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

EFEITOS DOS NANOMATERIAIS FULERENO E NANOPRATA SOBRE BACTÉRIAS ASSOCIADAS À SUPERFÍCIE CORPORAL DO POLIQUETO ESTUARINO *Laeonereis acuta* (POLYCHAETA, NEREIDIDAE)

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SUMÁRIO

RESUMO

Ambientes estuarinos e costeiros são um dos prováveis destinos finais dos nanomateriais, mesmo havendo ainda uma grande lacuna em relação a estudos que lidem com os efeitos dos nanomateriais sobre organismos estuarinos. Este trabalho analisou o crescimento e respostas bioquímicas de seis colônias expostas aos nanomateriais fulereno (nC_{60}, preparado sem uso de solventes) e nanoprata (nAg; não encapsulada), aplicados individualmente (0,01; 0,1; e 1 mg/L) e conjuntamente (nC_{60} + nAg: 0,01; 0,1; e 1 mg/L de nanoprata e 1 mg/L de fulereno), sobre colônias de bactérias isoladas do muco do poliqueto estuarino *Laeonereis acuta* (Nereididae), no escuro. As exposições ocorreram em 24 h, com agitação constante e, em seguida, as amostras foram inoculadas em agar por mais 24 h para analisar a biomassa produzida e as unidades formadoras de colônias (UFC). As UFC foram, então, contadas, e a biomassa úmida foi pesada, sendo este material utilizado para as análises bioquímicas. O crescimento (por biomassa úmida) foi inibido nas concentrações de 0,01 e 0,1 mg/L de nAg e para 0,01 e 0,1 mg/L de nAg + 1 mg/L constantes de nC_{60} ($p < 0,05$). O crescimento não foi afetado em termo de número de UFC ($p > 0,05$). A capacidade antioxidante total contra peroxi-radicais não mostrou diferenças significativas aos controles ($p > 0,05$). O dano por peroxidação lipídica foi significativo em relação ao controle nas concentrações 0,1 e 0,01 mg/L de nC_{60} e a atividade da GST foi significativamente maior em relação ao seu controle apenas concentração de 1 mg/L da co-exposição de ambos os nanomateriais ($p < 0,05$). A nanoprata demonstrou-se tóxica para as colônias bacterianas. Apesar do fulereno não ter inibido o crescimento bacteriano, ele intermediou peroxidação lipídica e induziu aumento nos níveis de GST quando em exposição conjunta com nAg. Tais resultados podem significar que nC_{60} induziu a produção de algum nível de ERO nos tratamentos, estando tanto em co-exposição com nAg ou sozinho, na ausência de luz, mas seu efeito no crescimento bacteriano quando em exposição conjunta com nAg foi inexistente. Por outro lado, nAg foi capaz de inibir o crescimento bacteriano aparentemente sob limitado intermédio de ERO.

Palavras-chave: fulereno, nanoprata, bacterias, poliqueto estuarino, *Laeonereis acuta*.

ABSTRACT

Estuarine and coastal environments are likely to become one of the final destinations for nanomaterials, and there is still a great lack of studies that deal with the effects of nanomaterials on estuarine organisms. This work analised growth and biochemical responses of six colonies exposed to the nanomaterials fullerene (nC_{60}, no solvents used in preparation) and nanosilver (nAg, not encapsulated) alone (0.01, 0.1, and 1 mg/L) and together (nC_{60} + nAg: 0.01, 0.1, and 1 mg/L of nanosilver and 1 mg/L of fullerene), in darkness, on bacterial colonies isolated from the mucus of the estuarine polichaeta *Laeonereis acuta* (Nereididae). Exposures were performed during 24 h, with constant shaking and then the samples were inoculated on agar during 24 h again to produce biomass or colony forming units (CFU). After growth analysis this material was employed for the biochemical tests. Growth (tested by wet biomass weight) was inhibited at the 0.01 and 0.1 mg/L nAg and 0.01 and 0.1 mg/L nAg + constant 1 mg/L nC_{60} ($p < 0.05$). Colonies growth was not affected in terms of CFU number ($p > 0.05$). Total antioxidant capacity against peroxyl radicals showed no significant variation ($p > 0.05$). Lipid peroxidation damage was significant from the control for the concentrations of 0.01 and 0.1 mg of nC_{60}/L and GST activity was significant from the control group at the concentration of 1 mg/L of both nanomaterials co-exposed ($p < 0.05$). Althoug nC_{60} did not induce inhibition in bacterial growth, it induced lipid peroxidation when alone and increased GST activity when coapled with nAg. These results could mean that nC_{60} induced some ROS levels in treatments, being alone or coupled with nAg, without light, but it did not induce bacterial growth inhibition when alone or affected nAg bacterial growth inhibition when together with the latter. On the other hand, nAg was capable of bacterial growth inhibition, without the detection of ROS influence by our biochemical tests.

Key-Words: fullerene, nanosilver, bacteria, estuarine polychaeta, *Laeonereis acuta*.

INTRODUÇÃO

1. Nanomateriais e nanotecnologia

Nanomateriais são utilizados há mais de 1000 anos em um método cerâmico chamado de “lustre”. A técnica surgiu provavelmente no Iraque, no início do século IX e resulta em um brilho metálico, iridescente sobre a peça sobre a qual é aplicada. Esta utilização dos nanomateriais era totalmente empírica, pois a tecnologia requerida para o reconhecimento dos mesmos é muito recente (Pérez-Arantegui & Larrea, 2003).

Os nanomateriais são definidos pela U.S. National Nanotechnology Initiative como materiais que apresentam, pelo menos, uma dimensão na faixa de 1 a 100 nm (Oberdörster, 2004). Seu tamanho diminuto lhes garante uma grande relação superfície/volume, que por sua vez é o principal contribuinte na grande reatividade molecular que lhes é característica (Oberdörster *et al.*, 2005). Nanomateriais são utilizados nos mais variados setores da indústria hoje em dia, podendo ser encontrados em pneus, roupas resistentes a manchas, protetores solares, cosméticos, células combustíveis, aparelhos eletrônicos, pastas de dente, desinfetantes de água e estão sendo testados (alguns já se encontram liberados) para uma série de aplicações médicas (Colvin, 2003; Kim *et al.*, 2010; Nel *et al.*, 2006; Wijnhoven *et al*., 2009).

Estima-se que a nanotecnologia possa ter um impacto maior que o da revolução industrial, e que, em 2014, mais de 15% de todas as mercadorias disponíveis no mercado global serão fabricadas com algum tipo de nanotecnologia em seus processos produtivos e que a nanotecnologia deve se tornar um mercado de 1 trilhão de dólares em 2015 (Dawson, 2008; Nel *et al*., 2006).

Com este cenário em mente, surge a preocupação acerca do possível impacto negativo desses nanomateriais, não apenas para a saúde humana, mas também para meio ambiente (Colvin, 2003; Nel *et al.*, 2006; Bystrzejewska-Piotrowska *et al.*, 2009;

Paschoalino *et al.*, 2010). Esse uso muito difundido dos nanomateriais pela indústria pode resultar em um aumento na liberação de nanopartículas no ambiente aquático, o que é especialmente preocupante, tendo em mente que seu destino, comportamento e toxicidade permanecem em grande parte desconhecidos (Navarro *et al.*, 2008).

2. Nanomaterial orgânico: fulereno

O fulereno (C_{60}) é uma molécula composta por 60 átomos de carbono e com aparência semelhante à de uma bola de futebol. A molécula foi descoberta pelo ganhador do prêmio Nobel Harold W. Kroto e colaboradores em 1985 e nomeado em homenagem ao famoso arquiteto Richard Buckminster Fuller, pela semelhança dos fulerenos com seu domo geodésico (Kroto *et al.*, 1985).

O composto é formado em eventos onde há muita energia envolvida, tornando-se depois extremamente estável (Kroto *et al.*, 1985). Dentre os processos naturais onde pode ocorrer a formação de fulerenos estão os relâmpagos, quedas de meteoritos ou processos metamórficos. O composto também pode ser encontrado em fuligem gerada pela combustão de hidrocarbonetos e oxigênio, no carvão comercial e em fuligem produzida na queima de velas (Isaacson *et al.*, 2009).

Os fulerenos foram produzidos pela primeira vez em quantidades macroscópicas em meados de 1990, após Wolfgang Krätschmer e seus colaboradores (1990) desenvolverem o método que o tornou possível. Apesar de ser muito hidrofóbico, o C_{60} possui a capacidade de se estabilizar em água, na forma de colóide. Em laboratório isso pode ser feito através do uso de solventes ou de agitação por um longo período de tempo, sendo esta forma suspensa em água denominada de nC_{60} (Andrievsky *et al.*, 1995; Deguchi *et al.*, 2001; Fortner *et al.*, 2005).

