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FISIOLOGIA ANIMAL COMPARADA

Estado redox no peixe *Danio rerio* (Cyprinidae) exposto via dieta a nanotubos de

carbono de paredes simples e múltiplas

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> **Tese apresentada no âmbito do Programa de Pós-Graduação em Ciências Fisiológicas: Fisiologia Animal Comparada da Universidade Federal do Rio Grande como requisito parcial para a obtenção do Título de DOUTOR em Ciências Fisiológicas**

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"A ignorância gera mais frequentemente confiança do que o conhecimento: são os que sabem pouco, e não aqueles que sabem muito, que afirmam de uma forma tão categórica que este ou aquele problema nunca será resolvido pela ciência."

Charles Darwin

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Dedico meus agradecimentos a todos que de alguma forma colaboraram com a realização deste trabalho que foi fruto de cooperação e dedicação de diversas pessoas:

Sumário

Lista de Abreviaturas

C_{60} **:** Fulereno

CNT: nanotubos de carbono (do inglês *Carbon Nanotubes*)

CVD: deposição química de vapor (do inglês *Chemical Vapor Deposition*)

DWCNT: nanotubos de carbono de parede dupla (do Inglês *Double Walled Carbon Nanotubes*)

LPO: peroxidação lipídica (do inglês *Lipid Peroxidation*)

MWCNT: nanotubos de carbono de parede múltipla (do inglês *Multi-Wall Carbon Nanotube*)

ROS: espécies reativas do oxigênio (do inglês *Reactive Oxygen Species*)

SWCNT: nanotubos de carbono de parede simples (do inglês *Single Wall Carbon Nanotube*)

TBARS: substâncias reativas ao ácido tiobarbitúrico

RESUMO

Os nanomateriais são compostos nos quais pelo menos 50% deles possuem uma de suas dimensões na faixa de 1 a 100 nm. Os nanotubos de carbono (CNT) são estruturas cilíndricas que podem ter diversas aplicações devido suas propriedades físicas e químicas diferenciadas. No entanto, seus potenciais efeitos tóxicos são ainda pouco conhecidos, incluindo a periculosidade que estes nanomateriais podem oferecer aos organismos aquáticos e ao meio ambiente. Com a grande produção mundial destes tipos de nanomateriais é importante prever o impacto no futuro próximo dos recursos hídricos, sendo assim importante saber como os organismos aquáticos vão ser afetados. Portanto, este trabalho teve por objetivos: **(1)** revisar o conhecimento científico existente sobre a nanoecotoxicologia em peixes; **(2)** investigar os possíveis efeitos dos nanotubos de carbono de parede simples (SWCNT) ou múltiplas (MWCNT) na exposição *in vivo* através da ração aos peixes *Danio rerio* (Cyprinidae, Teleostei) e **(3)** verificar possível interferência que os nanomateriais de carbono exercem no método de substâncias reativas ao ácido tiobarbitúrico (TBARS). Os resultados mostram que os CNT através da dieta não aumentaram o teor de TBARS nas brânquias, cérebro, intestino e músculos do *Danio rerio*. No entanto, nas brânquias e intestino dos peixes expostos a SWCNT e MWCNT foram observados uma diminuição dos níveis de TBARS em comparação com o controle, estes resultados motivaram a execução dos ensaios onde verificou-se a interferência dos ensaios fluorométricos e os confirmaram. Através do método histológico observou-se hiperplasia, edema e possível inflamação nas brânquias e proliferação de células da glia no cérebro dos animais expostos a SWNTC. Por imunodetecção foi verificado um aumento na concentração de grupos carbonila em diversos órgãos dos peixes expostos ao SWCNT e MWCNT em comparação ao controle indicando uma situação de estresse oxidativo. Além disso, verificamos que os SWCNT e fulereno interferem na fluorescência quando se utiliza o método de TBARS. Estes resultados evidenciam a importância da verificação de metodologias para ensaios de nanotoxicologia e, ainda, indicam que os CNT podem induzir danos num modelo de espécie aquático, *D. rerio,* sob situação de exposição ambientalmente realistas.

Palavras chave: nanotubos de carbono, nanotoxicologia, *Danio rerio*, dano oxidativo, imunohistoquímica

INTRODUÇÃO

Em sua palestra na reunião anual da *American Physical Society,* o físico Richard Feynman postulou em 1959 que existia a possibilidade de manipular os materiais em escala atômica para obter as características desejadas (Feynman,1960; Feynman, 1992). O postulado de Feynman atualmente é uma realidade e o crescimento tecnológico está transcendendo a escala dos dispositivos e equipamentos, que apresentam tamanhos e propriedades nunca antes vistas. Inseridos nesses estão os *nanomateriais* na "N^{-9}", uma indústria em pleno crescimento (Savolainen et al., 2010). As nanotecnologias estão representadas por qualquer dispositivo que apresentar escala nanométrica (McNeil, 2005). Pela sua vez, os nanomateriais que são substâncias de origem natural ou manufaturados que possuem no mínimo 50% das suas partículas em uma de suas dimensões entre 1 a 100 nm (European Commission, 2011). Nesta faixa de tamanho existem mudanças significativas nas propriedades químicas e físicas das moléculas, gerando novas propriedades, muitas das quais podem ter um aproveitamento tecnológico (Colvin, 2003; Oberdörster et al., 2005).

Os nanomateriais têm tendência a interagir com biomoléculas, pois são menores do que a maioria das células e organelas e de tamanho semelhante a muitas biomoléculas (McNeil, 2005). A capacidade potencial dos nanomateriais de atravessar as barreiras celulares e interagir com receptores, ácidos nucleicos, fatores de transcrição e proteínas de sinalização pode gerar alterações bioquímicas e fisiológicas que podem pela sua vez gerar condições patológicas (Eckert et al., 2013). Como já mencionado anteriormente, o tamanho é o fator de maior importância, quando se trata de nanomateriais, principalmente pela elevada área de superfície (Auffan et al., 2009; Seaton et al., 2010). No entanto, os fatores relativos à toxicidade dos nanomateriais dependem das condições de exposição. No caso específico dos CNT, a co-exposição deles com contaminantes ambientais pode causar aumento dos potenciais efeitos adversos (Campus-Garcia et al., 2015). O desenvolvimento de novas técnicas e conceitos para a nanotoxicologia são necessários, com o surgimento de novos nanomateriais acabam por tornar os modelos de previsão clássicos obsoletos.

NANOTUBOS DE CARBONO e FULERENOS

Em 1991, Sumio Iijima demonstrou pela primeira vez a existência de estruturas tubulares de carbono, que foram mais tarde denominadas nanotubos de carbono (CNT; Figura 1) (Iijima, 1991). Esta descoberta, combinado a dos fulerenos (C_{60}), estruturas de carbono que formam uma esfera perfeita (Kroto et al., 1985; Figura 2) deram origem a estudos que estão inseridos no que hoje se denomina nanomateriais. Estes novos materiais apresentam grande número de possibilidades em aplicações tecnológicas, devido às suas propriedades magnéticas, ópticas, mecânica e alta condutividade elétrica (Huczko, 2002).
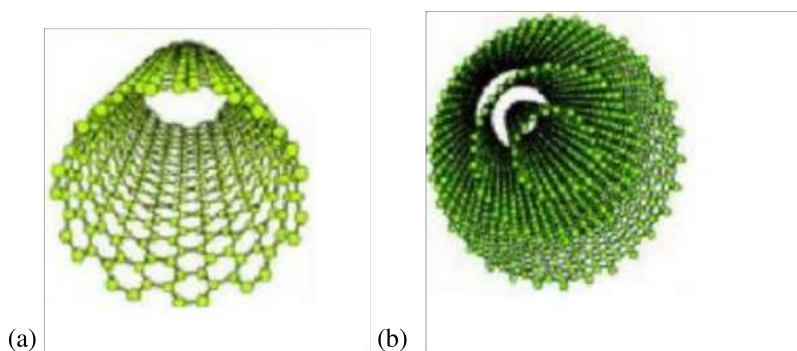

(a)   (b)

Figura 1: (a) CNT de parede simples (SWCNT), (b) CNT de paredes múltiplas (MWCNT). Extraído de Dresselhaus *et al.* (1998).
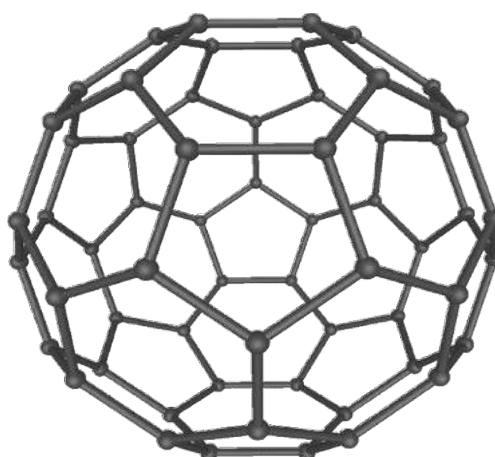

Figura 2: Modelagem gráfica computacional da molécula do fulereno C_{60}. Fonte: Wikimedia Commons, Wikipedia. http://en.wikipedia.org/wiki/File:C60a.png (acessado em 03/2015).

Os CNT são materiais extremamente resistentes por serem extremamente elásticos (Huczko, 2002). Apresentam interessantes propriedades eletrônicas, óticas e mecânicas. Estas características têm feito que estes nanomateriais tenham sido usados para produção de diferentes tipos de dispositivos, como emissores de elétrons, sensores de gases e sensores biológicos (Souza Filho e Fagan, 2007; Chen et al., 2012). Raffa et al., 2010; Zhao et al., 2011; Chen et al., 2012). Kuo e Lin (2009) encontraram uma possível aplicação para os CNT como filtro para remoção de metais tóxicos (cádmio) de efluentes industriais. Outra aplicação para os CNT foi apresentada por Upadhyayula et al., (2009) para o tratamento de águas superficiais, as quais podem ter contaminantes tóxicos que os tratamentos convencionais não conseguem retirar. Como alternativa, os CNT foram estudados principalmente por apresentarem uma alta capacidade de absorção e remoção de contaminantes biológicos (Li et al., 2011).

Com as propriedades apresentadas pelos CNT e a grande perspectiva de aplicações, já foram desenvolvidos uma variedade de procedimentos para a sua síntese, gerando moléculas que podem ser de paredes simples (SWCNT) (Figura 1a) ou de paredes múltiplas (MWCNT) (Figura 1b), com o objetivo de obter estruturas livres de defeitos e com alta pureza. No entanto, estas aplicações e características além de apresentar vantagens e aplicações, podem também significar riscos à saúde e meio ambiente, principalmente devido a estas mesmas características físicas e químicas. Atualmente estes nanomateriais apresentam uma produção anual na escala de toneladas,(Pérez et al., 2009) e já foi demostrado a presença do nanomaterial dióxido de titânio na água proveniente de tintas prediais (Kaegi et al., 2008). Uma estimativa da produção anual de nanomateriais para 2020 é de 58.000 toneladas (Maynard 2006; Nastassja et al., 2008).

As condições de síntese dos CNT são variadas e complexas, porém tais métodos apresentam alguns pontos em comum: os catalisadores que geralmente são metais de transição ou misturas deles, em escala milimétrica e que são utilizados como guias na formação dos CNT. Cada técnica parece possuir uma mistura ideal, sendo o níquel, o cobalto, o ferro e o cobre os elementos mais utilizados (Ferreira, 2003). Com isso, ao final da síntese, além dos CNT formados, também são encontrados carbono amorfo (grafite em escala milimétrica), fulerenos e resíduos de metais de transição utilizados como catalisadores.

Os metais de transição induzem estresse oxidativo nas células, nos tecidos e nos biofluídos (Kagan et al., 2005). Consequentemente, as respostas inflamatórias causadas pela presença combinada dos CNT com os metais podem ser particularmente prejudiciais (Kagan et al., 2005). A toxicidade aguda destas nanopartículas metálicas de cobre foi testada tanto em solução, como em

suspensão, utilizando o peixe *Danio rerio*, demonstrando que resíduos de cobre que possam existir como resquício de catalisadores podem apresentar toxidade para o peixe zebra com uma concentração de 1,5 mg/L (Griffitt et al., 2007). Isto salienta a importância de que os CNT estejam sem nenhum resquício de catalisadores para avaliar apropriadamente sua toxicidade intrínseca.

Em estudos realizados *in vivo* com ratos expostos por via respiratória, a SWCNT contendo contaminantes como Ni, Y, Al, Cu, Mo, Zn e Co, foram observadas respostas inflamatórias nos pulmões (Kagan et al., 2005). Quando ratos foram expostos por via intra traqueal a MWCNT sem resíduos de metais, apresentaram como efeito apoptose em macrófagos alveolares, sem evidências de resposta inflamatória (Elgrabi et al., 2008). Estes resultados levam a questionamentos de como este nanomaterial deve se comportar no meio ambiente atual, composto por diversos contaminantes ambientais. Para Pérez et al. (2009), os CNT funcionalizados são de difícil agregação e sedimentação em sistemas aquáticos, aumentando assim a persistência, estabilidade e probabilidade de serem incorporados pelos organismos aquáticos. Sendo assim, os CNT geram dúvidas no que diz respeito aos seus potenciais efeitos tóxicos (Cocco, 2008). Desta forma, há necessidade de determinar se estes materiais têm um impacto sobre as espécies aquáticas e qual o nível de exposição aceitável. Além disso, há a necessidade de desenvolver modelos para testar os potenciais efeitos dos diversos tipos de partículas em invertebrados e vertebrados aquáticos (Klaper et al., 2010) .

Em estudo realizado com larvas do anfíbio (*Xenopus laevis),* foram colocados DWCNT (nanotubos de carbono de parede dupla) em diferentes concentrações na água durante 12 dias em condições de laboratório, observaram a toxicidade aguda, analisando mortalidade e taxa de crescimento. Os resultados mostraram que a toxicidade dos DWCNT foi relacionada ao bloqueio físico das brânquias e trato digestivo (Mouchet et al., 2008). Em estudo *in vivo* com embriões de *Danio rerio* utilizando MWCNT com marcador fluorescente, verificou-se que nos primeiros 30 minutos após a injeção de MWCNT, este estava presente em todas as partes do embrião, e após 48 h não era mais visualizado. No entanto, a taxa de sobrevivência foi menor no grupo exposto ao MWCNT do que no grupo controle (Cheng et al., 2009), restando assim muitos questionamentos sobre os efeitos deste CNT.

Quando foi testada a toxicidade de SWCNT e C_{60} na truta arco-íris (*Oncorhynchus mykiss*) exposta através da ração, Fraser et al. (2011) não verificaram efeitos tóxicos significativos através do método de Tbars, no entanto, verificou patologias através da métodos histológicos.

Os nanomateriais de Carbono apresentam propriedades químicas e físicas que podem interferir em algumas metodologias que são utilizadas para avaliar a toxicidade destes materiais. Uma das principais metodologias empregadas por grupos que estudam estresse oxidativo é a diclorodihidrofluoresceína diacetato (H_2DCF-DA) um fluorocromo (Martin *et al*., 2011; Aranda *et al*., 2013; Kong *et al*., 2013). Devido a estes problemas relatados em diferentes metodologias que utilizam fluorescência, deve ser salientado que o estudo dos nanomateriais ainda carece de metodologias apropriadas, porque os métodos utilizados até o presente momento não contavam com as variáveis do tamanho e a maior área de superficial apresentadas pelos nanomateriais. O desenvolvimento de metodologias apropriadas são de extrema importância na atualidade, para poder fornecer respostas próximas da ocorrência natural em termos ecotoxicológicos, onde também pouco se sabe sobre o que acontece em níveis bioquímicos e moleculares. Sendo assim, estudos *in vivo* são de extrema importância para fornecer dados sobre os efeitos no meio ambiente e nos organismos exercidos pelos nanomateriais, aspecto que gera subsídios de conhecimento aplicados para o estabelecimento de normativa de uso seguro destes produtos.

JUSTIFICATIVA DO PRESENTE ESTUDO

A disposição para realizar esta pesquisa se justifica pela importância dos ambientes aquáticos em estudos de impactos ambientais, e os peixes representam um bom modelo para o estudo destes, toda vez que muitos contaminantes acabam escoando para ambientes aquáticos, onde causam grandes danos em todo ambiente.

O modelo biológico escolhido para o trabalho foi o peixe *Danio rerio* (Ciprinidae, Teleostei), chamado comumente pela comunidade científica de "zebrafish". É uma espécie de climas quentes endógena do sul-asiático (Lawrence, 2007) e é considerado um ótimo modelo para estudos ecotoxicológicos tanto *in vivo* quanto *in vitro,* devido a sua relativa facilidade de manutenção em biotério e ter todo seu genoma sequenciado (Best e Alderton, 2008). Estes animais são comumente utilizados para estudos com nanotoxicologia (Griffitt et al., 2007; Cheng et al., 2009; Reddy et al., 2011; da Rocha et al., 2013).

Portanto, a simulação de uma situação mais próxima da ocorrência natural é o principal foco em estudos ecotoxicológicos. Neste estudo, realizamos uma verificação de uma possível contaminação do corpo de água onde os animais acabam vindo a utilizar o CNT juntamente com o alimento.

