C_{60} treatments presented a more pronounced presence of vacuoles (possible steatosis) than other treatments. Also, only BaP (+) C_{60} treatments induced tissue disorganization in liver and intestine, compromising severely the functionality of the organs. In the eyes, co-exposure to BaP and C_{60} caused loss of the lens. This serious damage can be due to re-absorption or complete degeneration of crystalocytes, since the eye were in direct contact to the toxicants. Considering these findings, it can be inferred that BaP and fullerene could be acting in an additive way, or the nanomaterial is enhancing the uptake of BaP by larvae – the "Trojan Horse" effect, also observed in other studies (Baun et al., 2008; Azevedo Costa et al., 2012; Ferreira et al., 2014).

Conclusions

Overall results show that fullerene elicited several deleterious effects in zebrafish larvae as alone or co-exposed to BaP. Such damages were verified in terms of enhancing of protein oxidation, apoptosis and histopathological alterations in multiple larvae organs. Additionally, co-exposure to BaP and C_{60} induced more severe damages to morphology of larvae organs, indicating that a

interaction between the toxicants had occurred. The present findings suggest that a broader approach is advisable to investigate the toxicity of fullerene in virtue of the conflicting outcomes in literature.

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Table 1. Description of histopathological alterations in zebrafish larvae listed by organ. Symbols: "-": absence or normal occurrence of the alteration; "+": presence of the alteration; "++": higher severity of the alteration. Ctl – control; C0 – DMSO 0.1%; C1 – BaP 0.01 μg l^{-1}; C2 – BaP 0.1 μg l^{-1}; [C_{60}]: 1 mg l^{-1}.

Organ	Alteration	Treatment							
			(-) C_{60}				(+) C_{60}		
		Ctl	C0	C1	C2	Ctl	C0	C1	C2
Liver	- Large vacuoles (possible steatosis)	-	-	+	+	+	+	++	++
	- Disorganization on hepatocytes' conformation	-	-	-	-	-	-	+	+
	- Irregular borders between adjacent hepatocytes	-	-	-	-	+	+	+	+
Eyes	- Deformities (non-spherical shape)	-	-	-	-	+	+	+	+
	- Thickness of the retinal pigmented epithelium	-	-	-	-	+	+	+	+
	- Severe retinal disorganization	-	-	-	-	+	+	+	+
	- Absence of lens	-	-	-	-	-	-	+	+
Gills	- Aplasia in first portion of branchial arches	-	-	-	-	+	+	+	+
Brain	- Cranium deformed, rhombencephalon stretched, absence of telencephalon-mesencephalon boundary	-	-	-	-	+	+	+	+
Intestine	- Striated border augmented or absent	-	-	-	-	+	+	+	+
	- Catarrhal enteritis	-	-	-	-	+	+	+	+
	- Goblet cells absent in some animals	-	-	-	-	+	+	+	+
	- Disorganization on enterocytes' conformation, absence of nucleus	-	-	-	-	-	-	+	+
Other	- Kyphosis	-	-	-	-	+	+	+	+
	- Swim bladder deformed	-	-	-	-	+	+	+	+
	- Poor content or absence of vitellus	-	-	-	-	+	+	+	+
	- Muscular fibers presenting flaccidity	-	-	-	-	+	+	+	+

