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Genotoxic Potential of Copper Oxide Nanoparticles in the Bivalve Mollusk *Mytilus trossulus*

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Abstract Copper oxide nanoparticles (CuO-NPs) are among the most widely used metal oxide nanoparticles, which increases the chance of their being released into the marine environment. As the applications of these particles have increased in recent years, their potential impact on the health of marine biota has also increased. However, the toxicological effects of these NPs in the marine environment are poorly known. In the present study, the DNA damaging potential of CuO-NPs in the marine eastern mussel *Mytilus trossulus* was evaluated and compared to that of dissolved copper exposures. Genotoxicity was assessed by the single cell gel electrophoresis (comet) assay in mussel gill and digestive gland cells. The results showed that copper in both forms (CuO-NPs and dissolved copper) was accumulated to different extents in mussel tissues. The mussel exposed to the dissolved copper attained higher concentrations of copper in the gills than in the digestive gland. In contrast to these results, it was found that CuO-NPs could induce much higher copper accumulation in the digestive gland than in the gills. A clear and statistically significant increase in DNA damage was found in both tissues of the Cu-exposed group compared to the control mussels. Our results indicated that the CuO-NP exposure produced remarkable effects and increased DNA damage significantly in mussel gill cells only. It should be noted that the digestive gland cells were prone to accumulation following CuO-NPs when compared to the gill cells, while the gill cells were more sensitive to the genotoxic effects of CuO-NPs. These results also suggested the need for a complete risk assessment of engineered particles before its arrival in the consumer market.

Key words CuO nanoparticles; copper accumulation; DNA damage; genotoxicity; *Mytilus trossulus*

1 Introduction

Modern nano-technology provides a great variety of nanoparticles. Since it appeared, some materials have acquired unique physical and chemical properties being formulated as nanoparticles (NPs) compared with conventional formulations (Oberdorster, 2005; Bhatt and Tripathi, 2011; Arora *et al*., 2012). The unique properties of engineered nanomaterials (ENMs) have become beneficial in commercial applications in different fields: electronics, catalysis, water filtration, semiconductors, antibacterial materials, cosmetics and medicine (Nel *et al*., 2006).

NPs are known to exert a cytotoxic effect; therefore, a potential environmental risk related to contamination by NPs needs to be assessed. The beneficial properties of the ENMs can lead to unpredictable outcomes in terms of their interactions with biota. The NPs have a very high surface-area-to-volume ratio due to their extremely small size, which renders them highly reactive in terms of penetration and reaction catalysis. Possible negative properties of these NPs include their ability to penetrate dermal barriers, cross cell membranes, travel neuronal pathways and interact at the molecular level (Moore, 2006; Singh *et al*., 2009). Therefore, it is necessary to understand and assess the potential toxicity of NPs in the environmental conditions.

Copper oxide nanoparticles (CuO-NPs) are one kind of the most widely used nanoparticles. They are extensively used in microelectronics, cosmetics and catalysis (Bondarenko *et al*., 2013). Recent evidence showed that CuO-NPs induce toxic responses in different mammalian and human cell lines (Ahamed *et al*., 2010; Song *et al*., 2012; Bondarenko *et al*., 2013). Some studies reported the toxicity of CuO-NPs in bacteria, microalgae, crustaceans, protozoan and yeasts (Kasements *et al*., 2009; Blinova *et al*., 2010; Kahru and Dubourquier, 2010; Bondarenko *et al*., 2013). NPs released into the environment through industrial and domestic sewage waters tend to end up in

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the marine environment and can damage aquatic organisms (Blinova *et al*., 2010; Pang *et al*., 2012; Bondarenko *et al*., 2013; Isani *et al*., 2013; Mwaanga *et al*., 2014). Only few studies have evaluated the toxicity of CuO-NPs in marine invertebrates such as bivalve mollusks (Buffet *et al*., 2011; Gomes *et al*., 2012; Baker *et al*., 2014). Filter-feeding mollusks are prime candidates for the uptake of NPs from environmental releases because mollusks are known to accumulate various conventional pollutants (Moore, 2006; Canesi *et al*., 2012; Matranga and Corsi, 2012).

