UNIVERSIDADE FEDERAL DO RIO GRANDE – FURG
PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS:
FISIOLOGIA ANIMAL COMPARADA

Interação da cianotoxina microcistina e o nanomaterial de carbono fulereno (C_{60}) em brânquias do peixe *Cyprinus carpio* (Teleostei: Cyprinidae) sob incidência de radiação UVA.

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“É mais valente quem vence seus desejos do que quem vence seus inimigos, pois a vitória mais difícil é sobre si mesmo.”

(Aristóteles)

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Sumário

Resumo

A produção mundial de nanomateriais tem aumentado nos últimos anos, em função de suas variadas aplicações tecnológicas e, como consequência do seu crescente uso e demanda, poderão existir riscos ambientais sendo a água o ambiente onde muitas destas substâncias podem exercer efeitos deletérios. Um dos nanomaterias de carbono mais utilizados é o fulereno, um composto orgânico lipofílico que pode se comportar como carreador de moléculas tóxicas, potencializando a entrada de contaminantes ambientais em órgãos específicos, fenômeno conhecido como "cavalo de Troia". As microcistinas (MC) são cianotoxinas produzidas por cianobactérias durante episódios de floração, afetando aos organismos aquáticos e ao ser humano. Diversos estudos demonstram que organismos expostos tanto às MCs quanto ao fulereno podem causar produção excessiva de espécies ativas de oxigênio e alterar os níveis de antioxidantes. Além disso, outro fator que pode vir a intensificar o potencial tóxico de ambos é a incidência de radiação UVA. Sendo assim, procurou-se avaliar os efeitos em parâmetros de estresse oxidativo da co-exposição *ex vivo* da cianotoxina microcistina-LR (MC-LR) e o nanomaterial de carbono fulereno em brânquias do peixe *Cyprinus carpio* sob incidência de radiação UVA. Os resultados mostraram que: (a) houve uma perda da capacidade antioxidante no tratamento com MC-LR (baixa concentração) quando co-exposta com fulereno no UVA em relação com o tratamento realizado sem co-exposição com fulereno; (b) o fulereno no UV diminuiu a atividade da enzima glutationa-S-transferase (GST) quando comparado com o controle no UV; (c) a MC-LR (alta concentração) co-exposta com fulereno foi capaz de diminuir as concentrações do antioxidante glutationa (GSH) quando comparado com o mesmo tratamento tanto no UVA quanto no escuro sem a co-exposição ao fulereno; (d) o tratamento MC-LR (baixa concentração) com UVA aumentou o dano oxidativo lipídico quando comparado com o controle UVA; (e) o fulereno não causou uma maior bioacumulação da microcistina no tecido. Sendo assim, pode-se concluir que o fulereno não apresentou o potencial de carregador de moléculas nessas concentrações de microcistina, porém, a co-exposição dos compostos diminuem tanto capacidade antioxidante total, como a concentração da GSH, podendo gerar problemas a longo prazo na detoxificação da toxina.

Palavras chave: estresse oxidativo, nanotoxicologia, ambiente aquático, ultravioleta; microcistina.

1. Introdução

Os nanomateriais (NM) existem provavelmente a bilhões de anos, resultantes de vários fenômenos naturais como atividade vulcânica, deposição mineral e incêndios florestais. Além disso, são encontrados também em partículas virais, magnetita bacteriana e até mesmo em biomoléculas como a ferritina (Oberdörster *et al*., 2007).

A aplicação dos nanomateriais em processos tecnológicos fez com que sua produção aumentasse de maneira exponencial nos últimos anos devido à sua grande utilização em praticamente todos os campos da indústria, o que gera benefícios significativos tanto para a sociedade quanto para a economia (Adlakha-Hutcheon *et al*., 2009). Os NM fazem parte de muitas áreas da vida humana, que vão desde equipamentos eletrônicos, vestuário e cosméticos à combustíveis e catalisadores químicos. Além disso, sua aplicação tem sido bastante usada na medicina e na área ambiental, ajudando na remediação de ambientes contaminados (Nel *et al.,* 2006; Maynard, 2007; Cheng *et al.,* 2009; Savolainen *et al.,* 2010).

Os NM são definidos pela U. S. National Nanotechnology Initiative como materiais que possuem pelo menos uma dimensão espacial na faixa de 1a 100 nm, onde 1 nanômetro é um bilionésimo de metro, cerca de mil vezes menor que um glóbulo vermelho (Oberdörster, 2004). Devido à grande relação superfície/volume em função de sua escala nanométrica, a capacidade de interação com outras moléculas, incluindo biomoléculas, torna-se muito maior (Oberdörster *et al.*, 2005; Nuffer & Siegel, 2010; Sund *et al.,* 2011).

Apesar da produção mundial de NM estar em aumento, as informações dos possíveis impactos que eles podem ter são bastante limitadas, e pouco é sabido sobre os mecanismos de interação com os sistemas biológicos ou os potenciais efeitos tóxicos no ambiente (Oberdörster *et al*., 2007; Limbach *et al.,* 2009; Kahru & Dubourguier, 2010).

Além disso, é difícil determinar o dano potencial para os seres vivos, incluindo organismos aquáticos, pois as propriedades destes materiais dependem de muitas variáveis, incluindo a distribuição de tamanho, forma das partículas, a carga de superfície, a presença de matéria orgânica e de agregação, todas as quais são intimamente relacionadas com as características particulares de cada ambiente (Howard, 2010; Galego-Urrea *et al.*, 2011).

Entre os NM de carbono, o fulereno C_{60} é um dos mais produzidos no mundo (Spohn *et al.*, 2009). Ele apresenta sessenta carbonos em sua estrutura molecular esférica de ciclopentenos e ciclohexenos, semelhante a uma gaiola (Fig. 1), o que favorece a sua estabilidade e persistência na água na forma de agregados coloidais (Andrievsky *et al.*, 2005). As características apolares do fulereno também permitem a sua passagem através das membranas biológicas (Johnston *et al.*, 2010), podendo também atravessar inclusive a barreira hematoencefálica (Porter *et al*., 2007).

Figura 1. Representação de uma molécula do fulereno, uma estrutura formada por 60 átomos de carbono dispostos nos vértices de um icosaedro truncado, que tem a forma de uma bola de futebol. Sua forma é a de em domo geodésico composto por 12 pentágonos e 20 hexágonos. Fonte: Foldvari e Bagonluri (2008).

Como sua produção tem aumentado exponencialmente desde 1990, quando sua fabricação foi desenvolvida pela primeira vez para a produção em massa (Kratschmer *et al.*, 1990), a escassez de dados a respeito de sua potencial toxicidade tem sido alvo de

preocupação. Tanto o fulereno quanto outros NM tem demonstrado potencial de gerar estresse oxidativo em diversos organismos, incluindo aquáticos. Oberdörster (2004) reportou uma queda na concentração do antioxidante glutationa (GSH) em brânquias do peixe *Micropterus salmoides*. Zhu *et al.* (2008) demonstraram que quando o peixe *Carassius auratus* é exposto a concentrações sub-letais de fulereno (0,04 - 1,0 mg/L), ocorre um aumento da atividade de enzimas antioxidantes como superóxido dismutase (SOD) e catalase (CAT) e diminui a concentração do antioxidante glutationa (GSH). Utilizando brânquias do peixe *Cyprinus carpio*, trabalhos prévios mostraram que o fulereno foi capaz de induzir dano lipídico, diminuir a atividade da enzima limitante na síntese de GSH (glutamato cisteína ligase, GCL), refletindo-se numa queda de GSH (Britto *et al.,* 2012; Ferreira *et al.,* 2012).

Devido à elevada superfície relativa que possuem, existe a possibilidade de que contaminantes presentes no ambiente possam se ligar aos NM. Isso facilitaria a incorporação celular destes contaminantes, podendo exercer efeitos tóxicos até mesmo superiores aos do contaminante isolado, efeito esse conhecido como "cavalo de Tróia" (Limbach *et al.,* 2007; Baun *et al.,* 2008). Trabalhos como o de Limbach *et al.* (2007) mostram que a co-exposição de nanopartículas de sílica com metais como o cobalto e manganês em células de epitélio pulmonar, aumentam em até oito vezes a concentração de espécies ativas de oxigênio (EAO) em relação aos seus controles. Baun *et al.* (2008) expuseram o crustáceo *Daphnia magna* a contaminantes ambientais, e verificaram um aumento na toxicidade de fenantreno e pentaclorofenol, além de uma maior bioacumulação destes quando eram co-expostos junto com o fulereno. O Trabalho realizado com células de hepatócitos de zebrafish, também revelou uma maior acumulação de arsênito (As^{III}) quando esse era co-exposto com fulereno (Costa *et al.*, 2012).

Produzidas por cianobactérias e liberadas na água, as cianotoxinas exercem efeito tóxico para os organismos aquáticos e seres humanos (Oberemm *et al.*, 1997). As microcistinas (MCs) (Fig.2) são consideradas como um dos grupos mais comuns e perigosos de cianotoxinas (Sivonen *et al.*, 1999). Elas são heptapeptídeos monocíclicos principalmente produzidos por cianobactérias dos gêneros *Microcystis*, *Anabaena*, *Nodularia*, *Oscillatoria* (*Planktothrix*) e *Nostoc* durante episódios de florações (De Figueiredo *et al.*, 2004; Amado & Monserrat, 2010).

