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

EFEITOS TÓXICOS INDUZIDOS PELOS NANOMATERIAIS FULERENO (C_{60}) E NANOPRATA NO POLIQUETO *LAEONEREIS ACUTA* (NEREIDIDAE) E NAS COMUNIDADES BACTERIANAS QUE HABITAM SUA SUPERFÍCIE CORPORAL

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Sumário

RESUMO

O fulereno (C_{60}) pertence a uma família de nanomateriais (NM) constituída exclusivamente de átomos de carbono, sendo encontrado na forma de suspensão na água (nC_{60}). A nanoprata (nAg) possui um excepcional e amplo espectro bactericida e um custo de fabricação relativamente baixo. No entanto, pouco se sabe a respeito dos eventuais efeitos tóxicos induzidos por estes NM em organismos estuarinos. O poliqueto *Laeonereis acuta* tem o muco colonizado por comunidades bacterianas. Há registros de que *L. acuta* apresenta um gradiente corporal para concentração de EAO e capacidade antioxidante total. Neste estudo, os poliquetos foram expostos *in vivo* durante 24 horas ao nC_{60} e à nAg, separadamente. Após isso, as unidades formadoras de colônias (UFC) bacterianas foram contadas e pesadas, além de serem realizadas diversas medições bioquímicas nos poliquetos e nas bactérias. Os números de UFC bacterianas expostas ao nC_{60} foi menor na concentração de 0.01mg/L e os números de UFC bacterianas expostas à nAg foram similares aos dados de biomassa, diminuindo na maior concentração (1.0 mg/L) ($p<0.05$). A capacidade antioxidante contra radicais peroxil em homogeneizados bacterianos expostos ao nC_{60} foi menor na concentração de 0.1mg/L quando comparado ao controle ($p<0.05$). A região anterior apresentou menor capacidade antioxidante ($p<0.05$) nos poliquetos expostos a 1.0 mg/L, quando comparado ao controle. Os poliquetos expostos à nAg apresentaram menor capacidade antioxidante na região posterior na concentração de 1.0 mg/L quando comparado ao controle ($p<0.05$). O conteúdo de peróxidos lipídicos (TBARS) foi reduzido na região anterior dos poliquetos expostos nas duas menores concentrações (0.01 e 0.1 mg/L) de nC_{60} ($p<0.05$). Na região corporal posterior, somente os organismos expostos a maior concentração de nC_{60} (1.0 mg/L) mostraram aumento na concentração de TBARS quando comparado ao grupo controle ($p<0.05$). A atividade da enzima glutationa-S-transferase (GST) foi aumentada ($p<0.05$) na região média e posterior dos poliquetos expostos a 0.1 mg/L de nC_{60}. Como conclusões pode se dizer que os dois NM induziram efeitos tóxicos ainda numa situação (escuridão) onde o fulereno não é foto-excitado. O aumento na produção e comercialização de produtos com NM levanta a questão dos riscos ambientais associados ao desenvolvimento da nanotecnologia.

Palavras - chave: fulereno; nanoprata; estresse oxidativo; poliqueto; comunidades bacterianas.

ABSTRACT

Fullerenes (C_{60}) are a family of NM constituted exclusively by carbon atoms. The most environmentally relevant form of C_{60} is the fullerene water suspension (nC_{60}). nAg posses an exceptional broad spectrum bactericidal activity and relatively low cost of manufacturing. The polychaete *Laeonereis acuta* has the mucus colonized by bacteria. There are reports that ROS concentration and total antioxidant capacity showed a body gradient in *L. acuta*. The worms were exposed during 24 hours to nC_{60} and nAg. After this, the bacterial CFU were counted and weight. Biochemical analysis polychaetes and bacterial samples were made. The number of bacterial CFUs exposed to nC_{60} was lower in the concentration of 0.01mg/L and the number of bacterial CFUs exposed to nAg were similar to weight data, since decreased at the higher concentration (1.0 mg/L), in relation to the other concentrations and control ($p<0.05$). Antioxidant capacity against peroxyl radicals in homogenates of bacterial exposed to nC_{60} was lower in the concentration of 0.1mg/L when compared to the control ($p<0.05$).The anterior region presented lower antioxidant capacity ($p<0.05$) in worms exposed to 1.0 mg/L, when compared to the control. Worms exposed to nAg presented lower antioxidant capacity in the posterior region of those exposed to 1.0 mg/L when compared to the control ($p<0.05$). Lipid peroxides content was reduced in the anterior region of worms exposed to the two highest concentrations (0.1 and 1.0 mg/L) of nC_{60} ($p<0.05$). In the posterior body region, only worms exposed to the highest nC_{60} concentration (1.0 mg/L) showed augmented TBARS concentration when compared with the control group ($p<0.05$). The activity of GST revealed to be augmented ($p<0.05$) in the middle and posterior region of worms exposed to 0.1 mg/L of nC_{60}. As conclusions, can be said that the two NM induced toxics effects even in a situation (darkness) where the fullerene is not photo-excited. The increase in production and marketing of products with NM raises the question of the risks associated with the development of nanotechnology.

Key words: fullerene; nanosilver; oxidative stress; polychaeta; bacteria communities.

INTRODUÇÃO

Os nanomateriais (NM) são definidos como materiais que tem características estruturais com pelo menos uma dimensão na faixa de 1 a 100 nm; diferindo no tamanho, forma, composição e origem, e são classificados como orgânicos ou inorgânicos (Pérez *et al*., 2009). Os NM podem ocorrer naturalmente e estar no meio ambiente por milhões de anos (fulereno foi detectado em deposições geológicas a partir da fronteira do Terciário - Cretáceo) (Becker *et al*., 1994), serem produzidos involuntariamente durante muitos processos industriais ou serem conseqüência da poluição vinda dos motores de máquinas ou ainda podem ser fabricados intencionalmente para diversas finalidades (Pérez *et al*., 2009). Atualmente, os NM fabricados são usados em uma variedade de aplicações comerciais e industriais, incluindo bio-sensores, cosméticos, produtos têxteis, produtos farmacêuticos, na remediação ambiental, como transportadores de drogas, microeletrônicos e catalisadores (Oberholster *et al*., 2011).

O rápido crescimento da nanotecnologia levanta preocupações sobre o impacto dos NM na saúde humana e meio ambiente, proporcionando vários estudos que têm aliviam os efeitos da exposição dos NM em sistemas biológicos (Oberdörster *et al*., 2005; Baun *et al*., 2008; Zhao *et al*., 2005). O ramo de estudo que objetiva avaliar os efeitos dos NM nos organismos vivos é chamado de nanotoxicologia (Oberdörster *et al*., 2005; Oberdörster *et al*., 2007).

Desde a sua descoberta em 1985 por Kroto *et al*., o fulereno tem atraído grandes interesses em pesquisas devido as suas propriedades únicas e sua produção já está em curso desde 1990 (Krätschmer *et al*., 1990). A forma mais relevante ambientalmente de C_{60} é o fulereno em suspensão na água (nC_{60}), (Fortner *et al*., 2005), que pode ser formado através de suaagitação intensa na água, sonicação ou empregando um solvente

orgânico (Lyon *et al*., 2008). O mecanismo de toxicidade do nC_{60} tem sido atribuído, geralmente, à sua habilidade de gerar espécies reativas de oxigênio (ERO) quando foto-sintetizado (Miyata *et al*., 2000). Essas ERO podem provocar vários tipos de danos à célula, incluindo peroxidação lipídica e oxidação protéica (Imlay, 2003). Estudos com células de mamíferos revelaram que o fulereno pode causar dano de oxidativo e que sua citotoxicidade é relativa à sua lipofilicidade (Colvin *et al*.,2004). O C_{60} pode ser tóxico para linhagens de células eucarióticas (Isakovic *et al.*, 2006), crustáceos como *Daphnia magna* (Lovern e Klaper, 2006), diversas espécies de peixes (Oberdörster 2004; Oberdörster *et al.*, 2006) e bactérias (Fortner *et al*., 2005).