A genotoxicity is one of the aspects of a general cytotoxicity. Therefore, potential damage of genetic material by NPs might induce or promote carcinogenesis. However, it is little known about genotoxicity of NPs, especially in marine organisms. Given this context, the object of this study was to estimate the genotoxic potential effect of nano form of Cu on marine organisms.

Among the many sedentary aquatic organisms, bivalves are often used as an effective bioindicator to study the biological effects of environmental contaminants. These are filter feeders organisms, and so can accumulate high levels of heavy metals in their organs (Livingstone *et al*., 2000; Al-Subiai *et al*., 2011). For this reason we used the benthic species *Mytilus trossulus*, as one of the widespread and also commercial species along the coastal area of the Peter the Great Bay (the Sea of Japan).

In the present investigation, the comet assay was used to evaluate the DNA damaging potential of CuO-NPs ($0.020\,mg\,L^{-1}$) under laboratory exposure of the *Mytilus trossulus*. The concentration of dissolvent Cu $0.020\,mg\,L^{-1}$ is widely used in ecotoxicological studies (Canty *et al*., 2009; Al-Subiai *et al*., 2011). To investigate the genotoxic potential of CuO-NPs, gills and digestive gland were used. The gills are the initial barrier to pollutants which penetrate from the environment. Digestive gland is the key organ of heavy metal accumulation and detoxication (Gibson and Barker, 1979).

2 Materials and Methods

Normal melting point (NMP) and low melting point (LMP) agarose were obtained from LKB, Sweden. Ethidium bromide [CAS No. 1239-45-8] was obtained from Sigma. All other chemicals were of analytical grade. Millipore water was used as the solvent.

A stock solution of dissolved copper (Cu^{2+}) was prepared from $CuSO_4 \cdot 5H_2O$ dry powder.

Copper (II) oxide nanoparticle (CuO-NP) dry powder [CAS No. 1317-38-0] was purchased from Sigma-Aldrich, Saint Louis, MO, USA. The physical characteristics (as obtained from the supplier) were particle size (<50 nm), surface area ($25–40\,m^2g^{-1}$) and density ($4.26\,g\,mL^{-1}$ at 25 ℃). The dry powder of CuO-NPs was suspended in deionized water at a concentration of $1\,mg\,mL^{-1}$. It was sonicated using a sonicator bath at room temperature (20℃) for 15 min at 40 W to form a homogeneous suspension. Working solutions were made by serial dilution, followed by sonication and vigorous vortexing when required.

The hydrodynamic size of CuO-NPs ($200\,\mu g\,L^{-1}$ in filtered seawater) during a cycle of 24 h (corresponding to the period between water change and NPs redosing) was determined by ANALYSETTE 22 Nano Tec Plus, FRITSCH, Germany.

Mytilus trossulus were collected from an unpolluted site in Vostok Bay (Peter the Great Bay, the Sea of Japan, Russia). Adult mollusks of similar shell length (45±5 mm) were used in the experiments to reduce the risk of size-/age-related variability. The mussels were acclimated for at least 5 d in a holding tank at 18℃ with continuous aeration.

After the acclimation to the laboratory conditions, the mollusks were divided into three groups and placed into tanks containing 10 L of filtered seawater with (experimental tanks) or without (control tank) copper for up to 7 d, at 18℃ with continuous aeration.

The stock suspension of CuO-NPs in deionized water was prepared by sonication and immediately added to the tank to reach the desired concentration. Mussels (15 mussels for each condition) were exposed to CuO-NPs for 7 d at $0.020\,mg\,L^{-1}$ nominal concentration level. For comparative purposes, mussels were exposed to an ionic copper treatment in the form of Cu^{2+} ($CuCl_2$ solution) at a concentration of $0.020\,mg\,L^{-1}$. For each treatment, a parallel control group of mussels (untreated) was kept in clear seawater for 7 d. The experiments were repeated 4 times. The exposure to different copper forms did not result in significant mortality.

During the experimental period, the seawater was changed daily and copper dosing was repeated at each water change. Aeration at the bottom of the tank was used to minimize agglomeration and subsequent sedimentation of the contaminants. Mussels were not fed during the experiment to minimize the risk of the CuO-NPs being absorbed to food or fecal material and to help maintain water quality.