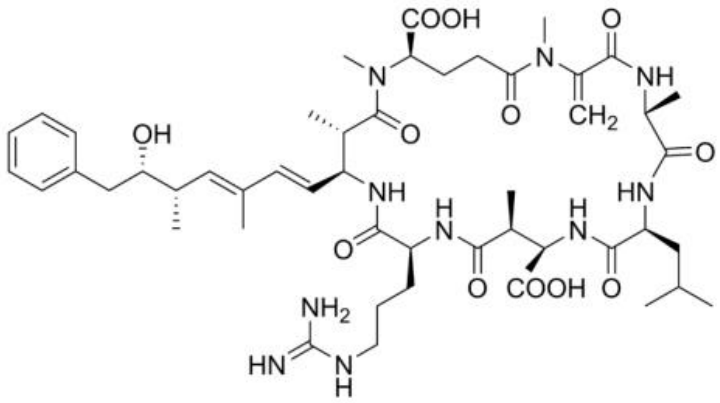

Figura 2. Estrutura química da [D -Leu]-Microcistina-LR.Fonte: Matthiensen *et al.*(2000).

Florações da cianobactéria do gênero *Microcystis* são regulares e recorrentes na Lagoa dos Patos e seu estuário, tendo seus primeiros registros científicos em 1987. Durante os meses de verão e outono as grandes florações de *M. aeruginosa* parecem estar associadas ao fluxo de enchente do estuário, à relação N:P (entre 10:1 a 16:1) e a temperaturas médias da água (acima de 20ºC) (Yunes, 2009).

Os peixes podem ser facilmente expostos as toxinas por ingestão ou respiração, fazendo com que toxinas passem através das brânquias (Zurawell *et al.*, 2005). Após a

exposição, passam a ocorrer diversos efeitos adversos nos animais, desde problemas no desenvolvimento embrionário, alterações em processos fisiológicos e bioquímicos até mudanças no comportamento (Radbergh *et al.*, 1991; Fischer & Dietrich, 2000; Zhang *et al.*, 2007).

Diversos estudos têm demonstrado que MCs são capazes de promover o aumento da geração de EAO (Zegura *et al.*, 2004; Wiegand & Pflugmacher, 2005; Amado & Monserrat, 2010), além de diminuir os níveis de antioxidantes (Ding & Ong, 2003; Jos *et al.*, 2005; Pinho *et al.*, 2005) e alterar a atividade de enzimas de detoxificação como a glutationa-S-transferase (GST), tanto no sentido de aumentar a atividade (Cazenave *et al*., 2008), quanto na redução desta (Cazenave *et al.*, 2006). No âmbito molecular, por serem inibidores específicos de fosfatases, favorecem a hiperfosforilação celular, desregulando variados processos bioquimicos, incluindo aqueles relacionados com a defesa antioxidante. Sun *et al.* (1996) e Toroser *et al*. (2006) mostraram que a fosforilação da subunidade catalitica da glutamato cisteína-ligase (GCL) por proteínas quinase A e C pode diminuir sua atividade, prejudicando as defesas antioxidantes, principalmente pela queda na síntese da GSH.

Até agora nenhum trabalho procurou avaliar a co-exposição de nanomateriais de carbono com cianotoxinas, o que é bastante relevante, visto que ambas por si só já tem um potencial tóxico, que podem gerar espécies ativas de oxigênio e induzir estresse oxidativo. Além disso, poucos trabalhos têm avaliado os efeitos da interação de nanomateriais de carbono conjuntamente com fatores físicos ambientais, como a incidência de radiação ultravioleta (UVA) solar (Kamat *et al.*, 2000; Britto *et al.*, 2012)

A radiação ultravioleta que incide na coluna de água do estuário da Lagoa dos Patos durante o verão é de aproximadamente 10 mW/cm^2 e de 7 mW/cm^2 no outono (Gouveia, 2009), períodos esses que coincidem com as florações de cianobactérias

nesses ambientes. A penetração da radiação ultravioleta nos ambientes aquáticos varia devido a muitos fatores, dentre eles a presença de matéria orgânica dissolvida (MOD) (Hader *et al.*, 2007). No entanto, mesmo em corpos de água com concentrações mais altas da MOD, ambos os comprimentos de onda UV-A e UV-B podem chegar até a uma profundidade de 1 m (Williamson e Rose, 2010) e, desta forma, impactar no plâncton, peixes e outros organismos aquáticos.

No nível molecular, a radiação UV causa danos ao DNA, como dímeros de pirimidina, danos nas estruturas de proteínas (que pode levar a inativação de enzimas) e danos lipídicos (alterando a fluidez de membranas biológicas) (Matsumura & Ananthaswany, 2004). Outros efeitos nocivos da exposição aos raios UVA estão associados à geração de EAO, tais como ânion superóxido ($O_2^{\cdot -}$), radical hidroxila ($HO^{\cdot}$), peróxido de hidrogênio (H_2O_2) e oxigênio singleto ($^1O_2$), bem como os hidroperóxidos lipídicos e suas formas radicalares (LOOH e $LOO^{\cdot}$) (Sakurai *et al.*, 2005).

Sabe-se que as moléculas de C_{60} são fotoativadas por incidência UVA (Taylor *et al.*, 1991; Creegan *et al.*, 1992). Kamat *et al.* (2000) mostraram que o C_{60} induz peroxidação lipídica em microssomas hepáticos de ratos exposta à luz UVA. Trabalhos que utilizaram o peixe *Cyprinus carpio*, mostraram também um aumento na peroxidação na presença de luz visível (Shinohara *et al*., 2009) bem como na presença da radiação UVA (Britto *et al.*, 2012).

A escolha do órgão a ser utilizado que demonstrem possíveis alteraçãos no animal é importante, no ambiente aquático, os poluentes na água podem ser absorvidos pelos peixes por quatro vias: alimentação, brânquias, ingestão de água e através da pele, sendo que alimentação e via branquial são as principais. (Heath, 1997). A utilização da brânquia como o orgão a ser utilizado no trabalho, se deve pelo fato delas estarem em

contato permanente com o ambiente aquático e apresentarem uma área superficial muito grande, representada pelas lamelas (Poleksić & Mitrović-Tutundžić, 1994). O Modo de exposição em *ex vivo,* já utilizados em trabalhos prévios (Britto et al., 2012) permite que a exposição seja a mais próxima do animal no ambiente onde o órgão inteiro fica submersa na água onde estarão presentes os tóxicos.

2. Justificativa

Levando em consideração a produção em massa de NM, o inevitável contato que irão ter com ecossistemas terrestres e aquáticos, bem como a falta de conhecimento a respeito do impacto na saúde humana e no ambiente, foi escolhida uma abordagem capaz de simular em parte uma condição ambiental, com o intuito de analisar os efeitos de NM de carbono e seu possível contato e interação com cianotoxinas presentes na água e sob radiação UVA em uma exposição das brânquias de carpas em *ex vivo*.

3. Objetivo geral

Avaliar os efeitos em parâmetros de estresse oxidativo resultantes da co-exposição *ex vivo* à cianotoxina microcistina e ao nanomaterial de carbono fulereno (C_{60}) em brânquias do peixe *Cyprinus carpio* (Teleostei: Cyprinidae), sob incidência de radiação UVA.

3.1. Objetivos específicos

(a) Verificar a capacidade antioxidante total contra peroxi-radicais em brânquias do peixe *C. carpio* expostas em *ex vivo* aos diferentes agentes tóxicos (fulereno e microcistina), levando em conta também o efeito da radiação ultravioleta;

(b) Determinar a atividade da enzima glutamato cisteína ligase (GCL) e concentração de glutationa reduzida (GSH) nas mesmas condições experimentais enunciadas no ponto **(a)**;

(c) Verificar dano oxidativo em termos de peroxidação lipídica nas mesmas condições experimentais enunciadas no ponto **(a)**;

(d) Determinar a atividade da enzima glutationa-S-transferase (GST) nas mesmas condições experimentais enunciadas no ponto **(a)**;

(e) Quantificar a bioacumulação de microcistina nas brânquias do peixe *C. carpio* expostos aos diferentes agentes tóxicos (fulereno e microcistina), levando em conta também o efeito da radiação ultravioleta.

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INTERACTION OF CARBON NANOMATERIAL FULLERENE AND MICROCYSTIN-LR IN GILLS FISH OF *CYPRINUS CARPIO* (TELIOSTEI: CYPRINIDAE) UNDER INCIDENCE OF ULTRAVIOLET RADIATION

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Abstract

One of the most widely used carbon nanomaterials is fullerene (C_{60}), a lipophilic organic compound that potentially can behave as a carrier of toxic molecules, enhancing the entry of environmental contaminants in specific organs. Microcystins (MC) are cyanotoxins very toxic for human and environmental health. Several studies show that exposure to MC or C_{60} generates excessive production of reactive oxygen species (ROS) and changes in antioxidant levels. Also, another factor that can come to enhance the toxic potential of both is UVA radiation. Therefore, it was evaluated the effects on oxidative stress parameters of *ex vivo* co-exposure of MC and C_{60} in gills of the fish *Cyprinus carpio* under UVA radiation incidence. The results showed that: (a) there was a loss of antioxidant capacity after L+C_{60} co-exposure under UVA; (b) C_{60} under UVA decreased glutathione-S-transferase (GST) activity; (c) H+C_{60} co-exposure decreased the concentrations of glutathione (GSH) under UVA or in the dark; (d) L under UVA increased lipid peroxidation; (e) the fullerene has not caused a higher bioaccumulation of microcystin in the tissue. Thus, it can be concluded that the fullerene not facilitate MC entry into gills. However, the lowering of GSH in H+C_{60} co-exposure should compromise MC detoxification.

Keywords: oxidative stress, nanotoxicology, aquatic environment, ultraviolet, microcystin.