Efeitos bactericidas de C_{60} em *Bacteria subtilis* foram registrados por Lyon et al (2006), um resultado que foi reforçado pelo uso do solvente orgânico (tetrahidrofurano, THF) utilizado para preparar a suspensão. Oberdörster (2004) mostrou que suspensões de fulereno (nC_{60}) preparadas com solvente orgânico (THF) induziram peroxidação lipídica em cérebro de peixes juvenies. Zhu *et al*. (2006) registrou que o método de preparação de nC_{60} pode afetar, significativamente a sua toxicidade, nC_{60} com THF mostrou ser mais tóxico que nC_{60} em agitação intensa na água quando exposto ao microcrustáceo *Daphnia magna.* Em contrapartida, Zhu *et al*. (2008), mostrou que o nC_{60} suspendido em agitação por longo tempo na água induziu altos níveis de peroxidação lipídicos em peixes e aumentou a atividade da enzima antioxidante superóxido desmutase. Recentemente, Lyon e Alvarez (2008) propuseram que nC_{60} exerce estresse oxidativo independente de ERO em bactérias. Outro estudo usando análise de ácidos graxos fosfolipídicos não mostrou evidências de peroxidação lipídica em bactérias expostas ao nC_{60} (Fang *et al*., 2007). Além disso, Lyon e Alvarez (2008) publicaram que nenhuma produção de ERO e nenhum dano mediado por ERO foi encontrado em bactérias expostas ao nC_{60} e postularam que nC_{60} é um NM que exerce

toxicidade como uma partícula via interação química em contato direto com a bactéria. Entretanto, dados sobre as propriedades oxidantes e ações biológicas do C_{60} são contraditórios. Vários estudos mostraram que o C_{60} pode exibir propriedades antioxidantes. Kam *et al*. (2004) encontraram que a água e derivados solúveis de gordura de C_{60} previnem a oxidação de peróxidos com maior eficiência do que antioxidantes naturais como a vitamina E.

Nanoprata (nAg) é o nanomaterial fabricado mais utilizado em produtos de consumo (Marambio-Jones e Hoek, 2010). A nAg possui um excepcional e amplo espectro bactericida e um custo de fabricação relativamente baixo (Fabrega *et al*., 2011). No entanto, se esse nanomaterial exercer impactos não-intencionais ou indesejados aos organismos deve ser interpretado como um perigo potencial (Marambio-Jones e Hoek, 2010). Asharani *et al*. (2008) observou um aumento dependente da concentração na mortalidade, e um atraso de incubação, quando embriões de *Danio rerio* foram tratados com nAg. Bilberg *et al*. (2011) demonstrou que até mesmo breves exposições de nAg são capazes de suprimir a resposta olfatória nos peixes *Carassius carassius* e *Perca fluviatilis*. Além disso, já foram comprovados efeitos tóxicos de nAg em ambas bactérias aeróbicas e anaeróbicas isoladas das estações de tratamento de águas residuais (Choi and Hu, 2008), os quais poderiam levar a severos rompimentos da comunidade bacteriana (Marambio-Jones e Hoek, 2010).

Marambio-Jones e Hoek (2010) demonstram em seu trabalho que a nAg pode liberar íons e gerar ERO; interagir com proteínas de membrana, afetando sua correta função; acumular-se dentro na membrana e afetar sua permeabilidade e entrar na célula onde pode gerar ERO, liberar íons e afetar o DNA.

Um estudo sobre os efeitos de toxicidade em diferentes formas de prata em bactérias nitrificantes mostrou que não somente Ag^{+} e AgCl, mas as nAg também são capazes de induzir geração de ERO intracelular (Choi *et al*., 2008).

A concentração estimada de de nAg no ambiente aquático é de aproximadamente 0.01µg L (Tiede *et al*., 2009), mas com o aumento de seu uso, a descarga de nAg aumentará, sem dúvida, em um futuro próximo (Bilberg *et al*., 2011). De fato, em suspensões com alta força iônica, a carga estabilizada de nAg tende a precipitar no sedimento dentro de algumas horas de exposição e se acumulam na camada de superior de sedimento em sistemas aquáticos (Bradford *et al*., 2009).

Os invertebrados bentônicos muitas vezes consomem, seletivamente, diferentes tamanhos de partículas que contém alto teor de carbono orgânico, bem como elevadas concentrações químicas (Oberholster *et al*., 2011).

O poliqueto da família nereididade *Laeonereis acuta* é uma espécie bentônica encontrada no estuário da Lagoa dos Patos (Sul do Brasil), sendo de acordo com Pagliosa e Barbosa (2006), uma das espécies dominantes em rios urbanizados do Sul do Brasil. É caracterizado como um consumidor de depósito seletivo e, portanto, vive em contato estreito com o sedimento (Raes e Vanreusel *et al*., 2006). Ferreira e Cravo (2007) registraram que a concentração de ERO e capacidade antioxidante mostraram um gradiente corporal em *L. acuta*. A epiderme desses poliquetos secreta muco que recobre e protege a cutícula. Este muco é uma secreção pegajosa usada para a locomoção, lubrificação e proteção contra partículas estranhas (Barnese Barnes, 1982). Um estudo de Rosa *et al* (2005b) mostrou a atividade da catalase no muco de *Laeonereis acuta*. O muco secretado por *L. acuta* é habitada por um grande número de

bactérias (Rosa *et al.*, 2005b). Desta forma, o muco poderia representar um substrato para o crescimento das bactérias (Wood e Sørensen, 2001).

Moraes *et al.* (2006) argumentaram que um nível de antioxidante basal no muco do poliqueto *L. acuta* pode ser devido ao conteúdo de carboidratos contido nas suas mucoproteínas, mas este nível basal pode ser modulado pela presença de bactérias com defesas antioxidantes adicionais que colonizam a secreção do muco. Sendo assim, é possível pensar que exista uma determinada condição ou condições fisiológicas para o poliqueto produzir muco com características favorecendo a colonização de grupos de bactérias, possivelmente com competência antioxidante. Se isso for verdade, pode ser postulada uma inter-relação entre as bactérias que poderiam crescer no muco secretado pelo poliqueto e ao mesmo tempo oferecer a este proteção antioxidante.

OBJETIVO

O presente estudo teve como objetivo avaliar os efeitos bioquímicos induzidos pelos nanomateriais nC_{60} e nAg, separadamente, em diferentes regiões corporais do poliqueto *L. acuta* e nas comunidades bacterianas que vivem na secreção do muco do poliqueto.

ARTIGO

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Toxicological effects induced by the nanomaterials fullerene and nanosilver in the polychaeta *Laeonereis acuta* (Nereididae) and in the bacteria communities living at their surface

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Abstract

Fullerene (nC_{60}) and nanosilver (nAg) are nanomaterials with bactericide properties. The increments in their use raise questions about their potential environmental impacts, including estuarine ones. The polychaete *Laeonereis acuta* (Nereididae) secretes mucus that is colonized by bacteria communities. We analyzed the biochemical responses of anterior, middle and posterior region of *L. acuta* and bacteria communities after nC_{60} or nAg exposure during 24 h. Molecular analysis showed a prevalence of *Vibrio* genera in the communities. Bacteria biomass was lowered in worms exposed to 1.0 mg/L of nAg. nC_{60} reduced total antioxidant capacity of bacteria from worms exposed to 0.1 mg/L. Worms anterior region presented lower antioxidant capacity after exposure to 1.0 mg nC_{60}/L, and the same was observed in the posterior region of worms exposed to 1.0 mg nAg/L. Lipid peroxidation was reduced in the anterior region of worms exposed to nC_{60} and the opposite was observed in the posterior region.

Key words: fullerene; nanosilver; oxidative stress; polychaeta; bacteria communities.

Capsule: Toxicity of fullerene (C_{60}) and nanosilver in estuarine polychaete and associated bacteria communities.

1. Introduction

Nanomaterials (NM) are being used in several applications due to their extremely small size, with correspondingly large surface-to-volume ratio. Furthermore, their properties can be modified by varying the size, shape and composition (Li et al., 2010). At present, manufactured NM are used in a variety of commercial and industrial applications (Oberholster et al., 2011). The rapid growth of nanotechnology raises concerns about the impact of NM on human and environment health, prompting several studies that have evaluated the effects of NM exposure on biological systems (Zhao et al., 2005; Baun et al., 2008).