Figure 1

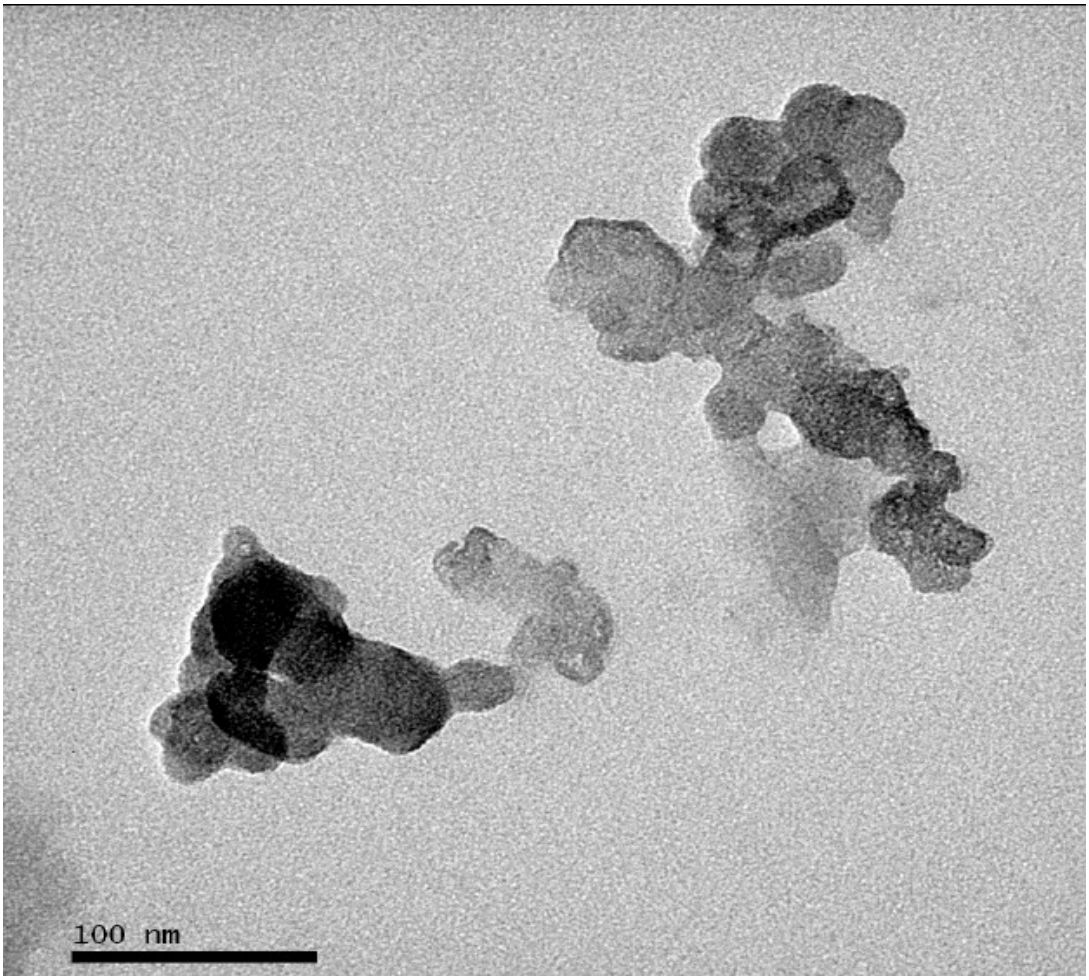

Figure 2

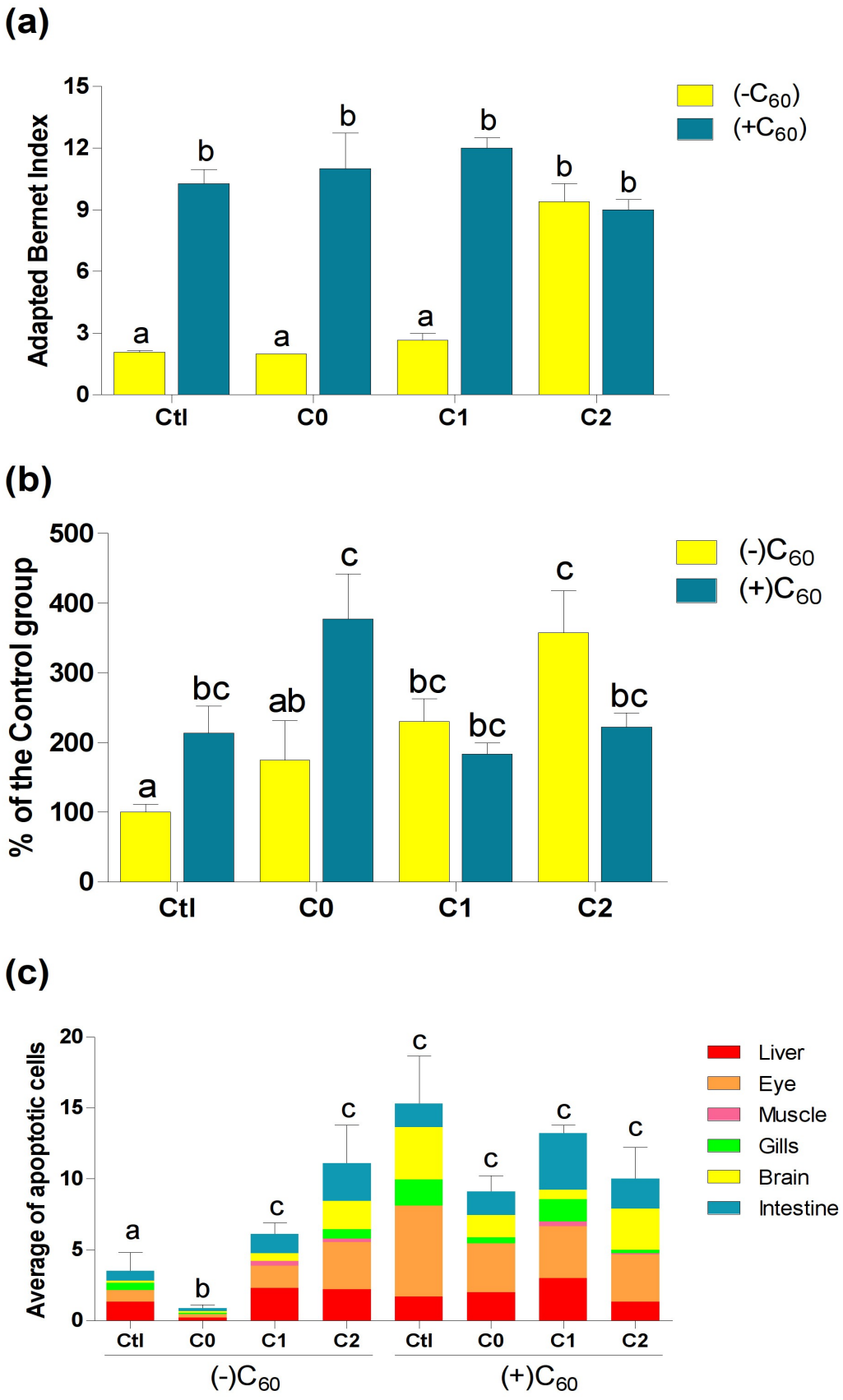
(a)
Adapted Bernet Index
15
12
9
6
3
0
a
b
a
b
a
b
b
b
Ctl
C0
C1
C2
(-C60)
(+C60)
(b)
% of the Control group
500
400
300
200
100
0
a
bc
ab
c
bc
bc
c
bc
Ctl
C0
C1
C2
(-)C60
(+)C60
(c)
Average of apoptotic cells
20
15
10
5
0
a
b
c
c
c
c
c
c
Ctl
C0
C1
C2
Ctl
C0
C1
C2
(-)C60
(+)C60
Liver
Eye
Muscle
Gills
Brain
Intestine