INTRODUCTION

Nanotechnology is based on the production of materials size range nanometer in order to obtain products with wide applications in various fields of industry [1]. Despite the global production of nanomaterials (NMs) to be growing, information about the mechanisms of interaction with biological systems or potential toxic effects on the environment is very limited [2,3].

The properties of NMs are dependent, among others on, size distribution, surface charge, particle shape and the presence of organic matter and aggregation, thus making it difficult to determine the potential harm to aquatic organisms [4,5]. Fullerene (C_{60}) is one of the many carbon nanomaterials produced in the world [6]. It presents sixty carbon atoms in its molecular spherical structure, favoring their stability and persistence in water in the form of colloidal aggregates [7]. Moreover, present non-polar characteristics, which allows to pass through biological membranes [8].C_{60} and other NMs have demonstrated potential to generate oxidative stress in several aquatic species [9-11].

Due to the relatively high surface, NMs have a great ability to interact with environmental contaminants, so that thay are incorporated together by some aquatic organisms and exert even higher toxic effects than the compounds alone, a phenomena known as ‘Trojan horse’ [12].

Previous studies showed that C_{60} is photoactivated by UVA radiation [13]. Kamat et al. [14] showed that C_{60} induces lipid peroxidation in rat liver microsomes exposed to UVA light and studies with fish *Cyprinus carpio*, also showed an increase in lipid peroxidation in the presence of visible light [9, 11] or under UVA radiation [10].Other harmful effects of UVA exposure are associated with the generation of ROS,

such as superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($HO^{\cdot}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) and lipid hydroperoxides and their radicalar species [15].

Cyanobacterial blooms, which frequently occur in freshwater, reservoirs, ponds, and coastal waters around the world due to exacerbated water eutrophication, posses ecological risks because they can release algal toxins [16]. Microcystins (MC) are considered one of the most common and dangerous of cyanotoxins [17], being produced by cyanobacteria of the genera *Microcystis*, *Anabaena*, *Nodularia*, *Oscillatoria* (*Planktothrix*) and *Nostoc* during bloom episodes [18].

One of the most important and relevant toxicity of microcystin is their potential inhibition of phosphatases PP1 and PP2A [16], moreover, can increased production of reactive oxygen species (ROS) [18], while decreasing the levels of antioxidants [19], and altering the activity of detoxification enzymes as glutathione-S-transferase (GST) [20].

Thus, due to the scarcity of studies on the behavior of carbon NMs in the aquatic environment, and little information about its interaction with other toxic substances from the environment, such as microcystin, the study aimed to analyze in oxidative stress parameters, effects of co-exposure of *ex vivo* gills of fish *Cyprinus carpio* to cyanotoxin microcystin and namomaterial C_{60} fullerene with the addition of a factor present in the environment, ultraviolet radiation.

MATERIALS AND METHODS

All procedures in this work were carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments and were approved by the CEUA (Comissão de Ética em Uso Animal) at the Federal University of Rio Grande – FURG (process P010/2011).

Biological model

Carp (*Cyprinus carpio*) specimens were obtained from local suppliers and acclimated to laboratory conditions for at least a week before the experiments. They were held in aquaria containing 200 L of freshwater with continuous aeration, temperature of 25 °C, 12:12 L:D cycle and pH between 6.8-7.0. During acclimation period the fish were fed once a day with commercial feed. This species was chosen due to its wide use in toxicological studies, including analysis of the toxic effects of C_{60} [9,10].

Preparation of fullerene suspensions

Two hundred mg of fullerene powder (SES Research, 99% purity) was diluted in 1 L Milli-Q water, leaving under constant stirring for 2 months in presence of artificial fluorescent light. After this period, the suspension was centrifuged at 25,000 x g for 1 hour at 15 °C and then sequentially filtered through nylon filters of 0.45 and 0.20 μm pore size, in order to obtain a suspension at the nanometer range. After filtration, the fullerene concentration was determined by the detection of organic carbon in a total carbon analyzer (TOC, Shimadzu) [10, 11]. The final fullerene concentration employed in the assays was 5 mg of fullerene per 1 L Milli-Q water. Note that previous studies have shown by transmission electron microscopy that this protocol for preparation of aqueous fullerene suspensions renders particles in the nanometric range (1-100 nm) [10,11].

Preparation of microcystin solutions

The cianotoxin microcystin-LR was obtained from Cayman Chemical Company, diluted in methanol. The solution in methanol was evaporated and re-suspended in Milli Q water. Two concentrations were tested in the assays: a low (L) with 50 μg of

microcystin per liter of solution, and another high (H) with 200 µg of microcystin per liter of solution.

Exposures doses to ultraviolet radiation

Ultraviolet radiation, to which the gills were exposed, was obtained through a lamp with a filter for light emission in the range of 365 nm (Vilber Lourmat VL 215 L, 30W) placed 35 cm above the organs. The radiation dose delivered was estimated to be 3.132 and 0.0108 J/cm^2 for UV-A and UV-B, respectively (the lamp also emits a small amount of light in the UV-B range).

This dose was chosen considering the previous studies of [21] with human lens epithelial cells, in which a similar UV-A dose (3.7 J/cm^2) did not alter cell viability, and also by previous work performed in our laboratory [10].

Organ exposure

The gill exposure methodology was based on the method previously described by Britto et al. [10]. Thirty fish were anaesthetized in tricaine (40 mg/mL) and the gills were dissected. Immediately after, the gills were submerged in saline for freshwater fish, composed of 125.00 mM NaCl, 2.50 mM KCl, 0.94 mM $MgSO_4$, 30.00 mM NaH_2CO_3, 1.00 mM NaH_2PO_4, 1.25 mM $CaCl_2$ and 10.00 mM $C_6H_{12}O_6$ with the pH adjusted to 7.40 [10]. The fullerene suspensions and microcystin solution used were diluted in this saline solution. The gills, after extracted, were divided into six treatment group. Each fish was considered a sample, being employed five replicates in each treatment. The treatments were: (1) Control - saline only (Ctr); (2) Fullerene - 5 mg/L (C_{60}); (3) Microcystin low concentration - 50 µg/l (L); (4) Microcystin higher concentration – 200 µg/l (H); (5) Fullerene (5 mg/l) plus microcystin low concentration (50 µg/l) (L+ C_{60}); (6) Fullerene (5 mg/l) plus microcystin higher concentration (200 µg/l) (H+ C_{60}). All treatments were performed in two sets: a “Dark” set (n=5), in which

the gills were exposed without light incidence, and in a "UVA" set (n=5), in which the exposure was performed under ultraviolet radiation.

The gills were placed in glass recipients containing 3 mL of saline solution plus respective solutions, under constant agitation for 1 hour at room temperature. After the elapsed time, the gills were removed, dried, weighed and stored at -80°C for later homogenization for biochemical measurements.

Biochemical measurements

Tissue sample preparation

A total of 60 samples (6 treatments x 2 light conditions x 5 replicates) were homogenized according to the methodology described by da Rocha et al. [22] in 1:5 (w/v) in Tris-HCl (100 mM, pH 7.75) buffer plus EDTA (2 mM) and Mg^{2+} (5 mM). The homogenates were centrifuged at 10,000 x g during 20 min at 4°C, and the supernatants were removed and used for biochemical determinations. Total protein content determination was performed with a commercial kit based on the Biuret method (Doles®), using a microplate reader (Biotek ELX 800) at a wavelength of 550 nm.

Measurement of total antioxidant capacity

Total antioxidant competence against peroxyl radicals was analyzed through ROS determination in organs samples treated or not with a peroxyl radical generator. Peroxyl radicals were produced by thermal (37 ºC) decomposition of 2,2´-azobis-2-methylpropionamidine-dihydrochloride (ABAP, 4 mM; Aldrich). For ROS determination it was employed the fluorogenic compound 2´,7´-dichlorofluorescein diacetate (H_2DCF-DA; Invitrogen) at a final concentration of 40 µM, according to the methodology described by Amado et al. [23]. Readings were carried through in a fluorescence microplate reader (Victor 2 Perkin), in a medium containing 30 mM HEPES (pH 7.2), 200 mM KCl, 1 mM MgCl 2, 40 µM DCF-DA. Total

fluorescence generation was calculated by integrating the fluorescence units (FU) along the time of the measurement. The results were expressed as the area difference of FU x min. The readings were performed in the same sample with and without ABAP addition and standardized to the ROS area without ABAP (background area). The relative difference between the ROS area with and without ABAP was considered to be a measure of antioxidant competence. A high area difference was considered to indicate a low antioxidant capacity, as high fluorescence levels were obtained after adding ABAP, suggesting a low ability to neutralize peroxyl radicals [23].

Determination of glutamate cysteine ligase (GCL) activity and glutathione (GSH) concentration

The level of reduced glutathione (GSH) and the activity of the enzyme glutamate cysteine ligase (GCL) were determined according to da Rocha et al. [22]. The method is based on the detection by reading the fluorescence generated by the complex that is formed by conjugation of naphthalene dicarboxaldehyde (NDA) and GSH or γ-glutamylcysteine. In one plate is added 25 µl of a reaction cocktail (400 mM Tris-HCl, 40 mM ATP, 20 mM glutamate, 2.0 mM EDTA, 20 mM sodium borate, 2 mM serine and 40 mM $MgCl_2$).Then, 25 µl of gill homogenates (protein concentration fixed at 4 mg/mL) was added, and the reaction was initiated by adding 25 µl of 2 mM cysteine. After 60 minutes elapsed, the reaction was stopped with 25 µl of 200 mM sulfosalicilic acid (SSA). After protein precipitation, the plate was centrifuged for 5 min at 1,500 × g, and 20 µl of supernatant from each well was transferred to a white plate. Then, a NDA solution was added to all wells, and after 30 min of incubation, the fluorescence intensity of the NDA-GSH complex was read at the excitation and emission wavelengths of 485 and 530 nm (Victor 2, Perkin Elmer).