OBJETIVOS

Objetivo geral

Verificar o potencial de toxicidade de nanotubos de carbono de paredes simples (SWCNT) e múltipla (MWCNT) através da dieta no organismo aquático *Danio rerio* (Cyprinidae).

Objetivos Específicos

Verificar se os nanotubos de carbono de paredes simples (SWCNT) e múltipla (MWCNT) adicionados via dieta no organismo aquático *Danio rerio* (Cyprinidae) causam:

- Realizar uma revisão bibliográfica sobre Nanoecotoxicologia em especies de peixes.

- Alteração no dano oxidativo lipídico em brânquias, cérebro, intestino e músculo.

- Alteração na concentração de proteínas oxidadas (grupos carbonila) através de cortes longitudinais onde observe-se todos os seus órgãos.

- Verificar alterações histológicas observando o organismo aquático *D. rerio,* através de cortes longitudinais onde observe-se todos os seus órgãos.

- Verificar possível interferência dos nanomateriais de carbono no método de determinação de dano lipídico através da concentração de substâncias reativas ao ácido tiobarbitúrico (TBARS).

Esta tese foi realizada em três partes sendo a primeira uma contribuição como co-autor no capítulo 11 "Nanoecotoxicology in Fish Species" do livro "Pollution and Fish Health in Tropical Ecosystems" publicado em (2013). Na segunda parte foi realizada a exposição de peixes *Danio Rerio* a SWCNT e MWCNT, originando o manuscrito submetido a revista *Chemosphere*, fator de impacto 3,34 com o título "Histology, immunohistochemistry and lipid peroxidation in the fish *Danio rerio* (Cyprinidae) fed with single and multi walled carbon nanotubes" este experimento apresentou resultados que foram primordiais para a concepção do próximo e terceiro trabalho no qual foi verificada a possível interferência gerada por nanomateriais de carbono a os métodos flurimétricos o qual gerou o manuscrito a ser submetido a revista Ecotoxicology and Environmental Safety, fator de impacto 2,76 com o título "Carbon nanomaterials interference in TBARS (thiobarbituric acid reactive substances) Test".

Capítulo do Livro: **Pollution and Fish Health in Tropical Ecosystems**

Capítulo 11 do livro "**Nanoecotoxicology in Fish Species**" (2013)

Editores: Eduardo Alves de Almeida e Ciro Alberto de Oliveira Ribeiro

CHAPTER **11**

Nanoecotoxicology in Fish Species

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Introduction

Nanotechnology has been developing rapidly in the last few years and, as a consequence, nanomaterials (NM) already are being used in several commercial products. As NM possess extremely low dimensions (up to 100 nm), they present a high surface/volume relationship, making possible their application in cosmetics, foods, medicine and electronics, among others (Wang et al. 2011). Owing to increased production and applications of NM, the aquatic environment is a potential sink and the exposure of these NM with aquatic biota is expected. In this context, some questions arise: (a) Do

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NM have the potential to induce toxicological effects in aquatic organisms? and (b) what are the toxicity mechanisms elicited by these NM?

Some studies have evaluated the effects of several NM in different aquatic organisms; however, the toxicity effects of materials at the nano-scale dimension has sometimes shown differences to those induced by bulk materials of the same composition (Li et al. 2008). Thanks to their unique characteristics, NM have been attracted great interest in application of many biological fields (Kim et al. 2011). However, the physical-chemical properties, large surface area, chemical activity and high penetration power of NM can be also regarded as potentially harmful to both environment and living organisms (Matranga and Corsi 2012).

The development of nanotechnology is ahead of the evaluation of the impact of NM on the environment, plants and animals. As a matter of fact, data about potential effects of NM are still limited, especially concerning aquatic organisms. Therefore, there is a need to raise investments (both economically and intellectually) in toxicological studies that assess the effects of NM on aquatic organisms from an ecotoxicological perspective. For this, it is necessary to consider some aspects related to the characteristics of NM, route and condition of exposure and mostly the species analyzed.

NM are grouped according to their chemical composition such as carbon (fullerene, graphene and nanotubes), metallic oxides (for example: titanium dioxide, zinc oxide, cerium dioxide), metals (silver, gold) and semiconductors (for example: quantum dots) (Krysanov et al. 2010). Characteristics such as shape, mass, surface area, aggregation, agglomeration, solubility, and surface chemical can influence the NM's biological fate; for example, if the particle will be dispersed, deposited or eliminated (Moore 2006). Consequently, these characteristics will influence the biological effects such as inflammatory responses, cytotoxicity, immunomodulation, genotoxicity, cell transformation and oxidative stress (Bergamaschi 2012).

Although nanotoxicological studies do not accompany the development of nanotechnology at the same scale, some studies have analyzed the effect of several NM in different species including fish. In this chapter, we will discuss some important topics such as sources and environmental fate, routes of exposure, toxicity of NM considering biotic and abiotic factors, interaction of NM with other pollutants, mechanisms of action and biological effects. This body of information certainly will contribute towards clarifying some aspects that are extremely important for the development of nanotoxicology.

In this context, the use of fish as animal models can be compared with other organisms. The review of Kahru and Dubourguier (2010), analyzed the toxicological knowledge of several biological groups, shown in Table 11.1.

Table 11.1. Sensitivity of different organisms to inorganic (nano TiO_2, nano ZnO, nano CuO, nano Ag) and organic (SWCNT, MWCNT, C_{60}) nanomaterials. Up arrows indicate the most sensitive organism group. Gray boxes stand for existing toxicological data. White boxes indicate absence of toxicological information. Data based from the study of Kahru and Dubourguier (2010). SWCNT: single-wall carbon nanotubes. MWCNT: multi-wall carbon nanotubes. C_{60}: fullerene.

Organism	NanoTiO$_2$	Nano ZnO	Nano CuO	Nano Ag	SWCNT	MWCNT	C$_{60}$
Crustaceans				↑		↑	
Bacteria							
Algae	↑	↑	↑		↑		
Fish							
Ciliates							↑
Nematodes							
Yeasts							

As can be seen, toxicological data for both organic and inorganic NM exist for fish organisms, although it seems less sensitive than other biological groups such as algae. It is also important to consider quantitative aspects of the published studies in econanotoxicology and the impact on the scientific community. Using SCOPUS (www.scopus.com) as a database of scientific publications, surveys were carried out on a number of papers and the *h* index registered (Hirsch 2005), using key-words like "nanoparticles", "toxicity" and the name of each organism group shown in Table 11.1.

It is important to note that, according to data from Fig. 11.1, fish is the second organism group in terms of number of published articles and
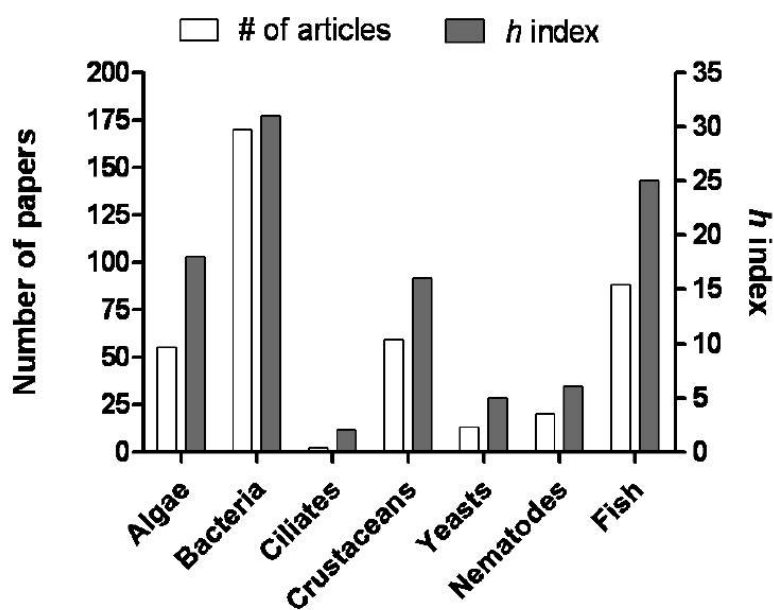

Figure 11.1. Number of published papers (white bars) and *h* index (grey bars) registered on 06/28/2012 in SCOPUS database (www.scopus.com).

h index, surpassed only by the bacteria group. However, it is important to state that several papers on bacteria deal with antibiotic responses induced by NM, more focused on biomedical than on ecotoxicological or environmental aspects. It is evident that nanotoxicology is still "crawling" and more studies should be performed with different species and NM to extend our knowledge about biological effects induced by NM in fish and other aquatic organisms and their interaction with the environment.

Variety and quality of toxicological information are thus needed in order to implement new policies considering the release of these NM into the environment and the associated risks. The current state of knowledge and scientific needs will also be discussed in this chapter.

Source and Environmental Fate of Nanomaterials

NM are abundant in nature, produced in processes as volcanic eruption, photochemical reactions, forest fires and simple erosion, for example. However, many NM are of anthropogenic origin, as they are by-products of cooking, ore refining and smelting, simple combustion, chemical manufacturing, welding, combustion in vehicles, airplane engines and others. In this context, Hoyt and Mason (2008) suggest that the term "nanoparticles" (NP) or "nanomaterials" (NM) should only be applied to those from engineered technology, such as fullerenes, metal oxides, carbon nanotubes, etc. (Buzea et al. 2007).

Engineered nanomaterials present many unique properties when compared to conventional formulations of the same material (Ferrari 2005; Vasir et al. 2005; Qin et al. 1999; Webster et al. 1999, 2000). These singularities led to their use in a wide range of fields, from medical applications to environmental sciences. However, there is no complete and comprehensive review of nanotechnology applications, likely due to the rapid development of this field (Arora et al. 2012).

NM are increasingly being used for commercial purposes and are commonly used in many products on the market as in sporting goods, cosmetics (Farkas et al. 2011), stain-resistant clothing, surface disinfectants, food additives, medical applications (Ferrari 2005; Vasir et al. 2005) besides their general use as microelectronics, synthetic rubber, catalytic compounds, photographic supplies, inks and pigments, coatings and adhesives, ultrafine polishing compounds, UV absorbers for sun screens, synthetic bone, hazardous chemical neutralizers, diesel and fuel additives, fluids iron, optical fiber cladding and other related products (Buzea et al. 2007).

A recent report from a project on Emerging Nanotechnologies shows more than 1,000 nanotechnology based consumer products were on the market in 2009 (Woodrow Wilson International Center for Scholars 2009), where silver nanoparticles (AgNP) were the most frequently used NM due

to their antibacterial/antifungal effects (Pal et al. 2007; Martínez-Castanõń et al. 2008). Following this study, major areas of use of AgNP in consumer products include fitness and health care products such as cosmetics, cloth and sporting devices, personal care products and others like air sanitizer sprays, socks, pillows, slippers, detergent, air filters, coatings of refrigerators, vacuum cleaners, washing machines, food storage containers, cellular phones, and even in liquid condoms (Buzea et al. 2007). Other engineered NM that have been in focus are titanium dioxide (nano-TiO_2), zinc oxide (nano-ZnO) and aluminum oxide (nano-Al_2O_3), which are widely used in many products such as white pigment, food colorant, sunscreens and cosmetic creams (Donaldson et al. 2004) and sterilizing equipment of environmental microorganisms in health care facilities (Shimtani et al. 2006).

With their large production and widespread application, quantities of NM are growing and it is expected that significant levels of such materials will inevitably enter the environment and end up in water either from industrial and domestic products, and wastes containing NM, both directly into rivers and lakes via outdoor use of sunscreens, or indirectly via surface run-off, domestic or industrial wastewater (Zhang 2003; Aitken et al. 2006; Moore 2006; Vaseashta et al. 2007; Baun et al. 2008; Wiesner et al. 2009; Gottschalk et al. 2011; Bhawana and Fulekar 2012). Besides, the use of NM foreseen for applications in environmental remediation techniques is another expected way of its direct release into the aquatic environment. However, little is known about the environmental fate and effects of NM leached from consumer products. Also, unfortunately, the quantitative detection of NM and the distinction between naturally NM occurring in the environment are still extremely limited (Limbach et al. 2008; Tiede et al. 2008; Hassellöv and Kaegi 2009; Gottschalk et al. 2011).

In this regard, scientific efforts have been particularly focused on aquatic pollution (Battin et al. 2009; Farre et al. 2009; Perez et al. 2009). Although it has shown that NM can promote some level of protection against oxidative stress (Ahlbom et al. 2009), many studies have observed that NM can accumulate and are toxic (Hussain et al. 2005; Vinardell 2005; Adams et al. 2006; Dhawan et al. 2006; Griffitt et al. 2007; Heinlaa et al. 2008). Thus, there are serious concerns over NM risks once released into the environment; understanding the transfer, transport and fate of NM in the environment is significant for evaluating their environmental and health impact (Helland et al. 2007; Nowack and Bucheli 2007).

Given its complexity, the mineral and organic compositions and structural heterogeneity of natural media must be taken into account to understand the transport and fate of NM under natural conditions. Almost nothing is known about how NM interact with soils and sediments (Oberdörster et al. 2006; Wiesner et al. 2006). Solid and dissolved matter

can be powerful geosorbents that affect NM mobility. Recently, it has been shown that organic matter can interact with NM in a way that may influence their transport and dispersion (Hyung et al. 2007; Yang et al. 2009), and some environmental factors, such as pH and ionic strength, may determine if NM are attached within or transported out of soils and sediments towards the water (Luthy et al. 1997; Brant et al. 2005). Furthermore, behavior of NM in the environment can be influenced by their structure, concentration, physical-chemical properties and technically intended modifications in functionalization or coating characteristics (Huuskonen 2002; Schwarzenbach et al. 2003; Mathivanan et al. 2012).

NM employed in laboratory assays have been so far characterized by using a multiplicity of techniques, but accurate analysis for detection in environmental samples still requires the development of new and effective methods, and the use of complex detection techniques of variable resolution power (nanometric, micrometric and metric). Clearly, available analytical methods for characterizing NM in the environment must be improved in terms of sensitivity and selectivity (Tiede et al. 2009; Simonet and Valcárcel 2009).

As addressed above, consumer products are a likely source of NM entering into the environment. According to Gottschalk et al. (2009), it must be assumed that different release pathways of these materials to the environment can occur during the product's life-cycle. Indeed, some studies have already shown the release of NP from commercially available products as nanosilver washing machines and nano-textiles products (Benn and Westerhoff 2008; Kaegi et al. 2008; Geranio et al. 2009; Kaegi et al. 2010; Farkas et al. 2011). At the same time, some other attempts have been made to predict future environmental concentrations of some of the most frequently used NP through computational models using probabilistic/stochastic methods of environmental exposure analysis (McKone and Bogen 1991; van der Voet and Slob 2007; Mueller and Nowack 2008). These approaches intend to consider all possible inputs in the models, also covering extreme events. They also provide an insight into the frequency probability of each simulated outcome. These probabilistic modeling approaches have been used to describe flows of environmental NM in Switzerland (Gottschalk et al. 2009; Gottschalk et al. 2010).

According to a few models also used to assess aquatic exposure to NM, probabilistic methods of environmental exposure analysis allow for explaining some inconsistency and variability of input parameters by using probability (or density) distributions. Those input distributions may be constructed based on empirical data, on expert judgment or on a combination of these sources (Scheringer et al. 1999; Blaser et al. 2008; Arvidsson et al. 2011; Tuoriniemi et al. 2012).

For the future, there is a need to check and amend the methodological variability of the data applied to existing models that assess the fate of NM in the environment. It is important that standardization of the methodology be developed and implemented so that variability may be maintained at a minimum, with results widely accepted and replicated.

Besides, design and development of models to assess the release of NM and their fate in the environment are essential to estimate environmental exposure. Such estimations have to cover diffuse emissions from a large number of relevant NM containing products and life-cycle stages. These include, but are not limited to, nanoparticles release into the environment from NM production, nanoparticles incorporation into products and storage, use, waste generation and disposal of such products (Gottschalk et al. 2011). These models need to be challenged with high quality data and modified as necessary to take into account any effect that is "nano-specific". This will be an iterative and continuous process, that should lead to improvements in models for faster evaluations.

Routes of Exposure

The exposure routes to xenobiotics, including NM, in juvenile and adult fish are the gills surface and food intake. In fish embryos, the egg surface is the target, although the chorion represents a protection, with pores of 0.5–0.7 μm in diameter (Rawson et al. 2000).

Exposure through Water

Three fish species have been the most studied using NM exposure via water: the medaka fish (*Oryzias latipes*), the rainbow trout (*Oncorhynchus mykiss*) and the zebrafish (*Danio rerio*). In the adult medaka fish exposed to latex nanoparticles, accumulation mainly occurred in the gills and intestine, but they were also detected in brain, testis, liver and blood (Kashiwada 2006). In another study, morphological and histopathological changes were observed in the gills and intestine of medaka fish exposed to nano-iron, besides a disturbance in the activity of the antioxidant enzyme superoxide dismutase (SOD) and levels of reduced glutathione (GSH) in liver and brain (Li et al. 2009). Adult rainbow trouts exposed to single-wall carbon nanotubes (SWCNT) also presented gill histopathologies and augmented ventilation rate, increased activity of Na^+K^+-ATPase in the gills and intestine, and pathologies in the brain and liver (Smith et al. 2007). TiO_2 nanoparticles injured rainbow trout gills (Federici et al. 2007) and Ag nanoparticles were also concentrated in the gills, inducing the expression of *cyp1a2* (Scown et al.