Figure 3

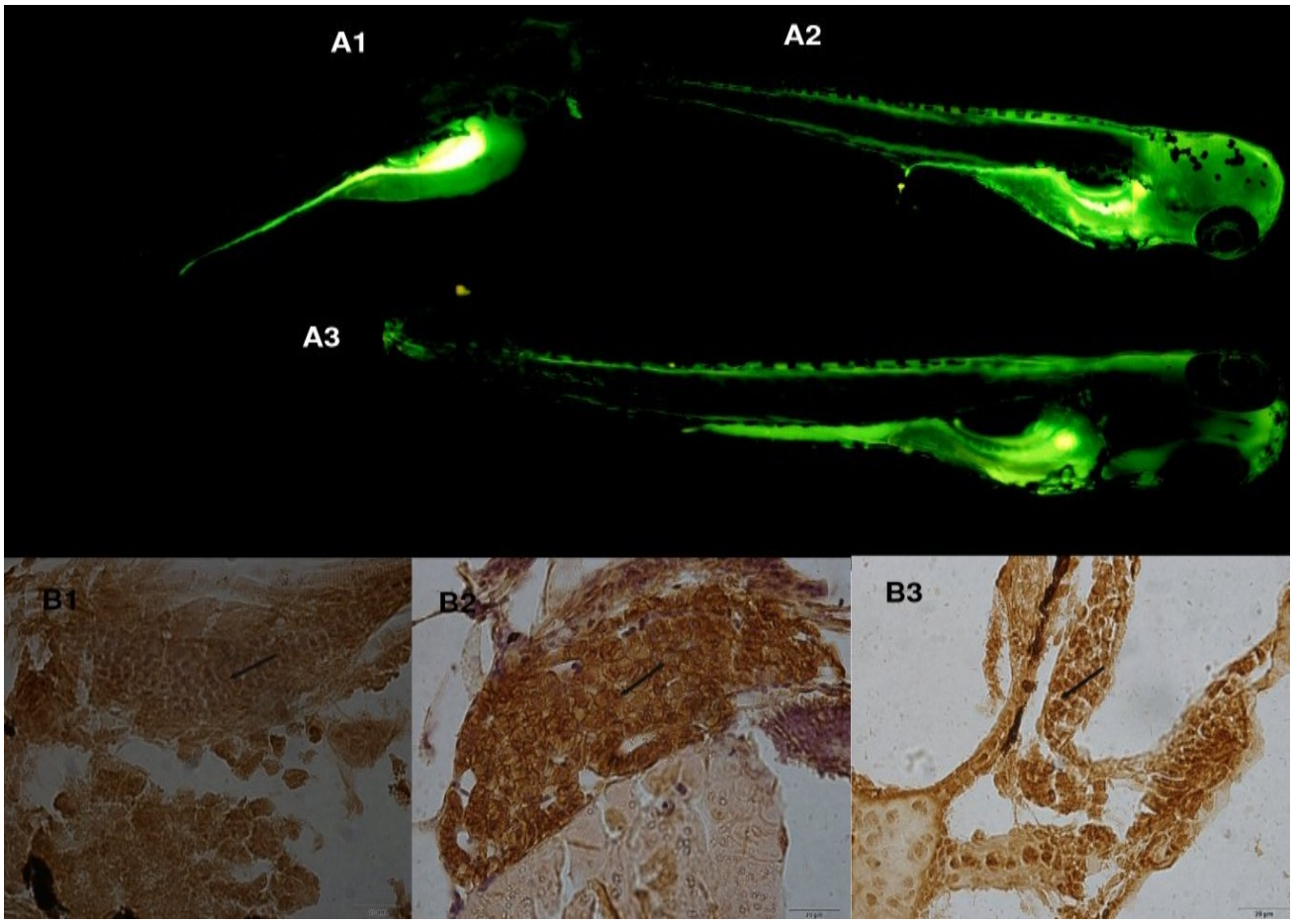

Figure 4

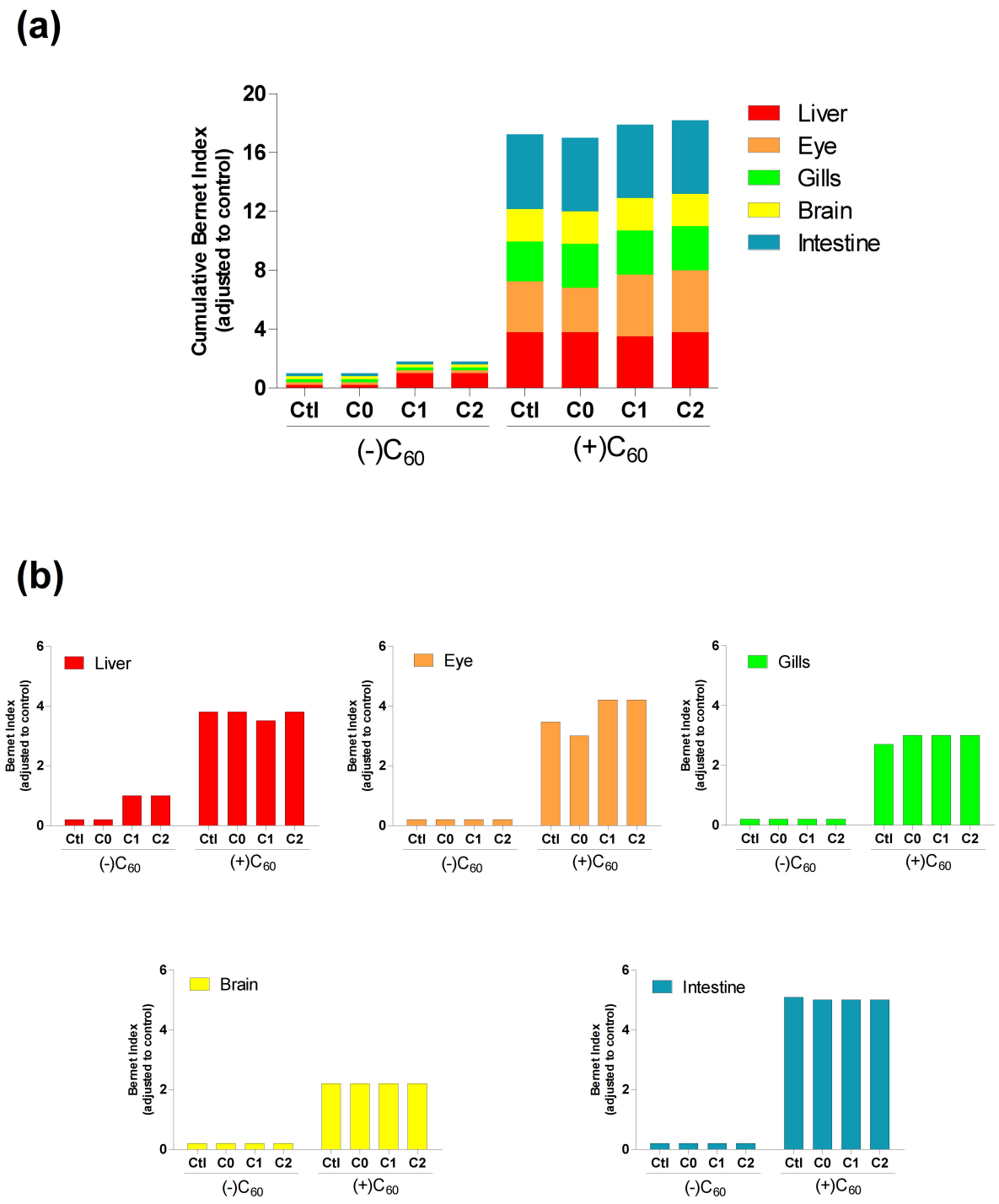
(a)
Cumulative Bernet Index (adjusted to control)
20
16
12
8
4
0
Ctl C0 C1 C2 Ctl C0 C1 C2
(-)C60
(+)C60
Liver
Eye
Gills
Brain
Intestine
(b)
Bernet Index (adjusted to control)
6
4
2
0
Liver
Eye
Gills
Brain
Intestine
Ctl C0 C1 C2
(-)C60
(+)C60