Fullerenes are a family of NM constituted exclusively by carbon atoms. The simplest member of this family and the most common fullerene known is C_{60} (Farré et al., 2011). Probably the most environmentally relevant form of C_{60} is the fullerene water suspension (nC_{60}), (Fortner, et al., 2005), which can be formed in water either through extensive stirring of C_{60} powder or by employing organic solvents (Lyon et al., 2006). The toxicity mechanism of nC_{60} has generally been attributed to its ability to generate reactive oxygen species (ROS) when photosensitized (Kamat et al., 2000; Shinohara et al., 2009), although other studies have reported bactericidal effects through a ROS-independent mechanism (Lyon and Alvarez, 2208; Lyon et al., 2008). Bactericidal effects of fullerene in *Bacteria subtilis* were reported by Lyon et al. (2006), a result that was enhanced by the use of an organic solvent (tetrahydrofuran, THF) to prepare the fullerene suspension. Oberdörster (2004) showed that nC_{60} suspensions prepared with the organic solvent tetrahydrofuran (THF) induced lipid peroxidation in brains of *Micropterus salmoide* fish. Zhu et al. (2006) reported that the method of nC_{60} preparation can greatly affect the toxicity of fullerene, with THF-nC_{60} being more toxic than water-stirred-nC_{60} when exposed to the microcrustacean *Daphnia magna*. Zhu et

al. (2008) showed that fullerene aggregates, suspended in water after long-term stirring, induced higher lipid peroxides levels in *Carassius auratus* fish and augmented the activity of the antioxidant enzyme superoxide dismutase.

As cited above, Lyon and Alvarez (2008) proposed that the nC_{60} exerts ROS-independent oxidative stress in bacteria. Another study using phospholipid fatty acid analysis (PLFA) showed no evidence of lipid peroxidation in bacteria exposed to nC_{60} (Fang et al., 2007). Furthermore, Lyon and Alvarez (2008) postulated that nC_{60} is a NM that exerts toxicity as a particle via a chemical interaction upon direct contact with the bacteria. However**,** data on the oxidizing properties and biological action of fullerenes are contradictory, indicating the need of scientific information for the safe use of nanotechnology.

Nanosilver (nAg) is one the engineered NM most commonly used in consumer products (Marambio-Jones and Hoek, 2010). This NM posses an exceptional broad spectrum bactericidal activity and relatively low cost of manufacturing (Fabrega et al., 2011). According to Marambio-Jones and Hoek (2010) some of the toxicity mechanisms elicited by nAg include, between others: (1) release of silver ions, generating ROS; (2) interaction with membrane proteins affecting their function; (3) accumulation in the cell membrane, affecting membrane permeability. In fact, a study of the toxicity effects of different silver forms on nitrifying bacteria showed that not only Ag^+ and AgCl, but also nAg are able to induce intracellular ROS generation (Choi et al., 2008).

The predicted concentration of nAg in the aquatic environment is estimated to be about 0.01µg/L, but given the increasing number of applications, the discharge of nAg will undoubtedly increase in the near future (Bilberg et al., 2011). Indeed, in high ionic strength suspensions, charge stabilized nAg tend to precipitate in the sediment within a

few hours of exposure, and accumulate in the uppermost sediment layer in aquatic systems (Bradford et al., 2009). This behavior point to benthic invertebrates as unintended targets of NM (Oberholster et al., 2011).

The polychaete *Laeonereis acuta* is a selective deposit feeder and, therefore, it lives in close contact with the sediment (Raes and Vanreusel, 2006). Moraes et al. (2006) registered high total antioxidant competence against peroxyl and hydroxyl radicals in the mucus of this species that showed to be colonized by rod-shape bacteria. Authors argued about an inter-relationship between bacterial communities and *L. acuta*, through of mucus that should aid in bacterial growth and, in turn, augmenting the antioxidant defense of the worm. Ferreira- Cravo et al. (2007) reported that ROS concentration and total antioxidant capacity showed a body gradient in *L. acuta,* where a lower ROS concentration was observed in the anterior region (first 20 settiger segments), augmenting in the middle (next 20 settiger segments) and posterior regions (rest of the body).

Taking into account the cited information, the objective of this study was to evaluate the biochemical effects induced by nC_{60} and nAg on different body regions of the polychaeta and on bacteria communities living at the mucus secretion of this organism.

2. Materials and method

2.1. Fullerene solution preparation and characterization

Previous evidences showed that preparation of fullerene stock solution with organic solvents induced toxicity related to degradation products of these solvents (Zhu

et al., 2006). Because of these, an aqueous fullerene suspension (nC_{60}) (200 mg/L; SES Research, 99% purity) was prepared by the constant stirring of C_{60} in Milli Q water during two months (photoperiod: 24 L). The aqueous suspension was sequentially filtered through 0.45 and 0.20 µm. Fullerene concentration was estimated by measuring total carbon concentration with a TOC-V CPH (Shimadzu) total organic carbon analyzer. Suspensions of fullerene were characterized using a JEOL JSM 1200 EX II transmission electron microscopy (TEM) operating at 100 kV. Samples of about 30 µl of nC_{60} were disposed onto 300 µm mesh TEM grids (SPI) coated with Formvar. Analysis was performed after 24 h in order to allow sample evaporation (Lyon et al., 2006).

2.2. Nanosilver suspension, preparation and characterization

A colloidal suspension of 1% w/v nanoparticulate silver was provided by Nanotek S.A., which manufacture the product under the brand name nanArgen®, by a proprietary method (patent AR053568 A1). One of the most used methods for preparation of transition metals nanoparticles is the chemical reduction of their salts under the presence of stabilizing chemicals and controlled operating parameters, such as above atmospheric pressure. The mechanism involves reduction of the element to its zerovalent state, followed by a tendency of these reduced atoms to act as nucleating centers of aggregates or nanoclusters. By controlling this aggregation process, different sizes and geometries of nanomaterials can be obtained. The sinterization process, in our case, is controlled by means of stabilizing organic polymers (for example starch) which form an "envelope" by adsorption on the particles surfaces. The main advantages of chemical synthesis are reproducibility and the ability to obtain monodispersed colloids

with a narrow size distribution. Thus, the synthesis path by which this colloidal suspension was prepared is chemical, in a pressurized reactor. The pro analysis quality (ACS) reactants employed were AgNO3 (silver nitrate), provided by Carlo Erba (code 423954, batch 5151928332), soluble starch Anedra (batch 16909-1) and Milli Q water. Scanning electron microscopy (SEM) was employed in order to characterize the nanoparticulate silver suspension.

2.3. Polychaetes sampling, maintenance and exposure design

A total of 105 species of *L. acuta* adult, weighing in average 250 mg were collected in a salt marsh ("Saco do Justino") around Rio Grande city (Southern, Brazil—32°S, 52°W), a site previously characterized as a unpolluted reference site (Geracitano et al., 2004). After sampling, worms were immediately transported to laboratory where they were transferred to glass tubes with 5 mL of autoclaved estuarine water. The glass tubes were capped with cotton in order to avoid the bacterial contamination from the environment. Abiotic parameters (salinity, pH and oxygen concentration) were measured in autoclaved estuarine water.

Concentrations chosen in the assays were 0.01; 0.10 or 1.00 mg/L of nC_{60} or nAg plus a control with saline water. Fullerene concentrations were selected taking into account a previous study from our group (Lettes et al., 2011). The same concentrations were selected for nAg in order to favor the comparison between the two NM. Nanomaterial suspensions were all filtered on 0.22 µm filters before exposure to avoid bacterial contamination. Five polychaetes per concentrations were assayed. Organisms were exposed during 24 h under darkness at 20 ºC.

2.4. Bacterial community isolation from L. acuta and counting of colony forming units.

After 24 h, bacterial communities living at the mucus of three worms at each concentration of nC_{60} or nAg plus the control group were isolated with disposable loops of 10 µL (passed twice over the animal surface) and transferred to eppendorfs with 280 µL autoclaved distilled water. The drop-plate method was done according to Herigstad et al. (2001). Samples were diluted between 10^{-1} to 10^{-4} and were inoculated in Petri dishes containing tryptic soy Agar (Hi-Media).

Each treatment dilution had four 10 µL drops representing its repetitions. A control test was carried out as well, consisting of a pair of plates inoculated with autoclaved distilled water and sterile autoclaved estuarine water (280 µL autoclaved distilled water and 20 µL autoclaved estuarine water). The plates were incubated for approximately 20 h at 20 ºC in the dark. After this time, the colony forming units (CFU) were counted.

2.5. Molecular identification of bacteria living at the mucus secretion of L. acuta

DNA was extracted from 1 mL of isolated bacteria cultures using the Wizard Genomic DNA Purification kit (Promega, Madison, WI) according to the supplier's instructions. Extraction products were visualized on 1% agarose gel with GelRed (Biotium). Primers of 16S segment (forward 5'-CCTACGGGAGGCAGCAG-3' and reverse 5'-GACTACCAGGGTATCTAATC-3') were designed as described previously (Ritchie et al., 2008). DNA samples were amplified through polymerase chain reaction (PCR), which was performed according to Ritchie et al. (2008), except for the primer annealing temperature, which was optimized for 58 ºC. PCR products (approximately 460-bp long) were analyzed on GelRed-stained 1% agarose gel, with

Low DNA Mass Ladder (Invitrogen) as molecular weight marker, and then purified using the enzymes exonuclease I and shrimp alkaline phosphatase. Purified PCR products were sequenced in both directions using a MegaBACE 1000 automated sequencer. The resulting chromatograms were analyzed, and DNA sequences were blasted using GenBank National Center for Biotechnology Information-BLAST searches.