2010). In zebrafish gills, Cu nanoparticles exposure enlarged gill filaments and induced a distinctive gene expression profile (Griffitt et al. 2009). These studies demonstrate that exposure to NP via water affects mainly gills and intestines at both morphological, biochemical and genetic level.

Exposure through Food Intake

Few studies have considered NM incorporation through food. Juvenile rainbow trouts that consumed ration containing SWCNT for six weeks had only one modification on the several parameters analyzed, an increase in brain TBARS (lipid peroxidation) at week four (Fraser et al. 2011). The crustacean *Daphnia magna*, treated with TiO_2 nanoparticles, transferred these NP via diet to zebrafish, although no biomagnification was observed (Zhu et al. 2010).

Nanomaterials Exposure to Embryos and Larvae

Although there are few studies in the area, they make it evident that exposure to nanoparticles may influence the development of embryos and larvae of fish. Zebrafish embryos exposed to fullerene showed an increase in body malformations, pericardial edema, necrotic and apoptotic death and mortality (Usenko et al. 2008). Furthermore, at 36 hours and 48 hours after fertilization, modifications occurred in the expression of several genes, like ferritin, α-tocopherol transport protein, heatshock protein 70, glutathione-S-transferase and glutamate cysteine ligase (Usenko et al. 2008). In addition, studies with TiO_2 nanoparticles were implicated in impairment of zebrafish reproduction (Wang et al. 2011), with a reduction in the number of zebrafish eggs (Wang et al. 2011) and affected larval swimming parameters (Chen et al. 2011). The medaka embryos exposed to TiO_2 nanoparticles also presented pericardial edema and other indicators of toxicity such as premature hatching and, after hatching, moribund swimming behavior and mortality (Paterson et al. 2011).

Toxicological Effects of Nanomaterials on Fish Species

Understanding the effects of NM on fish is therefore an important aspect when considering the effects of NM on the aquatic environment as a whole. Potential routes of uptake for NM in fish include absorption via the gill epithelia, via the gut epithelia as a result of dietary exposure and drinking or via the skin (Handy et al. 2008), as explained in the previous section.

Carbon-based Nanomaterials

One of the first *in vivo* exposure studies of fish to NM to emerge was one that analyzed fullerene toxicity responses in juvenile largemouth bass *Micropterus salmoides* (Oberdorster 2004). The finding that oxidative stress was detected in the brain alerted the scientific community to the potential hazards associated with the release of NM into the aquatic environment. Henry et al. (2007) conducted a similar study using larval zebrafish and found changes in gene expression and reduced survival in zebrafish exposed to fullerenes dispersed using the organic solvent tetrahydrofuran (THF), as well as in water to which THF had been added and evaporated off. Analysis of the THF-fullerene media and THF-water by gas chromatography-mass spectrometry did not detect THF but detected an oxidation product, butyrolactone, which has an LC_{50} of 47 mg/L for zebrafish, suggesting that the effects observed in fish exposed to fullerene prepared in this way may be attributable to this degradation product. As a result of the issues surrounding the preparation of stable suspensions of fullerene, the present literature concerning *in vivo* exposure of fish species to fullerene does not yet provide clear information regarding the effects, as so many studies have employed the use of solvents to aid dispersion. However, some studies have in fact showed that aqueous suspensions of fullerene can in fact induce oxidative stress in fish or even in bacteria associated with mucous fish surface (Letts et al. 2011; Britto et al. 2012; Ferreira et al. 2012).

A number of studies have assessed the effects of carbon nanotubes in zebrafish embryos. Embryos treated with simple-walled carbon nanotubes (SWCNT) and double-walled carbon nanotubes (DWCNT) caused hatching delay at concentrations over 120 mg/L and 240 mg/L, although 99% of embryos hatched by 72 hours post fertilization (hpf). Embryonic development was not found to be affected and the authors suggested that as chorion pores are nano-scaled, they may provide a protective barrier against penetration by the micro-scaled carbon nanotubes (CNT) aggregates. Hatching delay was therefore attributed to trace levels of residual cobalt and nickel catalysts in the CNT. Carbon black nanoparticles were found to have no effect on hatching at similar concentrations (Cheng et al. 2007). Embryos treated with multi-walled carbon nanotubes (MWCNT) exhibited dose dependent increases in mortality and decreased hatching as well as bradycardia, slowed blood flow and apoptosis. Mortality of 100% was reached at a concentration of 200 mg/L and at concentrations of 60 mg/L and above, embryos showed deformation of the notochord and increased mucus production in the intrachorion region (Asharani et al. 2008a). The authors did not provide details of trace metal concentrations in nanotubes preparations; however, the concentrations where hatching delay was observed were similar to that seen in the study by Cheng et al. (2007).

A further study by Cheng et al. (2009) found that fluorescent-labeled MWCNT microinjected into zebrafish embryos at the 1-cell stage was distributed to all blastoderm cells and excluded from the yolk cell; after being introduced into the circulation system, it was removed from the body after 96 hours. In juvenile rainbow trout *Oncorhynchus mykiss* exposed to SWCNT, a dose-dependent rise in ventilation rate was observed. Accumulation of SWCNT aggregates was associated with gill mucus (Smith et al. 2007).

The results of these studies suggest that carbon-based NM, such as fullerenes and nanotubes, have the capacity to induce toxicity in aquatic vertebrates both as a function of their chemistry by inducing oxidative stress and as a result of their aggregation, probably causing physical damages.

Titanium Dioxide Nanoparticles

Exposure of zebrafish embryos to TiO_2 nanoparticles have so far been shown to be relatively non-toxic when compared with effects seen in exposures to carbon-based nanoparticles. No toxicity was observed in zebrafish embryos exposed to 30 nm TiO_2 comprised of 20% rutile and 80% anatase crystals at concentrations of up to 10 mg/L (Griffitt et al. 2008); a further study by the same research group found exposure of adult zebrafish to 1.0 mg/L of the same TiO_2 nanopartices caused no changes in molecular or histological parameters in zebrafish gills (Griffitt et al. 2009). In contrast, however, a 14-day semi-static exposure of rainbow trout resulted in gill oedema and thickening of the gill lamellae as well as decreases in Na^+, K^+-ATPase activity in the gills and intestine (Federici et al. 2007).

A subsequent 8-week oral exposure to rainbow trout by the same research group found that concentrations of up to 100 mg kg^{-1} in the food had no impact on growth. An increase in TiO_2 content in the liver and spleen was observed in the early stages of the exposure but no effect was seen on haematological parameters or TBARS (lipid peroxidation), suggesting that adverse effects of TiO_2 nanoparticles on rainbow trout are more severe as a result of water-borne exposure than by exposure via the diet (Handy et al. 2008). In a study using a rainbow trout gonadal cell line (RTG-2), no adverse effects were observed in cells at concentrations of up to 50 µg TiO_2 nanoparticles/mL, although increased levels of DNA strand breaks were reported after exposure to TiO_2 under UVA radiation (3 kJ/m^2) (Vevers and Jha 2008).

Very few studies in fish have examined the uptake and partitioning of TiO_2 nanoparticles within the body as result of exposure, probably due in part to the difficulties involved in measuring low levels of TiO_2 and limitations in analytical equipment. A study by Moger et al. (2008), however, used coherent anti-Stokes Raman Scattering (CARS) to examine the gills of rainbow trout exposed to 5,000 µg/L TiO_2 nanoparticles and confirmed the

presence of small numbers of particle aggregates within the gill tissue. Two other studies have demonstrated the enhanced accumulation of the heavy metals arsenic and cadmium in the viscera and gills of carp when exposed to the presence of TiO_2 nanoparticles (Sun et al. 2007; Zhang et al. 2007).

Silver Nanoparticles

To date, the majority of *in vivo* exposures of silver nanoparticles to fish have been in zebrafish models. Effects in embryo exposures have shown similarities between effects seen with carbon-based nanoparticles although the exact mechanisms of toxicity have not yet been elucidated.

Zebrafish embryos exposed to 5–20 nm silver nanoparticles, capped with starch or bovine serum albumin (BSA) to aid dispersion, exhibited a dose-dependent increase in mortality and hatching delay and dose-dependent toxicity which was typified by larvae with deformations of the notochord, slow blood flow, pericardial oedema and cardiac arrhythmia. Distribution of silver nanoparticles in the brain, heart, yolk and blood was demonstrated by transmission electron microscopy (TEM) and apoptosis was also seen in 50% of the embryos treated with 50 µg/mL and above (Asharani et al. 2008b). Another study also found decreased hatch rate, notochord abnormalities and weak heart beat in embryos treated with 10–20 nm silver nanoparticles. Catalase activity was found to be increased in exposed embryos and the expression of Sel N1, a gene associated with notochord development and heart disease in zebrafish, was significantly lower in exposed fish. The study also confirmed the presence of silver ions (Ag^+) in the exposure media to which the authors attributed the detrimental effects seen (Yeo and Kang 2008).

Unlike the aggregates of carbon nanotubes that were unable to pass through chorion pores, silver nanoparticles of 5–46 nm have been shown to be transported in and out of chorion pore channels by Brownian diffusion (Lee et al. 2007). Another study that evaluated the effects of silver nanoparticles in zebrafish fry found that although 26 nm silver particles exhibited toxicity, silver ions were found to be over 300 times more toxic to zebrafish fry on a mass basis (Griffitt et al. 2008). This led to a further study where zebrafish were exposed to 26 nm silver nanoparticles at their previously ascertained no observable effect concentration (NOEC) of 1,000 µg/L. Analysis of global gene expression in the gills found differences in response between nanoparticle exposed fish and fish exposed to soluble silver ions, suggesting that the biological effects of exposure to silver nanoparticles do not appear to be driven solely by the release of silver ions (Griffitt et al. 2009).

Effects of Nanomaterials on Nervous System

In general, most molecules cannot cross the blood-brain barrier (BBB) but NM made of certain materials and with varying sizes can cross this barrier and enter into the brain or enter by olfactory bulb and these NM can be administered to the animal body via several routes including inhalation, oral administration and injection (Hu and Gao 2010).

Experiments performed with rats and fish have arisen the idea that carbon NM can be taken up by olfactory neurons and are translocated to the brain (Oberdorster 2004; Simkó et al. 2008; Belyanskaya et al. 2009); it was also shown that some NM can cross the BBB, affecting brain signaling linked to Alzheimer's and Parkinson's diseases and decrease of cognitive function (Wu et al. 2012).

The blood-brain-barrier (BBB) protects the central nervous (CNS) system from harmful xenobiotics and endogenous molecules but it has been shown that NM from the blood circulation may influence endothelial cell membrane and/or disrupt the BBB, inducing vesicular transport to gain access into CNS (Chen et al. 2008). Moreover, NM can induce oxidative stress generating free radicals that could disrupt the BBB and cause dysfunctions (Simkó and Mattsson 2010). According to Smith and colleagues (2007), SWCNT may damage the cardiovascular system of fish or alter the permeability of the BBB and when vascular lesions are associated with a vital organ such brain, these responses will increase the risk of stroke and mortality. Altered neurotransmitter levels (dopamine and serotonin) were observed in zebrafish larvae exposed to Ag^+ (released by silver nanoparticles), neurotransmitters that play important roles in reward, anxiety and sensorimotor integration such the deficits in swimming behavior (Powers et al. 2011). Also, inhibition of Na^+, K^+-ATPase activity in the rainbow trout brain was observed after exposure to TiO_2 nanoparticles (Ramsden et al. 2009) .

Abiotic Factors Influencing Nanomaterials Toxicity

The aggregation state, morphology and surface charge of NP in the aquatic environment determines its reactivity, transportation, fate and distribution, and consequently, its bioavailability. From an environmental point of view, the inter-relationship between NM and abiotic variables is a key issue because abiotic environmental factors influence and control the stability, aggregation dynamics and surface chemistry of NM and, consequently, its effects on fish and other organisms (Handy et al. 2008; Kusk et al. 2008; Peralta-Videa et al. 2011). Some representative abiotic factors are discussed below.

Ionic Strength and pH

The persistence of NM in the water column strongly depends on the formation of stable colloidal particles. The diffuse electrostatic double layer surrounding the particles which prevents aggregation is maintained by a balance between the van der Waals attraction forces and the electrostatic repulsion forces, as stated by the classic DLVO theory (Derjaguin and Landau 1941; Verwey and Overbeek 1948). The presence of ions in the medium compress the diffuse layer, increasing the attraction forces, decreasing the zeta potential and causing aggregation and sedimentation (Zha et al. 2002; Cosgrove 2005). The valence of the surrounding ions also determines the rate and size of the aggregation. Depending on the surface charge density of the NM, bivalent cations can cause faster and more pronounced aggregation than monovalent cations in the same concentrations. Likewise, ionic strength and pH of the medium interrelate with the particle surface charge in a way that changes in the pH decrease the electrolytes concentration necessary to cause aggregation (Chen and Elimelech 2006; Lowry and Wiesner 2007; French et al. 2009; El Badawy et al. 2010). These factors must be taken into account when predicting the impacts of NM in aquatic environments that present particularities as variations in pH, salinity and water hardness as seen in estuarine, coastal and some surface waters.

Organic Matter

Natural dissolved organic matter (DOM) is ubiquitous in aquatic environments and can reach up to 100 mg/L in fresh water (Paul et al. 2006). Depending on their sources, such as degradation of lignins, tanins and algae, DOM is composed by a complex mixture of humic substances (humic and fulvic acids) and non-humic compounds (as polysaccharides) that can react with ions forming colloids in water (Uyguner-Demirel and Bekbolet 2011). Surface coating of NM by DOM regulates the behavior of NM by enhancing the colloidal stability, mobility and deposition in water (Keller et al. 2010; Jiang et al. 2012; Kim et al. 2012). For organic NM, whose main mechanism of reaction is through π-π interactions, DOM coating increases steric repulsion and avoids particle aggregation. This is due, in part, to the content of phenolic substances as phenolate groups that enhance the colloidal stability. Other properties of DOM include the ability to promote redox reactions, which can alter NM chemistry in the environment (Hyung et al. 2007; Lin and Xing 2008; Chappell et al. 2009).

Effects of DOM on NM toxicity in aquatic organisms apparently depend on the species analyzed and are linked to the chemical nature of both humic and non-humic compounds. For example, harmful effects of multi-walled carbon nanotubes on zebrafish embryos, and quantum dots on daphnids

have been reported to be ameliorated only by typically hydrophobic DOM (Lee et al. 2011; Kim et al. 2012). For microorganisms, humic acids also mitigated the toxicity of zero-valent iron and silver nanoparticles (Fabrega et al. 2009; Chen et al. 2011). In all of these cases, the protection performed by DOM was attributed to NM coating that should prevent the direct contact of NM with organisms. However, it was also found that deleterious effects of CuO nNP to algae *Microcystis aeruginosa* were enhanced by pond water with high organic matter content, and this finding was ascribed to the intake of CuO NP bound to nutrients present in DOM (Wang et al. 2011).

Ultraviolet Radiation Incidence

Ultraviolet (UV) radiation is among the factors which may contribute to the increased toxicity of NM. These radiations comprise a range of electromagnetic wavelengths ranging between 200 and 400 nm. The organic NM fullerene (C_{60}) has strong light absorption within the solar spectrum, especially in the UV range and it was demonstrated that C_{60} undergoes surface modification or decomposition under sunlight or UV irradiation (Hwang and Li 2010; Hou and Jafvert 2009; Lee et al. 2009), suggesting that surface-oxidized C_{60} may be an important form of C_{60} in the aquatic environment. Authors like Kamat et al. (2000) have already reported that C_{60} generates reactive oxygen species in the presence of both UV and visible light.

The manufacture of NP such as zinc oxide, aluminum and titanium dioxide (TiO_2) finds wide use in cosmetics and sunscreens because of its ability to reflect, scatter and absorb UV radiation, preventing sunlight related skin disorders. Although TiO_2 has been considered to be biologically inert, recent reports have demonstrated that when TiO_2 is under UVA or UVB radiation, oxidative, genotoxic and cytotoxic effects are observed in cells. UV radiation by itself is known to cause several deleterious effects, ranging from molecular and tissue damage to population level effects and include alterations of relevant biological molecules such as proteins, lipids and DNA (Sinha and Häder 2002; Gouveia et al. 2005).

In this sense, some authors have been concerned with the understanding of ecotoxicological interactions between radiations and TiO_2 NP. Hund-Rinke and Simon (2006) did not registered algae and daphnid mortality using pre-illuminated TiO_2 to examine potential phototoxicity. More recently, Miller et al. (2012) showed that relatively low levels of ambient UV radiation can induce toxicity of TiO_2 to marine phytoplankton, increase overall oxidative stress and cause decreased resiliency of marine ecosystems.