Determination of glutathione-S-transferase (GST) activity

Activity of the enzyme GST was determined through the conjugation of 1-chloro- 2,4 di-nitrobenzene (CDNB, from Sigma) diluted in ethanol 100% at a final concentration of 1 mM with reduced glutathione (GSH, from Sigma) diluted in phosphate buffer at a final concentration of 1 mM. GST catalyzes the reaction and form a conjugate (GSH-CDNB) which is read at absorbance of 340 nm [22].

Measurement of lipid peroxidation

The quantification of lipid peroxidation was measured the according to da Rocha et al. [22]. This methodology quantifies malondialdehyde content, one of thiobarbituric acid reactive substances (TBARS). Gill extracts (10 µl) were added to a reaction mixture containing 20 µl of BHT solution (1.407 mM), 150 µl of 20 % acetic acid, 150 µl of thiobarbituric acid (0.8 %), 50 µl of Milli Q water and 20 µl of sodium dodecyl sulfate (SDS, 8.1 %). The samples were heated at 95 ^{o}C for 30 min, and after100 µl of Milli-Q water and 500 µl of buthanol were added. After centrifugation (3,000 x g for 10 min at 15 ^{o}C), the organic phase (150 µl) was placed in a microplate reader, and the fluorescence was registered after excitation at 520 nm and emission at 580 nm. The concentration of TBARS (nanomoles/mg of wet tissue) was calculated employing a standard curve of tetramethoxypropane (TMP,Acros Organics) as malondialdehyde equivalents.

Quantification of accumulated microcystin in gills

Gills has exposed were lyophilized and then it was added 36 ml of buthanol:methanol:Milli Q water (1:4:15, v:v:v). After, samples were put in an ultrasonic bath for 3 min (30% amplitude, 180VA, 25 kHz) and then centrifuged (3,200 xg for 20 min at room temperature). The supernatant was removed and evaporated in a water bath at 60 °C under N_2 gas flow and finally re-suspended in 200 ul of H_20 Milli Q

[24]. Microcystin concentration in the samples was analyzed through a specific ELISA kit (Envirologix)..

Statistical analysis

The biochemical variables were analyzed by analysis of variance (ANOVA), nested design. This statistical design was adopted since for each treatment (Ctr., C_{60}, L, H, L+ C_{60}, and H+ C_{60}) four branchial arches were exposed to the dark condition and the other four branchial arches of the same fish were submitted to UVA radiation. So, the light factor (dark or UVA exposure) was nested in the main treatment factor [10]. The assumptions of ANOVA (normality and variance homogeneity) were previously evaluated and a logarithm transformation applied if at least one of them was violated. Mean comparisons were performed by Newman-Keuls and orthogonal contrasts. In all cases, the type I error was fixed in 5%.

RESULTS

Total antioxidant capacity

No significant differences ($p > 0.05$) were found between the gills exposed to UVA compared to the exposed dark environment. Gills exposed to L+ C_{60} UVA showed a decrease in antioxidant competence (higher relative area) ($p < 0.05$) compared to the gills treated only with L UVA (Fig. 1).

Activity of glutamate cysteine ligase (GCL) and glutathione (GSH) concentration

There were no significant differences ($p > 0.05$) in the GCL enzyme activity between the different treatments (Fig. 2), and also no effect of radiation was observed ($p > 0.05$, Fig. 2). In terms of glutathione concentration, H+ C_{60} showed a decrease when compared to gills from H group, both in dark and under UVA radiation ($p < 0.05$; Fig. 3).

Activity of glutathione-S-transferase (GST)

No significant differences ($p>0.05$) were found in treated groups between gills exposed to UVA and gills kept in the dark environment. However, lower GST activity in C_{60} UVA groups was observed when compared to Control UVA ($p<0.05$; Fig 4). This result was not observed in the dark treatment ($p>0.05$).

Levels of thiobarbituric acid reactive substances (TBARS)

As Fig.5 shows, the treatment MIC_B UVA presented higher levels of TBARS than the control UVA and this effect was reversed for the $L+C_{60}$ UVA treatment.

Quantification of microcystin

There were no significant differences ($p> 0.05$) between treatments in microcystin LR accumulated in the gills (Fig. 6).

DISCUSSION

It is very important to study the effects that a NM can generate on the environment, since the industry has intensified their production. A plant of large scale production of fullerenes has been opened for some years ago in Japan, allowing the production of a significant amount per year (40 tons / year) [25,26]. It is known that the solvent of organic NM suspensions influence their toxicity. For example, solvent are capable of altering NM penetration into the skin, as seen in study *in vivo* and *in vitro* [27], where fullerene nanoparticles were readily absorbed when dissolved in toluene, cyclohexane or chloroform. In fact, some studies indicate that water fullerene suspensions possess lower toxicity than in organic solvents suspensions as tetrahydrofuran [28]. These evidences determined gill exposure to aqueous fullerene suspensions in order to avoid another toxicity source due to the solvent.

In addition, Yang et al. 2013 [29] showed in his work that salts as NaCl concentrations above 100 mM are able to make nano-sized particles to aggregate and remain in larger sizes, being able to get to micrometer scale. The saline solution used in the work in which the gills are submerged around these NaCl concentrations used, however the use of it and also the other salts is essential for the exhibition, for an hour without being with salts lose their integrity. You should also consider that within these bodies also exist salts.

Besides the solvent employed for preparation of fullerene suspensions, the generation of ROS by C_{60} has been related to other factors such as: the presence of the associated water and exposure to light (UVA and visible) [28]. The light promotes fullerene transition to a triplet state of long lifetime and the subsequent energy transfer to molecular oxygen, resulting in the generation of the highly reactive singlet oxygen ($^1O_2$) [30].

In this present work, the effect of UVA on the toxicity of C_{60} was evidenced by the lowering of GST activity under this condition, an effect that was not observed in the dark (Fig. 4). Furthermore, the concentration used in the study (5 mg/L) was equal to that used by Canesi et al. [31] in mussels, where it also was observed a drop in the activity of this enzyme.

The loss of GST activity (Fig.4) is highly relevant in the context of microcystin toxicity. The primary route of detoxification of MC is by conjugation with reduced glutathione (GSH). The conjugation MC and GSH is enzymatically catalyzed by glutathione S-transferase (GST) [32-33]. The generation of a MC-GSH conjugate has been reported in detoxification processes in different aquatic organisms such as plants (*Ceratophyllum demersum*), invertebrates (*Dreissena polymorpha, Daphnia magna*) and fish (*Danio rerio*) [33]. Despite the co-exposure MC+C_{60} does not reduce the GST

enzyme activity, the decrease caused by C_{60} demonstrates a possibility of deficiency of other detoxification compound that need this reaction phase II to facilitate their elimination.

Following this line, the results showed that co-exposure of fullerene and microcystin led to changes in the total antioxidant capacity (Fig.1) too in UVA and concentration of glutathione (Fig.3) compared to treatment with microcystin alone. The decrease of GSH, have at least two consequences: (1) impairs MC detoxification process, and (2) impairs antioxidant capacity, since GSH is known to neutralize free radicals, preventing chain reactions [35]. The decrease of total antioxidant capacity against peroxyl radicals also indicates that *C. carpio* gills are prone to suffer a pro-oxidant condition as consequence of MC and fullerene co-exposure. However, the lowering of total antioxidant capacity and GSH concentration was not paralleled by higher TBARS levels, since only gills exposed to low MC concentration showed a statistical significance (Fig. 5), suggesting a different time frame for the different treatments.

Although previous studies showed that fullerene acts as a "Trojan horse" and thus facilitating the entry of toxicants into the cell [12, 36], in present work it was observed that the amount of MC within the gills in MC+C_{60} treatment was not greater than the MC treatment (Fig.6). The fact that co-exposure does not lead to a greater microcystin accumulation does not denies the fact of MC and fullerene interaction since, as mentioned above, the co-exposure of these compounds leads the gills of *C. carpio* to a pro-oxidant condition. Also it is important to point that Baun et al. [37] found that fullerene augmented phenanthrene toxicity but antagonized pentachlorophenol, with no effects on atrazine and methyl parathion toxicity. At

present, studies establishing the relationship of toxicants structure with their capacity to be sorbed to fullerene suspensions are lacking.

Synergistic interactions are well known in toxicology and occurr when there is an increase in the effect produced by the combined exposure of two or more toxicants. Synergistic effects of microcystin and other compounds have already been addressed in other studies, such as [38], found with Linear Alkylbenzene Sulfonate (LAS) in species of duckweed (*Lemna minor*) causing changes in growth, activity of superoxide dismutase (SOD) and GSH concentration. Another study conducted jointly by microcystin exposure to ammonia in carp larvae, showing a synergistic effect to changes in the activity of SOD and lipid damage [39].

The concentration of the toxic substance and time that the animals are exposed can have different effects on the organs, giving different answers. The present work fixed exposure time of 1 hour, but made exposure to different concentrations of microcystin. When analyzed gill lipid damage, major damage was observed when they were exposed at the lowest concentration of microcystin. Concentration of toxic and time that are exposed can have different effects on the organs, giving them different answers. In the case of lipid damage effects, possibly the time window differs in two concentrations of microcystin (high and low). In the work was set time of 1 hour, and made exposure to different concentrations of microcystin at that time the low concentration of microcystin proved able to induce damage lipids, an effect not observed when the concentration of microcystin was higher (Fig. 5), suggesting that at higher concentrations cause damage more quickly between this first hour, then is reversed and can no longer be seen.