2.6. Homogenization of the bacterial samples

After the counting, Petri plates were incubated during two weeks and at the end of this period, they were scrapped and the material transferred to eppendorfs and weighed, in order to estimate bacteria biomass. The bacterial samples were diluted 1:10 in phosphate-bufered saline (PBS) (137 mM NaCl and 3 mM of sodium phosphate, with pH adjusted to 7.4) and then frozen in liquid nitrogen and then thawed, three times, in order to promote their homogenization. Then they were centrifuged at 9,000 x g for 40 min at 4 ºC. The supernatant was used for biochemical analysis. Total protein content was measured using a commercial diagnostic kit (Doles, Brazil), based on the Biuret method and read in an ELISA plate reader (Biotek ELx 800) at 550 nm.

2.7. Homogenization of L. acuta samples

After exposure, worms were sacrificed by cold, dissected and subdivided in three regions: anterior region (A: first 20 settiger segments), middle region (M: next 20 settiger segments) and posterior region (P: the rest of the body, approximately 57 segments) (Rosa et al., 2005). Samples were weighed and homogenized (1:3) in cold (4^{o}C) buffer solution containing 20 mM Tris-Base, 1 mM EDTA, 50 mM sacarose, 150

mM KCl and 0.1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma), the pH adjusted to 7.6 (Geracitano et al., 2004). Homogenates were then centrifuged at 9,000 x g during 45 min at 4 ºC and the supernatants kept. Pools of A, M or P sections were formed to make one sample. Total protein content of the supernatant was measured in the same way as for the bacteria homogenates.

2.8. Biochemical measurements

Total antioxidant competence against peroxyl radicals, levels of by-products from lipid peroxidation (TBARS assay) and activity of glutathione-S-transferase (GST) were performed according to Oakes and Van Der Kraak (2003), Amado et al. (2009), Díaz-Jaramillo et al. (2011). Total antioxidant competence and TBARS were determined through fluorometry. GST activity was estimated by the conjugation of 1 mM glutathione (Sigma) with 1 mM of 1-chloro-2, 4-dinitro-benzene (CDNB; Sigma), which it is measured as absorbance increments at 340 nm (Habig and Jakoby, 1981).

2.9. Measurement of silver accumulation in different body regions of L. acuta

Anterior, middle and posterior regions of *L. acuta* were dissected as stated in **Section 2.7**. Samples were lyophilized and then the samples were digested by using an acidic oxidant mixture containing HNO_3 and H_2O_2. Firstly, the weighted solid samples were added of 500 µL concentrated HNO_3 and heated at 90 °C for 6 h (without boiling). After cooling, the sample was added of 5 mL concentrated HNO_3 and refluxed once again for 30 min (95 °C). This sample volume was then added of 50 µL of H_2O_2 30% to the digestion tubes. The solution was heated for 1 h, cooled down and diluted to 1mL with ultrapure water. The determination of Ag in the digested samples were made

by using an extern calibration curve ranging from 5.0 to 30.0 µg/L of Ag. Here, a stock solution of As 1.0 g/L was diluted to 10 mg/L, so that it was used as a working intermediate solution for preparing the calibration curve. Measurements were performed in a Analytikjena-ZEEnit 600 atomic absorption spectrometer (Analytikjena, Germany), equipped with an auto-sampler MPE 60.

2.10. Statistical analysis

Statistical differences between various parameters were tested through analysis of variance (ANOVA), followed by Newman–Keuls test (α= 0.05). Assumptions of normality and variance homogeneity were previously checked and mathematical transformations applied if necessary (Zar, 1984).

3. Results

Figure 1 shows the TEM images for nC_{60} (a) and nAg (b). Micrographs show rather regular spherical particles of nanosilver gathered in aggregates in form of chains. At a greater magnification focused on a less aggregated cluster, the closer view allows observation of the spherical morphology of isolated nanoparticles of an average size ranging between 20 and 25 nm. Analysis using Zetasizer indicated the existence of particles populations at the microscale (data not shown), an effect that can be related to the high ionic strength of the saline water employed. The average values of parameters were as follows: pH = 8.62; salinity = 13.33; oxygen concentration = 4.8 mg/L.

The number of bacterial CFU coming from worms exposed to nC_{60} was lower for the concentration of 0.01 mg/L when compared with the other concentrations and the

control group ($p<0.05$) (Figure 2a). No difference ($p>0.05$) of bacteria biomass weight was found for fullerene treatment (Figure 2b). The results of bacterial CFU coming from worms exposed to nAg were in accordance to biomass weight data, as they decreased for the highest concentration (1.0 mg/L) in relation to the other concentrations and control ($p<0.05$) (Figure 2c and 2d).

Total DNA was extracted from the eight isolates obtained and the 16S rDNA sequences were partially determined. One isolate was identified as *Shewanella*, and seven isolates were identified as belonging to *Vibrio* genera (Table 1).

Antioxidant capacity against peroxyl radicals in homogenates of bacteria exposed to nC_{60} was lowest (highest relative area) in the concentration of 0.1 mg/L when compared to the control ($p<0.05$) (Figure 3a). Treatment with nAg did not alter ($p>0.05$) the antioxidant competence against peroxyl radicals (Figure 3b).

Although several attempts with different extracts volumes were tested, no GST activity was detected in the majority of the samples. In control group only one sample from five differed from a blank and in bacteria samples from NM-exposed worms were also few. Although no attempt to analyze the few data obtained was done, a trend to higher GST activity was observed in bacteria communities coming from NM exposed worms, particularly nAg (data not shown). However homogenates of bacteria exposed to nC_{60} and nAg showed no significant differences in TBARS content ($p>0.05$; data not shown).

Antioxidant capacity was different along the body regions of *L. acuta* exposed to nC_{60} As showed in Figure 4a, the anterior region presented lower antioxidant capacity ($p<0.05$) in worms exposed to 1.0 mg/L, when compared to the control. The other body

regions did not presented statistical differences ($p>0.05$) between treatments (Figure 4b and 4c). Worms exposed to nAg showed absence of effect in the anterior and middle region (Figure 4d and 4e), but presented lower antioxidant capacity (higher relative area) in the posterior region of those exposed to 1.0 mg/L when compared to the control ($p<0.05$; Figure 4f).

Lipid peroxides content (TBARS) was reduced in the anterior region of worms exposed to the two highest concentrations (0.1 and 1.0 mg/L) of nC_{60} ($p<0.05$) (Figure 5a). In the middle region (Figure 5b), no statistical differences were observed between all treatments ($p>0.05$). Finally, in the posterior body region (Figure 5c), only worms exposed to the highest nC_{60} concentration (1.0 mg/L) showed augmented TBARS concentration when compared with the control group ($p<0.05$). Worms exposed to nAg showed no significant differences in TBARS content ($p>0.05$; data not shown).

Activity of GST in worms exposed to nC_{60} revealed: (1) no differences in the anterior region ($p>0.05$; Figure 6a); (2) to be decreased ($p<0.05$) in the middle region of worms exposed to 1.0 mg/L (Figure 6b); and (3) to be increased ($p<0.05$) in the posterior region of worms exposed to 0.1 mg/L of nC_{60} (Figure 6c). No effect of nAg ($p>0.05$) was observed (data not shown).

In terms of silver accumulation, only in the posterior region (that accounts for almost the whole worm) were obtained readings different from the blank. The posterior regions of worms exposed to the lowest nAg concentration (0.01 mg/L) showed the highest silver accumulation (5.58 ± 1.23 µg/g) when compared with worms exposed to 0.1 mg/L (1.24 ± 0.62 µg/g) or 1.0 mg/L (0.48 ± 0.35 µg/g) ($p<0.05$).