Figure 5

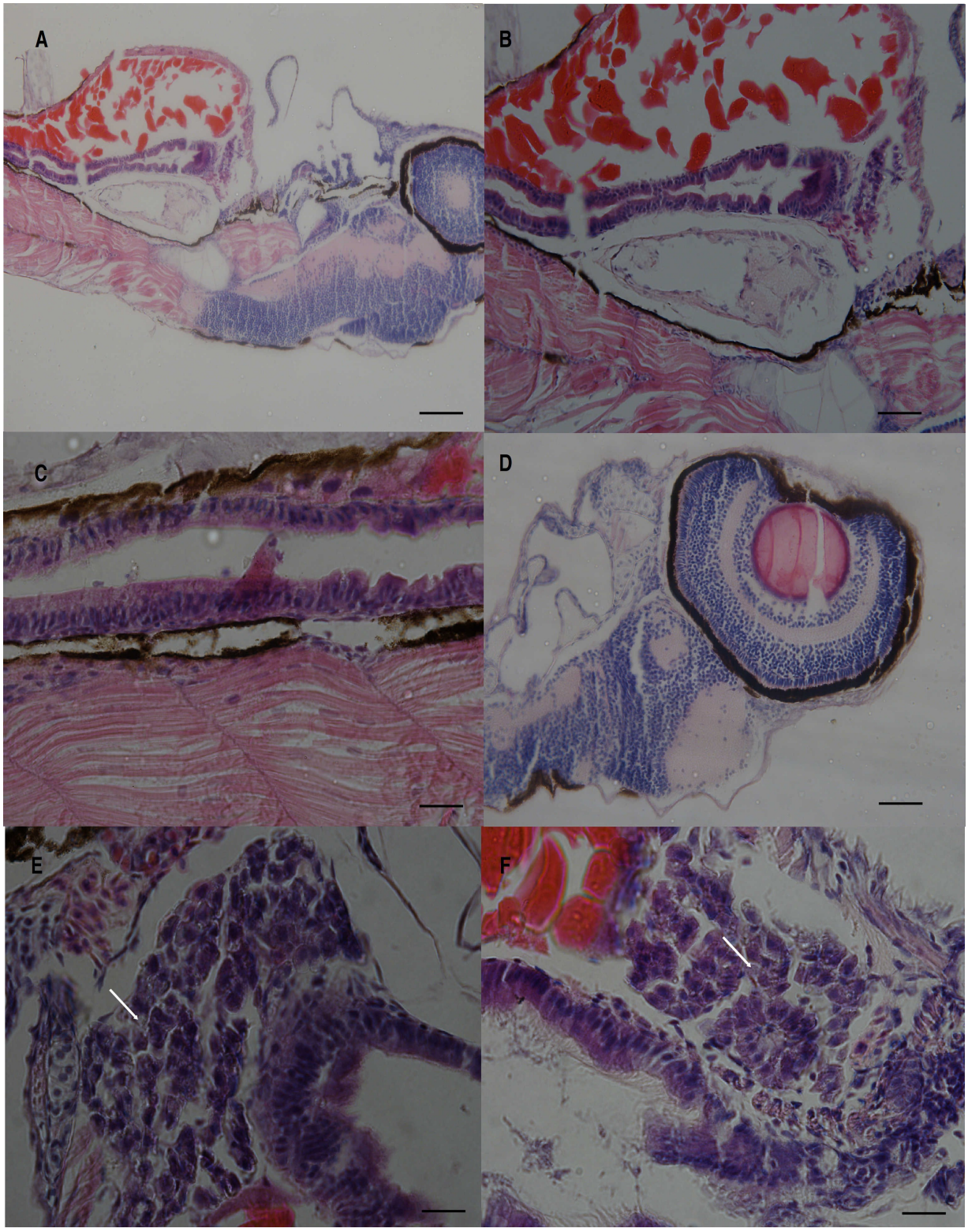

Figure 6

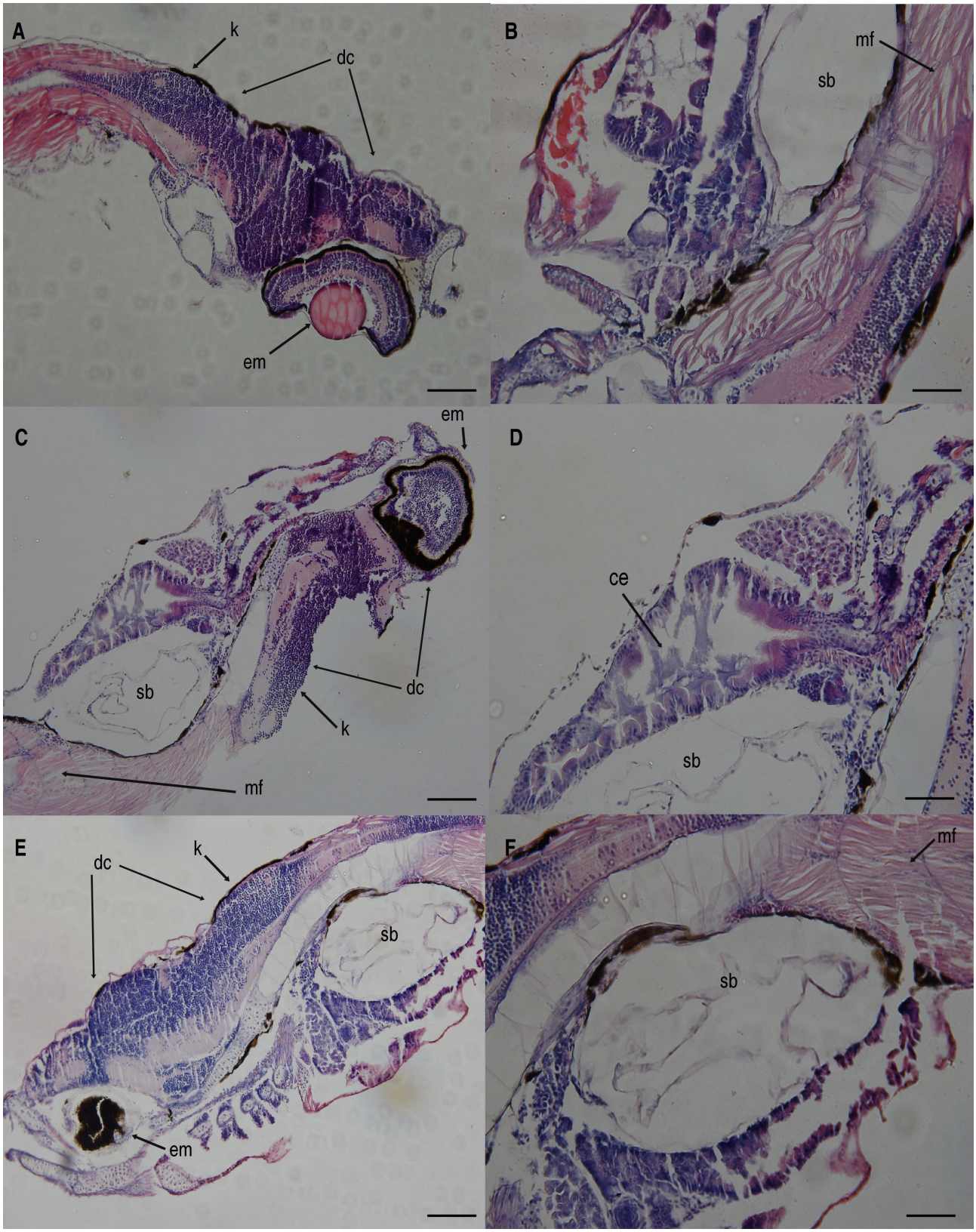

Figure 7

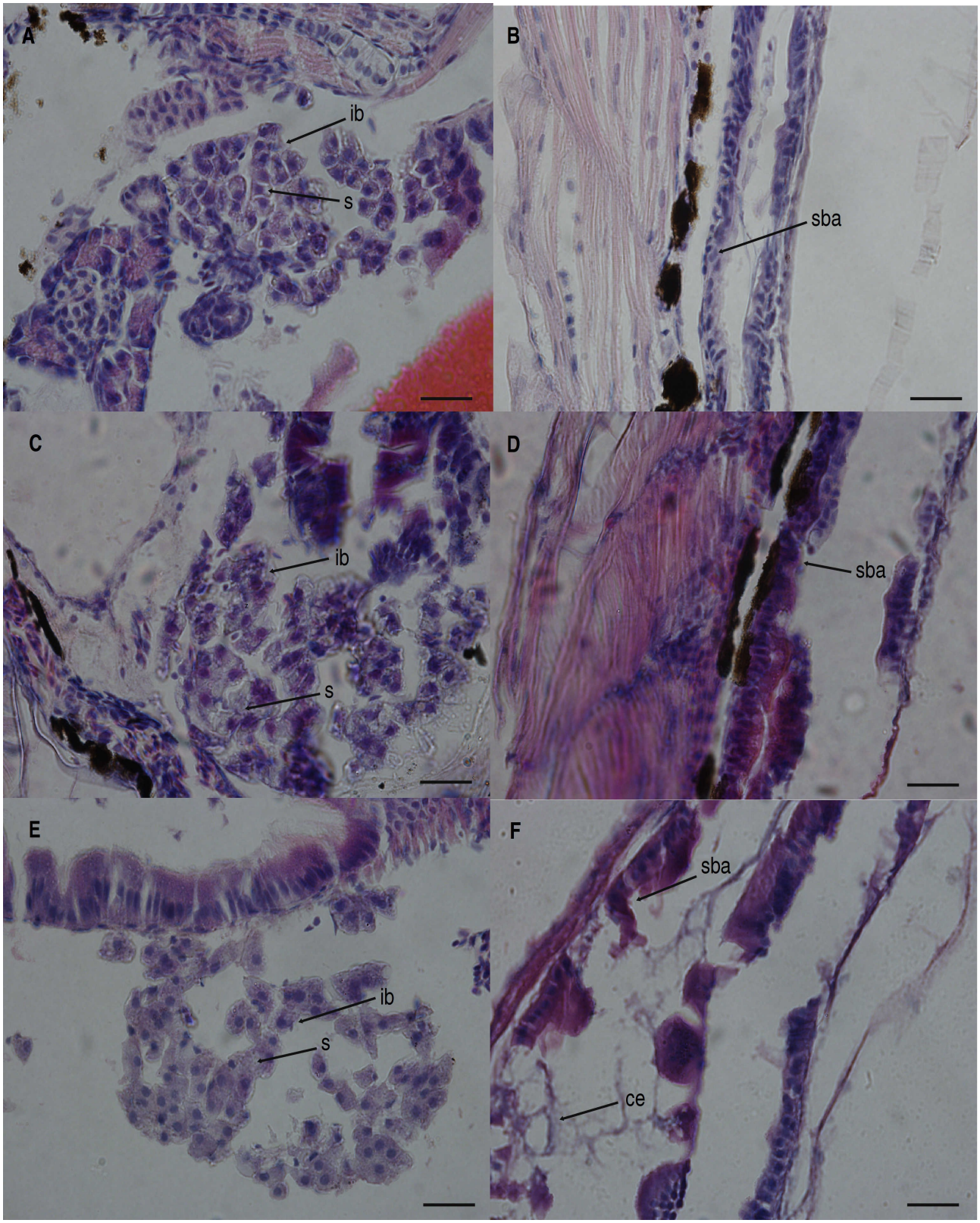

Figure Captions

Figure 1. Transmission electron microscope (TEM) image of fullerene nanoparticles obtained through the aqueous-stirred suspension, as described in **Methods**.