O fulereno e suas contrapartes funcionalizadas possuem inúmeras aplicações cosméticas, eletrônicas e ópticas (Isaacson *et al*., 2009). O composto, quando em suas formas solúveis em água, possui várias qualidades que lhe conferem possíveis aplicações na medicina. O fulereno parece ser um bom agente bactericida quando na presença de luz (Kai *et al*., 2003) e agente antioxidante quando ligado a grupos malonil (Dugan *et al*., 1997). Este nanomaterial possui ainda potencial terapêutico contra a meningite bacteriana (Tsao *et al*., 1999), além de inibir atividade virótica em sistemas biológicos infectados (incluindo atividade do vírus HIV) (Käsermann & Kempf, 1998). O nC_{60} também parece possuir a capacidade de carrear fármacos para órgãos específicos e de atravessar a barreira hemato-encefálica (Vogelson, 2001).

Tem-se conhecimento de que o fulereno apresenta efeitos nocivos para vários organismos, como *Daphnia magna* (Lovern *et al*., 2007; Oberdörster *et al*., 2006), algumas espécies de peixe como *Micropterus salmoides* (Oberdörster, 2004) e *Carassius auratus* (Zhu *et al*., 2008), células eucarióticas (Sayes *et al*. 2005, Isakovic *et al*., 2006) e procarióticas (Fortner *et al*., 2005; Lyon *et al*., 2006; Lyon & Alvarez, 2008), mas seus mecanismos de toxicidade são controversos. O C_{60} é conhecido por produzir espécies reativas de oxigênio (ERO), principalmente quando foto-excitado (Hotze *et al*., 2008; Oberdörster, 2004; Sayes *et al*., 2005; Yamakoshi *et al*., 2003). Brunet e colaboradores (2009), no entanto, não encontraram geração significativa de ERO por nanopartículas coloidais (nC_{60}) foto-excitadas em bactérias. A existência de um mecanismo de toxicidade independente de ERO foi sugerida por Lyon e Alvarez (2008), com evidências de oxidação protéica, mudanças no potencial de membrana e interrupção da respiração celular em bactérias. Os autores propuseram que o mecanismo poderia ser decorrente do contato direto das nanopartículas contra as estruturas afetadas.

Ainda, a toxicidade de C_{60} pode ser relevante se considerada a capacidade de interação entre C_{60} e outros contaminantes (Costa *et* al., 2011; Henry *et al*., 2011).

O fulereno, de uma forma geral, é muito resistente a degradação, podendo se acumular facilmente tanto em meio aquoso quanto terrestre, ou mesmo em organismos vivos (Johansen *et al.*, 2008). A quantidade de fulereno que pode ser encontrada em sua forma em suspensão é de cerca de 10 ppm, o que corresponde a 100 vezes a solubilidade em água dos hidrocarbonetos aromáticos (PAHs). Mesmo em baixíssimas concentrações, os PAHs são capazes de causar impactos significativos, portanto é possível que o fulereno, mesmo em baixas concentrações, também possa ter propriedades toxicológicas importantes (Colvin, 2003).

3. Nanomaterial metálico: nanoprata

Já faz séculos que se tem conhecimento das propriedades bactericidas da prata, e apesar do metal ser altamente tóxico para procariotos, é considerado praticamente inócuo para organismos superiores, tornando-o um agente bactericida ao mesmo tempo seguro e eficiente (Janardhanan *et al*., 2009; Pshennikova *et al*., 2011). A nano-prata (partículas com menos de 100 nm e consistindo de cerca de 20-15.000 átomos de prata e que será chamada de nAg nesta dissertação por motivo de simplificação), por sua vez, é tóxica para bactérias em poucas unidades de mg/L, afetando inclusive cepas consideradas multi-resistentes (Kvitek *et al*., 2011; Wijnhoven *et al*., 2009). Além das suas propriedades bactericidas, a nAg também é um fungicida efetivo, possui propriedades anti-inflamatórias e parece ser capaz inibir a replicação do vírus HIV-1 (Wijnhoven *et al*., 2009). A nanoprata é tóxica para células de mamíferos (Braydich-Stolle *et al*., 2005; Gopinath *et al*., 2008; Wen *et al*., 2007), mas as concentrações necessárias para afetá-las são geralmente muito maiores do que as concentrações para

procariotos, ficando em torno de 60 mg/L para células de mamíferos e 30 mg/L para alguns microorganismos eucarióticos (Kvitek *et al*., 2011).

Os mecanismos pelo qual nAg é tóxica podem estar relacionados a danos a membrana celular, produção de ERO ou liberação de íons Ag^{+}, que por sua vez podem interagir com proteínas e enzimas (Navarro *et al*., 2008), sendo sugerido que as ERO geradas tanto por nAg quanto por Ag^{+} podem ser as maiores responsáveis pela alta atividade bactericida atribuída a nanoprata (Choi & Hu, 2008).

Por causa de suas fortes propriedades bactericidas, a nano-prata é a principal nano-partícula em uso pela indústria atualmente (Luoma, 2008). Ela é aplicada em inúmeros produtos como têxteis, contêineres diversos para armazenamento de alimentos, aditivos para lavagem de roupas, tintas e até mesmo em suplementos alimentícios, tornando-a uma forte candidata a liberação no ambiente aquático (Navarro *et al*., 2008). Conclui-se que há riscos, portanto, no uso indiscriminado da nano-prata, em tantas mercadorias, tanto para as comunidades microbianas benignas ao ambiente natural quanto para a biota em geral.

Geralmente a nano-prata em pó é estabilizada em forma coloidal pela adição de agentes encapsulantes, o que acaba por diminuir a área superficial das partículas e conseqüentemente suas propriedades bactericidas (Janardhanan *et al.*, 2009). No presente trabalho foi utilizado um novo tipo de nano-prata coloidal que possui um núcleo de amido, mas nenhum agente encapsulante, deixando assim sua superfície livre.

4. Modelos biológicos: o poliqueto *Laeonereis acuta* e as bactérias ambientais

Laeonereis acuta (Treadwell, 1923) (Nereididae: Polychaeta) é uma espécie de poliqueto, encontrada desde a Peninsula de Valdéz, Argentina, até o estado Piauí, no Brasil (Pamplin *et al*., 2007), sendo uma espécie bentônica constituinte da infauna

(Rosa & Bemvenuti, 2004). Esta espécie tem sido utilizada em vários testes toxicológicos (Geracitano *et al*., 2004). O poliqueto sobrevive na ampla faixa de salinidade de 1 a 40 ppm e faz parte da dieta de vários animais, de formigas a pássaros (Palomo *et al*., 2003). *L. acuta* apresenta em seu muco uma comunidade bacteriana, formando assim um biofilme (Fernandez da Silva *et al*., 2008). O muco por si só aumenta a distância de difusão para espécies reativas de oxigênio permeáveis como o peróxido de hidrogênio (H_2O_2) (da Rosa *et al*., 2005), enquanto que as bactérias que crescem nesse substrato possuem enzimas antioxidantes (Dowds, 1994; Wood & Sorensen, 2001). Moraes *et al*. (2006) considera que o biofilme secretado por *L. acuta* é responsável pela interceptação ou degradação de H_2O_2 e radicais peroxil e hidroxil, garantindo assim uma importante capacidade antioxidante ao animal. Microorganismos são importantes para a saúde de todos os ecossistemas conhecidos (Lyon *et al*., 2006), possuindo funções fundamentais, como as de produtores primários, facilitadores na reciclagem de nutrientes e agentes degradadores de poluentes (Smith, 2007). O conhecimento a respeito da toxicidade dos nanomateriais sobre microorganismos pode ser utilizado para prevenção, regulação e remediação de ambientes impactados por esse tipo de composto (Lyon & Alvarez, 2008).

JUSTIFICATIVA

Ambientes estuarinos e costeiros representam, possivelmente, o destino final para as nanopartículas (Cannesi *et al*., 2011) e ainda são raros os trabalhos que tratem de nanomateriais em ambientes estuarinos e sua respectiva biota. O conhecimento acerca dos efeitos destes compostos sobre a biota que habita tais ambientes é de fundamental importância para que tanto a prevenção quanto a remediação possam ter dados nos quais futuramente se embasarem para que as estas devidas ações possam ser tomadas.

OBJETIVOS

O presente trabalho teve como objetivo avaliar o crescimento de colônias de bactérias associadas ao corpo do poliqueto *L. acuta*, quando expostas aos nanomateriais nC_{60} (preparado sem solventes) e nAg (não encapsulado), sem a presença de luz. O efeito dos compostos juntos também foi testado, na tentativa de identificar alguma inter-relação toxicológica entre ambos. A capacidade antioxidante total de cada colônia isolada, a capacidade de detoxificação envolvendo a enzima glutationa-S-transferase (GST) frente aos nanomateriais e o dano por peroxidação lipídica também foram avaliadas.