Studying cell lines, Vevers and Jha (2008) showed that TiO_2 engineered nanoparticles plus UVA radiation increases the cytotoxic potential of established fish cell line derived from rainbow trout (*Oncorhyncus mykiss*).

In an *ex vivo* study with gills extracted from carps, Britto et al. (2012) also attributed generation of oxidative stress by singlet oxygen when the organs were exposed to fullerene under UVA incidence. Park et al. (2011) saw that TiO_2 NP in the absence of photoactivation are cytotoxic to HaCaT cells (human keratinocyte) and this effect becomes more pronounced in the simultaneous irradiation of UVA dependent on photocatalytic potential of TiO_2 NP, also causing oxidative stress. Yin et al. (2012) studying the same cell line showed that phototoxicity is mediated by reactive oxygen species (ROS) generated during UVA irradiation. The same phototoxic damage is true in retinal pigment epithelial cells (ARPE-19) (Sanders et al. 2012). Tu et al. (2012) showed that nitrite increased the photo-toxicity of TiO_2 NP in a dose dependent manner, and generated protein tyrosine nitration in keratinocyte cells. Murrat et al. (2012) saw that co-exposure to UVB and superparamagnetic iron oxide (SPIONs) was associated with induction of oxidative stress and release of inflammatory mediators, showing the need to evaluate dermal toxicity of engineered NP on human skin.

Therefore, these results illustrate that phototoxicity must be considered when evaluating impacts the level of organism or environment of NM, many of which are photoactive. Moreover, some scientists also have been concerned about the use of TiO_2 NP in photodynamic therapy for the treatment of cancer cells that has been proposed following studies of cultured cancer cells (Yu et al. 2011).

In aquatic environments, abiotic factors are obviously not isolated, and the interactions among them result in a buffered medium that constantly modifies and is modified by the organisms in a dynamic interchange. Such interactions rule NP fate and effects on aquatic life. For example, it was demonstrated that sunlight incidence in the presence of DOM can induce reduction of $AuCl_4^-$ and Ag^+ forming nano-Au and nano-Ag (Yin et al. 2012). This is important because nanoscaled forms of these metals sometimes are more toxic than their ionic counterparts. DOM can also increase the colloidal stability of CeO_2 NP, but the increase in water pH and ionic strength thwarts DOM adsorption to the NP, causing particle aggregation, and this modulates the NP toxicity to algae (Van Hoecke et al. 2011). These examples reinforce the urgency of understanding the actual consequences to fish, and aquatic life in general, of the entrance of NM in aquatic environments.

Effects of Co-exposure of Nanomaterials with other Pollutants on Fish

NP are diverse substances that, when released in the environment, can interact with each other and other kinds of pollutants (Baun et al. 2008; Handy et al. 2008; Sánchez et al. 2011; Costa et al. 2012), so it is of special importance to take into consideration not only the impact a NP can cause

in the environment, but the interactions it can have with other pollutants possibly present in that environment as well.

Several studies have been performed aiming to analyze the interactions of NM with other kinds of compounds, taking into account the adsorptive interactions between them, generally aiming for their employment as decontaminants of polluted waters (Filho et al. 2007; Pourata et al. 2009; Chen et al. 2011; Bikshapathi et al. 2012). Some of these works focus on the use of NM for other objectives, like increasing the efficiency of antifoulings, mainly trying to reduce their release into the water column from the surface on which they would be applied (Shtykova et al. 2009; Karmali et al. 2012).

It is interesting to note, however, that the interactions between NM and other kinds of substances may cause especially harmful effects, even if at first glance these may appear positively promising. Although, for example, the adsorption between a NM like fullerene (C_{60}) and a pollutant, like arsenic (As), may mean that nC_{60} can be used in the remediation of an environment polluted with this semi-metal, it is known that C_{60} has the propriety of carrying As inside living organisms, raising its incorporation under a "Trojan horse" effect. (Costa et al. 2012). A similar effect seems to happen with C_{60} and phenanthrene (Baun et al. 2008). It is of great importance to first investigate if the cleaning product is toxic by itself or if it interacts negatively with the pollutant it is intended to remove or degrade or even with other substance present in the effluent treated, because depending on these factors, the benefits of using a NM under this context can be very limited (Sánchez et al. 2011).

Taking into account the "Trojan horse" concept of, where contaminants can have their input in organisms enhanced by association with a NM (Limbach et al. 2007), there is a real need to evaluate the consequences of these associations and if they can cause bioaccumulation (Costa et al. 2012). The study of Costa and co-workers (2012), carried out with zebra fish hepatocytes, analyzed the interaction of C_{60}, and arsenic. The results showed higher arsenic (as As^{III}) accumulation in cells co-exposed to C_{60} and As^{III}, although no alterations in cell viability were observed. C_{60} (1 mg/L) when co-exposed to arsenic (2.5 µM and 100 µM) decreased the levels of lipid peroxidation and a peak of reduced glutathione (GSH) registered in cells exposed to the lowest As^{III} concentration (2.5 µM). Thus, although C_{60} seems to act as a "Trojan Horse", increasing the concentration of intracellular arsenic, no evidence of higher toxicity was observed, suggesting that the dynamics of absorption and further releases of toxic molecules once inside the cells should be a critical point for the onset of toxicity under the "Trojan Horse" paradigm. Also, Sun et al. (2009) reported that carp (*Cyprinus carpio*) co-exposed to As^{III} and TiO_2 NP showed significant increase (44%) in the accumulation of arsenic when compared with carps only exposed to As^{III}.

Other studies conducted with carp showed that animals exposed TiO$_2$ NP and cadmium presented higher intracellular concentration of cadmium, confirming that NP have the ability to deliver compounds to the intracellular environment. In fact, the significant increase in cadmium caused biochemical changes which can lead to cell damage (Zhang et al. 2007). The study of Reeves et al. (2008) in goldfish skin cells exposed to TiO$_2$ NP and UVA showed that cells exposed only to TiO$_2$ NP did not show a significant reduction in its viability, even at the highest concentration (1,000 µg/ml). However, cells exposed to TiO$_2$ NP and UVA presented a dose dependent reduction in cell viability, with further increases in DNA damage.

Although there is a good deal of study on bioaccumulation of pollutants in organisms like fish, after co-exposure with different kinds of NM, it seems that studies on the biochemical toxicological interactions between these compounds in fish is being at least comparatively overlooked. This is an important issue, as the uses of NM can be limited in some situations, depending on the toxic interactions these can present with other kind of compounds (Sánchez et al. 2011).

Nanomaterials Contamination Policies

Nanotechnology development has been growing exponentially in the lasts years and consequently, the use of NM in commercial products has accompanied this development. However, little is known about risk assessment in biological systems with these NM (Moore 2006; Kahru and Dubourguier 2010). Inevitably, different kinds of NM should reach the environment and depending on their chemical properties, volume and mode of use, they can affect human and environmental health in the long term (Guillén et al. 2012). Once into the environment, these NM can be incorporated by living organisms and bioaccumulated, with the potential to induce several deleterious effects through many biological pathways.

Thus, it is clearly necessary to increment the knowledge about the risks and benefits of several NM that are being launched in the global market at present. As commented in Section 1, Kahru and Dubourguier (2010) revised the toxicity of several NM in different species considering LC(E)$_{50}$. These authors classified different NM according to the degree of toxicity: extremely toxic (< 0.01 mg/L); very toxic (0.1–1 mg/L); toxic (1–10 mg/L) and harmful (10–100 mg/L). It is important to underline that some of compounds considered very toxic, for example, carbon nanomaterials, are widely used in several products (Krysanov et al. 2010). Although some existing studies have evaluated the toxicological effects of NM, they are still are behind the

rapid development of nanotechnology. As a consequence, the potential risk of these NM being released into the environment, without the knowledge about their effects on living organisms, cannot be disregarded. Perhaps many of the effects that might be caused by NM exposure will be known only in the future. So, there is a need for new legislation that stipulates a mandatory compliance with standards of risk assessment considering several types of NM and organisms (Lapresta-Fernández 2011).

From this, the importance is evident of biological studies using biomarkers, which are early signs, mostly reversible, that indicate an actual or potential condition of exposure, effect and susceptibility that can trigger damages in several organisms (Bergamaschi 2012). Data of exposure biomarkers can help to understand the behavior and the risks that NM offer to the environment and organisms being, in this sense, useful in evaluating risk assessments of these compounds. Once the early effects triggered by NM can be established, security procedures can be defined before these NM may reach the environment, affecting several ecologically relevant parameters such as social behavior and reproduction.

In this context, there is a need to establish regulatory norms, considering the toxicity of NM. Some efforts have been undertaken to ensure the safety of living organisms; however, much remains to be done. Obviously, for the implementation of new legislation to regulate the development of nanotechnologies, breakthroughs in toxicological studies are necessary. .

Acknowledgements

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Manuscrito 1: **Histology, immunohistochemistry and lipid peroxidation in the fish *Danio rerio***

(Cyprinidae) fed with single and multi walled carbon nanotubes

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Histology, immunohistochemistry and lipid peroxidation in the fish *Danio rerio* (Cyprinidae) fed with single and multi walled carbon nanotubes

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Abstract

The increase in the production of carbon nanotubes (CNT) arises potential scenarios of exposure to these nanomaterials for several organisms including aquatic species. Experiments were conducted to determine the toxicity of single wall (SWCNT) and multi-walled (MWCNT) carbon nanotubes to the fish *Danio rerio* (Cyprinidae) exposed to these CNT via diet (500 mg/kg) during 28 days, Levels of lipid peroxidation (TBARS assay), protein carbonyl groups (by immunohistochemistry), and histopathological alterations were evaluated. Results indicated that carbon nanotubes contributed to histological and oxidative damage in proteins, whereas no effects in terms of lipid peroxidation were observed. The effects were observed mainly in gills and intestine, a route that should be considered and studied properly, taking into account that agreggation and deposition in sediment is an expected behaviour of nanomaterials in the environment.

1. INTRODUCTION

In 1991, Sumio Iijima [1] was the first to demonstrate the existence of tubular carbon structures, which were later called carbon nanotubes (CNT). This discovery, together with the fullerenes C_{60} [2] prompted the development of nanosciences and their technological applications (nanotechnologies). The extent of applications of nanomaterials makes the nanoscience clearly a multidisciplinary subject. Nanomaterials are natural or synthetic substances with 50% or more of their size distribution in the range up to 100 nm [3]. In this size range, significant changes in chemical and physical properties of molecules are exhibited, many of which may have a technological utilization [4; 5].

CNT show magnetic and optical properties, and high mechanical strength and electrical conductivity [6]. Currently these nanomaterials have an annual production in tons, meaning that in the future they probably will be present in the environment [7]. Because they are lipophilic in its pure form, CNT are one of the least biodegradable nanomaterials [8] with a tendency to bioaccumulate [9]. Poland and colleagues (2008) noted similarities in the toxicity of CNT and asbestos [10]. The geometric shape of CNT should be one the key factors of its cytotoxicity promoting necrosis and apoptosis [11].

In vivo assays with CNT in various aquatic animals showed induction of oxidative damage [12; 13], an increase in mortality rates [14; 15], CNT accumulation in the digestive tract [16; 17], and alterations in reproduction [18; 19] In fact, *in vivo* studies are of utmost importance to provide data about nanomaterials effects on the environment and organisms, a key regulatory issue. According to Velzeboer et al. (2011) aquatic sediments are a major sink of manufactured nanomaterials like fullerenes and CNT and these may cause adverse effects in benthic species or organisms that fed sediments [20].

The objective of this study was to investigate potential damages to the fish *Danio rerio* by nanotubes offered via diet, in way to evaluate potential exposure to this CNT under an environmental realistic condition.

2. MATERIALS and METHODS

2.1 Experimental design

Prior to testing, adult zebrafish (*Danio rerio, Cyprinidae)* were bought from a local store and acclimated to laboratory conditions for at least three weeks. The photoperiod was fixed at 12 h light/12 h dark, with water temperature maintained at 26 ºC and the pH between 6.8-7.0.

All procedures in this study were approved by the committee of ethics of animal use (CEUA FURG- n° Pq004/2013). Fish were fed twice a day, with a commercial food Brand Tetra[R], originally presented in very thin sheets that were macerated with water Milli-Q and dried at 60°C by 8 hours with new maceration. The daily average amount of food eaten by fish was previously determined. With this information, the final CNT concentration in the food was established. It was used 60 fish, equally divided in 3 treatments, 20 fish received the control diet (no CNT added), 20 fish were given feed containing SWCNT and 20 fish were given feed containing MWCNT, both at a dose of 500 mg/kg of food [13]. Each fish was maintained in a single aeration flask containing 300 ml of freshwater at a temperature of 26 °C, with a photoperiod of 12 h light:12 h dark. Each fish received half of the total amount of feed at 10:00 h in the morning and the other half in the afternoon at 16:00 h. The food was offered during one day, next day the single aeration flask was clean and water was exchanged. This procedure was followed for 28 days and then the animals were dipped into the methane sulfonate solution tricaine (TMS MS222); ≥ 250 mg / L to death and tissue removed, frozen or fixed and stored for later analysis.

2.2 Nanotubes carbon

Nanotubes used were purchased from commercial supplier (SES Ressearch: SWNTC lot ps-09607 and MWNTC lot gs-1802) with 10-30 nm diameter with 99.9% purity. To ensure purity and absence of metal catalysts, it was employed the technique described

by Chen et al. (2004), doing a acid bath to remove traces of metals, using a solution containing sulfuric and nitric acid (3:1, v/v) mixed and sonicated for 6 hours and further centrifuged for 3,000 g for 20 minutes. The centrifugation procedure was repeated 5 times and then CNT samples were further oven dried for 48 hours at 50 ° C [21].

2.2.1 Characterization of the SWCNT and MWCNT

The technique chosen to characterize these materials was mirroring Raman spectroscopy. This technique characterizes carbonaceous materials by identifying the types of links and provides information regarding the disorder of the crystal lattice of the material and identifying the various crystalline and amorphous forms present in the sample, and thereby exhibiting characteristic peaks in the spectra in the region between 1000 and 1800 cm-^{1}. CNT are indetified mainly by the shapes of the bands. For example, when analyzing MWNTC, there is a peak intensity of G-band with frequency of about 1582 cm^{-1}. The peak frequency ($\sim$ 1618 Raman Shift/cm-1) is typical of defective materials, so bands of MWCTC and SWCNT samples of good quality should be lower [22; 23]. Raman analyzes were performed on equipment inVia Renishaw Raman Spectrometer, experiments were performed at room temperature in the range of 0-2500 cm^{-1} using a laser of 532 and 785 nm wavelength.

2.3 Biochemical analyses

2.3.1 Determination of lipid oxidative damage

To determine lipid peroxidation [24], TBARS method was used in accordance with Oakes e Van der Kraak (2003), which involves the reaction of malondialdehyde (MDA), a degradation product of peroxidized lipids, with thiobarbituric acid (TBA) under conditions of high temperature and acidity, generating a chromogen that is quantified by fluorimetry (excitation: 520 nm; emission: 580 nm). For these analyzes, pieces of gills, brain, muscle, and intestines were frozen and stored at -80 ° C until the

time of analysis. The tissues were homogenized in 1.15% KCl solution, 35 μM BHT and after a solution is added of 0.8% TBA, the concentration of TBARS (namoles / mg wet tissue) was calculated employing recorded tetramethoxypropane default.

2.4 Histopathology

Histological analysis was used protocol similar to those described by [12; 25]. The fish were anesthetized and after killed with an overdose of methane sulfonate tricaine (TMS, MS222 ≥ 250 mg / L.). Whole fish were preserved in Methacarn fixing solution: (Methanol/Chloroform/Acetic Acid in a proportion of 6:3:1) for 12 hours, after they were stored in 70% ethanol. Was used an automatic processor PT05 LUPE, where the samples underwent serial dehydration (alcohol 70%, 80%, 90%, 96%, absolute), diaphanization or clarification (xylene bath 1 and 2 bath) and finally impregnating in paraplast (Sigma-P3558) (first bath and second bath). Subsequent to this procedure, fish were placed in paraplast blocks for cutting (5 μm) with microtome LUPE MRP03. Samples were mounted slides and stained with haematoxylin and eosin (H & E) or for immunohistochemistry. H & E staining followed the protocol described Carson and Hladik [26]. In this case the slides were washed by xylene (2 baths of 15 minutes) to remove the paraffin and after hydration were bathed in a series of absolute alcohol, 96%, 90%, 80%, 70% and water, and subsequently stained H & E and dehydrated again by the inverse process for mounting the blade histological using balm.

2.5 Immunohistochemistry

For localization of protein carbonyls, a common indicator of protein damage via oxidative stress, it were used histological slides without H & E staining and the immunohistochemistry kit protocol, Millipore catalog number S7450, "Oxidative stress detection kit". The test involves chemical derivatization of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH). This results in a chemical reaction with proteins covalently coupled to DNP at their carbonylation places. The DNP-derivatized proteins

were detected by a specific antibody that binds to the DNP molecule, with a subsequent incubation with a secondary antibody conjugated to biotin, and streptavidin-HRP and stained using a 3,3' diaminobenzidine (DAB) that allows immunohistochemical detection of carbonylated proteins. For the implementation of statistical calculations relating to these data we used the adapted Bernet protocol [27].