4. Discussion

Mucus from marine invertebrates may play different functions, although the information about this issue is still scarce (Stabili et al., 2011). Mucus from the polychaeta *Laeonereis acuta* presents high density of bacteria communities (Moraes et al., 2006). In present study we isolated eight colonies living at the mucus from *L. acuta*, where seven belong to *Vibrio* genera and the other was identified as *Shewanella* (Table 1). The genus *Vibri*o sp. are Gram-negative bacteria, pathogenic to vertebrate and invertebrate animals (Kim and Bang, 2008), whereas the *Shewanella* genus comprises an group of Gram-negative bacteria that have been isolated from marine environments, sediments and marines organisms like abalone *Haliotis discus hannai* (Kim et al., 2007). Authors like Stabili et al. (2011) have observed a lysozyme-like activity in the mucus of the polychaeta *Sabella spallanzanii* to different *Vibrio* genera, suggesting that the community composition and bacteria density are controlled by the worms. Fang et al (2007) observed that both Gram-negative and Gram-positive bacteria can change its lipid composition and membrane phase behavior when exposed to nC_{60}. The effect of nC_{60} on bacterial physiology suggests the potential environmental impact (Fang et al., 2007).

The sediment is a sink for many contaminants in water ecosystems (and probably also for aggregated nanoparticles). Thus sediment feeders can accumulate high contaminant concentrations. In these instances, it may be more appropriate to study the effects of the NM on benthic organisms rather than on water-dwelling species. In fact it is important to focus the toxicological responses triggered by NM in aquatic organisms in general, including invertebrates, since there are very few studies which have addressed this issue (Baun et al., 2008). From this point of view, the invertebrate *L.*

acuta seems to be an appropriate model to analyze the potential risks elicited by NM on estuarine organisms.

Fullerene (nC_{60}) upon contact with water can form negatively charged nanoscaled colloids, which are stable over time (Fortner et al., 2005). The conditions that lead to ROS production by nC_{60} in water and the reactive species formed are less well understood. According to Lee et al. (2008), differences in the aggregation state of nC_{60} as well as functionalization of then C_{60} contained within these aggregates may affect the ability for ROS production. In the present study bacteria CFU from worms exposed to nC_{60} was lowered at the concentration of 0.01 mg/L when compared with the other concentrations and the control group (Figure 2a). This result can be explained by the degree and kinetics of aggregation and the size range of the aggregates that depends on characteristics of the particle and its concentration, as well of the characteristics of the environmental system (Farré et al., 2011). Nel et al. (2006) showed that lower NM concentrations should have smaller aggregates that could affect bacterial communities, as we observed. According to Tiede et al. (2009), it is possible to observe higher toxicity at lower test concentrations because the extent of aggregation at these concentrations can be likely reduced, occurring more nanoparticles (NP) in the free particulate (un-aggregated) form.

The antioxidant capacity against peroxyl radicals in homogenates of bacteria exposed to nC_{60} was lower (higher relative area) for the concentration of 0.1 mg/L when compared to the control (Figure 3a), indicating that even in darkness (where nC_{60} is not expected to be photo-excited) this NM was able to reduce the antioxidant competence of the bacteria communities living at the mucus secretion of *L. acuta*. Sayes et al. (2005) found that over a range of pH values (3.75-10.25) nC_{60} was formed and that the pH of

the water did seem to influence the process as the average particle size decreased with an increase in the pH. We found that the concentration of 1.0 mg/L of nC_{60} showed lower antioxidant capacity in the anterior region of worms (Figure 4a). This effect can be evidenced at a pH of 8.62 of the water; the higher concentration can suffer alterations in its particle size or aggregation state, changing the toxicity.

The content of organic matter (OM) in estuarine environments should be considered as a factor that influences fullerene toxicity. Xie et al. (2008) reported that changes in nC_{60} size and morphology correlated with OM content. The presence of organic matter (OM) in ecotoxicity tests is known to affect the bioavailability and toxicity of, especially, hydrophobic organic substances (Baun et al., 2008). Thus, it can be considered that nC_{60} may have caused alterations in the antioxidant capacity in the anterior region *L. acuta*, through of ROS, due to OM presence in the water that we sampled and used in our assays. Another non-excluding possibility to be considered is the interaction of nC_{60} with ROS produced by the cells where this nanomaterials entered, rendering more reactive species.

Enzymes of glutathione-S-transferase (GST) group play a key role in cellular detoxification, protecting cells against pollutants or toxicants by conjugating them to glutathione and other endogenous molecules. No GST activity detected in most of bacteria communities sampled. This result was similar to Moraes et al. (2006), since these authors were unable to detect GST activity in mucus sampled from *L. acuta*. However GST activity decreased in the middle and posterior region of worms exposed to 1.0 mg/L of nC_{60} (Figure 6a) and increased in the posterior region of worms exposed to 0.1mg/L of nC_{60} (Figure 6b and 6c) when compared with other treatments, suggesting a double effect of nC_{60}.

It is difficult to predict whether fullerene will act as an anti- or pro-oxidant in vivo (Zhu et al., 2006). In our study, in fact, we verified both conditions, since lipid peroxides content was reduced in the anterior region of worms exposed to the two highest concentrations (0.1 and 1.0 mg/L) of nC_{60}, indicating an antioxidant behavior (Figure 5a). Studies like that in Kam et al. (2004) showed that water and fat soluble derivatives of fullerenes prevented lipid peroxidation more efficiently than did natural antioxidants like vitamin E. Díaz-Jaramillo et al. (2011) found absence of toxic responses mediated by oxidative stress in estuarine worms *Perinereis gualpensis* exposed to fullerene mixed in sediments. In fact these authors observed that under this exposure condition fullerene elicited an antioxidant response triggering higher total antioxidant competence against peroxyl radicals in exposed worms.

Contrary to the antioxidant effect observed in the anterior effect, a pro-oxidant behavior of nC_{60} was registered in the posterior region of worms exposed to the highest nC_{60} concentration (1.0 mg/L) that showed augmented TBARS concentration when compared with the control group (Figure 5c). A previous study of Ferreira-Cravo et al. (2007) reported that *L. acuta* presents a body gradient of ROS concentration, being lowest at the anterior region and highest at the posterior region. It is hypothesized that nC_{60} internalized in the posterior region, with high ROS concentration should react with these species, generating more reactive ones that should promote lipid peroxidation. The pro-oxidant effect of nC_{60} has been reported in other aquatic species, since Zhu et al. (2008) showed that lipid peroxidation levels increased in the fish liver after being exposed to 1.0 mg/L of this nanomaterial.

As mentioned previously, environmental conditions such as pH, ionic strength, presence of complexing agents, and natural organic matter affect the toxicity of nAg (Marambio-Jones and Hoek, 2010). The high salts concentration promote nanoparticle

aggregation (Marambio-Jones and Hoek, 2010), an important fact to consider when evaluating NM risks in estuarine environments. In our study, some evidences of nAg being agglomerated at the micrometric scale was obtained (Zetasizer measurements), a result expected taking into account the high ionic strength of saline water. This in fact was also supported by data of silver accumulation in worms, since an inverse relationship was observed between silver accumulation in posterior region with the exposure concentration, meaning lower bio-availability at higher concentrations. However the higher concentration of nAg (1.0 mg/L) decreased the bacterial CFU and also the weight of bacterial communities living at the surface of *L. acuta* decreased (Figure 2c and 2d). This result can be due to bactericidal or bacteriostatic effect on the bacterial communities living in estuarine organisms like *L. acuta* even when the exposure conditions (high concentration of nAg in saline water) should impose the presence of silver particles in the micrometric range. Previous studies using the same concentration have reported growth inhibition of nAg (Choi et al., 2008).

Roh et al. (2009) found that nAg affected reproduction potential and induced high levels of oxidative stress in *Caenorhabditis elegans*. In our work, the concentration of 1.0 mg/L presented lower antioxidant capacity (higher relative area) in the posterior region of polychaete *L. acuta.* (Figure 4f). Knowledge about toxicity mechanism of nAg in invertebrates is limited, but research on invertebrate species has also shown how the type of capping of the nAg, ionic strength and concentration of organic carbon of the biological media are crucial for predicting and understanding toxicity (Fabrega et al., 2011). Nanosilver (nAg) is the engineered nanomaterial most commonly used in consumer products (Marambio-Jones and Hoek, 2010), consequently, its potential releasing into the environment deserves more attention.

As conclusions can be said that the two NM, nC60 and nAg, induced toxic effects in the polychaete *L. acuta* and in the bacteria still in a situation (darkness) where the fullerene is not photo-excited. The increase in production and marketing of products with NM raises the question of the risks associated with the development of nanotechnology.

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Figures captions

Figure 1. Transmission electronic microscopic image of the aqueous fullerene (nC_{60}) (a) and nanosilver (nAg) (b) suspensions employed in the biossays.