ARTIGO

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Effects of fullerene and nanosilver nanomaterials against bacteria associated to the body surface of the estuarine worm *Laeonereis acuta* (Polychaeta, Nereididae)

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Abstract

Estuarine and coastal environments are likely to become one of the final destinations for nanomaterials, and there is still a great lack of studies that deal with the effects of nanomaterials on estuarine organisms. This work analised growth and biochemical responses of six bacterial colonies, which were isolated from the mucus of the estuarine polichaeta *Laeonereis acuta* (Nereididae), after been exposed to the nanomaterials fullerene (nC_{60}, no solvents used in preparation) and colloida nanosilver (nAg) alone (0.01, 0.1, and 1 mg/L) and together (0.01, 0.1, and 1 mg/L of nanosilver and 1 mg/L of fullerene added to each nAg concentration), in darkness. Exposures were performed during 24 h, with constant shaking and then the samples were inoculated on agar during 24 h again to produce biomass or colony forming units (CFU). After growth analysis this material was employed for the biochemical tests. Growth (tested by wet biomass weight) was inhibited at the 0.01 and 0.1 mg/L of nAg and 0.01 and 0.1 mg/L nAg + constant 1 mg/L of nC_{60} ($p < 0.05$). Colonies growth was not affected in terms of CFU number ($p > 0.05$). Total antioxidant capacity against peroxyl radicals showed no significant variation ($p > 0.05$). Lipid peroxidation damage was significant from the control for the concentrations of 0.01 and 0.1 mg/L of nC_{60} and GST activity was significant from the control group at the concentration of 1 mg/L of both nanomaterials co-exposed ($p < 0.05$). Althoug nC_{60} did not induce inhibition in bacterial growth, it induced lipid peroxidation when alone and increased GST activity when together with nAg. These results could mean that nC_{60} induced some ROS levels in treatments, being alone or together with nAg, without light, but it did not induce bacterial growth inhibition when alone or affected nAg bacterial growth inhibition when together with the latter. On the other hand, nAg was capable of bacterial growth inhibition, although

the biochemical measurements did not suggest that this response is due to ROS generation.

Key-Words: fullerene, nanosilver, bacteria, estuarine, *Laeonereis acuta*.

1. Background

Nanomaterials are used in the most varied sectors of industry today. They are found in tires, stain resistant clothes, sun screens, cosmetics, fuel cells, electronics, tooth pastes, water disinfectants and have being considered useful for several medical applications [1-4]. Some estimations consider that nanotechnology could have a greater impact than the industrial revolution, suggesting that in 2014, more than 15% of all products available at the global market will be produced with some kind of nanotechnology into their manufacturing processes and that nanotechnology may become a 1 trillion dollar market in 2015 [3, 5]. This widespread use of nanomaterials by the industry tends to raise its release in the aquatic environment, which is especially concerning since their fate, behavior and toxicity remain largely unknown [6].

This study analyzed two distinct nanomaterials: a carbon based one, fullerene (C_{60}), and a metallic one, nanosilver. It is know that C_{60} is toxic against some organisms, like *Daphnia magna* [7, 8], some fish species, like *Micropterus salmoides* [9] and *Carassius auratus* [10], eucariotic cells [11, 12] and prokariots [13-15], but its mechanisms of toxicity are somewhat controversial. C_{60} is known to produce reactive oxygen species (ROS), mainly when photo-excited [9, 11, 16, 17]. Brunet and co-workers [18], however, did not find significant ROS generation by photo-excited nC_{60} (fullerene forming clusters in suspension in water) in bacteria. The existence of a ROS-independent mechanism of toxicity was proposed by Lyon and Alvarez [15], with evidence of protein oxidation, changes in cell membrane potential, and interruption of cellular respiration in bacteria. Authors proposed that this mechanism of toxicity could occur by direct contact of C_{60} nanoparticles against membrane proteins involved in

respiration. Still, fullerene can interact with other contaminants, possibly raising their toxic potential [19, 20].

For centuries it is known that silver has bactericidal properties, and although it posses high toxicity to prokaryotes, it is considered that higher organisms are not susceptible to it, making silver a secure and effective bactericide [21, 22]. Nanosilver (particles with less than 100 nm consisting of about 20-15,000 silver atoms; they will be called nAg when in water suspension in this paper for simplification) is toxic to bacteria at concentrations in the range of mg/L, even against multi-resistant strains [4, 23]. In addition to its bactericidal properties, nAg is an effective fungicide, possesses anti-inflammatory properties and seems even able to inhibit HIV-1 virus replication [4]. Nanosilver is toxic to mammalian cells [24-26], but the concentrations are generally much higher than the needed to affect prokaryotes, being around 60 mg/L for mammalian cells and about 30 mg/L for some eukaryotic microorganisms [23].

Nanosilver toxicity is generally considered to be related to ROS production, damages at cell membranes or Ag^+ ions interaction with proteins and enzymes [6, 27]. ROS production, induced by both nAg and Ag^+ ion, can be responsible by the high bactericidal activity attributed to nanosilver [27].

Because of its strong bactericidal properties, nanosilver is the main nanomaterial currently in use by industry [28]. Nanosilver is applied today in numerous consumer products like textiles, food storage containers, laundry additives, paints and food supplements, making it very likely to be released in the aquatic environment [6]. Generally, nanosilver powder is stabilized in colloidal form by the addition of capping

agents, diminishing its surface area and reducing its bactericide properties [21]. In present study we employed colloidal nanosilver with a starch nucleus but with no capping agents, leaving its surface free.

Laeonereis acuta (Nereididae: Polychaeta) [29] is polychaeta species occurring from Peninsula de Valdéz, Argentina, to Piauí State, Brazil [30] and is a benthonic, infaunal animal [31]. The animal has being used in various toxicological experiments [32]. Its estuarine habitat includes a variety of sediment types, under the wide range of salinity of 1 to 40 ppm, being part of the diet of various animals, ranging from ants to birds [33]. Over the mucus secreted by this worm exists a microbial community capable of producing enzymatic and non-enzymatic antioxidants [34]. This microbial community might give an important antioxidant capacity to *L. acuta*, since its mucus even presents higher antioxidant competence against peroxyl and hydroxyl radicals than the worm itself [34]. Microorganisms are important to the health of all known ecosystems [14], so the knowledge about the toxicity of engineered nanomaterials on bacteria may be used for prevention, regulation and remediation of impacted environments by this kind of molecules [15].

This work analised growth and biochemical responses of six colonies exposed to the nanomaterials fullerene (nC_{60}, no solvents used in preparation) and colloidal nanosilver (nAg) alone (0.01, 0.1, and 1 mg/L) and together (nC_{60} + nAg; 0.01, 0.1, and 1 mg/L of nanosilver and 1 mg/L of fullerene added to each nAg concentration), on bacterial colonies isolated from the mucus of the estuarine polichaeta *Laeonereis acuta* (Nereididae). Considering the water turbidity that is frequently found in estuarine

waters and the worm behavior of burrowing itself into the sediment [33], nanomaterials exposures and bacterial growth were realised without the incidence of any kind of light.

2. Results

The estimation of fullerene and nanosilver particle sizes, alone or together in the same suspensions, employing laser diffractometry indicated that a sub-population of the particles were at the micro scale, indicating that the bacteria were exposed to a mix of nano and micro particles (data not shown). TEM images did suggest particles at the nanoscale as predominant in the nAg treatments and around the nanoparticle definition limit of 100 nm for at least one dimension for nC_{60} (**Figure 1**).

Inhibition of bacterial growth by nanomaterials was analyzed by wet mass weighting and colony forming units (CFU) counting. In the wet mass weighting test, no significant ($p > 0.05$) bacterial growth inhibition was induced by fullerene in any concentration (**Figure 2a**). In this same test, nAg reduced ($p < 0.05$) bacterial growth at 0.01 and 0.1 mg/L nAg treatments, when comparing these data with their control but did not affect ($p > 0.05$) the 1 mg/L nAg treatment (**Figure 2b**). Bacteria exposed to 0.01 and 0.1 mg/L nAg + constants 1 mg/L nC_{60} showed reduction in growth ($p < 0.05$) as well, when comparing these with their respective control, but the 1 mg/L nAg + 1 mg/L nC_{60} treatment did not affect ($p > 0.05$) bacterial growth (**Figure 2c**). All treatments presented no significant differences ($p > 0.05$) in bacterial growth inhibition from their controls, in any concentration, by CFU number (**Figure 3**).

Total antioxidant capacity against peroxil radicals showed no significant variation ($p > 0.05$) for nC_{60}, nAg or both compounds together (**Figure 4**). In the evaluation of lipid peroxidation (TBARS) (**Figure 5**), fullerene alone showed no induction of lipid peroxidation at the concentration of 0.01 mg/L C_{60} when compared with the control group ($p > 0.05$), but at the concentrations of 0.1 and 1 mg/L it was observed higher ($p < 0.05$) TBARS levels when compared with the other two treatments (control and 0.01 mg/L of fullerene). Exposure to nAg or to nAg + nC_{60} did not alter TBARS levels ($p > 0.05$).