CONCLUSIONS

The Co-exposure of fullerene (C_{60}) with microcystin LR showed a reduction in the levels of GSH and decrease in total antioxidant capacity in the gills of *C. carpio* exposure when compared to only microcystin, impotante effect since the long run this could cause problems in the detoxification process of toxina. Moreover, the fullerene was not capable of carrying larger amount of microcystin into the organ. The activity of GST was reduced after exposure to C_{60}, this decrease can cause deficient detoxification of other compounds that require this reaction stage II to facilitate their removal. Finally, ultraviolet radiation appears to enhance the toxic effects of microcystin and C60 since most of them were found on gills exposed to UVA.

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Figures captions

Figure 1. Total antioxidant capacity against peroxyl radicals (ROO•) in gills of *Cyprinus carpio* submitted to the different treatments. Data are expressed as the difference in area with and without ABAP relative to the fluorescence area measured without ABAP. Low relative area means high total antioxidant capacity against ROO•. Ctr: Control; C_{60}: fullerene; H: microcystin high concentration; L: microcystin low concentration; H+C_{60} and L+C_{60}: fullerene co-exposed with microcystin at high and low concentrations, respectively. Asterisk (*) indicate significant differences ($p<0.05$) after applying orthogonal test.

Figure 2. Activity of glutamate-cysteine ligase (GCL) in gills of *Cyprinus carpio* submitted to the different treatments. Ctr: Control; C_{60}: fullerene; H: microcystin high concentration; L: microcystin low concentration; H+C_{60} and L+C_{60}: fullerene co-exposed with microcystin at high and low concentrations, respectively. Data are expressed as nanomoles of the dipeptide glutamyl-cysteinyl produced per mg of proteins per hour.

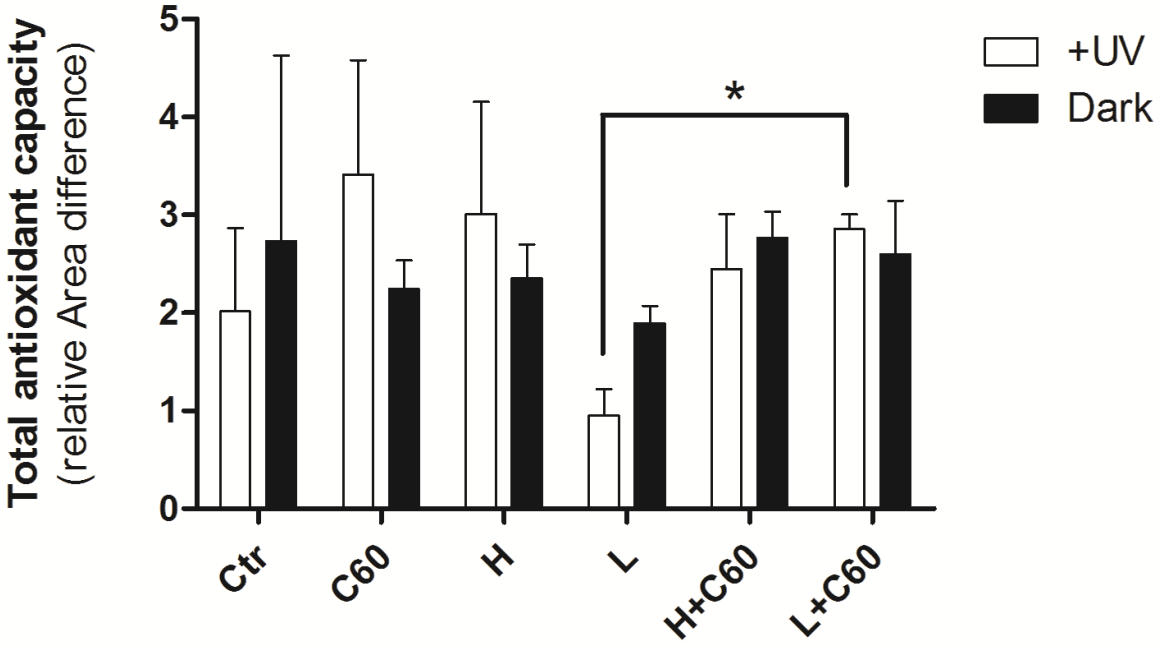
Figure 3. Concentration of the tripeptide glutathione (GSH) in gills of *Cyprinus carpio* submitted to the different treatments. Data are expressed as nanomoles of glutathione per mg of proteins. Ctr: Control; C_{60}: fullerene; H: microcystin high concentration; L: microcystin low concentration; H+C_{60} and L+C_{60}: fullerene co-exposed with microcystin at high and low concentrations, respectively. Same letters mean no statistically significant differences ($p> 0.05$).

Figure 4. Activity of glutathione-S-transferase (GST) (in nmol CDNB-GSH conjugate/min/mg of proteins) in gills of *Cyprinus carpio* submitted to the different treatments. Ctr: Control; C_{60}: fullerene; H: microcystin high concentration; L: microcystin low concentration; H+C_{60} and L+C_{60}: fullerene co-exposed with microcystin at high and low concentrations, respectively. Asterisk (*) indicates significant differences ($p<0.05$) after applying orthogonal test.

Figure 5. Concentration of thiobarbituric reactive substances (TBARS) (nmol of TMP/mg of tissue) in gills of *Cyprinus carpio* submitted to the different treatments. Ctr: Control; C_{60}: fullerene; H: microcystin high concentration; L: microcystin low concentration; H+C_{60} and L+C_{60}: fullerene co-exposed with microcystin at high and low concentrations, respectively. Asterisk (*) indicates significant differences ($p<0.05$) after applying orthogonal test.

Figure 6. Amount of microcystin-LR accumulated (µg/g of wet tissue)in the gills of *Cyprinus carpio* submitted to the different treatments. Ctr: Control; C_{60}: fullerene; H: microcystin high concentration; L: microcystin low concentration; H+C_{60} and L+C_{60}: fullerene co-exposed with microcystin at high and low concentrations, respectively. Same letters mean no statistically significant differences ($p> 0.05$).

Figure 1.


Total antioxidant capacity
(relative Area difference)
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2
1
0
*
+UV
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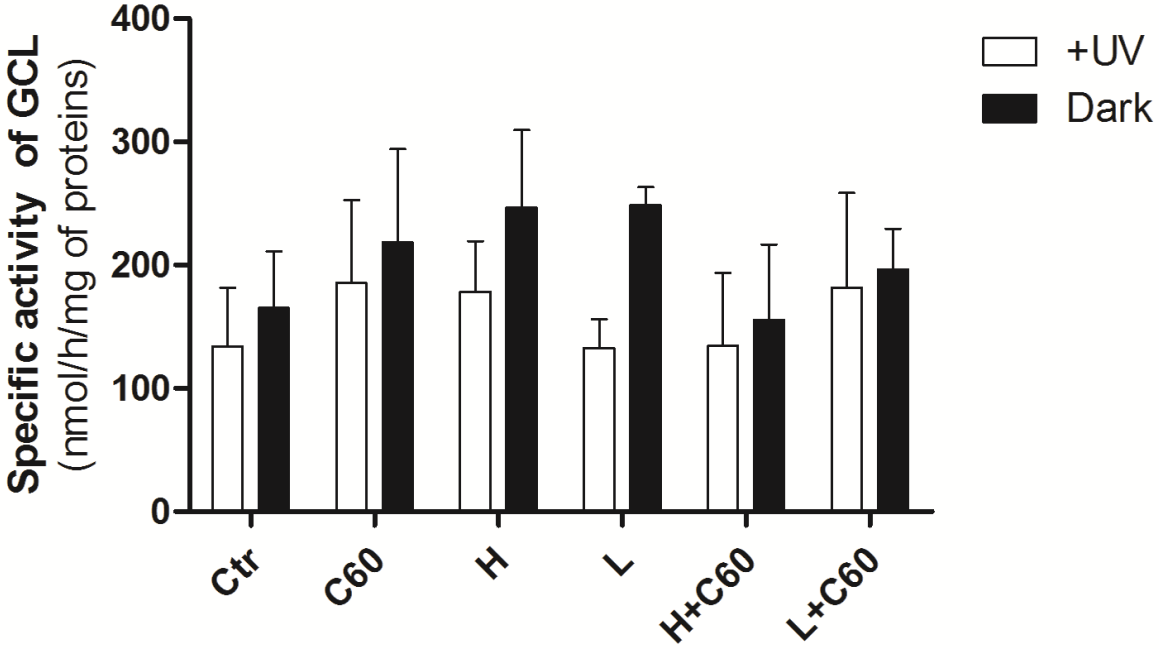
Ctr
C60
H
L
H+C60
L+C60

Figure 2.


Specific activity of GCL
(nmol/h/mg of proteins)
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200
100
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C60
H
L
H+C60
L+C60
+UV
Dark

Figure 3.