Figure 2. Parameters of oxidative stress and apoptosis in zebrafish larvae. Ctl – control; C0 – DMSO 0.1%; C1 – BaP 0.01 μg l^{-1}; C2 – BaP 0.1 μg l^{-1}; [C_{60}]: 1 mg l^{-1}. **(a)** Content of oxidatively modified proteins (carbonyl groups) in larvae. Data are expressed as mean±1 standard errors of Bernet Index values adapted to protein damage (n=3). **(b)** Concentration of ROS in living zebrafish larvae. Data are expressed as mean±1 standard errors of fluorescence units in percentages of the control group (n=15). **(c)** Occurrence of apoptotic cells in zebrafish larvae. Bars represent the cumulative incidence in the different organs. Data are expressed as mean±1 standard errors (n=3).

Figure 3. Microscopy images of zebrafish larvae. Ctl – control; C0 – DMSO 0.1%; C1 – BaP 0.01 μg l^{-1}; C2 – BaP 0.1 μg l^{-1}; [C_{60}]: 1 mg l^{-1}. **A1 to A3**: fluorescence microscopy images of living larvae showing ROS content in treatments Ctl(-)C_{60}, C1(+) C_{60} and C2(+)C_{60}, respectively. **B: to B3**: images of the content of carbonylated proteins in zebrafish larvae assessed by immunohistochemistry. In **B1**, arrow shows a normal liver from control; in **B2** and **B3** arrows indicate carbonylated proteins staining in livers of larvae from treatments C1(+) C_{60} and C2(+)C_{60}, respectively.

Figure 4. Bernet Index (BI) estimate of histopathological modifications in zebrafish larvae. Data are expressed as 1 + BI adjusted to control group. Ctl – control; C0 – DMSO 0.1%; C1 – BaP 0.01 μg l^{-1}; C2 – BaP 0.1 μg l^{-1}; [C_{60}]: 1 mg l^{-1}. **(a)** Cumulative BI of organs in treatments. **(b)** BI of each organ in which morphological alterations were observed.

Figure 5. Images of morphological alterations in larvae exposed without addition of fullerene. Ctl – control; C0 – DMSO 0.1%; C1 – BaP 0.01 μg l^{-1}; C2 – BaP 0.1 μg l^{-1}; [C_{60}]: 1 mg l^{-1}. **A**, **B**, **C** and **D**: control group (no abnormalities); **E** and **F**: C1(-)C_{60} and C2(-)C_{60} treatments, respectively. Arrows indicate lipid droplets as evidence of steatosis in the liver.

Figure 6. Overview of morphological alterations in various organs of fullerene-treated larvae. Ctl – control; C0 – DMSO 0.1%; C1 – BaP 0.01 µg l^{-1}; C2 – BaP 0.1 µg l^{-1}; [C_{60}]: 1 mg l^{-1}. **A** and **B**: Ctl(+) C_{60} treatment; **C** and **D**: C1(+) C_{60} treatment; **E** and **F**: C2(+) C_{60} treatment. Legend: **ce** – catarrhal enteritis; **dc** – deformed cranium; **em** – eye malformation; **k** – kyphosis; **mf** – muscular flaccidity; **sb** – swim bladder deformed.

Figure 7. Morphological alterations of fullerene-treated larvae focusing on liver (left column) and intestine (right column). Ctl – control; C0 – DMSO 0.1%; C1 – BaP 0.01 µg l^{-1}; C2 – BaP 0.1 µg l^{-1}; [C_{60}]: 1 mg l^{-1}. **A** and **B**: Ctl(+) C_{60} treatment; **C** and **D**: C1(+) C_{60} treatment; **E** and **F**: C2(+) C_{60} treatment. Legend: **ce** – catarrhal enteritis; **ib** – irregular borders in hepatocytes; **s** – hepatic steatosis; **sba** – striated border augmented in intestine.

Normas da Revista

Nanotoxicology
Instructions for Authors

Table of Contents

About the Journal

Aims and Scope

Nanotoxicology invites contributions addressing research relating to the potential for human and environmental exposure, hazard and risk associated with the use and development of nanomaterials. In this context, the term nanomaterials includes 'materials with at least one dimension in the nanometer size range'. This therefore includes a variety of structures such as nanoparticles, nanofibres or nanoflakes, as pure chemicals, in mixtures and matrices throughout their lifecycle. Contributions about materials generated for purposeful delivery into the body (food, medicines, diagnostics and prosthetics), for consumer products (e.g. paints, composite materials, cosmetics, electronics and clothing), or for environmental applications (e.g. remediation) are welcome. It is the nano-size range of these materials that unifies them and defines the scope of ***Nanotoxicology***. The scope of the journal is broad but all manuscripts must have a link to investigation of hazard/toxicology.

While the term 'toxicology' indicates risk, the journal ***Nanotoxicology*** also aims to encompass studies that enhance safety during the production, use and disposal of nanomaterials. Well-controlled studies demonstrating a lack of exposure, hazard or risk associated with nanomaterials or studies aiming to improve biocompatibility are welcomed and encouraged, as such studies will lead to an advancement of nanotechnology. Studies on safety of nanomaterials in medical applications are encouraged.

The editors will prioritise publications that include: 1) material characterisation that complements a testable hypothesis and the objectives of the study; 2) an explanation of the chosen doses in terms of realistic exposures[a]; 3) the use of benchmark or reference materials and; 4) an explanation of the biological model employed, such as the cell type(s) in the case of *in vitro* studies or the route and method of exposure in the case of *in vivo* studies.