Glutathione-S-transferase (GST) activity was not significant ($p > 0.05$) affected neither by nC_{60} nor by nAg exposure (**Figures 6**). Co-exposure to both nanomaterials at the concentrations of 0.01 and 0.1 mg/L also did not affected GST activity ($p > 0.05$), but at the highest concentration (1 mg/L), a significantly higher GST activity was found ($p < 0.05$).

Total DNA was extracted from the six isolates obtained and the 16S rDNA sequences were partially determined (**Table 1**). Two isolates were identified as *Listonella anguillarum*, three isolates were identified as belonging to *Vibrio* genera and one to *Cupriavidus* genera. All identified colonies are gram-negative.

3. Discussion

Fullerene and nanosilver are important nanomaterials with several industrial and medical applications. They have being produced in industrial scale for considerable amount of time, although without a corresponding knowledge of their toxicity or

potential environmental impacts [1, 3, 35]. It is expected that under the growth of nanomaterials synthesis and production, estuarine and coastal environments become likely to be the final destiny for nanoparticles [36] and there is, still, a great lack of studies that deal with the effects of nanomaterials on estuarine organisms.

Of all bacterial colonies isolated in this work, five of them (*Vibrio* spp. and *Listonella anguillarum*) are from the Vibrionaceae family [37], a diverse group of facultative anaerobes with a broad ecological distribution that is one of the most well studied families of heterotrophic bacteria that inhabit marine and estuarine ecossistems [38]. One member of the Burkholderiaceae family, *Cupriavidus* sp., an aerobic, heterotrophic organism, was also identified [39]. The genus has been used in toxicity tests, and is considered resistant to various heavy metals and metalloids, but it had its growth impaired by quantum dots in a previous work [40].

The lack of growth inhibition by nC_{60} in terms of wet mass weigh (Fig. 2a) presented an expected result, since fullerene is generally considered toxic through the generation of ROS, which is considered to occur mainly by photo-excitation [41]. Altough it is know that fullerene may be also toxic without ROS generation [15], colonies growth was not affected by fullerene in the present work, in accordance with the study of Tong and co-workers [42], in which nC_{60} did not induce any toxic effect in a soil microbial community after 180 days of exposure. Authors stated that these results may be caused by nC_{60} becoming unavailable when in contact with organic matter and salts from soil. It is possible that the lack of toxicity of nC_{60} in the present work, in terms of bacterial growth, was consequence of nC_{60} adsorption by constituents of the nutrient broth, where the experiment was realized. The same lack of toxicity was found by Fortner *et al.* [13].

In this case, authors tested nC_{60} toxicity in two liquid media: a “rich” (Luria Broth) and a “minimal” one (minimal Davis media with 10% of the recommended potassium phosphate). They found a reduction in bacterial growth in the minimal media at the lowest nC_{60} concentration, but no effect in any “rich” media concentrations. Authors stated that this result was probably due to salt or protein concentration in its “rich” media, which could turn nC_{60} unavailable. Data obtained using laser diffractometry showed that although nanoscale particles were present, microscale particles did exists in all samples as well (data not shown).

A previous study from our group [43] consisted in the exposure of several bacterial isolates from the fish *Cyprinus carpio* during 3 h to nC_{60} in the same liquid nutritive media that was used in the present work. Authors found growth inhibition by nC_{60} at the three assayed concentrations (0.1, 1 and 10 mg/L). However, exposure was performed under fluorescent light, which leads to fullerene photo-excitation and consequently, ROS production, and a much shorter exposure time, which could means that fullerene has a fast growth inhibition effect, but that is reduced or nullified over time, as consequence of particles agregation.

Also, the apparent lack of toxicity at the highest nAg concentration (1 mg/L; Fig. 2b) could be explained by the substances present in the nutritive media, as cited for nC_{60}, since it is known that the behavior of nanoparticles in general in aqueous suspensions are strongly affected by ionic strength, calcium concentrations and other factors [36]. Organic, humic acid, carboxyl, phenolic, amino, and sulfidic ligand interactions and salinity can also lead to an increase in aggregation of nAg, with a consequent loss in antibacterial activity [44]. It is expected of this effect to be more conspicuous at higher

nAg concentrations, as observed in the present study, where a higher particle aggregation and, consequently, surface area reduction and unavailability should render the nanomaterials less toxic. Lower and intermediary nAg concentrations (0.01 and 0.1 mg/L of nAg; Fig. 2b) both presented expected antibacterial results, given nAg high antibacterial activity [2, 27, 45-47].

Despite of the growth inhibition found in some treatments by the weighting test, growth by CFU number (n = 3) was not afected in any treatment or nanomaterials concentration. Total antioxidant capacity (Fig. 4) showed no significant variations, meaning that bacteria possibly did not elicited an antioxidant response, a behavior that resulted in oxidative stress, since lipid peroxidation was detected in bacteria exposed to 0.1 or 1 mg/L of nC_{60} (Fig. 5). There were augmented levels of TBARS in bacteria exposed to nC_{60}, but this effect was not enough to reduce colonies growth after 24 h of exposure (Fig. 2a). The lack of light incidence and the proposed interaction of nanoparticles with nutrient broth constituents, like salts and proteins, are all factors expected to reduce the toxic effects induced by these nanomaterials [44].

On the other hand, as already stated, nAg inhibited colonies growth at concentrations of 0.01 and 0.1 mg/L of nAg (Fig. 2b). These results were contrasted by the lack of any significant variations in the biochemical variables (Figs. 4b, 5b and 6b), sugesting that nAg toxicity was unrelated or had low influence of ROS. Nanosilver ROS generation appears to be related to photocatalysis [27], which could explain the lack of effect in the biochemical tests. Although nAg toxicity against bacteria is not completely understood, it has being considered that nAg would be able to attach to their membranes, disturbing their respiration, permeability and ultimately resulting in cell death [4, 46]. But nAg can

enter inside bacteria as well, interacting with molecules containing phosphorus and sulfur, including DNA [48]. Despite of the mechanism responsible for nAg toxicity observed here, bacterial growth was inhibited, possibly under minimal ROS influence.

Although some statistical differences from its control were found for GST activity in the co-exposure treatment and no significant variation was found for GST activity when nAg was applied alone (Fig. 6), growth treatments measured by wet mass weight with nAg and nC_{60} together seemed to mirror the same tests with nAg alone, suggesting that nC_{60} did not affect nAg toxicity in a significant way. If all biochemical variables are analyzed together, it seems that ROS was not the main toxic mechanism responsible for the growth inhibition found in nAg and nAg + nC_{60} exposures, and even when lipid peroxidation damage was detected, in the case of nC_{60}, it was not enough to promote reduction in bacterial growth after 24 h of the nanomaterial exposure.

4. Conclusions

Estuarine and coastal environments possibly represent the ultimate destination for nanoparticles [36], therefore the knowledge about the effects of these compounds on the biota inhabiting these environments is of fundamental importance for acquiring data that might be useful for proper prevention or remediation decisions in these habitats. Nanosilver is proved to be potentially dangerous to environmental bacteria, even in the absence of light and even when part of it was agglomerated at the microscale. Although fullerene did not induce inhibition in bacterial growth and apparently did not affect nanosilver toxicity, lipid peroxidation was found even in the absence of light for fullerene alone, as well as an increase in GST activity in treatments with both

nanomaterials together. These results could mean that, although nC_{60} may have induced some degree of ROS in treatments, being alone or coupled with nAg, in the absence of light, this was not suficient to inhibit the growth of colonies. nAg, on the other hand, was capable of inhibit bacterial growth, possibly with minimal influence of ROS.

5. Methods

5.1. Nanoparticles preparation and characterization

Fullerene might probably form colloids in the water column, dissolved in a solvent or as powder, even with its hidrophobicity [49], so turning it soluble for experimentation is important. The problem is that some solvents used in the preparation of fullerene may affect its toxicity [49, 50]. Because of this, we used an aqueous C_{60} suspension in this study. A fullerene suspension (200 mg/L; SES Research, 99% purity) was prepared by constant stirring of C_{60} in Milli-Q water during 2 months (photoperiod 24h L). The aqueous suspension was sequentially filtered through 0.45 and 0.20 µm mesh. The C_{60} concentration was estimated by measuring total carbon concentration with a TOC-V CPH (Shimadzu) total organic carbon analyzer [19, 43]. Filtered C_{60} suspension in Milli-Q water was analysed by laser diffractometry (Mastersizer 2000, Malvern Instruments, UK) and characterized using a JEOL JSM 1200 EX II transmission electron microscope operating at 100 kV. Samples of approximately 30 L C_{60} suspension were disposed onto 300-µm mesh TEM grids (SPI) coated with Formvar. Analysis was performed after 24 h to allow sample evaporation [14].