The procedure followed for gill and digestive gland cells isolation was generally described by Slobodskova *et al*. (2012).

The alkaline version of the Comet assay (single cell gel electrophoresis) was applied to the mussel gill and digestive gland cell suspensions to determine the level of DNA strand breakage. The Comet assay was based on the procedure described by Slobodskova *et al*. (2012). The mollusks in the control and experimental groups were analyzed with 15 slides per group (1 slide=1 mussel). Each slide contained no less than 50 comets.

A total of 100 nuclei from each of the two replicates were examined and classified in one of the five damage classes, as described by Mitchelmore and Chipman (1998), according to the migration distance and the fluorescence rate between the head and the tail of the nucleus: Class 0, intact nucleus with no migrated fragments (<5% fragmented DNA); Class 1, dense nucleus with slight DNA migration forming a small tail (5%–20% fragmented DNA); Class 2, tail extending from the nucleus, with a weaker fluorescence than Class 2 (20%–40% fragmented DNA); Class 3, comets with a clear tail

that may reach full length (40%–75% fragmented DNA); and Class 4, nucleus, when present, being small and completely separated from the tail (>75% fragmented DNA).

The results are presented as the percentage distribution of nuclei in the various damage classes and summarized in an index of DNA integrity or genetic damage index (GDI) (Cavas and Kohen, 2008).

The mean and median comet parameter values were compared between treatments with a one-way ANOVA and a Dunnett's test at a $P=0.05$ level.

The copper content was determined after the mineralization of gill and digestive gland pieces from each mollusk with a mixture of nitric and perchloric acids (3:1) using atomic absorption flame spectroscopy with flame atomization and deuterium background correction (Shimadzu AA-6800). The copper concentration was calculated on the basis of the dry weight of the tissue piece, which was dried to a constant value at 85℃. Each mean represented individual tissue samples ± standard error. A one-way ANOVA (analysis of variance) was applied to reveal the differences between groups, taking a probability limit of $P<0.05$ as significant. Individual samples were compared by Student's *t*-test, using the standard error estimate derived from the corresponding ANOVA.

3 Results and Discussion

In the present study, the particle size of CuO-NPs specified by the manufacturer was approximately 50 nm. The mean particle size was also determined in seawater during a 24-h cycle using dynamic light scattering (DLS). In seawater, the CuO-NPs were highly aggregated and the hydrodynamic size obtained ranged from 206 nm to 383 nm (mean value 290±32 nm).

The contents of Cu in two tissues (digestive gland and gills) of the mussel *M. trossulus* after exposure to CuO-NPs and Cu^{2+} according to the determinations of 30 individuals are shown in Fig.1, where the mean concentrations ± standard errors are listed. Metal contents are expressed as μg copper per g organ dry weight (dw).

Mussels exposed to both forms of copper (CuO-NPs and soluble Cu) showed significantly higher concentrations when compared to the control mussels by the end of the experiments. However, significant differences were observed depending on the form of Cu in the exposure medium (Fig.1).

In the mussels exposed to dissolved copper (ionic Cu^{2+}), the gills attained higher concentrations of copper than the digestive gland. The gills reached a level of 146.8±12.6 $\mu g\,Cu\,(gdw)^{-1}$ and the digestive gland reached a level of 91.2±8.7 $\mu g\,Cu\,(gdw)^{-1}$ at the end of the experimental period. In contrast to these results, CuO-NPs were able to induce much higher copper accumulation in the digestive gland than in the gills (Fig.1). Copper levels in the digestive gland and the gills increased to 114.7 ± 9.4 $\mu g\,Cu\,(gdw)^{-1}$ and 31.9±6.8 $\mu g\,Cu\,(gdw)^{-1}$ after 7 d of exposure, respectively.

The results indicated that both copper forms (ionic and CuO-NPs) are accumulated to a greater or lesser extent by mussel tissues.

The Comet assay was used to measure endogenous DNA damage in gill and digestive gland cells from unexposed and exposed mussels. The percentage of DNA in the tail region (%DNAt) was used as the criterion for quantifying DNA strand breakage. This parameter is one of the best indices of induced DNA damage among the various parameters calculated by this method. The percentage of DNA in the tail was used to categorize the grade of damage in unexposed and exposed mussels according to the classification in Mitchelmore and Chipman (1998).