[a] We greatly value studies that explore the full range of the dose-response relationship, including the identification of a lowest-observable adverse effects level or a no adverse effects level. While this is desirable, we recognize that there are ethical and economic considerations with respect to *in vivo* studies that must be taken into account.

Nanotoxicology publishes 8 online issues throughout the year, combined in two print archive copies in June and December. Manuscripts will be pre-published online as early as 2-3 days after acceptance as 'Just Accepted' articles (see p. 11). These unedited author versions will be replaced by the copy edited, typeset and proofread manuscripts when ready. Access to the online version is included in all subscriptions.

Editors

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- Review articles
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- Book reviews
- Short communications

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If required sections are missing in the body of the paper or figures/tables are inadequate, the Editor reserves the right to return any manuscript to the contact author without scientific review.

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Original articles should be no more than 7000-word long including abstract and references. In addition, illustrations should not exceed three printed pages (typically six figures or tables). Authors can submit non-essential, supplementary data alongside the manuscript (see Supplementary Material p.6).

The body of the article should include the following distinct sections: introduction; methods; results; discussion; conclusions. Articles with a combined results and discussion section will be returned to the authors for editing.

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Discussion: This should include implications of the findings and their limitations, with reference to other relevant studies and the possibilities for future research.

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Commentaries should be knowledge-based or consensus-type articles (e.g. working group statement) expressing objective opinions, experiences or perspectives on an important area related to nanotoxicology.

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- *Contribution to a Book:* Chandrasekaran SK, Benson H, Urquhart J. (1978). Methods to achieve controlled drug delivery: The biochemical engineering approach. In: Robinson JR, ed. Sustained and Controlled Release Drug Delivery Systems. New York: Marcel Dekker, 557–593.

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Considerações Finais

Os resultados da presente Tese levam a algumas observações. Em primeiro lugar, a revisão de trabalhos sobre a toxicidade dos nanomateriais em organismos aquáticos revela que várias questões devem ser consideradas em estudos de nanotoxicologia. Tais questões são de natureza metodológica, como a obtenção e caracterização das suspensões de uso e também a escolha do organismo modelo estudado, já que alguns organismos (como dafnídeos e algas) mostram-se mais sensíveis do que outros aos efeitos dos nanomateriais. Na presente Tese, o peixe *D. rerio* demonstrou ser um excelente modelo para testes toxicológicos com o uso do fulereno C_{60}, uma vez que foi bastante responsivo aos efeitos deste nanomaterial, tanto *in vitro* como *in vivo.*

Os resultados gerais confirmam as hipóteses iniciais da presente tese. Os efeitos registrados do fulereno C_{60} *per se* no estudo com larvas de *D. rerio* revelaram o potencial tóxico do nanomaterial mesmo quando não está fotoexcitado, uma vez que os ensaios foram feitos em condições de escuridão. Em cultura de hepatócitos, o potencial de inibição da glutationa-S-transferase (GST) *in silico* chama a atenção para os efeitos indiretos sobre a detoxificação de outros contaminantes, além do óbvio prejuízo para o sistema de defesa antioxidante, dada a importância da enzima. Além disso, foi confirmado o efeito “Cavalo de Tróia”, uma vez que o nanomaterial aumentou a captação de BaP nos hepatócitos e isto se refletiu na diminuição da viabilidade celular.

Em resumo, pode ser concluído que o fulereno C_{60} tem o potencial de interferir na biodisponibilidade de outros contaminantes e causa efeitos deletérios graves em larvas de peixe. Tais evidências, em vez de agregarem consistência ao que já se sabe sobre o nanomaterial, aumentam a controvérsia a respeito dos seus efeitos nocivos. O presente trabalho traz novamente à tona a preocupação com o destino dos produtos e resíduos de fulereno que são produzidos em escala global, uma vez que os nanomateriais já estão

presentes como contaminantes nos ambientes aquáticos.

Como continuação desta linha de trabalho, são necessários mais estudos avaliando todos os estágios da vida de *D. rerio*, além de investigações sobre o efeito do fulereno na expressão e conteúdo de CYP1A e GST-π. Hepatócitos de *D. rerio* também podem ser usados como modelo para testar os efeitos da co-exposição do nanomaterial com outros contaminantes ambientais, avaliando-se diversos outros parâmetros como marcadores de sinalização redox, por exemplo.

É evidente a necessidade de que as pesquisas sobre novas tecnologias e as Ciências Ambientais andem em paralelo para que se possa estimar e, se possível, evitar ou mitigar os impactos sobre o ambiente. O uso moderado e planejado de quaisquer compostos ou dispositivos cujo potencial nocivo seja desconhecido – o que nos diz o Princípio da Precaução – vai na direção oposta das demandas tecnológicas atuais. Isso gera uma assimetria radical entre as pesquisas sobre desenvolvimento e os trabalhos que avaliam os efeitos tóxicos dos nanomateriais (Kahru e Dubourguier, 2010). Para corrigir tal descompasso, é necessário um esforço coordenado da comunidade científica com o intuito de padronizar técnicas e desenvolver metodologias para a produção de resultados genuinamente reprodutíveis na Nanotoxicologia. Uma vez que exista um corpo considerável e consistente de informações, finalmente poderão ser construídas diretrizes legais básicas para a regulamentação da fabricação, uso e descarte dos nanomateriais.

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