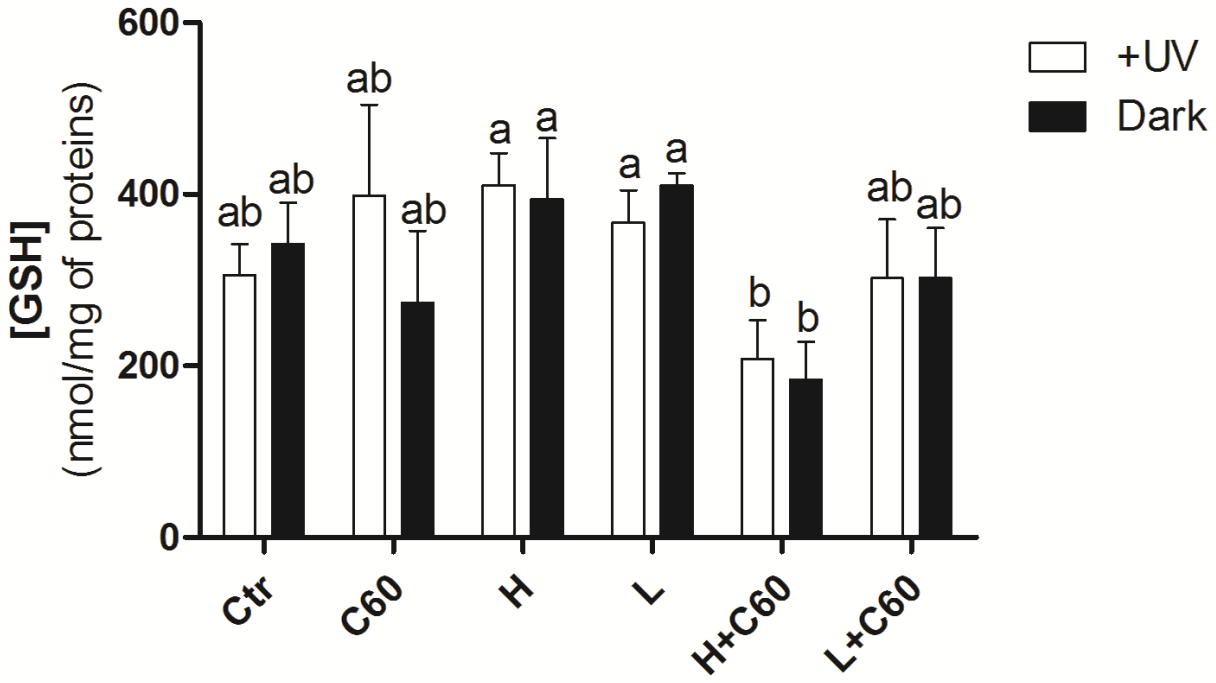
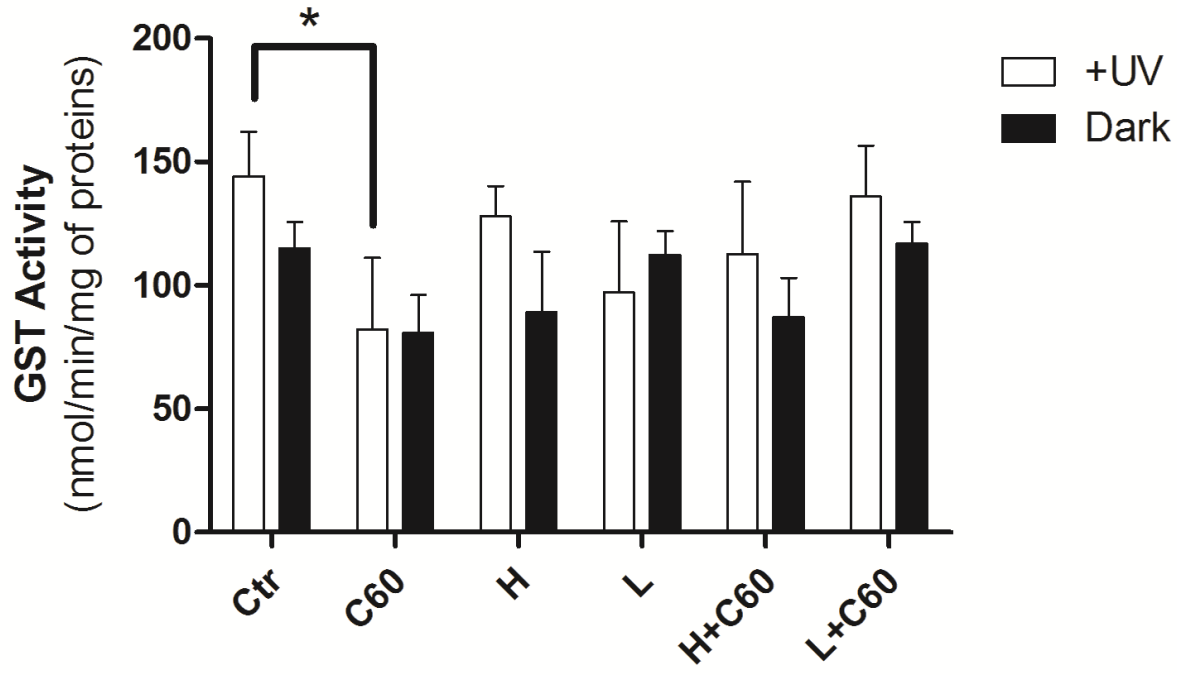

[GSH]
(nmol/mg of proteins)
600
400
200
0
ab
ab
ab
ab
a
a
a
a
b
b
ab
ab
Ctr
C60
H
L
H+C60
L+C60
+UV
Dark

Figure 4.


GST Activity
(nmol/min/mg of proteins)
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100
50
0
*
Ctr
C60
H
L
H+C60
L+C60
+UV
Dark

Figure 5.

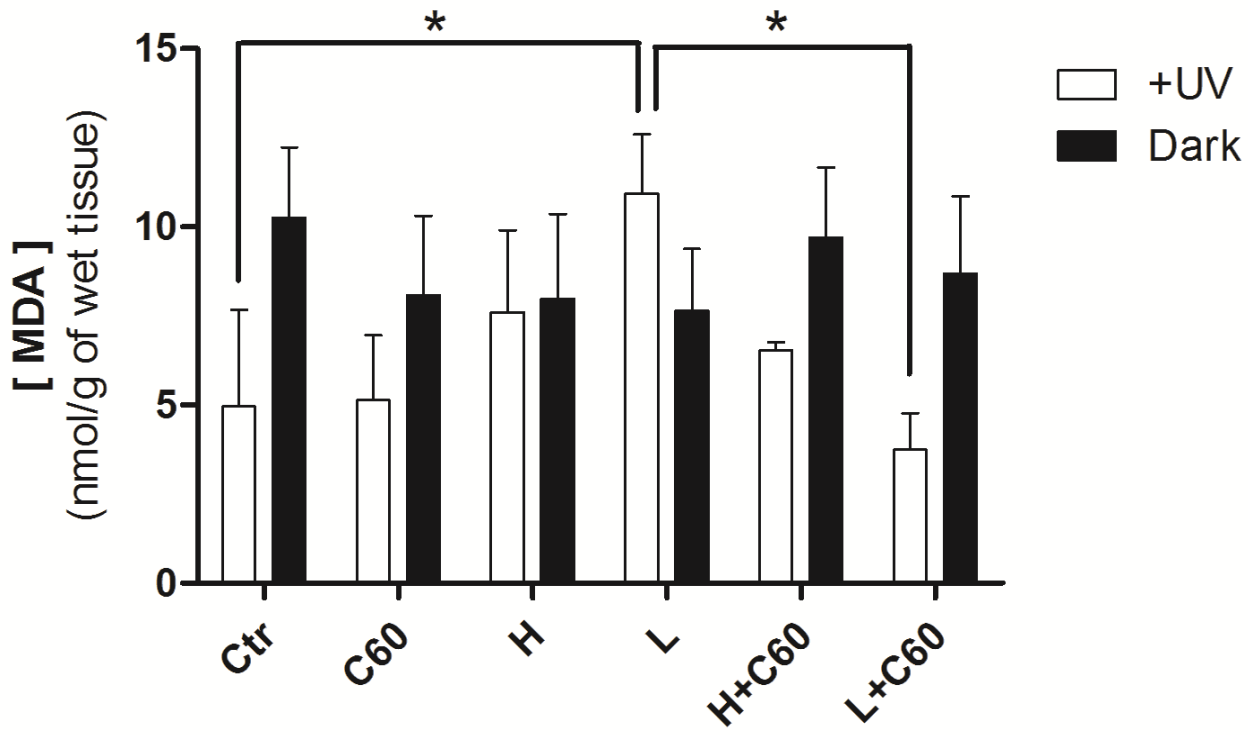
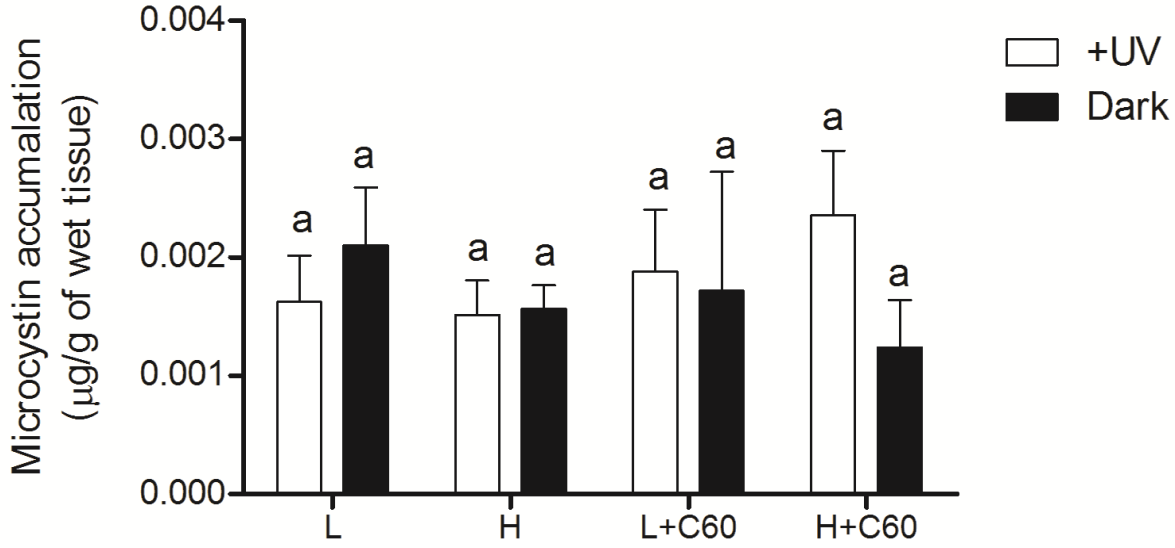


Figure 6.