The colloidal suspension of nanoparticulate silver was provided by Nanotek S.A. (Argentina), 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 method involves the reduction of the element to its zerovalent state, followed by a tendency of atoms in this state to form. Nanoparticles characterization in the exposure solutions were performed through laser diffractometry and TEM analysis as for fullerene assays.

5.2. *Laeonereis acuta* collection and bacterial colony isolation

Specimens of *L. acuta* were collected in a non-impacted area (‘‘Saco do Justino’’) of the Patos Lagoon, Southern Brazil [32]. Worms were transferred to the laboratory transported in a plastic bag with water, which was taken from their habitat immediately before the start of the collection. Once in the laboratory, samples from their body surfaces were taken and inoculated on disposable plastic Petri plates with tryptic soy agar (Hi-Media) as the growth medium [51]. After 24 h incubation, without light and at 20 ºC, 12 colonies, from 12 different plates, were selected and then inoculated in 10 mL nutritive liquid medium (Hi-Media) in plastic cuvettes. The incubation took 2 weeks at 20 ºC, at constant shaking and under a photoperiod of 12 L/12 D. After incubation, their OD_{540} were adjusted to 0.1, which was the lowest value achieved from the best growing colonies. They were maintained in a refrigerator at 4 ºC after this process.

5.3. Molecular analysis for bacteria characterizarion

DNA was extracted from 1 mL cell 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'-GTGTGCCAGCMGCCGCGGTAA-3' and reverse 5'-GGGGACTACVSGGGTATCTAAT-3') were designed as described previously [52]. PCR reactions were performed in a total volume of 25 µL using approximately 100 ng of genomic DNA, 0.24 µM of each primer, 50 µM of dNTP, 3 mM MgCl2 and 0.25 U Taq DNA polymerase (Invitrogen) under the following conditions: initial 2 min denaturation step at 94 ºC; 45 s at 94 ºC, 45 s at 55 ºC, and 1 min at 72 ºC for 35 cycles. Post-extension at 72 ºC was performed for 6 min. For each set of PCR reactions, negative and positive controls were included. PCR products 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.

5.4. Nanomaterials exposures and bacterial colony forming units (CFU) counting

Assay Test concentrations were 0.01, 0.1 and 1 mg/L for nC_{60} and nAg exposures. nC_{60} concentrations were chosen using previous knowledge about its toxicity on bacteria communities living at the surface of common carp, *Cyprinus carpio* [43] and by other studies that also analyzed nC_{60} toxicity on bacteria [15, 53]. nAg concentrations were chosen taking nC_{60} concentrations into account and using previous studies that analyzed its bactericidal properties [4, 23]. Treatments of 0.01, 0.1 and 1 mg of nAg/L plus 1 mg of nC_{60}/L added to each nAg concentration were also included to analyze the interaction effects of both nanomaterials on each of the six colonies that were exposed to the treatments cited above. Nanomaterials suspensions were all filtered on 0.22 µm filters

before exposure in order to avoid bacterial contamination. Exposures were run in duplicate. In the treatments where a single nanomaterial was assayed, it was employed 180 µL of nutritive media with each isolated bacteria and 20 µL of the nanomaterial suspension. Treatments with both nanomaterials were performed using 160 µL nutritive media with each isolated bacteria and 40 µL of the nanomaterials mix (20 µL nAg + 20 µL nC_{60}). Two kinds of controls (180 µL of nutritive media + 20 µL of Milli-Q water and 160 µL of nutritive media + 40 µL of Milli-Q) were also run. All samples were then incubated for 24 h (no light), at 20 ºC at constant shaking.

The drop-plate method was an adaptation of a previous work [54]. After diluting all treatments in a range of 10^{-1} to 10^{-7} with sterile distilled water, they were then inoculated in Petri dishes containing tryptic soy Agar (Hi-Media). Each treatment dilution had four 10 µL drops as replicates and each plate had two replicates. A control test was carried out as well, consisting of a pair of plates inoculated with sterile Milli-Q ultrapure water used in nanoparticles dilutions and sterile nutritive liquid medium at the same exposure proportions after being diluted with sterile distilled water and left for 24 h at 20 ºC in the dark. CFU were counted after that time.

5.5. Bacterial samples weighting and preparation for biochemical analysis

After exposure, the bacterial material on the agar surfaces were removed with a platinum inoculating loop and put inside sterile eppendorfs. Samples where all weighted in order to estimate the bacterial biomass growth. Then, these were homogenized (1:5 w/v) in phosphate-buffered saline (PBS) (0.137 M NaCl; 0.0015 M $Na_2HPO_4.12\ H_2O$ and 0.0015 M $NaH_2PO_4.H_2O$, pH adjusted to 7.4) [55]. Samples were then frozen in liquid nitrogen and then thawed, 3 times, in order to promote bacteria lysis.

Homogenates were then centrifuged at 1,000 X g during 40 min at 4 ºC and the resulting supernatants were employed for the biochemical measurements (see below). Total protein content was determined through Biuret method (550 nm), in duplicate, using a microplate reader (BioTex LX 800). Samples were adjusted to 1 mg of total protein content per ml for each biochemical assay test.

5.6. Antioxidant capacity against peroxyl radicals

Antioxidant capacity against peroxyl radicals was determined according to Amado *et al.* [56], through ROS determination with or without adding the peroxyl radical generator 2,2´-azobis (2 methylpropionamidine) dihydrochloride (ABAP; 4 mM; Aldrich), which decompose at 35 ºC to generate peroxyl radicals [57]. It was added H_2DCF-DA (40 µM) that is primarily deacetilated by samples esterases and later, by the action of ROS, oxidized to DCF, which is fluorescent and can be measured by a fluorimeter (Victor 2, Perkin Elmer) at excitation and emission wavelength of 485 and 520 nm, respectively. The difference between ROS area with ABAP minus the area without it, relative to area without ABAP, was considered a measure of antioxidant capacity. High area differences indicated low antioxidant capacity because high fluorescence levels were obtained after adding ABAP, meaning there was low competence to neutralize peroxyl radicals [56].

5.7. Determination of concentration of thiobarbituric acid reactive species (TBARS)

This measurement was realized according to Oakes and Van Der Kraak [58]. The method quantifies products of peroxidative damage to lipids. Malondialdehyde (MDA) is molecule that results from the peroxidative damage to lipids, and reacts with 2-thiobarbituric acid (TBA), which is added in the test, generating a chromogen that is

measured by fluorometry (Victor 2, Perkin Elmer) at a wavelength of 520 nm for excitation and 580 nm for emission. This method prevents the overestimation of TBARS by adding an antioxidant (butylated hydroxytoluene - BHT) [54].

5.8. Measurement of glutathione-S-trasferase (GST) activity

Glutathione-S-transferase (GST) activity was determined following the method described by Habig and co-workers [59] and Habig and Jakoby [60]. This method is based on the conjugation of 1 mM glutathione (GSH) with 1 mM 1-cloro-2,4 dinitro benzene (CDNB), where the absorbance of the conjugate was measured at 340 nm in a microplate reader (Victor 2, Perkin Elmer) at 25 ºC and pH 7.00. Enzyme activity values were expressed in nanomoles of conjugate/min/mg of total proteins.

5.9. Statistical analysis

Data were expressed as mean ± 1 SE. Statistical analysis was performed by way of analysis of variance followed by Newman–Keuls test ($\alpha = 0.05$). The assumptions of normality and variance homogeneity had being verified previously and mathematical transformation applied if at least one of them was violated [61].

List of used abbreviations

ABAP: 2,2’- azobis(2-metilaminopropano)

BHT: butylated hydroxytoluene

C_{60}: fullerene

nC_{60}: fullerene forming clusters in colloidal form

nAg: nanosilver forming clusters in colloidal form

DCFH-DA: diacetate 2’,7’-diclorofluesceina

GSH: glutathione

GST: glutathione-S-transferase

MDA: malondialdehyde

PBS: phosphate buffered saline

ROS: reactive oxygen species

SDS: sodium dodecyl sulfate

TBA: thiobarbituric acid

TBARS: thiobarbituric acid reactive substances

Competing interests

Authors declare no competing interests.

Authors' contributions

L.F.C.: Responsible for *L. acuta* collecting, bacterial colonies isolation and maintenance, nC_{60} preparation, performing the growth inhibition and biochemical tests and for the writing of the manuscript.

B.F.M.: Responsible for *L. acuta* collecting, bacterial colonies isolation, nC_{60} preparation, performing the growth inhibition and biochemical tests.

J.M.M.: Responsible for planning the experiments, nC_{60} and nAg characterization by TEM, *L. acuta* collecting, performing the statistical analysis and revising the manuscript.

L.W.K.: Responsible for the molecular identification of bacteria colonies employed in the study.

M.R.B.: Responsible for the molecular identification of bacteria colonies employed in the study and manuscript revising.

G.P.: Responsible for the production of nanosilver.

G.L.: Responsible for the production of nanosilver and for revising the manuscript.