2.6 Statistical analysis

TBARS differences between groups were analyzed through one way analysis of variance (ANOVA). Previously, assumptions of normality and homogeneity of variances were verified through the Shapiro-Wilks and Levene were tested, respectively. Mean pairwise comparisons were performed using Newman-Keuls test. Bernet index of carbonylated proteins was analyzed through Kruskal-Wallis test [28].

3. RESULTS

3.1 Characterization of the SWCNT and MWNTC

The SWCNT and MWCNT used were characterized by Raman spectroscopy frequency (~ 1618 Raman Shift/cm-1) and laser of 532 and 785 nm wavelength.

The employed technique showed the existence and quality of SWCNT and MWCNT (figure 1)
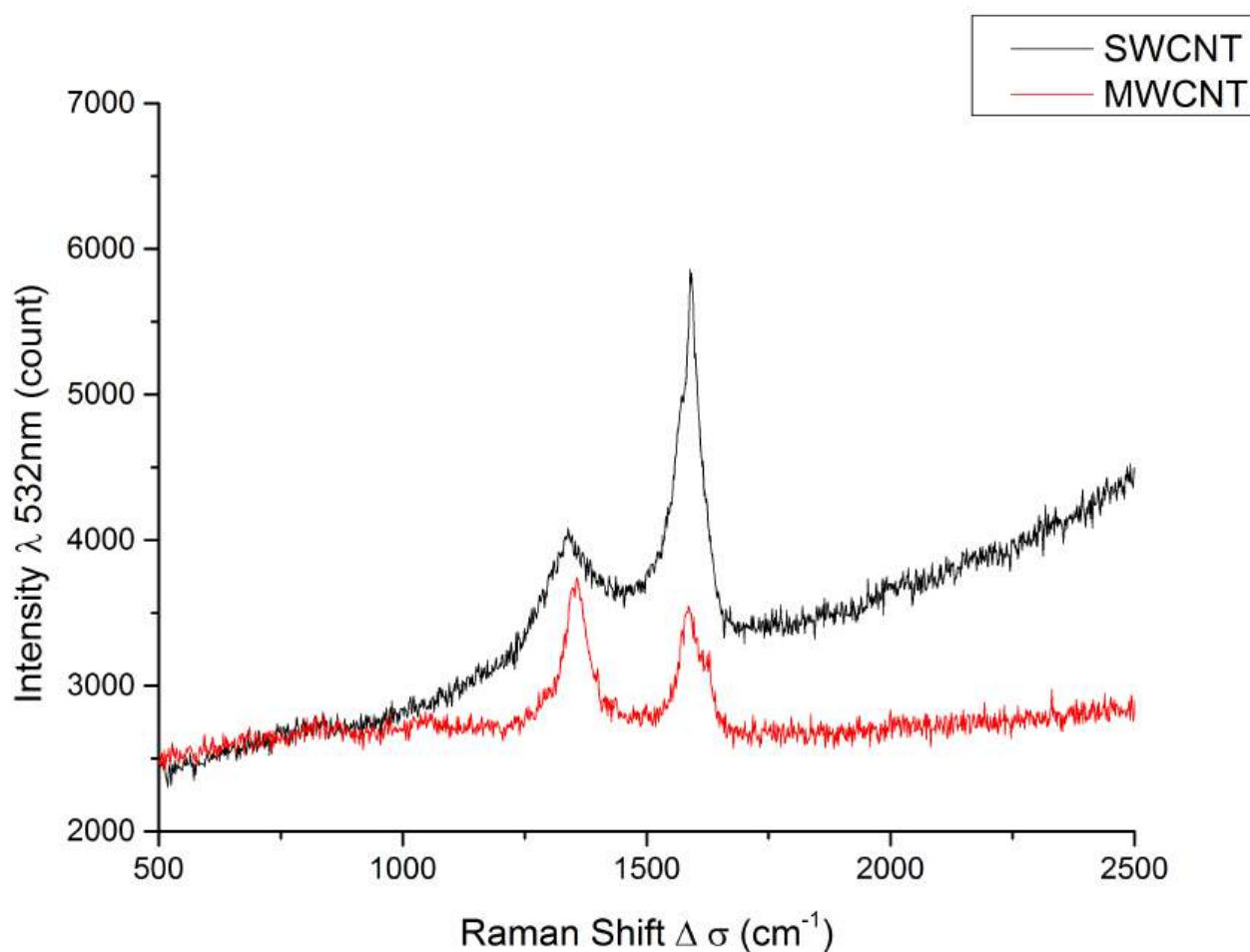

Figure 1. Raman spectroscopy of SWCNT and MWCNT.

3.2 Dietary exposure to carbon nanotubes

Mortality rate was 15 % in the group fed with rations containing SWNTC. The group fed with MWCNT showed no mortality, the same for the control group. It was observed that food with or without CNT was equally accepted by fish, and no apparent differences in the feeding behaviour were detected.

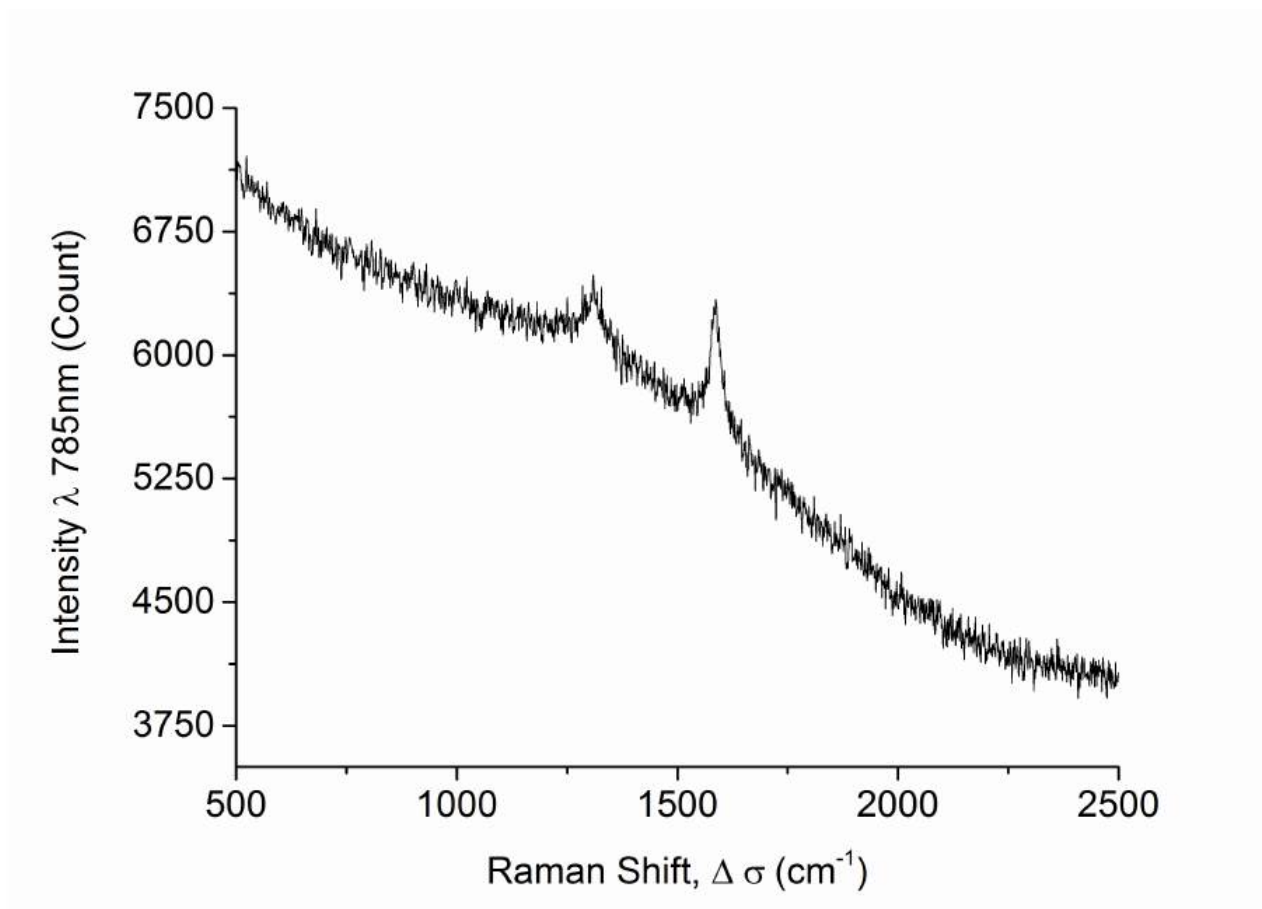The employed technique showed the existence of these CNT in the food offered to fish (Figure 2).

Figure 2. Raman spectroscopy of feed offered to the animals SWNTC containing in its composition. Raman spectroscopy feed given to the animals containing MWCNT did not find this characteristic peak MWCNT.

3.3 TBARS

The results showed that exposure to CNT through diet did not result in an increase of TBARS content in *Danio rerio*. In brain and muscle from zebrafish exposed to SWNTC or MWNTC, the TBARS levels were statistically similar ($p>0.05$) to that from control group (**Table 1**). For zebrafish intestines and gills, a reduction of TBARS levels were observed in SWNTC and MWNTC groups when compared to the control group ($p <0.05$) (**Table 1**).

	Gills	Brain	Intestines	Muscles
Control group	0.03± 0.009	0.02 ± 0.004	0.03 ± 0.014	0.005 ± 0.003
SWNTC	0.011 ± 0.002*	0.03 ± 0.005	0.02 ± 0.003*	0.008 ± 0.002
MWNTC	0.012 ± 0.004*	0.02 ± 0.005	0.012 ± 0.002*	0.013 ± 0.02)

Table 1: Levels of thiobarbituric reactive substances (TBARS) in different organs of zebrafish *Danio rerio,* expressed in nmol of TMP/mg of tissue. Data are showed as mean ± 1 standard error (n = 4). Asterisks (*) indicate significant differences ($p < 0.05$) with the control group. TMP stands for tetramethoxypropane, the standard employed in the assay.

3.4 Histopathology

It was observed that CNT induced macrophages death in the gills, due to granuloma generation and neutrophil infiltration. This characterized an inflammatory process observed both in SWNTC and MWNTC, In the control group the tissue is healthy. In brain, the proliferation of glial cells was observed in animals exposed to SWNTC (**Figure 3b**), however, was not observed in the control group (**Figure 3a**).

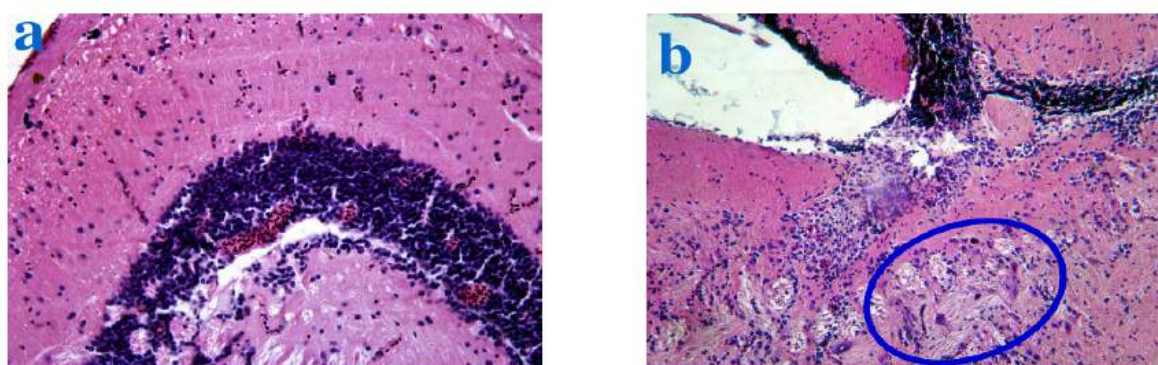The signs of tissue damage observed by microscopic examination:

Figure 3. Zebrafish *Danio rerio* brains from: (**a**) fish fed with the control diet, 40x magnification, (**b**) fish fed exposed to SWCNT, 20x magnification. Glial cells proliferation was observed in animals exposed to SWNTC, indicated by the ellipses.

3.5 Immunohistochemistry

In **Figure 4**, it is showed the results for protein carbonyl groups in gills of zebrafish *Danio rerio*. The results indicate that exposure to CNT in the diet increased oxidative damage to proteins, resulting in a higher detection of levels of carbonylated proteins. Similar results were obtained in liver, pancreas, kidney, intestine, stomach and eyes in fish exposed to SWCNT and MWCNT. The results of Bernet index for these organs are showed in Figure 5.
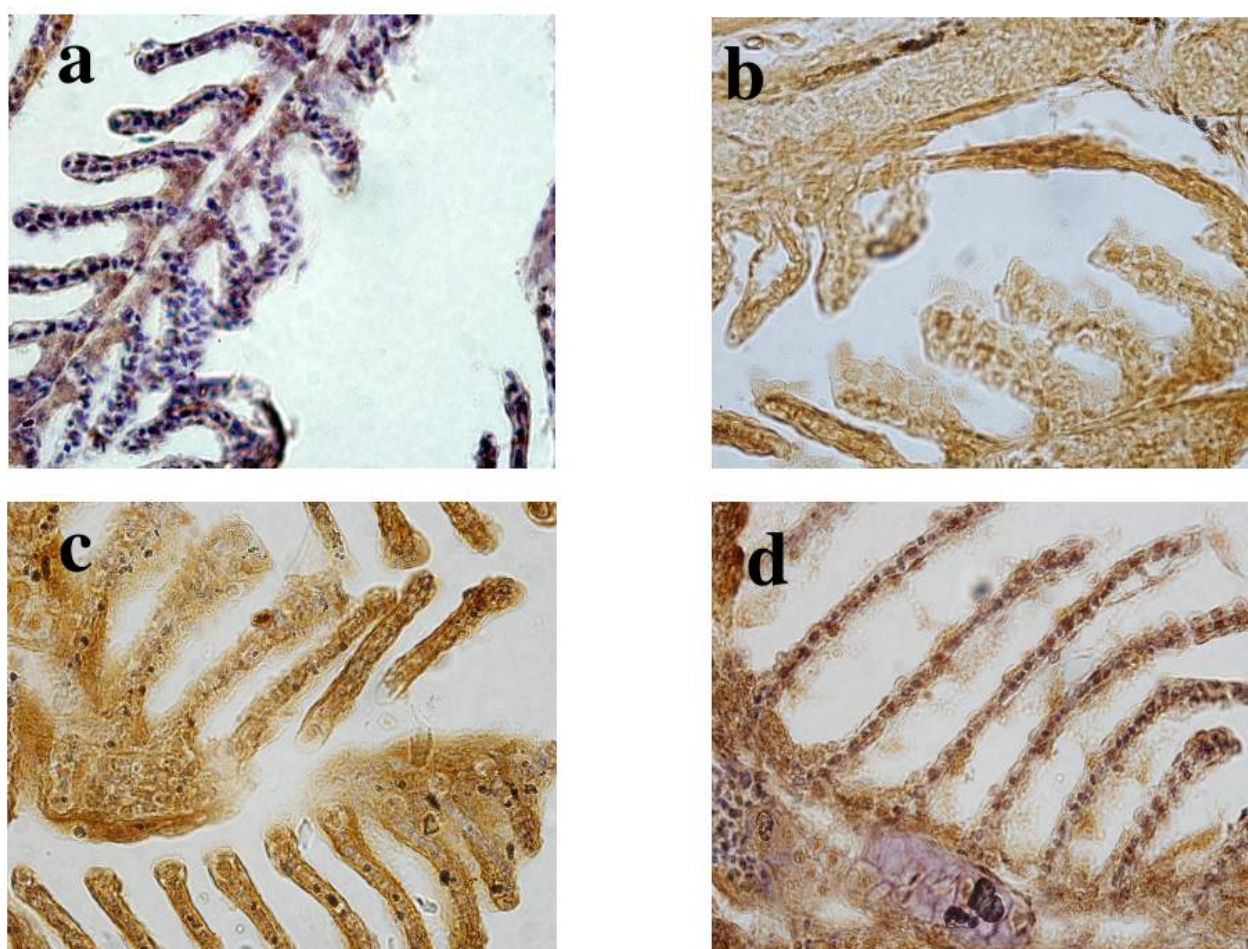