Microcystin accumalation
(μg/g of wet tissue)
0.004
0.003
0.002
0.001
0.000
a
a
a
a
a
a
a
a
L
H
L+C60
H+C60
+UV
Dark

4. Conclusões Gerais

(a) O fulereno não apresentou o potencial de carregador de moléculas tóxicas (efeito cavalo de Troia) quando co-exposto com microcistina-LR, tanto na baixa quanto na alta concentração;

(b) A co-exposição do fulereno com a microcistina na alta concentração diminuiu a concentração do antioxidante glutationa (GSH) nas brânquias de *C. carpio*, tanto na condição UVA quanto no escuro, efeito importante visto que a longo prazo isso poderia trazer problemas no processo de detoxificação da toxina.

(c) A co-exposição do fulereno e a microcistina na baixa concentração causou uma baixa na capacidade antioxidante total nas brânquias de *C. carpio* em relação as expostas somente à microcistina na radiação ultravioleta, não tendo o mesmo potencial no tratamento no escuro;

(d) O fulereno induziu uma queda na atividade da GST nas brânquias de *C. carpio* quando expostas a radiação ultravioleta. Embora sua co-exposição não mostre o mesmo, a baixa atividade induzida pelo fulereno pode causar deficiência de detoxificação de outros compostos que necessitam desta reação de fase II para facilitar sua eliminação.

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Environmental Chemistry—Papers should emphasize how chemistry is applied to measuring, assessing, or predicting chemical fate (abiotic and biological transformations) and environmental exposure (bioavailability, bioaccumulation) including indoor environments. The work should provide insights into toxicological responses of exposed organisms (including humans). The applicability of the work to environmental assessment, environmental toxicology, or ecological risk assessment must be clear. Papers on analytical methods must demonstrate meaningful and useful advancements over existing methods,

potential to influence current practice, and application to environmental samples and environmental assessment in general. Theoretical or modeling studies oriented toward predicting environmental behavior, chemical properties, or toxicity must show direct applicability to environmental or ecological risk assessment.

***Environmental Toxicology*—**Papers may deal with the harmful effects of chemical stressors on organisms, populations, communities, and ecosystems. Papers that use data or models to elucidate mechanisms and advance the ability to extrapolate toxicological information across species, chemicals, levels of biological organization, or ecosystems are particularly desirable. In addition, papers that show the interactions of multiple stressors and their resulting effects are also a priority. Studies that report in vivo toxicity endpoints intended to establish exposure- or dose-response relationships to be used in hazard or risk assessment should include analytical confirmation of exposure or dose concentrations. The exception to this requirement is on confirmation of nanomaterial concentrations that are in complex matrices, such as soils, sediments and periphyton, because the state-of-the-science makes this exceedingly difficult.

***Non-Chemical Ecological Stressors*—**Papers may deal with the harmful effects of a wide range of biological and physical stressors that may (or may not) interact with naturally occurring or anthropogenic chemicals to affect organisms, populations, communities, and ecosystems. This includes stressors such as alterations of habitat, climate change, and invasive species. Papers that show the interactions of multiple stressors and their resulting effects are also a priority.

***Hazard/Risk Assessment*—**Manuscripts should describe hazard or risk assessments, or provide methods or models to use in such assessments. Case studies must have sufficient scope and impact to be of interest to a broad audience. While most hazard and risk assessments focus on metals and synthetic organic chemicals, we are particularly interested in assessments that also include other cumulative stressor effects, including interactions with non-chemical stressors, such as alteration of habitat, climate change, or invasive species. Hazard/risk assessments should focus on the science of hazard/risk assessments; papers delving deeply into policy, regulation, value judgments, or public, social, or legal issues should be submitted to SETAC's second journal,*Integrated Environmental Assessment and Management (IEAM)*.

Manuscript Preparation

Double space and left justify *everything*, including tables, figure legends, and references. Place page numbers in the upper right-hand corner and leave liberal side margins of at least 1 inch. Format documents to US letter size (8.5 × 11 in), and number the lines of the text continuously from the first page through the figure legends. Consult recent issues for proper placement of main headings, subheadings, and paragraph headings. Titles and subheadings should be brief (55 characters or fewer) and should not be complete sentences, but words, phrases, or brief clauses. Only the first word of a title or subheading should be capitalized.

Order of Manuscript Pages

Arrange the manuscript in the following order:

***Page 1*—**Running head (not to exceed 60 characters and spaces), name, address, telephone and fax numbers, and email address of the corresponding author (author to whom copyright and page proofs should be sent); and the total number of words in the text, references, tables, and figure legends.

***Page 2*—**Title of article followed by authors' complete names and institutional affiliations, city, state/province, and country. Use the following symbol order to designate authors' affiliations: †, ‡, §, ‖, #. When more are needed, double them in the same sequence ††, ‡‡, §§, ‖ ‖, ##. *All persons listed as authors should have been sufficiently involved in the research to take public responsibility for its content.* The affiliation listed for an author MUST be the institution at which the research was conducted.

***Page 3*—**Footnote listing the email address of the corresponding author, and the present address of the corresponding author if different from the address on page 2.

***Page 4*—**Abstract describing the research, results, and conclusions (maximum of 220 words; 70 words for short communications) and no more than five key words. The abstract contains no citations.

***Text*--**Followed by acknowledgement (not to exceed 150 words), references, tables, figure legends (grouped on one page) and figures. Supplemental data such as very long tables and datasets may be submitted in PDF format for Web publication only. Submit all supplemental data with the manuscript.

Style

Write in simple declarative sentences. *ET&C* does not have a technical editorial staff to rewrite manuscripts; therefore, submissions must conform to the accepted standards of English style and usage. The title should be brief and informative. With the exception of references, the journal conforms to *Scientific Style and Format*, Council of Science Editors, Reston, VA, USA.

Length

Limit Letters to the Editor to two journal pages, Short Communications to four journal pages, Critical Reviews and Reviews to 12 journal pages, and Research Papers to 10 journal pages. One journal page equals about 3.2 double-spaced pages or about 1,100 words. The number of references should not exceed 40 (more are allowed for Critical Reviews); the number of tables 6, and the number of figures 6. Publication of excessively long manuscripts will be delayed and will incur substantial added cost.

References and Citations

Number all references in order of mention in the text, listing references in the table and figure legends last. Group full references at the end of the paper. Cite references by number in square brackets. Basic style is as follows:

- *Book:* Author AB, Author CD. 2007. *Title of Book.* Publisher, City, ST, Country.
- *Book Article:* Author AB, Author CD. 2007. Title of article. In Adams AB, Smith DC, eds, *Title of Book*, 2nd ed, Vol 1-Toxicology. Publisher, City, ST, Country, pp 1-5.
- *Journal Article:* Author AB, Author CD. 2007. Title of article. *Title of Journal* Vol:Pages.

- *Proceedings:* Author AB, Author CD. 2007. Title of article. *Proceedings*, Name of Conference, City, ST, Country, date (month, days, year), pp 00-00 (if no page numbers are available, cite parenthetically in the text).
- *Report:* Author AB. 2007. Title of report. EPA 600/334/778. Final/Technical Report. U.S. Environmental Protection Agency, Washington, DC.
- *Dissertation:* Author AB. 2007. Title of thesis. PhD thesis. University, City, ST, Country.
- *Website:* Author AB. 2007. Title of website. City (ST or country): Publisher. [cited 29 January 2013]. Available from: http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1552-8618

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Use capitalized zip-code abbreviations for states and Canadian provinces. Spell out names of countries except USA. For abbreviations of journal titles, consult BIOSIS *Serial Sources*(http://www.library.uq.edu.au/endnote/biosciences.txt)*;* spell out the full names of journals not listed in BIOSIS or in recent issues of *ET&C.*

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Tables

First, decide whether a table is needed; Tables are frequently overused in scientific publications, and presenting all data collected is rarely necessary. Tables should not duplicate information in the text or data presented in graphic forms and should stand alone without referring back to the text.

Tables must have at least three columns; the center and right columns refer back to the left column. All columns require brief headings that accurately describe the entries listed below. Include explanatory matter such as abbreviation definitions in the footnotes. Identify footnotes with superscript, lowercase letters ($^{a, b, c,}$ etc.), and list them below the table starting with the title, then upper left footnote designation,

proceeding to the right across a row, then down to the next row and proceeding again from left to right. Superscript, lettered footnotes are followed by asterisks for significance (*p* values), then by a list of acronyms. Designate significant differences using on-line full-size capital letters. An example (adapted from the *Scientific Style and Format*, Council of Science Editors, Reston, VA, USA) can be found here:http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1552-8618/homepage/SampleTableForET_C.pdf

Define all acronyms used in your table; refer to previous tables if a lengthy list of acronyms is used in successive tables. Avoid lengthy footnotes.

In your manuscript, double-space all information in tables, and place page breaks between each table. Number tables using consecutive Arabic numerals. *Environmental Toxicology and Chemistry* does not use designations of Table 1A and 1B, etc. Give each table a separate number or combine into one table. In your running text, indicate the first mention of each table and figure in red. For more guidance in constructing tables, see *Scientific Style and Format*, Council of Science Editors, Reston, VA, USA.