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Figures

Figure 1. Transmission electronic microscopy (TEM) images of the suspensions employed in the bioassays.

(a) Aqueous fullerene suspension (nC_{60}). **(b)** Aqueous nanosilver suspension (nAg).

Figure 2. Growth inhibition analised by wet mass weighting

Nanomaterial concentrations are 1, 0.1 and 0.01 mg/L for the single nanomaterial exposures, and constant 1 mg/L nC_{60} + 1, 0.1 or 0.01 mg/L nAg for the exposures with both nanomaterials together (n = 5). Panel **(a)** shows nC_{60} treatment, **(b)** shows nAg treatment and **(c)** stands for the combined nanomaterials treatment, with only nAg concentrations being decribed in the last panel. Identical letters mean absence of statistical differences ($p > 0.05$).

Figure 3. Growth inhibition analised through colony forming units (CFU) count

Nanomaterial concentrations are 1, 0.1 and 0.01 mg/L for the single nanomaterial exposures, and constant 1 mg/L nC_{60} + 1, 0.1 or 0.01 mg/L nAg for the exposures with both nanomaterials together (n = 3). Panel **(a)** stands for nC_{60}, **(b)** for nAg and **(c)** for nC_{60} + nAg treatment, with only nAg concentrations being decribed in the last panel. Identical letters mean absence of statistical differences ($p > 0.05$).

Figure 4. Total antioxidant capacity against peroxil radicals in bacterial colonies exposed to nanomaterials

Nanomaterial concentrations are 1, 0.1 and 0.01 mg/L for the single nanomaterial exposures, and constant 1 mg/L nC_{60} + 1, 0.1 or 0.01 mg/L nAg for the exposures with both nanomaterials together. Panel **(a)** refers to nC_{60} treatment (n = 5), **(b)** shows nAg

treatment (*n* = 2-5) and **(c)** stands for nC_{60} + nAg treatment (*n* = 3-5), with only nAg concentration being decribed in the last panel. Identical letters mean absence of statistical differences ($p > 0.05$).

Figure 5. Concentration of thiobarbituric acid reactive substances (TBARS) in bacterial colonies exposed to nanomaterials

Nanomaterial concentrations are 1, 0.1 and 0.01 mg/L for the single nanomaterial exposures, and constant 1 mg/L nC_{60} + 1, 0.1 or 0.01 mg/L nAg for the exposures with both nanomaterials together. TBARS is measured in nmol/mg of proteins. Panel **(a)** refers to nC_{60} treatment (*n* = 5-6), **(b)** shows nAg treatment (*n* = 3-6) and **(c)** shows the nC_{60} + nAg treatment (*n* = 3-6), with only nAg concentrations being decribed in the last panel. Asterisk (*) indicates significant differences ($p < 0.05$) between treatments connected by solid lines. Identical letters mean absence of statistical differences ($p > 0.05$).

Figure 6. Activity of glutathione-S-transferase (GST) in bacterial colonies exposed to nanomaterials

Nanomaterial concentrations are 1, 0.1 and 0.01 mg/L for the single nanomaterial exposures, and constant 1 mg/L nC_{60} + 1, 0.1 or 0.01 mg/L nAg for the exposures with both nanomaterials together. GST is measured in nmoles of GSH conjugated/min/mg of proteins. Panel **(a)** refers to nC_{60} treatment (*n* = 5-6), **(b)** shows nAg treatment (*n* = 4-5) and **(c)** stands for nC_{60} + nAg treatment (*n* = 4-6), with only nAg concentrations being decribed in the last panel. Asterisk (*) indicates significant differences ($p < 0.05$) between treatments connected by solid lines. Identical letters mean absence of statistical differences ($p > 0.05$).