Figure 4. Zebrafish *Danio rerio* gills from**: (a)** fish fed with the control diet, **(b)** fish exposed to SWCNT, **(c)** fish exposed to MWCNT, **(d)** fish exposed to hydrogen peroxide, (positive control). Immunodetection of protein carbonyl groups is characterized by its light brown color, 100x magnification.
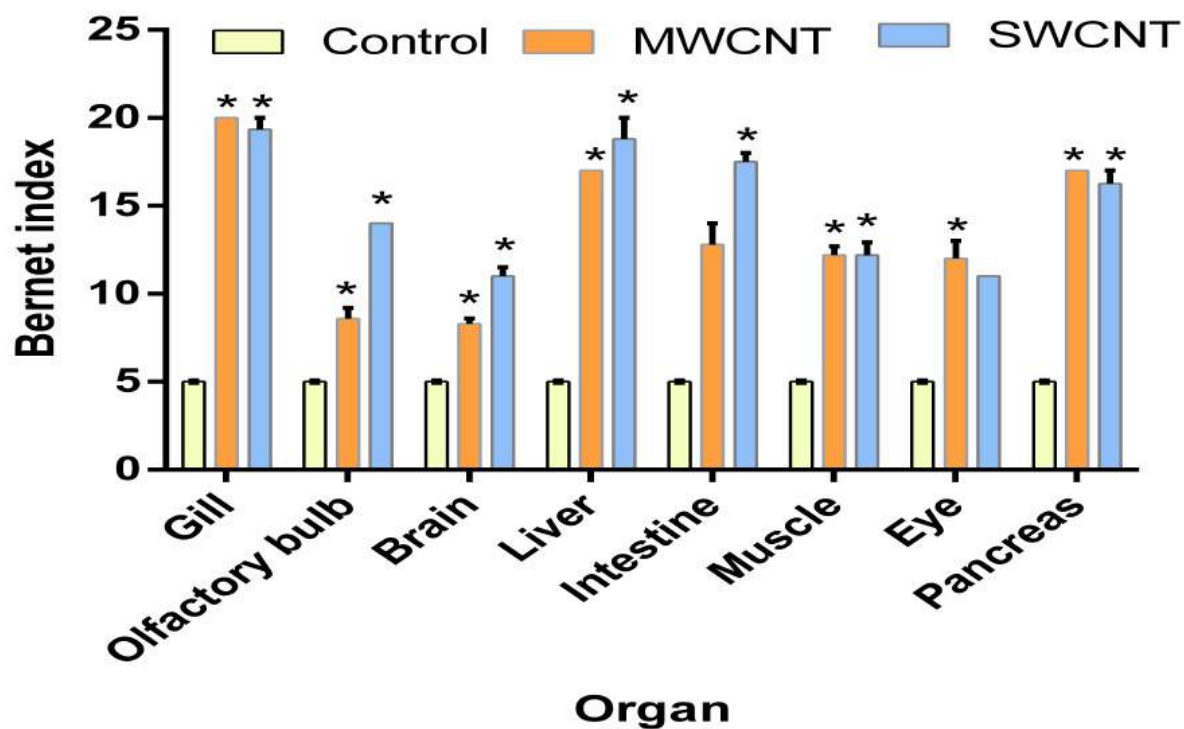
Figure 5. Carbonylation index of proteins in different organs of zebrafish exposed to single walled carbon nanotubes (SWCNT), or to multi walled carbon nanotubes (MWCNT). Control: refers for zebrafish non-exposed either to SWCNT or MWCNT. Data are expressa as mean + 1 standard error (n=3-10). * Significantly different (p<0.05) from Control after Kruskal-Wallis test.

4. Discussion

One of the most promising in vivo model systems for toxicity studies is *Danio rerio*, because. Utility of zebrafish for biotoxicity screens is largely based upon the close homology with the human genome [29]. The genetic parallels impart physiological and anatomical similarities [30;31;32;33]. When introduced to xeno-substances, zebrafish and mammals demonstrate a similar physiologic response such as the induction of metabolizing enzymes for foreign bodies and oxidative stress [34;35].

The physicochemical properties of the CNT are important to their possible applications, however, these properties can also be responsible to generate toxicity [36; 37].

Fish and other animals has antioxidant defense, where the lipid peroxidation levels has been used as a marker of oxidative stress [38], which is determined by the balance of production, the removal and elimination of oxidants by antioxidants [39]. When animals are exposed to CNT several responses have been reported, included inflammation, DNA damage and granuloma formation. A likely mechanism of CNT toxicity is oxidative stress generation, thus disturbing the homeostasis of the intracellular environment [40].

As the generation of reactive oxygen species (ROS) induced by CNT has been to transition metals released by these nanomaterials [40], it was employed a protocol to eliminate trace metals from the tested CNT. Other authors that employed purified CNT have reported ROS generation and activation of molecular signaling associated with oxidative stress, among them the activator protein-1 (AP-1) and nuclear factor kB (NF-kB) and MAPK [37]. Overall these response can alter the redox balance in biological environments [40].

The results present study showed that lipid peroxidation were not increased in zebrafish exposed during 28 days to SWCNT and MWCNT in their food. It is interesting to note, however, that the zebrafish gills exposed to SWCNT or MWCNT was reduced 2.5-fold when compared with the control group. A potential artifact of these results can not be discarded. For example, Ren and Zhong (2010) reported that the fluorescence of fluorochrome H_2DCF can be partially absorbed by SWNTC [41;42].

Also, depending on the electronic properties of CNT, they can act as antioxidant agents [43]. However the results obtained with protein oxidation levels seems deny this last possibility.

Smith et al. (2007) have reported absence of TBARS induction on gills, liver and intestine of rainbow trout (*Oncorhynchus mykiss*) exposed to SWCNT or fullerene C_{60} through diet [12]. SWCNT but behaves as a pro oxidant in zebrafish brain when exposed via intra peritoneal injection [42].

However the histological results were quite different, where conspicuous effects were observed in gills, even when fish were exposed to CNT through food.

In rats, exposure to MWCNT resulted in alveolar fibrosis and thickening of the alveolar septum [44]. The inflammatory responses observed in gills has been also reported in rats, where intratracheal instillation of SWCNT caused a persistent and progressive inflammatory response in the lung, as well as formation of blood clot and thrombosis in the arteries [45].

Long term (1 year) exposure to MWCNT (treated or not with acid)resulted in inflammation pulmonary, liver steatohepatitis and fibrosis [46]. However the exposure of MWCNT untreated with acid caused greater amount of damage and a more severe liver disease and greater lung inflammation and bronchial hyperplasia [46]. This point again to the need of acid treatment in order to isolate the influence of trace metals when toxic responses induced by CNT are being evaluated [47]. To verify generically oxidative stress induced by CNTs labeling immunohistochemistry was performed to be considered as a complementary technique in this study, the increase in intracellular ROS can react with cellular macromolecules, including DNA, lipids and proteins and disrupt homeostasis the intracellular medium in the presence of CNTs by these present the potential to cause metabolic conversion product of cellular oxygen [48]. These changes are associated with a number of disorders including inflammatory and pathological processes [49;50], even when living organisms are exposed to free radicals generated by their cellular functions [51], but the body defends itself through the presence of antioxidants, and thus leading to equilibrium [52].

When this imbalance occurs is affected attack the cellular components and proteins are a major target protein and modify the oxidation of the side chains of

cysteine, methionine, histidine, and tyrosine [53], and that catalyzes the introduction of other groups such as aldehydes and ketones and thus modifying the protein by oxidation [54], these modifications of the carbonyl group is to use the biomarker in this study to identify oxidative stress by immunohistochemistry.

As a conclusions of this study it can be considered that: **(1)** histological examination of whole animals seems to be an interesting strategy to detect CNT effects; **(2)** protein carbonyl groups detected by immunohistochemistry also showed to be a good option to evaluate responses in organs that usually are not considered in nanotoxicology studies as kidney, stomach and eyes; and **(3)** TBARS results needs further evaluations in order to establish if the apparent antioxidant responses observed in gills are not artifacts of the employed fluorometric techniques. Current studies are being conducted in our laboratory to evaluate this possibility.

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Manuscrito 2: **Carbon nanomaterials interference in TBARS**

(thiobarbituric acid reactive substances) Test

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CARBON NANOMATERIALS INTERFERENCE IN TBARS (THIOBARBITURIC ACID REACTIVE SUBSTANCES) TEST

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Abstract

Nanomaterials exhibit unique properties due their size and relative area, but the mechanisms and effects in the living organisms are yet to be unfold in their totality. Possible toxicities mechanisms concerning nanomaterials, including carbon nanotubes and fullerene-C_{60} have been investigated since the 90s. Fluorimetric and colorimetric methods have being systematically used to measure nanomaterials toxicity, and controversial results have been found. One of the problems associated variables and contradictory results should be that nanomaterials, including single-wall carbon nanotubes (SWCNT) and fullerene (C_{60}), can interfere in fluorometric assays. Through *in vitro* assays it was determined that these nanomaterials can decrease the fluorescence signal when using the method of the thiobarbituric acid reactive species (TBARS) to determine lipid peroxidation. This constitutes a problem to determined properly the antioxidants or pro-oxidants effects elicited by nanomaterials.

Keywords: carbon nanotubes, fullerene, fluorescence, interference, oxidative stress.

1. Introduction

Nanomaterials are substances of natural origin or human made (incidental or manufactured) that possess at least 50 % of their particles in one dimension under 100 nm (European Commission, 2011). In these dimensions, significantly changes in the chemical and physical properties arise (Colvin, 2003; Oberdörster et al., 2005; Balandrán-Quintana et al., 2008). The nanomaterials tend to interact with biomolecules from cells and organelles due of their size compatibility (McNeil, 2005). They show a potential to cross the cellulars barriers and thus interact with receptors, nucleic acids, transcription factors and signaling proteins (Eckert et al., 2013) that may lead to altered biochemical and physiological mechanisms, creating pathological conditions (Eckert et al., 2013).

Among these materials we can mention the carbon nanomaterials, these nanomaterials were first described in the late 80s, in the case of fullerene C_{60} (Kroto et al., 1985) and in beginning of the 90s, carbon nanotubes (CNT) (Iijima, 1991). In recent years, due the increase of CNT utilization in different products and an expected augmented presence in the environment, numerous studies have been conducted to evaluate the ecotoxicity of these materials (Maynard et al., 2006), in virtue of a a growing concern about the risk of nanomaterials on human health and the environment (Service et al., 2000).

A series of nanotoxicology studies has been done with considerable progress on the mechanisms comprehension of their toxicity (Templeton et al., 2006; Roberts et al., 2007; Smith et al., 2007; Cheng et al., 2007; Scott-Fordsmand et al., 2008; Petersen et al., 2008; Petersen et al., 2009; Fraser et al., 2011). Oxidative damage on fish exposed to CNT via water (Smith et al., 2007; Fraser et al., 2011), augmented mortality rate in crustaceans exposed to CNT (Templeton et al., 2006; Roberts et al., 2007), CNT accumulation in the digestive tract of annelids and crustaceans (Petersen et al., 2008; Petersen et al., 2009), and reproductive impairment in fish and annelids

(Cheng et al.,2007; Scott-Fordsmand et al., 2008) have been found. Other authores reported growth inhibition and death of bacteria *Escherichia coli* and *Bacillus subitilis* exposed to C_{60} (Mashino et al., 2003; Lyon at al., 2006) and biochemical alterations and oxidative damages in fish species exposed to C_{60} (Oberdörster et al., 2006; Zhu et al., 2006; Zhu et al., 2008).

The generation of reactive oxygen species (ROS) has been debated as a key mechanisms of nanomaterials toxicity (Nel et al., 2006). Still, others evidences show that chemical reactions are not the only path to nanotoxicity, as many interactions proteins-CNT that may conduct to toxicity and damages (Cedervall et al., 2007; Zou et al., 2012).

Due to their chemical and physical properties, the CNTs and fullerene-C_{60} may interfere with some methodologies that are used to evaluate the toxicity of these materials. One of the main fluorescence probes used by groups studying oxidative stress is the diclorodihidrofluorescein diacetate (H_2DCF-DA) (Martin et al, 2011; Aranda et al., 2013; Kong et al., 2013). Previous studies have suggested that some nanomaterials such as CNT interact with H_2DCF-DA, leading to erroneous interpretation of the obtained results (Martin et al., 2011; Aranda et al., 2013; Kong et al., 2013). Because of these problems reported on different methodologies that use fluorescence methods (Monteiro-Riviere et al., 2009; Aranda et al., 2013), it was decided to evaluate if misleading results could occur in the thiobarbituric acid reactive substances methodology (TBARS), which is widely used to quantify the levels of lipid peroxidation (Fraser et al., 2011; da Rocha et al., 2013).

Materials and Methods

Carbon nanomaterials

Single-wall carbon nanotubes (SWCNT) were purchased from SES research (Houston – USA) with 10-30nm diameter. The SWCNT were purified according to the purification technique

described by Chen et al. (2004) in order to ensure high level purity. After purification, SWCNT were dried at 50°C and stored in conical centrifuge tubes until use. For utilization, the SWCNT poured in deionized water and sonicated in ultrasonic bath for 10 minutes. The characterization of SWCNT was performed through Raman spectroscopy (Ibanez et al., 2014 e Landois et al., 2014) (Figure 1A). The fullerene employed in the aqueous suspension was purchased from SES research (Houston – USA). It was poured in deionized water and kept with constant agitation and illumination during 60 days. After, the aqueous suspension were centrifuged (25,000 x g, 60 min) and sequentially filtered using 0.45 and 0.22 μm nylon filters, respectively. This protocol was based on Lyon et al. (2006), with minor modifications. The quantification of fullerene-C_{60} in the aqueous suspension was through the measurement of the total organic carbon content using a total organic carbon analyzer (TOC-V CPH, Shimadzu Corp., Japan) (Ferreira et al., 2014). The C_{60} suspension was characterized in a JEOL JSM 1200 EX II transmission electron microscope, in which 10 μl of the suspension was put onto Formvar-coated 300-mesh TEM grids (SPI). To allow sample evaporation, analysis was done after 24 h.(Figure 1B).
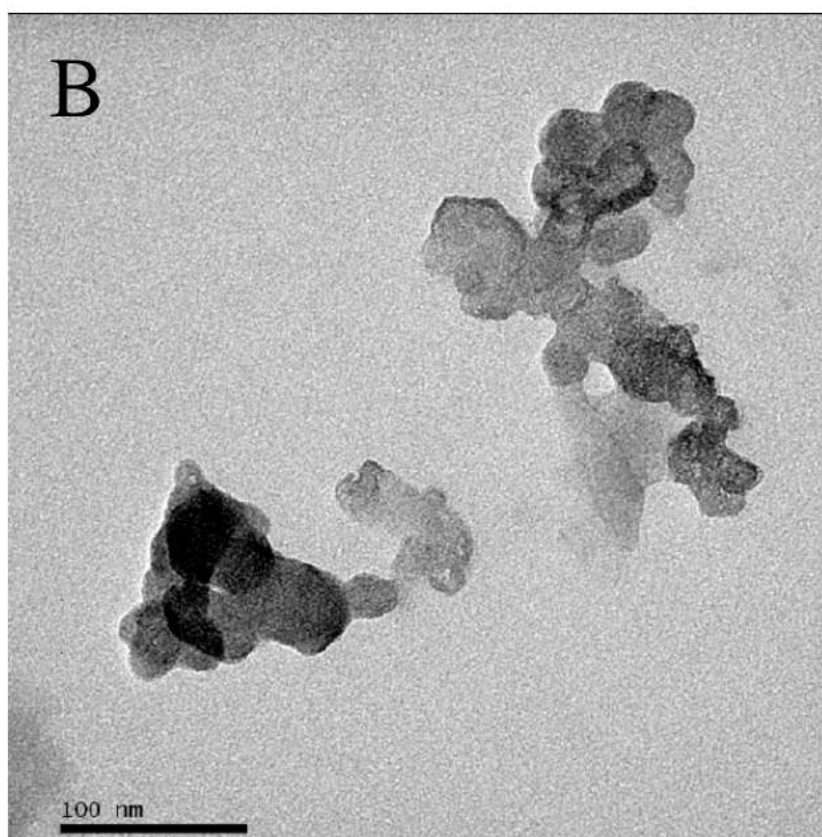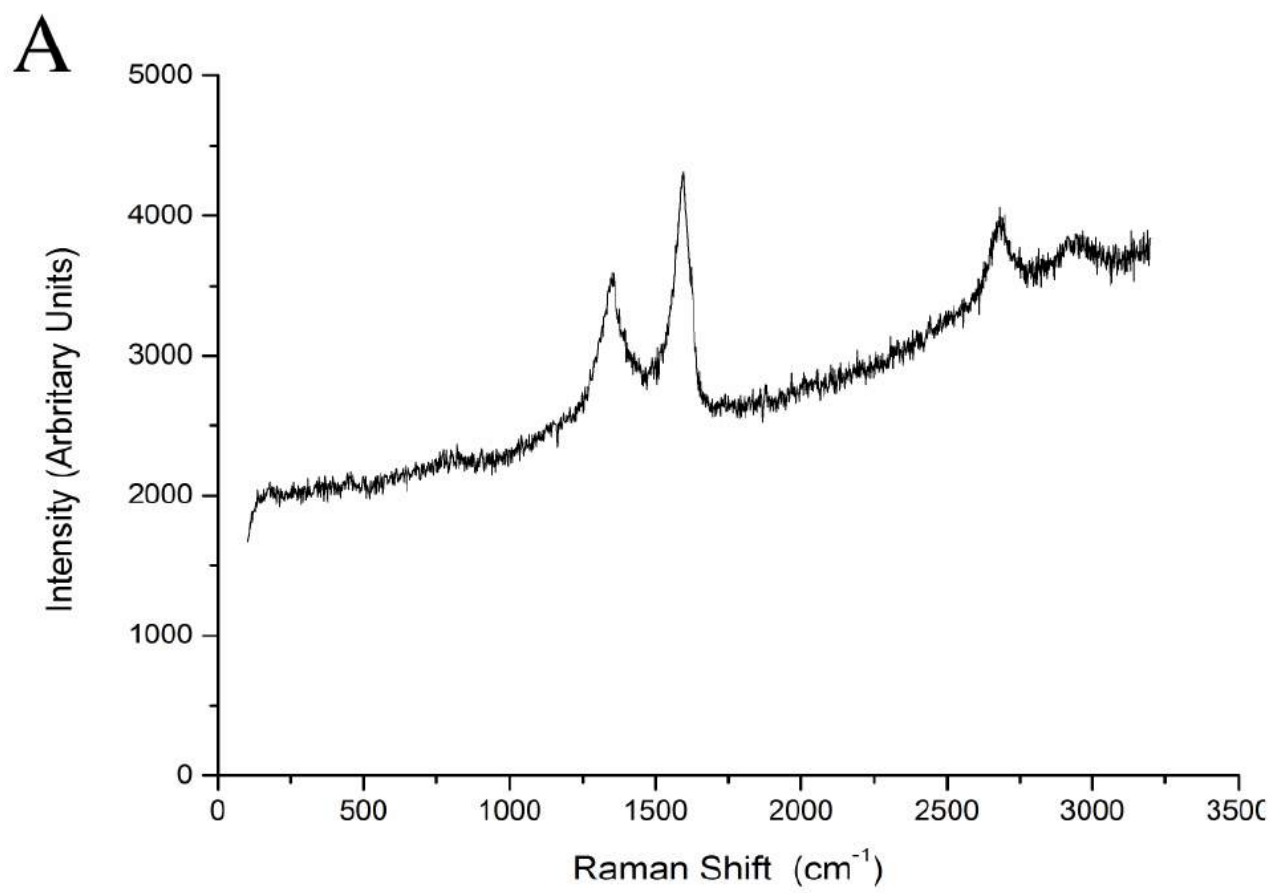
Figure 1. (**A**) Raman spectroscopy of SWCNT. Raman analyzes were performed on equipment inVia Renishaw Raman Spectrometer, experiments were performed at room temperature in the range of 0-2500 cm-1 using a laser of 532 nm wavelength; (**B**) Transmission electron microscopy (TEM) image of fullerene-C_{60} from the suspension obtained by the solvent-free method.