Figures and Illustrations

- General Appearance
- Size and Proportion
- Shading
- Symbols and Lines

Well-chosen and carefully executed illustrations will aid readers in the comprehension of the text. Illustrations should not duplicate information in tables or text and should be limited to no more than six per paper. Ensure that all are necessary to explain the research. Care should be taken to make sure that the figures are clear and can be interpreted without reference to the text.

Include titles and brief explanatory legends for all illustrations on a separate page after the References in your main document. Include symbol and acronym definitions in the figure legend, not on the figure itself. Label multipart figures with consecutive letters of the alphabet, using an upper case letter (A, B, C, etc.). Place this letter in the upper left corner of the figure, outside the figure itself (not on the figure). For examples, please see previous issues of *ET&C*.

In addition:

•Ensure that the figure will be legible when reduced to the width of a column of text (80 mm).
•Use sentence case (capitalize the first word ONLY) for axis titles, labels, and legends.
•Place the legend inside the figure or label the lines.
•Use Arial font and the same font size for all figures in the manuscript.
•Describe what the error bars mean (SE, SD).
•Use minor ticks, especially on log-scales.
•Avoid textures or shading that will not reproduce well or will not be distinguishable in your legend.
•Do not use a grid on graphs and do not use three-dimensional bar charts.
•If the graph is a characterization of correlation, add the coefficient of correlation to the graph.
•Use full words and avoid abbreviations. If you do use an abbreviation, define it in the figure legend.
•If graphs are stacked (preferably vertically) consider eliminating tick labels on all but the bottom graphic.

•Label each stacked graphic in full; do not use abbreviations.
•If graphs are meant to be compared, use the same scales on the *x* and *y* axes.
•If concentration– or dose–response is being characterized, use an arithmetic or log scale, not a categorical scale.

General Appearance--Halftones (gray scale images) do not reproduce well. Avoid small dotted lines, shading, and stippling. For bar graphs, use black, white, striped, or hatched designs, but only if they are sufficiently wide or separated in order to appear distinct from one another. If no important information will be lost, consider placing fewer numbers on the axes to achieve an uncluttered look. Make lines on maps bold and distinct and eliminate information not pertinent to the subject. Naming too many rivers, towns, and geographical elements results in a cluttered map that is difficult to read.

Size and Proportion--When possible, submit figures in the size they are to appear in the journal. Most illustrations, except some maps and very wide graphs, should be 1-column size (3.5 inches) and a resolution of 300 dpi. If the graph is composed in that size, legibility will be easy to determine. The font size on the *x* and *y* axes should not be larger than that of the title, and the same font (Arial or Times New Roman is preferred) should be used throughout. Numbers on the *x* and *y* axes should be smaller than the descriptive title, which should be 12-point font. Fonts smaller than 12 points are generally not legible when reduced to 1 column size. Use boldface type with care; if illustrations are to be reduced, the letters with open spaces will disappear.

Shading--Half-tones (gray scale) and stippling do not reproduce well. Occasionally, graphs are composed with four or more half tones that are barely discernible in the original; invariably the difference is lost entirely in print. Diagonal and horizontal stripes, checks, and solid black or white bars reprint well. If many differences must be presented, a color illustration may be the best alternative.

Symbols and Lines--Avoid very small symbols (no smaller than 2 mm) on line graphs; squares and circles look remarkably alike after being reduced to page size. Dotted lines often become invisible, and very complicated combinations of dots and dashes are difficult to read and even more difficult to define in the legend. Print all elements of the graph with the same degree of intensity. Figures with headings in boldface type but very light lines and symbols appear incongruous. Placing symbol definitions in a box to the right or left of the figure will make placement of the figure in one column impossible. Place definitions in the figure legend if at all possible or on the figure itself if only a few definitions are required.

Abbreviations

Use acronyms and abbreviations *sparingly* to avoid impeding comprehension of the text, and use only those that are well known. Too many acronyms make your manuscript difficult to read. Define each acronym at first introduction at the text, and on each table and figure legend, giving the abbreviation or acronym in parentheses. Spell out acronyms that begin a sentence. Do not use an acronym for words or phrases if that word or phrase is used fewer than five times. Symbols and abbreviations commonly used can be found here:http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1552-8618/homepage/ETCSymbolsandAbbrevs.pdf

Technical Information

Equations, mathematical formulas, flow diagrams

Simple equations should be written as A/B on one line. Decimals are preferred to fractions. Write out and hyphenate simple fractions (two-thirds), except in figures, graphs, legends, and in parentheses. Refer to the document Math to Type.pdf for specific guidelines.

Gases

Express parts per million (ppm) as microliters per liter µl/L) or parts per billion (ppb) as nanoliters per liter (nl/L). Use metric system only.

Ions

Represent ions as follows: Na+, Mn^{3+}, Br-, and PO^{3-}

Isotopes

An isotopically labeled compound is indicated by placing the isotopic symbol in square brackets attached to the name or the formula [^{14}C]ethanol; [^{32}P]ATP; [^{2}H]C_2H_2; [^{3}H]DNA. The specific position of the isotope should be given at the time of first mention; thereafter, it can be abbreviated to the less specific notation. The symbol indicating configuration should precede the isotopic symbol, and the position of isotopic labeling is indicated by Arabic numerals as in D-[^{14}C]lactate;D-[^{14}C]glucose 6-P; sodium D-[^{14}C]acetate; L-[1,2-^{14} C]alanine.

The term U indicates uniform labeling, as in [U-^{14}C]sucrose, where the isotope is uniformly distributed among all 12 carbons. Preference is given to [$^{14}C_2$] and ^{32}Pi rather than to [^{14}C]CO_2 or [^{14}C]CO_2 and [^{32}P]Pi.

Numbers

The metric system is standard, and SI units should be used as far as possible. Spell out all numbers or fractions that begin a sentence. If this is awkward, rephrase the sentence to avoid beginning with a numeral. Do not use a hyphen to replace the preposition "to" between numerals: 13 to 22 min, 3 to 10°C. Exception: Use the dash in tables, figures, graphs and in parentheses. Write out numerals one through nine except with units of measure.

Check tabular data, as well as numerical values, reported in the text for the proper number of significant figures. For decimals smaller than one, insert a zero before the decimal point: 0.345.

Powers in tables and figures

Care is needed in tables and figures to avoid numbers with many digits. The unit should be followed by the power of 10 by which the actual quantity was multiplied to give the reported quantity; the unit may be changed by the use of prefixes such as "mM" or "m." For example, an entry "5" under the heading "g x 10^{-3}" means that the value of g is 0.005; and entry "5" under the heading "g x 10^{3}" means that the value of g is 5,000. A concentration of 0.0015 M may be expressed as 1.5 under the heading "concn. (mM)" or as 1,500 under the heading "concn. (mM)" as 15 under the heading "10^{-4} x concn. (M)."

Ratios

Mixtures use "to" when general words are used, i. e., "the chloroform to methanol" ratio. Always use a colon with words when numerical ratio is provided, i. e., chloroform:methanol (2:1,v/v). Always use a

colon with a number ratio. Use a hyphen with a mixture only if a numerical value is not given, i. e., "used in chloroform-methanol."

Scientific names

The complete scientific name (genus, species authority for the binomial, and cultivar or strain), when appropriate, of all experimental organisms should be included in the *Abstract* and*Materials and Methods* sections. Following this initial citation, the generic name may be abbreviated to the initial, except when confusion could arise by reference to other genera with the same initial. The algae and microorganisms referred to in the manuscript should be identified by a Collection number or that of a comparable listing. Scientific names (genus and species) should be italicized.

Soil classification

Measured values for soil physicochemical characteristics having a bearing on the research must be reported in the manuscript for each individual type of soil used and may be reported in table format. Authors are strongly encouraged, whenever feasible, to give the soil type/name, texture, and scientific classification of each soil. This scientific nomenclature for soils must be consistent with a modern published soil classification system, and the system must be cited.

Solutions

Solutions of common acids and bases should be described in terms of normality (N) , and salts in terms of molarity (M), thus 1 N NaOH, 0.1 N acetic acid and 0.1 M Na_2SO_4. Fractional concentrations should be expressed in the decimal system: 0.1 N acetic acid and not N/10 acetic acid. The term % must be defined as w/w, w/v, or v/v; 10% (w/v) signifies 10 g/100 ml. Express concentrations as ng/L, mg/L, mg/L, ng/g, mg/g, etc.

Statistical treatment

When appropriate, statistical analysis should be included. Define all statistical measures clearly and use lower-case letters for abbreviations such as *r, f, and t.*

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The names of the manufacturers or suppliers of special material should be given in parenthesis following the name. If desired, include the superscript copyright, trademark, or registered designation ($^{©}$, ^{TM}, or $^{®}$) after the first mention in the text and first mention in table or figure legends. Trade names must be capitalized. The use of trade names and code numbers of experimental chemical compounds used in experimentation should be avoided. Such compounds should be identified by common name (ASA), if such a name exists, or by chemical name and structural formula. Lot numbers, purity, impurities, etc., may be appropriate.

Manuscript Processing

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Each manuscript is assigned to an editor with expertise in the field discussed, who, in turn, sends it to a minimum of two reviewers. Reviewers give evaluations, suggest improvements, and recommend acceptance or rejection of the paper. Reviewing should be completed within two months. If reviewers disagree, the paper may be sent to a third reviewer. The editor sends a decision letter and the critiques of reviewers to the corresponding author, and the editorial office prepares a detailed list of editorial issues to be amended or improved. Authors who do not follow*ET&C* style and format will experience a delay in publication.

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