Figure 1

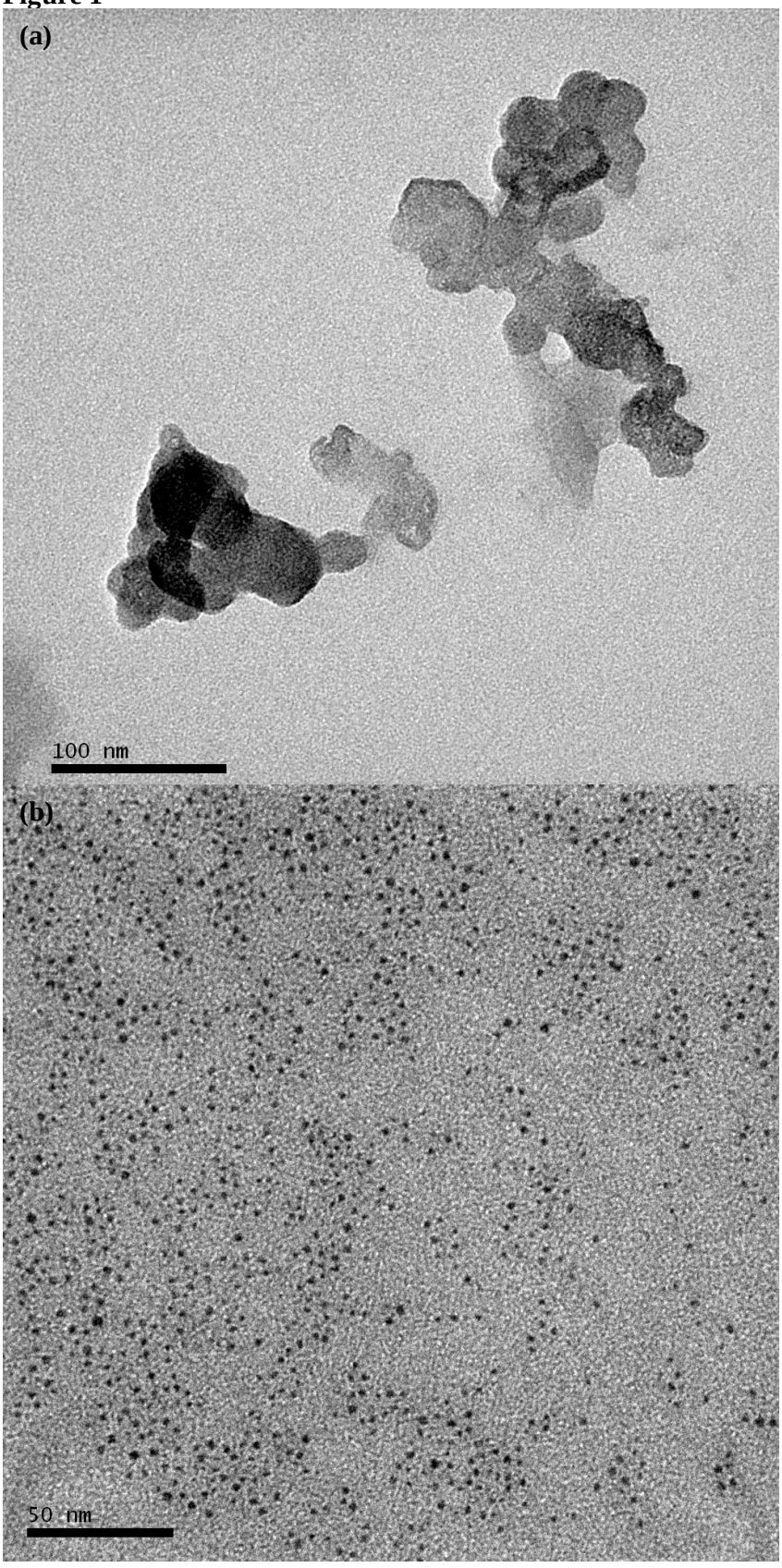


Figure 2

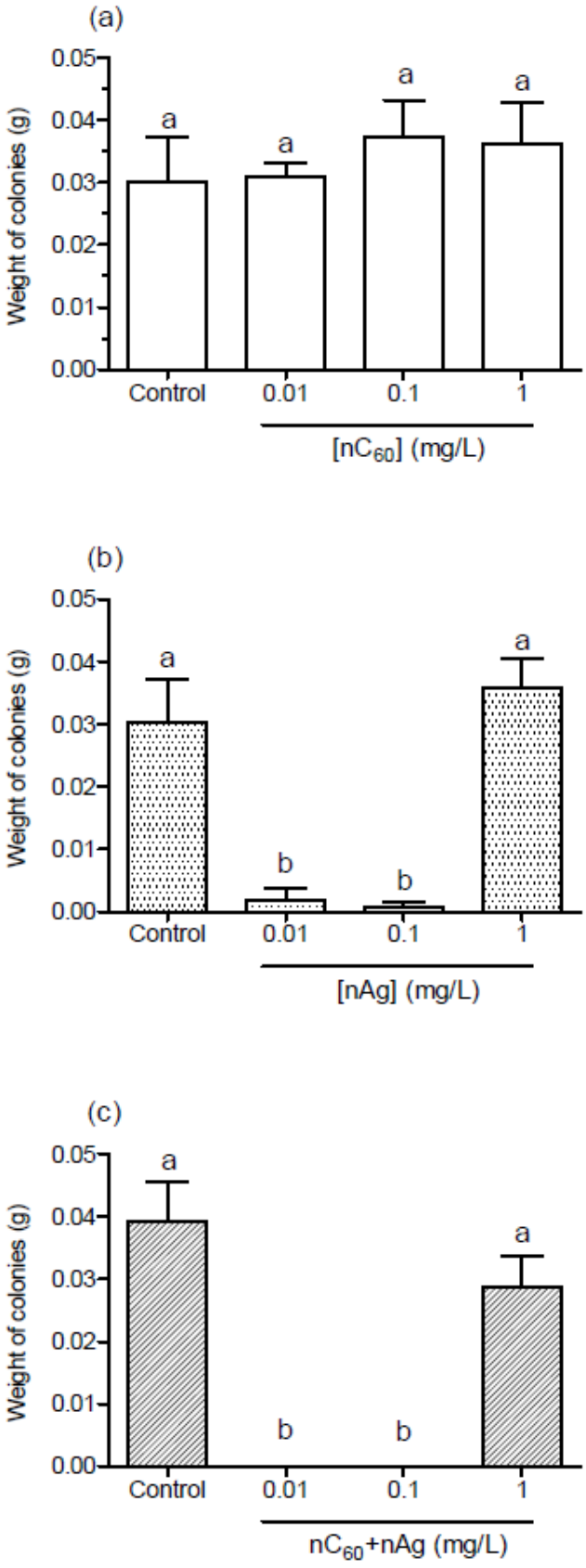

(a)
Weight of colonies (g)
0.05
0.04
0.03
0.02
0.01
0.00
a
a
a
a
Control
0.01
0.1
1
[nC60] (mg/L)
(b)
Weight of colonies (g)
a
b
b
a
Control
0.01
0.1
1
[nAg] (mg/L)
(c)
Weight of colonies (g)
a
b
b
a
Control
0.01
0.1
1
nC60+nAg (mg/L)

Figure 3

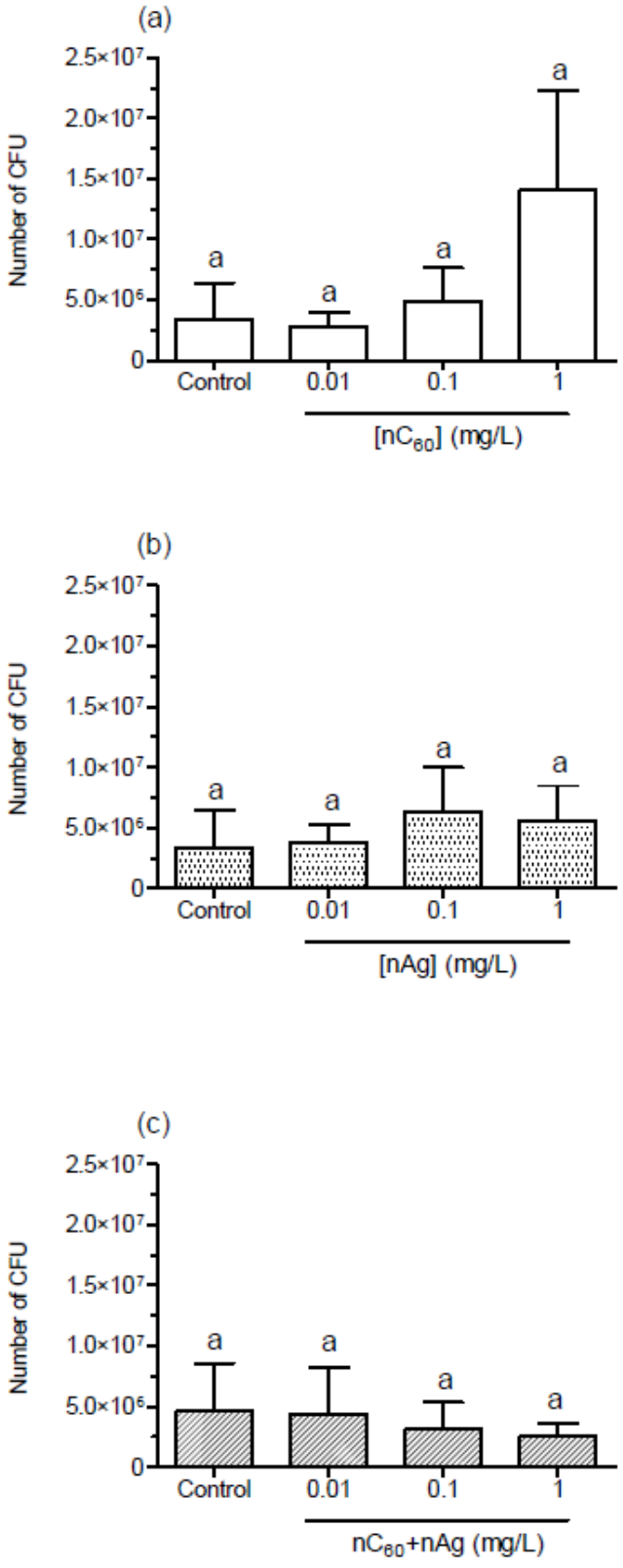

(a)
Number of CFU
2.5×10⁷
2.0×10⁷
1.5×10⁷
1.0×10⁷
5.0×10⁶
0
a
a
a
a
Control
0.01
0.1
1
[nC60] (mg/L)
(b)
Number of CFU
2.5×10⁷
2.0×10⁷
1.5×10⁷
1.0×10⁷
5.0×10⁶
0
a
a
a
a
Control
0.01
0.1
1
[nAg] (mg/L)
(c)
Number of CFU
2.5×10⁷
2.0×10⁷
1.5×10⁷
1.0×10⁷
5.0×10⁶
0
a
a
a
a
Control
0.01
0.1
1
nC60+nAg (mg/L)

Figure 4

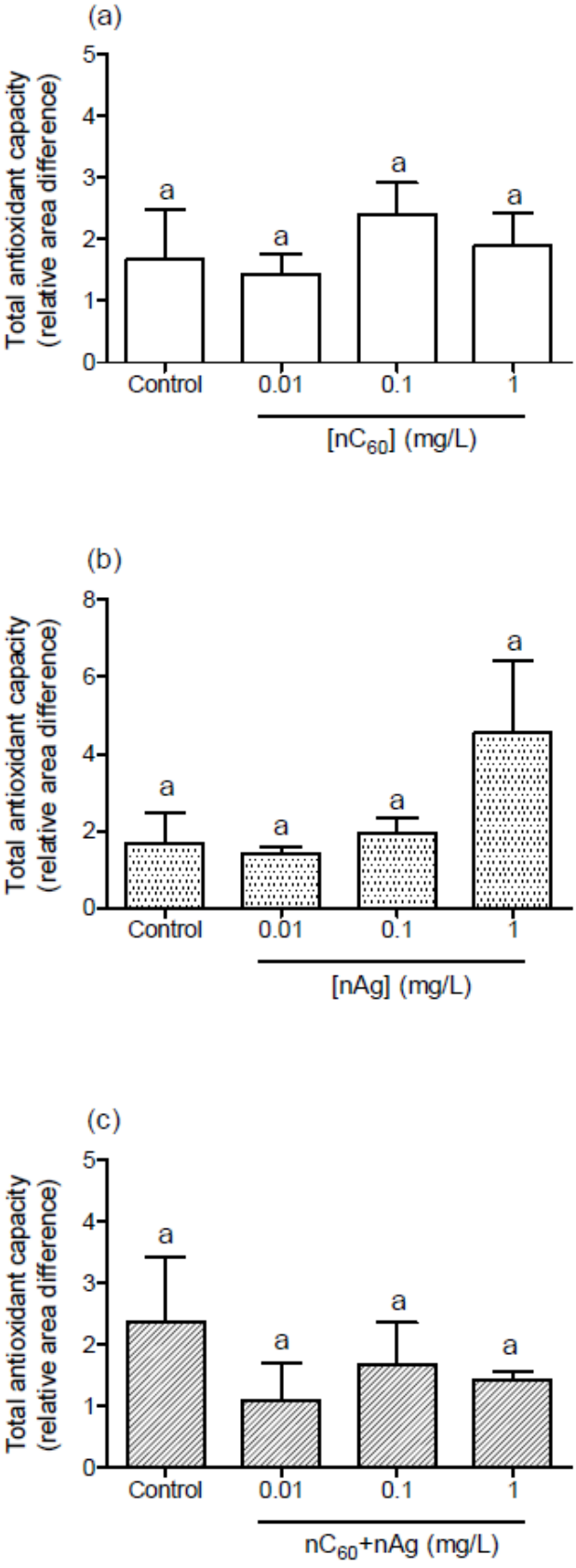

(a)
Total antioxidant capacity (relative area difference)
0
1
2
3
4
5
a
a
a
a
Control
0.01
0.1
1
[nC60] (mg/L)
(b)
Total antioxidant capacity (relative area difference)
0
2
4
6
8
a
a
a
a
Control
0.01
0.1
1
[nAg] (mg/L)
(c)
Total antioxidant capacity (relative area difference)
0
1
2
3
4
5
a
a
a
a
Control
0.01
0.1
1
nC60+nAg (mg/L)