Figure 2. Colony forming units (CFU) exposed to different fullerene (nC_{60}) (a). Bacteria biomass weight exposed to different fullerene (nC_{60}) (b). Colony forming units (CFU) exposed to different nanosilver (nAg) (c). Bacteria biomass weight exposed to different nanosilver (nAg) (d).Different letters indicate significant differences ($p < 0.05$) conducted three independent experiments (n = 9).

Figure 3. Total antioxidant capacity against peroxyl radicals (relative area) of the bacterial samples exposed to different fullerene (nC_{60}) (a) and nanosilver (nAg) (b) concentrations. Asterisk (*) indicates significant differences ($p <0.05$) between treatments connected by solid lines. Number of analyzed samples were 3 for each treatment being conducted three independent experiments (n = 9).

Figure 4. Total antioxidant capacity against peroxyl radicals (relative area) in the anterior (a), middle (b) and posterior (c) region of *Laeonereis acuta* exposed to different fullerene (nC_{60}) concentrations. Figures (d), (e) and (f) shows total antioxidant capacity against peroxyl radicals (relative area) in the anterior, middle and posterior region, respectively, of *Laeonereis acuta* exposed to different nanosilver (nAg) concentrations. Asterisk (*) indicates significant differences ($p<0.05$) between treatments connected by solid lines. Number of analyzed samples were 5 for each treatment being conducted three independent experiments (n = 15).

Figure 5. Concentration of thiobarbituric acid reactive substances (TBARS; nmol/mg of proteins) in anterior (a), middle (b) and posterior (c) region exposed to different fullerene (nC_{60}) concentrations. Asterisk (*) indicates significant differences ($p < 0.05$) between treatments connected by solid lines. Number of analyzed samples were 5 for each treatment being conducted three independent experiments (n = 15).

Figure 6. Activity of glutathione-S-transferase (GST) omega (nmol NADPH/min/mg of proteins) in anterior (a) middle (b) and posterior region (c) exposed to different fullerene (nC_{60}) concentrations. Number of analyzed samples were 5 for each treatment, being conducted three independent experiments (n = 15). Different letters indicate significant differences ($p < 0.05$) between means of different treatments. Number of analyzed samples were 5 for each treatment, being conducted three independent experiments (n = 15).

Figure 1.

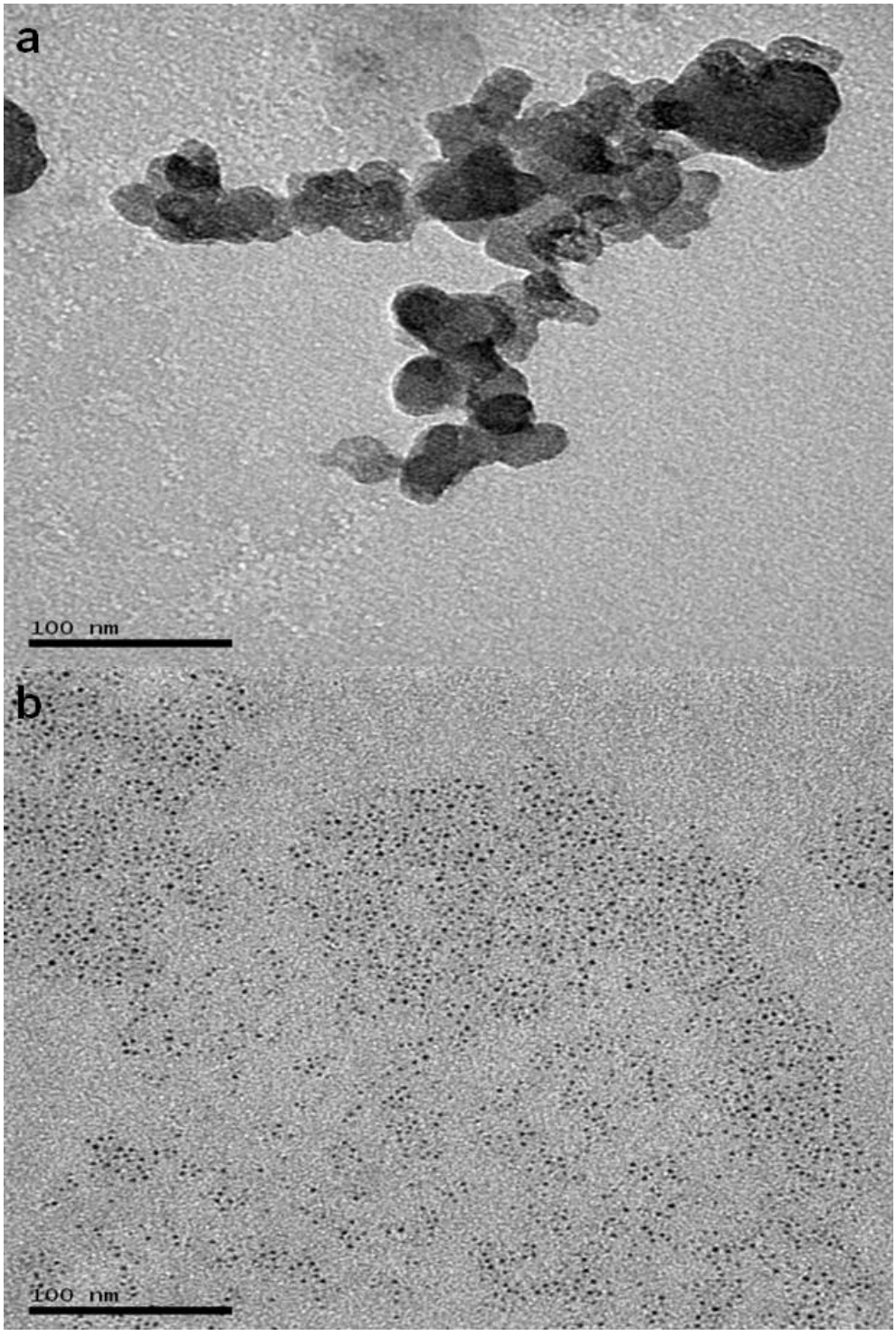

Figure 2.

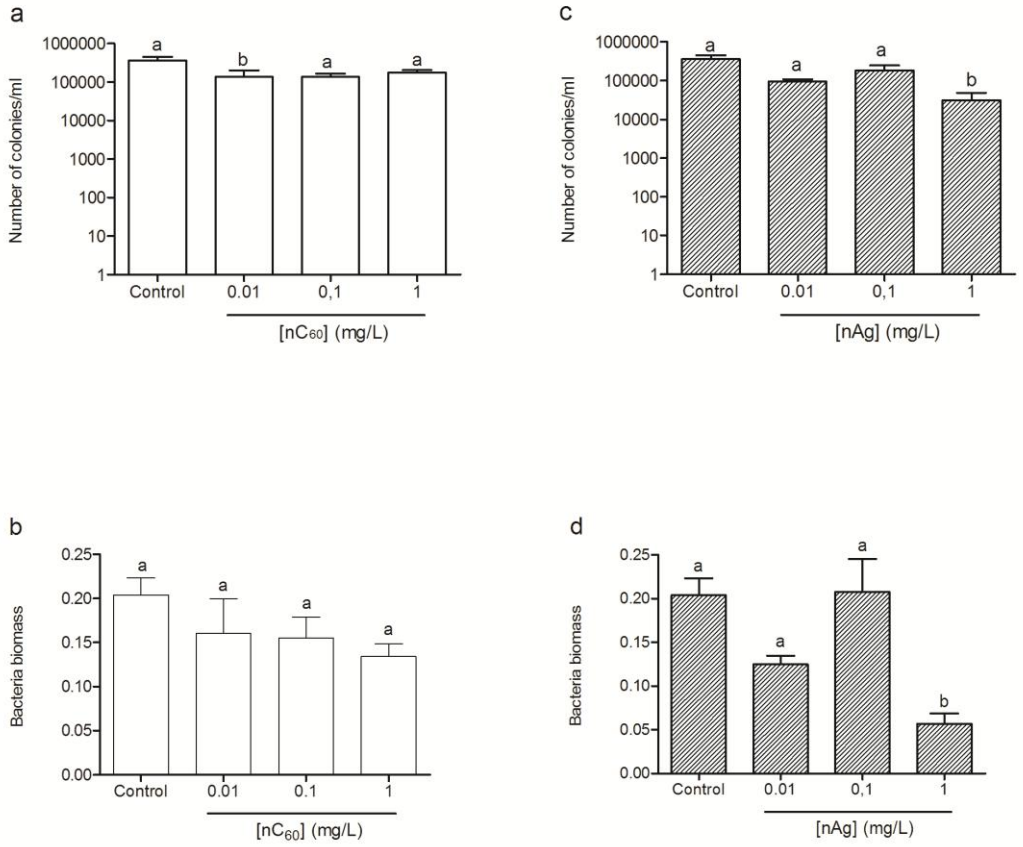
a
Number of colonies/ml
1000000
100000
10000
1000
100
10
1
a
b
a
a
Control
0.01
0,1
1
[nC60] (mg/L)
c
Number of colonies/ml
a
a
a
b
Control
0.01
0,1
1
[nAg] (mg/L)
b
Bacteria biomass
0.25
0.20
0.15
0.10
0.05
0.00
a
a
a
a
Control
0.01
0.1
1
[nC60] (mg/L)
d
Bacteria biomass
a
a
a
b
Control
0.01
0,1
1
[nAg] (mg/L)

Figure 3.