2.2 Biological samples

For the tests with the biological matrix, it was employed two animal species: the fish *Danio rerio* (brain and gill) and the polychaete *Laeonereis acuta* (divided into anterior, medium and posterior region of the body, according to Ferreira-Cravo et al., 2007). The tissues were frozen in liquid nitrogen and stored at -80°C, for subsequent homogenization and analysis. The use of vertebrate species (*D. rerio*) was approved by the Ethis Comitte of Federal University of Rio Grande – FURG (CEUA-FURG n° Pq004/2013).

The five types of animal tissues were divided as: control groups (CTR); fullerene-C_{60} groups in two concentrations (0.005 and 0.01 mg/L); and SWCNT groups in six concentrations (0.5; 1.0; 5.0; 10.0; 50.0; and 500.0 mg/L). In the different CNT groups, it was used the same animal sample, so the fluorescences differences should not be due to differences in the redox status of the sample, but by interactions between the biological matrix and the nanomaterials. The samples were incubated for five minutes prior the beginning of the tests.

2.3 TBARS

The thiobarbituric acid reactive substances (TBARS) assay quantifies the adduct malondialdehyde-thiobarbituric acid (MDA-TBA_2) (Halliwell e Gutteridge, 2007). The fluorometric version of TBARS method was done according to Oakes e Van der Kraak (2003). Butylated hydroxytoluene (BHT) was used as an antioxidant for the samples and 1,3,3-tetramethoxipropane (TMP) as the generating source of MDA in the tests without biological samples and as standard in all TBARS tests.

2.4 FOX

In order to compare the results obtained with a fluorometric method, the ferrous oxidation-xylenol orange assay for lipid hydroperoxides (FOX) was performed as described by Monserrat et al. (2003) and Ferreira-Cravo et al. (2009) with some modifications. The assay mixture was measured at 580 nm in a microplate reader right after the reagents were displayed and after half hour of incubation at room temperature. Cumene hydroperoxide (CHP) was used as the source of lipid hydroperoxides.

2.5 Statistical analysis

All values were stated as means ± standard error mean. Statistical analysis was executed through analysis of variance followed by Newman-Keuls post-hoc comparisons (a=0.05) for all determinations (Zar 1999).

3. RESULTS And DISCUSSION

In the TBARS assay, biological samples incubated with 500.0 mg/L of SWCNT showed a significant (p<0.05) lower fluorescence compared to the other groups. In the anterior region of *L. acuta,* the fluorescence of samples incubated with 0.5 mg/L of SWCNT showed a significant higher fluorescence (p<0.05) when compared with all the other treatments, expect for 50.0 mg/L of SWCNT (Figure 2A). In the middle region of *L. acuta,* fluorescence recorded in groups C_{60} (0.01mg/L), SWCNT (0.5-10.0 mg/L) was statistically higher (p<0.05) than control group (Figure 2B). In the posterior region of *L. acuta,* a fluorescence increase was observed in SWCNT at different doses (0.5-10.0 mg/L) which were statistically different (p<0.05) in relation to the other groups (Figure 2C).

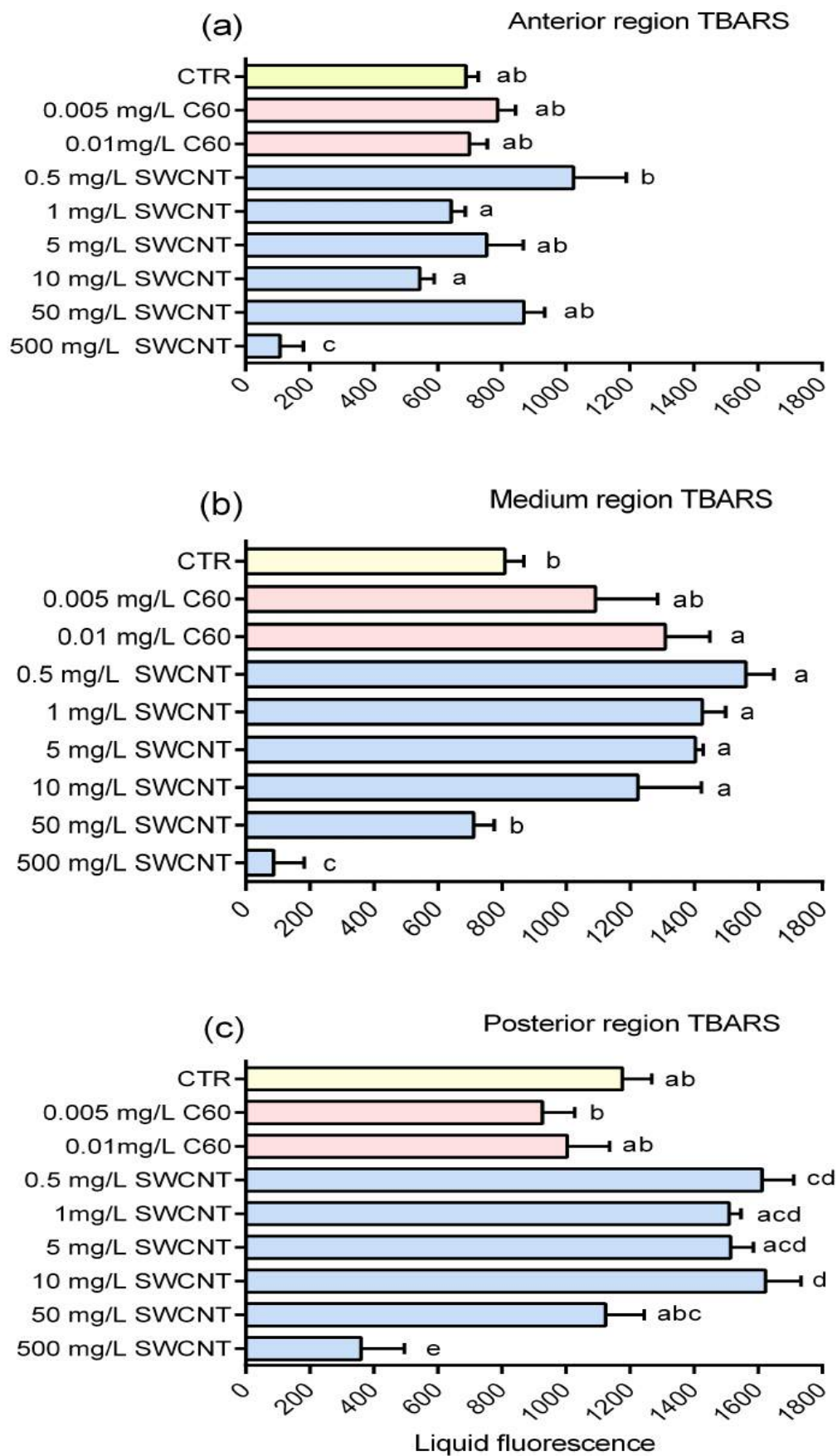(a) Anterior region TBARS

(b) Medium region TBARS

(c) Posterior region TBARS

Liquid fluorescence

Figure 2. Liquid fluorescence of *in vitro* using C_{60} and SWCNT at various concentrations and the *L. acuta* as biological matrix. In all cases data are expressed as media ± 1 standard error (n= 4-10). **(A)** anterior part. b significantly different ($p < 0.05$) from fluorescence recorded at 0.05 mg/L of SWCNT. Letters different are significantly different ($p < 0.05$) the other groups. **(B)** middle part media b significantly different ($p < 0.05$) from fluorescence recorded at control group and 50 mg/L of SWCNT. Letters different are significantly different ($p < 0.05$) the other groups. **(C)** posterior part b Groups presents insignificantly differences ($p > 0.05$) other groups. Letters different are significantly different ($p < 0.05$) the other groups.

In *D. rerio* brain, two concentrations of SWCNT (50.0 and 500.0 mg/L) showed negative fluorescence, lower than the background fluorescence, indicating a major influence of nanomaterials in the method (Figure 3A). When using gills of zebrafish as biological matrix, it was observed that the fluorescences registered at 50.0 and 500.0 mg/L of SWCNT were statistically lower ($p < 0.05$) than the other groups. However, the groups SWCNT (0,5; 1; 5mg/L) have statistically higher fluorescence than the groups: Control; (C_{60} 0,005 and C_{60} 0,01mg/L); (SWCNT 50 and SWCNT 500mg/L) (Figure 3B), These results indicate that depending on the nanomaterial and its concentration occurred fluorescence of exacerbation, which may be interpreted as a pro-oxidant action of the nanomaterial and the decrease in fluorescence may be interpreted as an antioxidant action nanomaterials.

The results obtained with brain samples from zebrafish showed a strong interference of the fluorescence emitted. Similarly, a study *in vitro* from our group (da Rocha et al., 2013) reported antioxidant capacity of SWCNT at the highest concentration (10 mg/L) using an *in vitro* fluorometric method. These results can be interpreted in terms of SWCNT to quench fluorescence, as reported by Ren and Zhong (2010).

In present study, it was observed a dualistic effect of SWCNT: at some concentrations (low and intermediate), high fluorescence values were recorded and at high concentration, low fluorescence or even negative (lower than the background fluorescence) was observed. The both

situations indicates a clear problem for data interpretation, since with a high fluorescence it should be concluded a pro-oxidant effect of SWCNT and the opposite for situations where the fluorescence was low or even negative.

It is possible that differences in CNT synthesis type, purification and functionalization should result in nanotubes with different reduction potentials and fluorescence quenching capabilities, points that need to be explored theoretically and experimentally (Aranda et al., 2013).
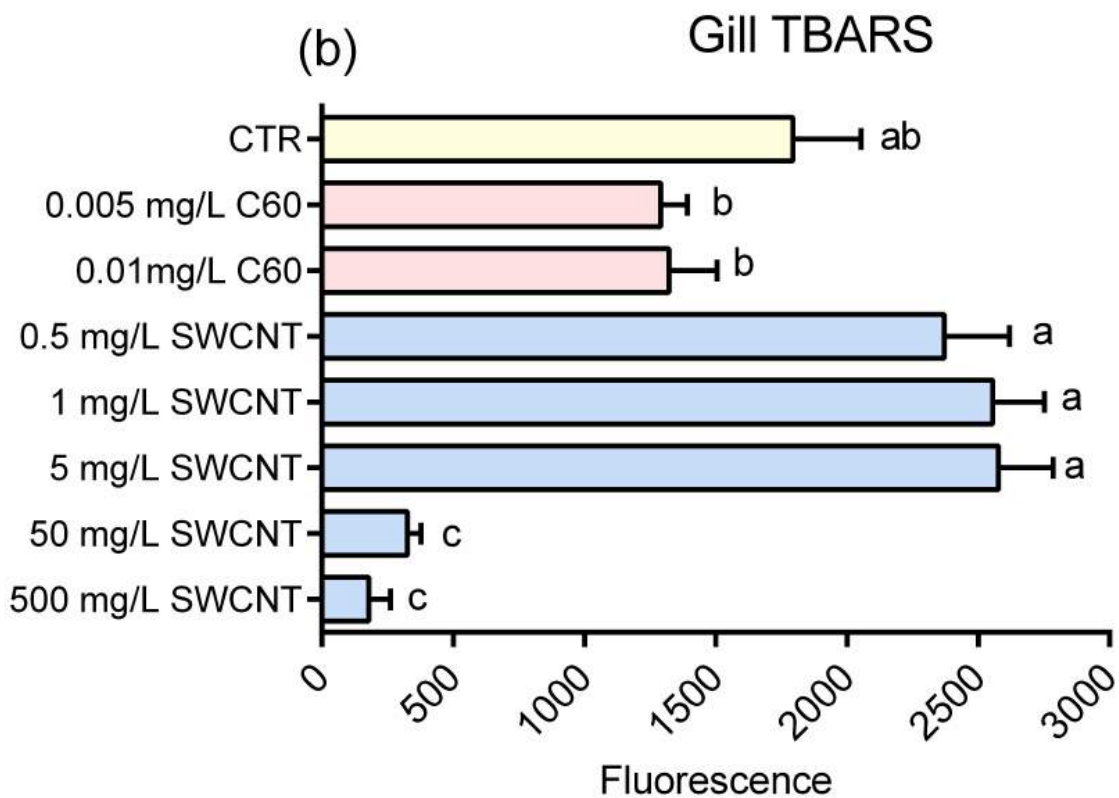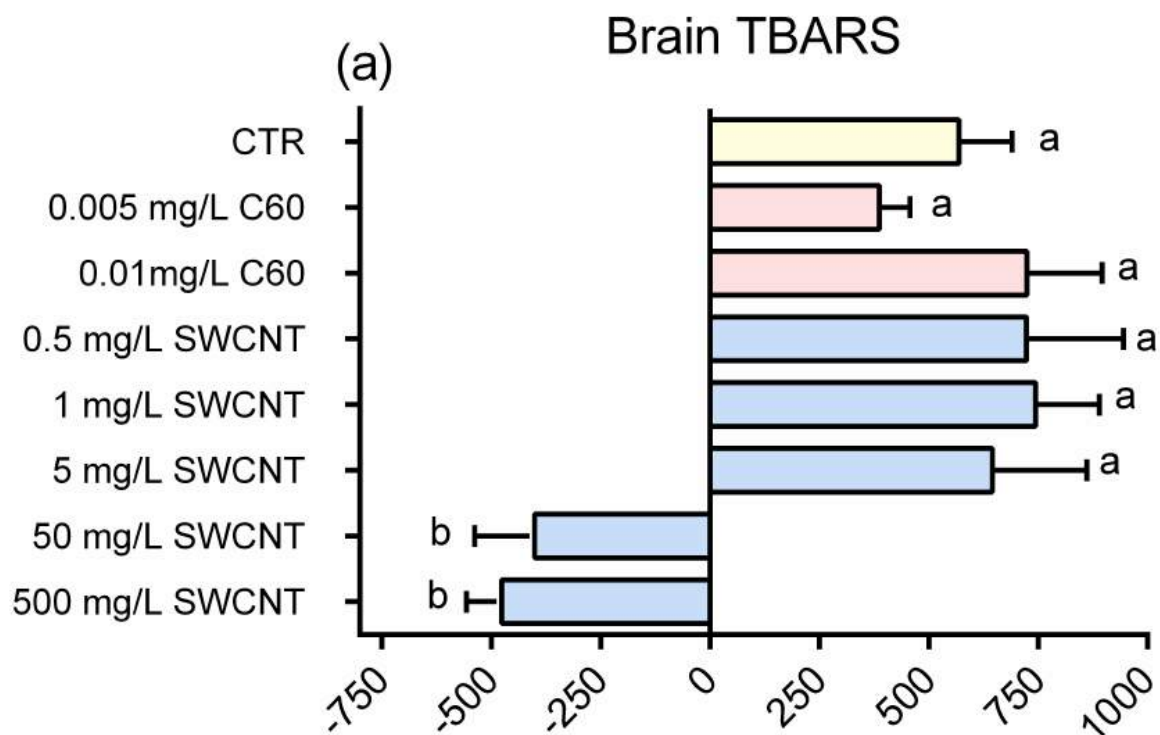
(a) Brain TBARS

(b) Gill TBARS

Figure 3. Liquid fluorescence of *in vitro* test with C_{60} and SWCNT at varying concentrations. In all cases, data are expressed as media ± 1 standard error (n= 5-11). Letters different are significantly different (p< 0.05) the other groups. (**A**) Brain of zebrafish *Danio rerio*. (**B**) Gills of zebrafish *Danio rerio*.