Figure 5

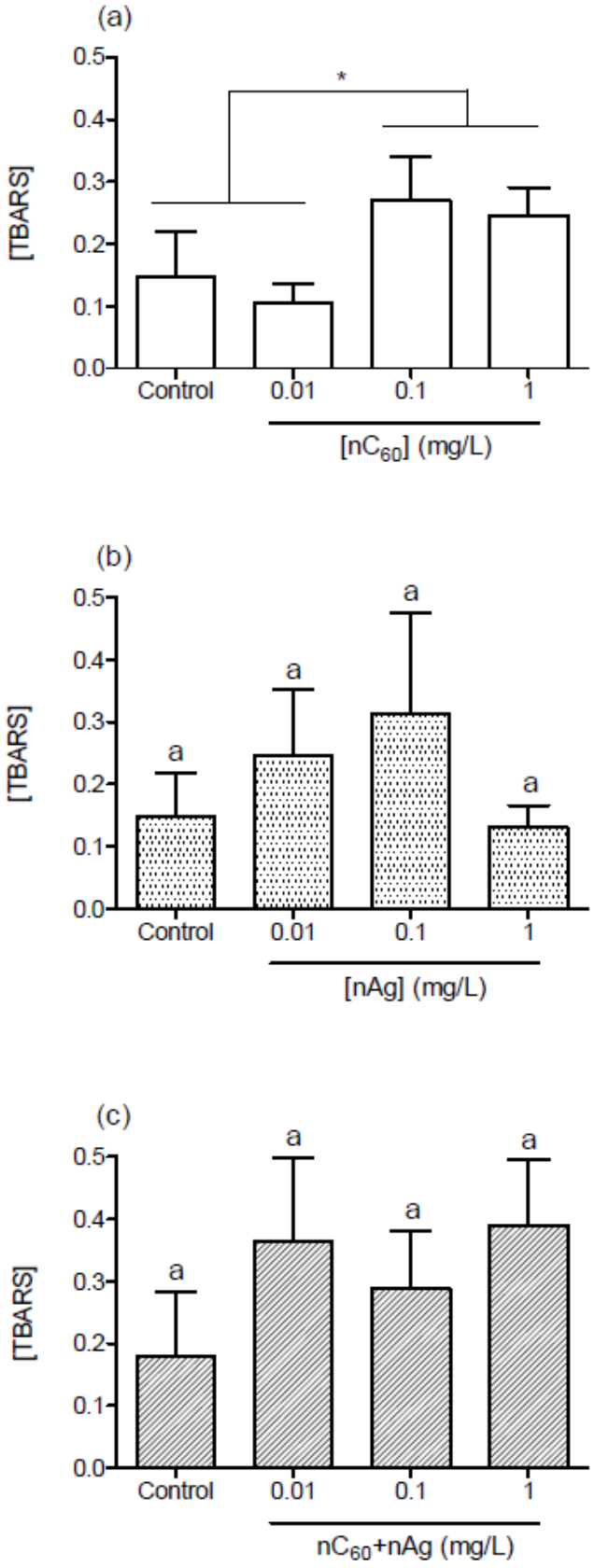
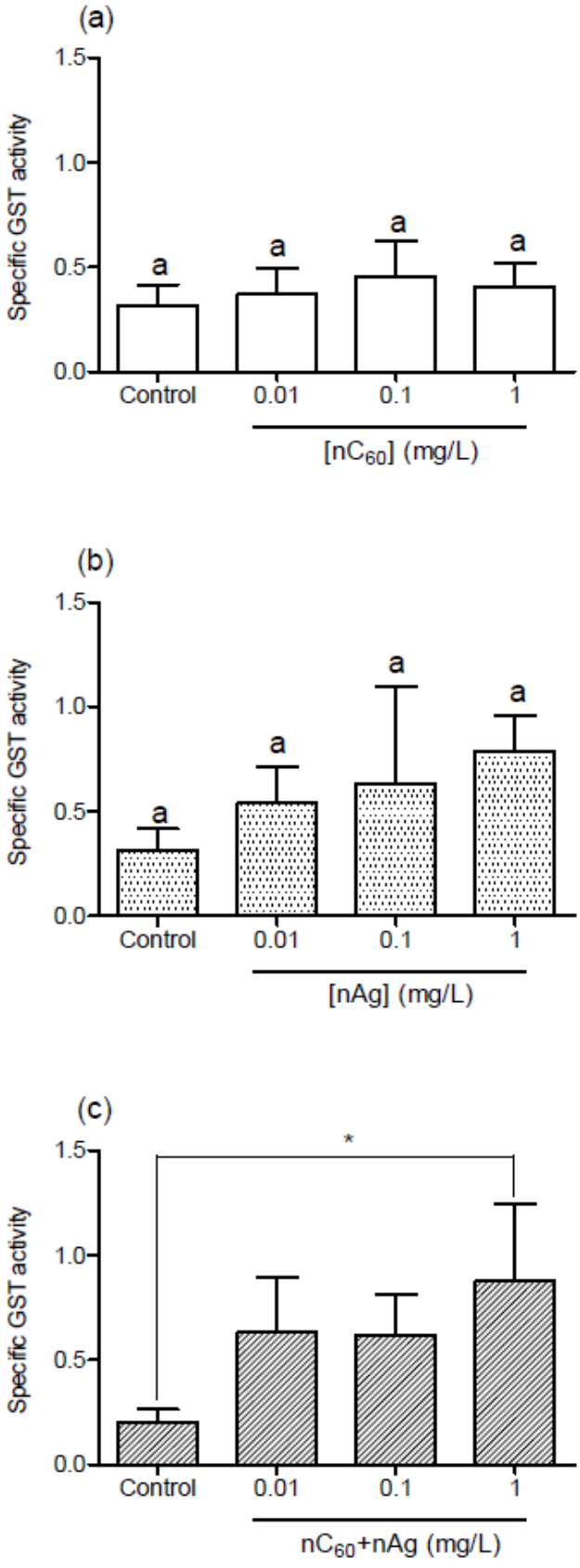

(a)
[TBARS]
0.5
0.4
0.3
0.2
0.1
0.0
*
Control
0.01
0.1
1
[nC60] (mg/L)
(b)
[TBARS]
a
a
a
a
Control
0.01
0.1
1
[nAg] (mg/L)
(c)
[TBARS]
a
a
a
a
Control
0.01
0.1
1
nC60+nAg (mg/L)

Figure 6


(a)
Specific GST activity
1.5
1.0
0.5
0.0
a
a
a
a
Control
0.01
0.1
1
[nC60] (mg/L)
(b)
Specific GST activity
1.5
1.0
0.5
0.0
a
a
a
a
Control
0.01
0.1
1
[nAg] (mg/L)
(c)
Specific GST activity
*
1.5
1.0
0.5
0.0
Control
0.01
0.1
1
nC60+nAg (mg/L)

Tables

Table 1. Identification of bacteria isolated from mucus of *L. acuta* based on 16S rRNA gene sequence.

Identification	Organism	GenBank accession no.
C3	*Cupriavidus* sp.	JN835403
C4	*Vibrio* sp.	JN835404
C6	*Vibrio* sp.	JN835405
C8	*Listonella anguillarum*	JN835406
C9	*Listonella anguillarum*	JN835407
C10	*Vibrio* sp.	JN835408

CONCLUSÕES

A nanoprata provou ser potencialmente perigosa para as colônias bacterianas neste trabalho, mesmo na ausência de luz e com parte das partículas encontrando-se na escala micrométrica. Apesar do fulereno não induzir a inibição do crescimento bacteriano e, aparentemente, não afetar fortemente a toxicidade da nanoprata, foi identificada peroxidação lipídica, mesmo na ausência de luz para nC_{60}. Foi também observada uma redução no crescimento bacteriano na co-exposição de ambos os nanomateriais (nC_{60} + nAg).

Tais resultados podem significar que nC_{60} induziu a produção de algum nível de ERO nos tratamentos, resultando em peroxidação lipídica, na ausência de luz, mas que isso não foi suficiente para inibir o crescimento das colônias. nC_{60} também não pareceu afetar a capacidade de nAg inibir o crescimento bacteriano. Por outro lado, nAg foi capaz de inibir o crescimento bacteriano aparentemente sem ou sob intermédio mínimo de ERO. Há, portanto, certo grau de periculosidade destes nanomateriais ainda em situações onde não haveria foto-excitação, com conseqüente produção de ERO altamente reativas.

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