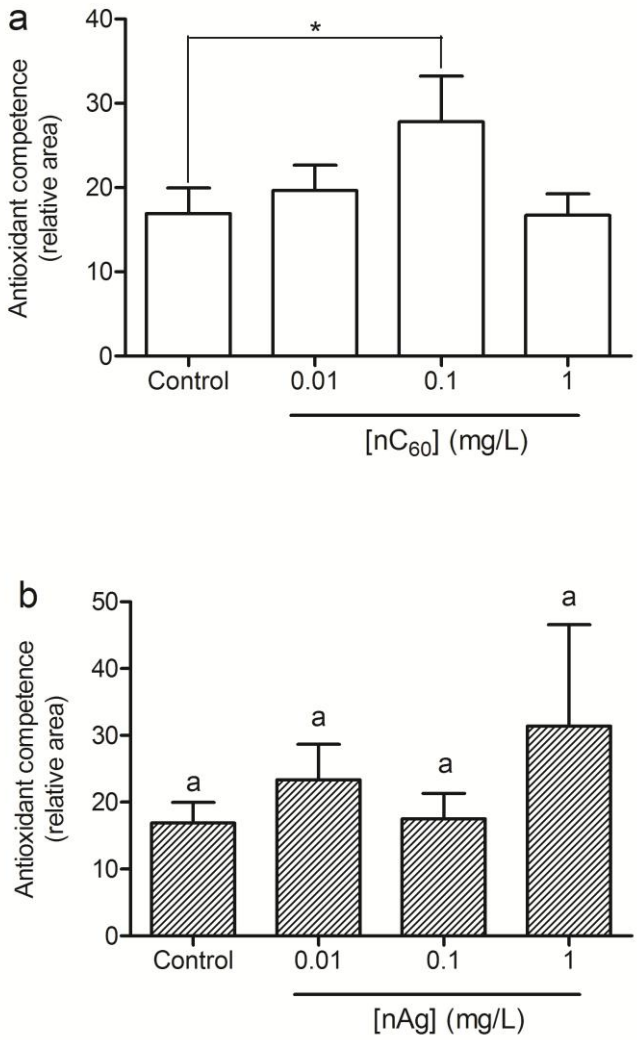
a
Antioxidant competence (relative area)
40
30
20
10
0
*
Control
0.01
0.1
1
[nC60] (mg/L)
b
Antioxidant competence (relative area)
50
40
30
20
10
0
a
a
a
a
Control
0.01
0.1
1
[nAg] (mg/L)

Figure 4.

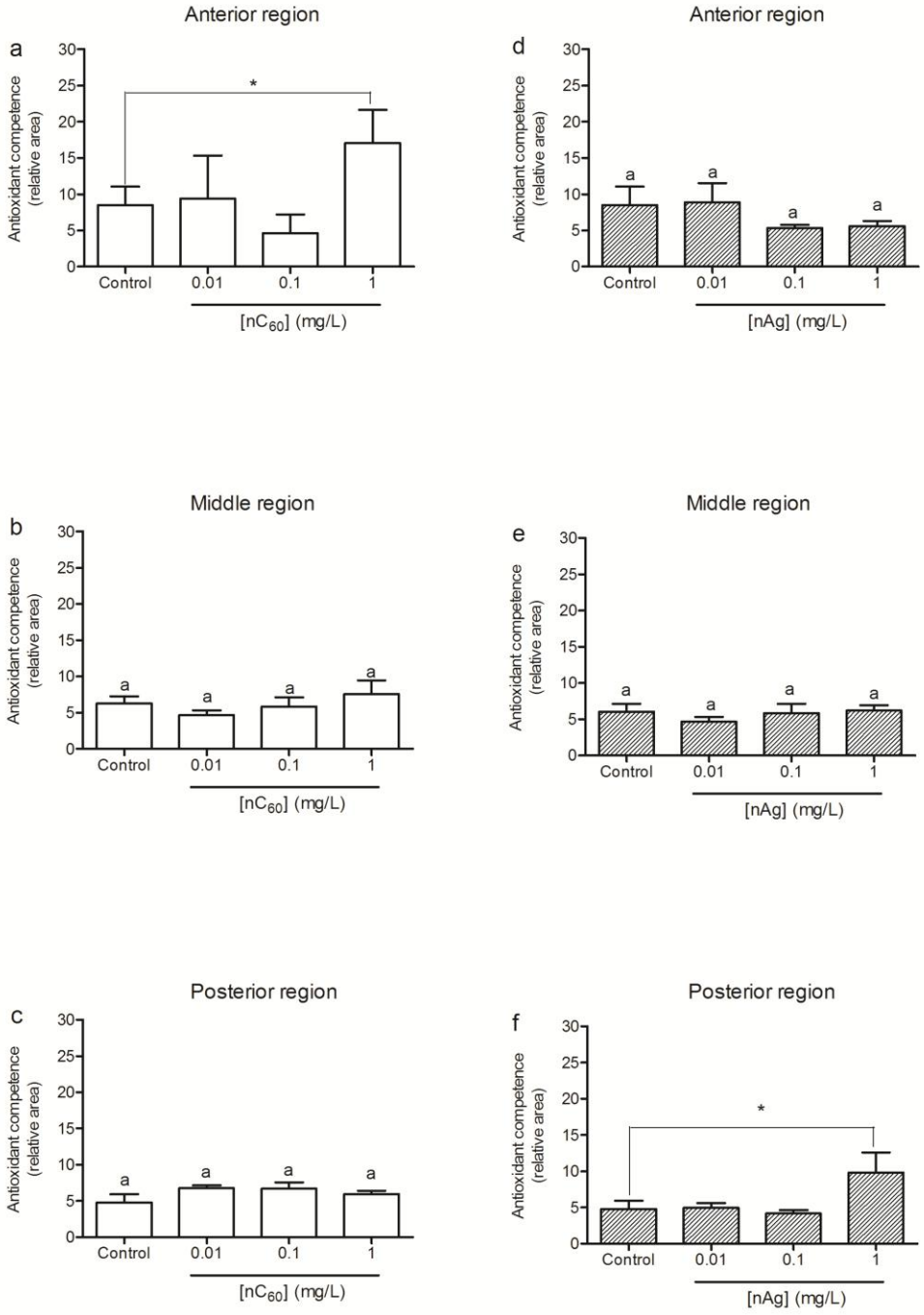
a
Anterior region
Antioxidant competence (relative area)
30
25
20
15
10
5
0
*
Control
0.01
0.1
1
[nC60] (mg/L)
d
Anterior region
Antioxidant competence (relative area)
a
a
a
a
Control
0.01
0.1
1
[nAg] (mg/L)
b
Middle region
Antioxidant competence (relative area)
a
a
a
a
Control
0.01
0.1
1
[nC60] (mg/L)
e
Middle region
Antioxidant competence (relative area)
a
a
a
a
Control
0.01
0.1
1
[nAg] (mg/L)
c
Posterior region
Antioxidant competence (relative area)
a
a
a
a
Control
0.01
0.1
1
[nC60] (mg/L)
f
Posterior region
Antioxidant competence (relative area)
*
Control
0.01
0.1
1
[nAg] (mg/L)

Figure 5.