We also found the interference of a chemical called **b**utylated **h**ydroxy**t**oluene "BHT" and the results show that in the groups C_{60} (0,005; 0,01 mg/L) with BHT fluorescence increased compared with control groups and treatment groups with SWCNT (50; 500 mg/L).

When we analyze the groups C_{60} (0,005; 0,01 mg/L) BHT without the same behavior was seen only in relation to groups SWCNT (50; 500 mg/L). In the SWCNT groups, only in the lower concentration (0,5 mg/L) without the addition of BHT there was an increase in fluorescence compared to control groups and SWCNT (50; 500 mg/L), already in groups SWCNT (0, 5, 1, 5, 10 mg/L) with BHT there was an increase in fluorescence compared to the control groups and SWCNT (50; 500 mg/L).

Fluorescence of the group SWCNT (500 mg/L) is the smallest statistically compared to all other groups, regardless of the presence or absence of BHT; since the fluorescent group SWCNT (50 mg/L) BHT is no less than the fluorescence of the other groups (less SWCNT 500 mg/L), since the fluorescent group SWCNT (50 mg/L) BHT is similar to the fluorescence of the control group, less than the fluorescence of the other groups (less SWCNT 500 mg/L), Figure: 4A.

When we analyze the BHT found that help in the dispersion of nanomaterials, increasing the reaction surface at one excitation wavelength used (515 nm) and the consequent emission of light, so the fluorescence of the groups treated with BHT are higher than fluorescence in the control group and the group SWCNT (50 mg/L) with BHT there was the black hole effect.

(a) TBARS assay (no BHT)
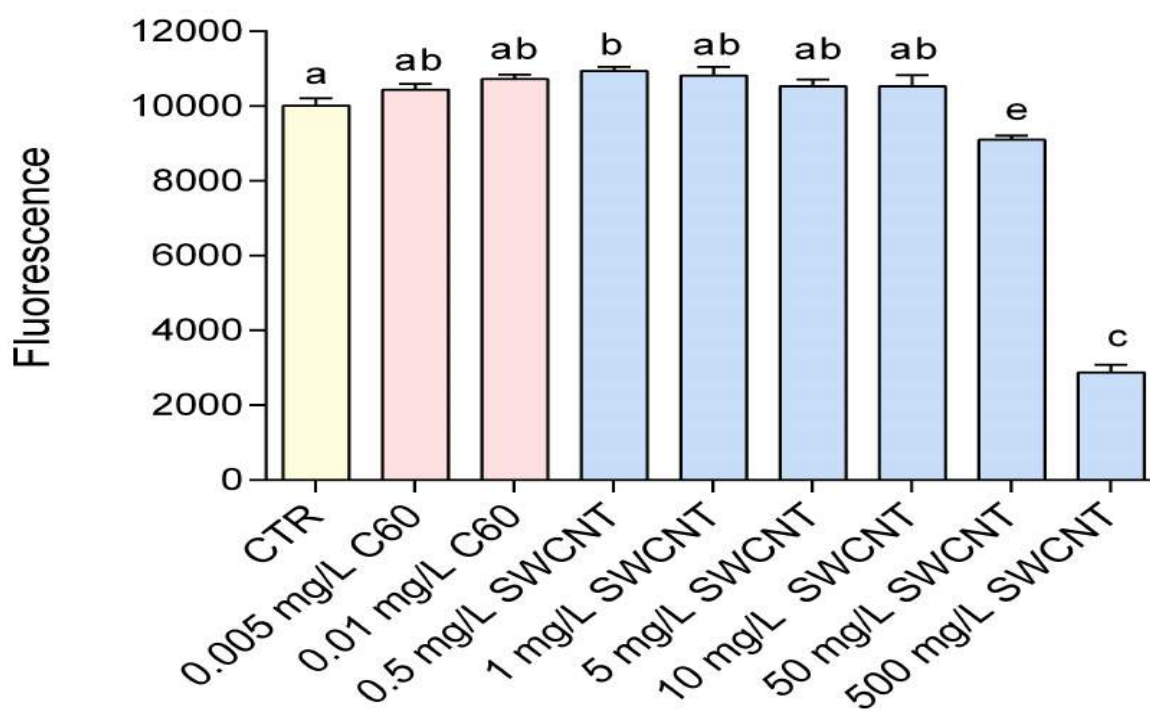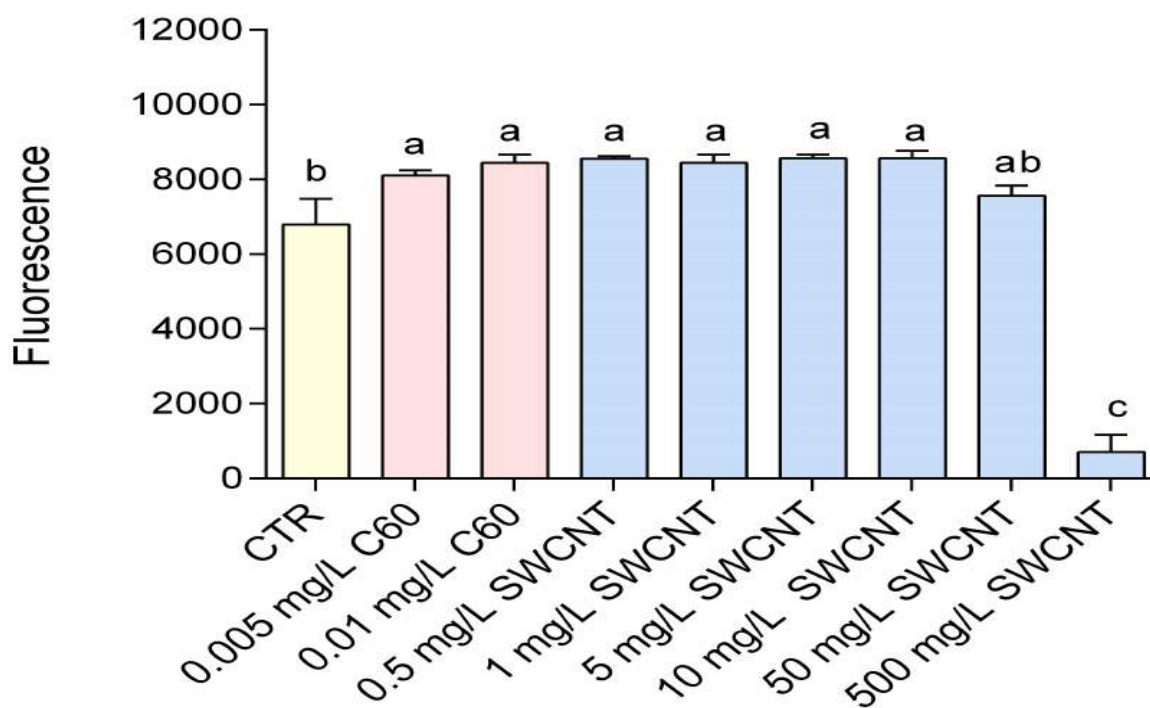

(b) TBARS assay (with BHT)

Figure 4. Liquid fluorescence of *in vitro* test using C_{60} and SWCNT in varying concentrations, with and without BHT, media ± error pattern, n=5. (**A**) Nanomaterials without adding BHT, (**B**) Nanomaterials with BHT, Letters different are significantly different (p< 0.05) the other groups.
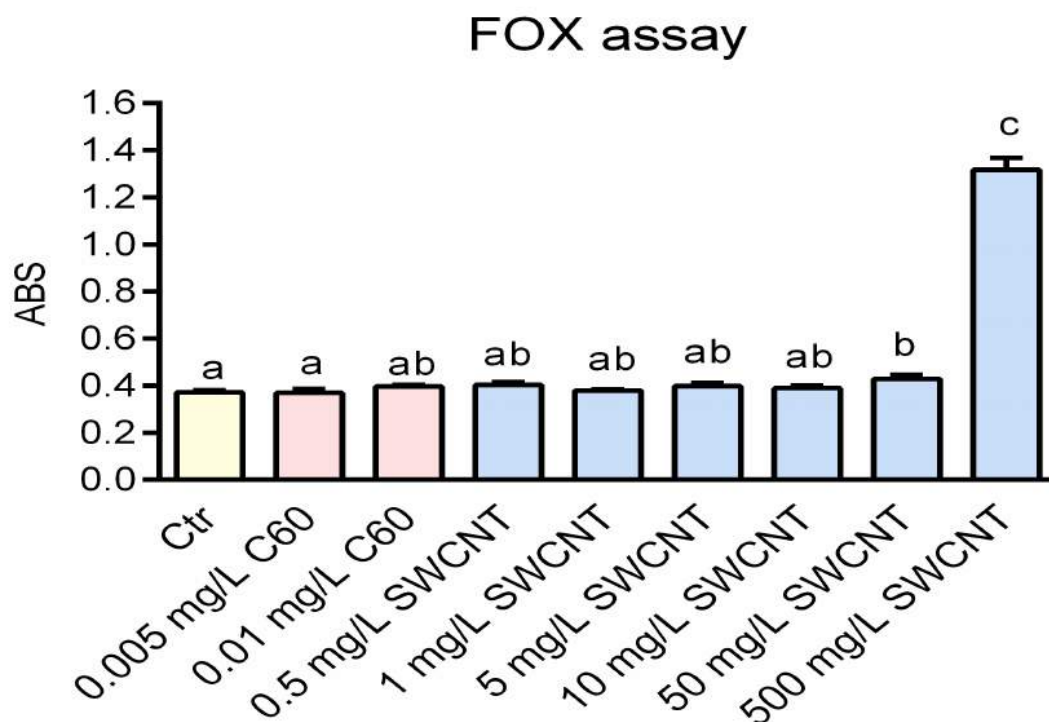
Figure 5. Liquid absorbance of *in vitro* test using C_{60} and SWCNT in varying concentrations, media ± standard error mean, n=5,Letters different are significantly different (p< 0.05) the other groups.

The nanomaterials may interact with testing means, modifying the result of this (Sathya et al., 2010 ; Greim & Norppa 2010). The strength of this interaction depend basically the type of chemical used and CNT. For example, carbon-based nanomaterials with them, have a surface able to absorb the dye, and thus modify its properties. Therefore, single wall carbon nanotubes (SWCNT) appear to interact with certain tetrazolium salts such as 3- (4,5-dimetiltiazol-2-il) -3,5-diphenylformazan employed in the colorimetric assay MTT, (Wörle-Knirsch et al, 2006;.. Davoren et al, 2007).

To, Kroll et al., 2012 interference occurs when a high concentration of nanomaterials is used, particles used test DCFH-DA and other cytotoxicity assays (MTT, LDH, IL-8) thus indicating that most particles interference can be avoided by changing assay protocols and decreased particle concentration. When there is the toxicity of CNT there is no conclusive answer, but several factors must be observed where the form of synthesis, surface, structure and impurities affect the toxicity of CNT (Liu et al., 2013). Given the variability of the physical and chemical properties (e.g., surface area, surface charge, morphology and surface chemistry) and the large number of combinations thereof (Ayres et al., 2008;. Bello et al., 2009;. Lu et al., 2009;. Meng et al., 2009;.. Xia et al., 2009).

We found a few of the possible interactions of carbon nanomaterials (C_{60} e SWCNT) may have due to their chemical and physical properties. These properties may interfere with some analytical methods which exhibit luminescence techniques, fluorescence and absorbance (Breznan et al., 2015). There are reports of chemical interaction of SWCNT (Casey et al., 2007; Isobe et al., 2006; Monteiro-Riviere et al., 2009; Worle-Knirsch et al., 2006). This interaction phenomenon appears mostly of CNT with these colorimetric and fluorescent dyes.

As we can see in this work the binding of CNTs are linked to the physical chemical characteristics of water, It may even take feature apolar, facilitating passage through biological membranes (Porter et al., 2007), Ideal SWCNTs are classified according to three possible configuration crystallography zigzag, armchair, and chiral, depending on the orientation of the rings benzene the walls of SWCNT. In zigzag conformation two opposing carbon-carbon bonds of each hexagon are parallel to the tube axis, whereas the conformation in the armchair carbon-carbon bonds are perpendicular to the axis. In all other arrangements, the opposing carbon-carbon bonds are at an angle to the tube axis, resulting in what is called helical nanotube chiral (Grobert 2007).

Thus, understanding the ways of nanomaterials toxicity, it is important not only checking the physicochemical properties of materials, but it is also necessary to consider the specific differences

in the type of biological interaction can also modify the results and lead to new hypotheses (Shvedova et al., 2012).

For (Ivask et al., 2014), the results of the environmental impact of nanomaterials, It is still small because of a lack of validation against data, there is still a lack of standardization with regard to the test protocols.

CONCLUSION

(1) Exacerbation of fluorescence and a decrease in fluorescence could be misinterpreted as a pro-oxidant and antioxidant action respectively of nanomaterials in this assay samples.

(2) Probably the results must be due to the physical properties of nanomaterials, which must somehow reduce the net emission of fluorescence when they are in high concentrations and over grouped and at lower concentrations and more dispersed emit more light.

(3) Our results indicate that different interference patterns according to the concentration of SWCNT and tissues analyzed, Therefore, the TBARS test is not suitable for samples treated with SWCNT.

(4) The fullerene aqueous interfered in TBARS assay with less intensity, however, it is believed that because of their dispersion.

(5) Fluorometric other measures need to be evaluated for potential interference biological nanomaterials and samples, as well as other animal tissues.

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Considerações finais

Os resultados do presente trabalho são: uma revisão sobre Nanoecotoxicologia em especies de peixes, apresentando diversas questões que devem ser consideradas quando se estuda os efeitos dos nanomateriais em organismos aquáticos, tais como métodos de síntese e caracterização do nanomaterial utilizado, metodologia adequada e escolha do organismo modelo estudado. Na segunda parte, foi realizada a exposição de peixes *D. rerio* a SWCNT e MWCNT, o qual demonstrou ser um excelente modelo para testes toxicológicos com o uso de nanomateriais, uma vez que apresentou respostas aos efeitos deste nanomaterial, em exposições *in vivo*. Como Algumas conclusões deste estudo, pode ser considerado que: (1) um exame histológico de animais inteiros parece ser uma estratégia interessante para detectar efeitos CNT; (2) grupos de proteínas carboniladas detectadas por imuno-histoquímica mostrou-se também uma boa opção para avaliar as respostas em órgãos que, geralmente, não são considerados nos estudos nanotoxicologia como rim, estômago e os olhos; e (3) resultados do TBARS apresentou uma contraditória resposta antioxidante aparente observadas nas brânquias, os resultados apresentados neste segundo trabalho revelaram o potencial tóxico do NTC aos peixes *D. rerio* quando expostos juntamente com a ração. Existem controvérsias a respeito dos efeitos nocivos, mas, o principal é a preocupação com descarte de produtos contendo CNT no ambiente, uma vez que já estão presentes no ambiente aquático. Este experimento apresentou resultados que foram primordiais para a concepção do próximo e terceiro trabalho no qual foi verificada a possível interferência gerada por nanomateriais de carbono a o método de TBARS, onde foram testados C_{60} e SWCNT e verificou-se a exacerbação de fluorescência e uma diminuição na fluorescência, fato comprova que os resultados estão ligados com as propriedades físicas dos nanomateriais, reduzindo a emissão de fluorescência quando os nanomateriais estão em elevadas concentrações e mais agrupados, em concentrações mais baixas e mais dispersa emitindo mais luz. Portanto apresentando diferentes padrões de interferência de acordo com a concentração de SWCNT e tecidos analisados, Por conseguinte, o ensaio TBARS não é apropriado para amostras tratadas com CNT. A fase aquosa do C_{60} também interferiu no ensaio TBARS com menos intensidade, no entanto, acredita-se que por causa da sua melhor dispersão. Como continuação desta linha de trabalho, outras medidas fluorimétricas precisam de ser avaliadas para possível interferência nanomateriais e amostras biológicas.

É importante que as novas tecnologias observem *o que diz o princípio da precaução*, e a comunidade científica que não pode prever tudo, mas, pode reduzir o risco.

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