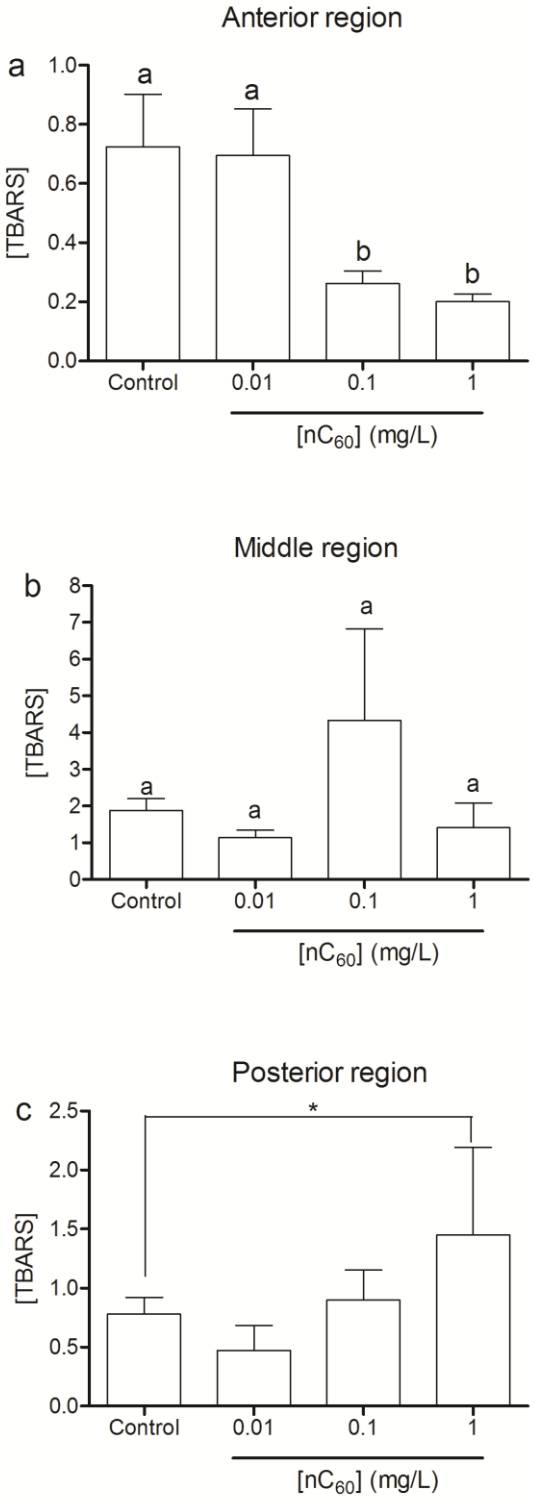
Anterior region
a
[TBARS]
1.0
0.8
0.6
0.4
0.2
0.0
a
a
b
b
Control
0.01
0.1
1
[nC60] (mg/L)
Middle region
b
[TBARS]
8
7
6
5
4
3
2
1
0
a
a
a
a
Control
0.01
0.1
1
[nC60] (mg/L)
Posterior region
c
[TBARS]
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
0.01
0.1
1
[nC60] (mg/L)

Figure 6.

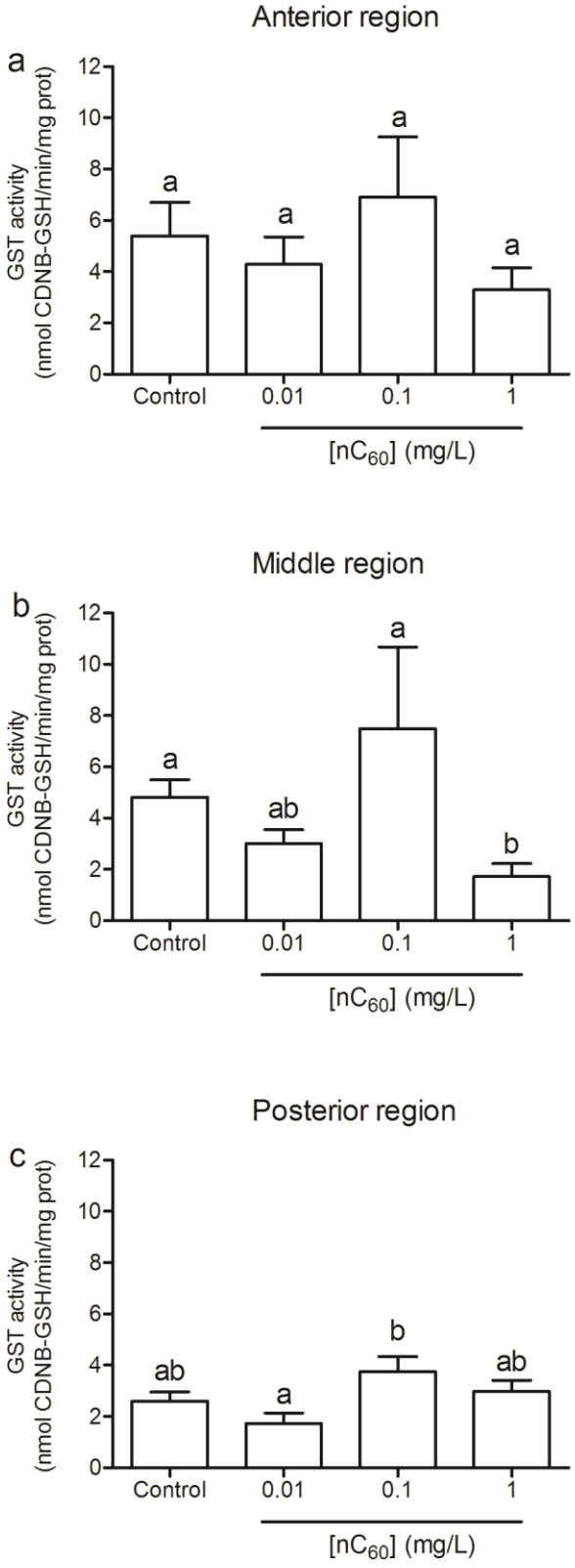
Anterior region
a
GST activity
(nmol CDNB-GSH/min/mg prot)
12
10
8
6
4
2
0
a
a
a
a
Control
0.01
0.1
1
[nC60] (mg/L)
Middle region
b
GST activity
(nmol CDNB-GSH/min/mg prot)
12
10
8
6
4
2
0
a
ab
a
b
Control
0.01
0.1
1
[nC60] (mg/L)
Posterior region
c
GST activity
(nmol CDNB-GSH/min/mg prot)
12
10
8
6
4
2
0
ab
a
b
ab
Control
0.01
0.1
1
[nC60] (mg/L)

Table 1. Molecular identification of bacteria living at the mucus of *L. acuta*.

Isolated colony	Identity	GenBank acession number
C.1	*Vibrio sp*	JN835409
C.2	*Vibrio sp*	JN835410
C.3	*Shewanella sp.*	JN835411
C.4	*Vibrio sp*	JN835412
C.5	*Vibrio sp*	JN835413
C.6	*Vibrio sp*	JN835414
C.7	*Vibrio sp*	JN835415
C.8	*Vibrio sp*	JN835416

CONCLUSÕES

A diminuição das UFC na concentração de 0.01mg/L de nC_{60} pode ser explicada pela diminuição e cinética de agregação e proporção de tamanho dos agregados, que são dependentes da concentração,das características da partícula e do sistema ambiental envolvido.

O efeito encontrado na concentração de 1.0mg/L de fulereno, pode ter sido evidenciado pelo pH de 8.62 da água, uma concentração maior pode sofrer alterações no tamanho da sua partícula ou no seu estado de agregação, alterando a toxicidade.

A concentração de matéria orgânica na água também pode ter afetado a toxicidade de nC_{60}, visto que há trabalhos mostrando que nC_{60} pode muda seu tamanho e morfologia quando há concentração de matéria orgânica no mesmo meio.

O nC_{60} mostrou-se antioxidante, com a redução de conteúdo lipídico na região anterior nas concentrações 0.1 e 1.0 mg/L, sendo que é comprovado na literatura que nC_{60} previne a peroxidação lipídica com maior eficiência que antioxidantes como a vitamina E.

A região posterior do poliqueto apresenta maior concentração de ERO, através do efeito pró-oxidante, o que pode ter interagido com essas ERO do poliqueto e ter gerado umas espécies mais reativas que acabaram provocando peroxidação lipídica na concentração de 1mg/L de fulereno.

A diminuição das UFC e do peso na comunidade bacteriana exposta a uma concentração de 1.0mg/L de nanoprata pode ter sido conseqüência de um efeito bactericida ou bacteriostático.

Os dois NM induziram efeitos tóxicos ainda numa situação (escuridão) tanto no poliqueto, quanto na comunidade bacteriana associada ao seu muco, onde o fulereno não é foto-excitado.

O aumento na produção e comercialização de produtos com NM levanta a questão dos riscos ambientais associados ao desenvolvimento da nanotecnologia.

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