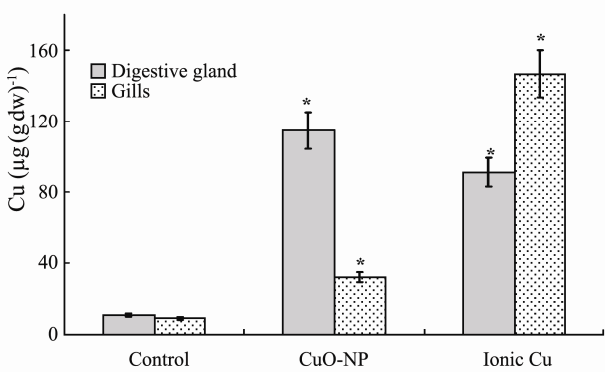

Fig.1 Copper concentrations in the gills and digestive gland of mussels *M. trossulus* after exposing to CuO-NPs and ionic Cu^{2+} for 7 d. Values are means ± S.D. N=3. n = 15. Bars with asterisk denote significant difference from control (* $P\leq0.05$).

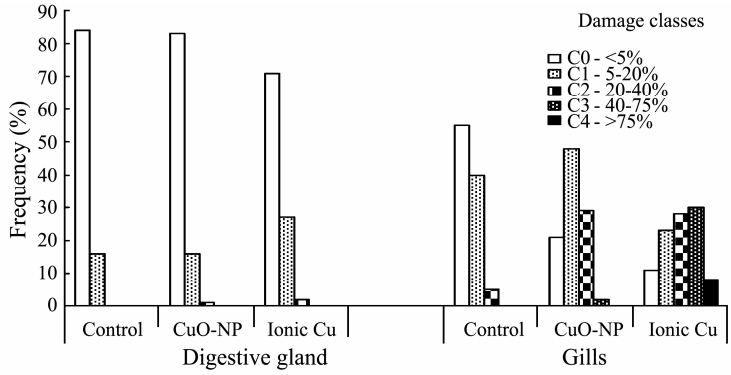

Fig.2 Percentage of cells distribution by grade of DNA damage in digestive gland and gills of mussels *M. trossulus* from control and those exposed to CuO-NPs and ionic Cu (n=750).

Fig.2 shows the distribution of cells according to the five classes of DNA damage. The data for mussel tissue cells are displayed using histograms of the frequency distribution of comet classes to demonstrate that the response was heterogeneous among the individual cells within the same tissue sample. The majority of digestive gland cells from unexposed mussels showed minimal and low-grade damage (C0 and C1 classes), characterized by zero or minimal DNA migration at the electrophoresis. A small proportion (> 3%–5%) of the control gill cells exhibited damage (C2 class), probably related to some strand breaks occurring in the field (*e.g*., environmental stress) or being originated by a special pathway of gill cells. For mussels exposed to dissolved copper (Cu^{2+}), the digestive gland cells showed mid-DNA damage with increasing levels of C1 and C2 comet classes (Fig.2). For the CuO- NP exposure, there was a trend of increased DNA damage in the cells relative to controls, but this was not statistically significant.

Exposure to both of the copper forms resulted in a statistically significant increase in DNA damage in the gill cells (Fig.2). The distribution of cells according to the damage classes clearly demonstrated that after exposure to CuO-NPs, the majority of cells showed more DNA damage (C1 and C2 classes) than cells from the control mollusks.

Significantly elevated DNA damage was observed in the gill cells from the mussels exposed to ionic copper. These mollusks had higher frequencies in the number of cells in classes 3 and 4. In addition, the summarized DNA damage produced by two copper forms in mussel tissue cells showed that accumulated copper caused a statistically significant ($P<0.05$) increase in the genetic damage index (GDI) when compared to the unexposed control group of mollusks (Fig.3). The GDI values measured in both groups of mollusks were generally reflected by a different percentage distribution of nuclei in the five classes of damage (Fig.2). The GDI was mostly predominated by classes 0 and 1 for the unexposed mussels, being representative of a low nuclear fragmentation. On the contrary, the greater values for exposed mussels were measured and increased with the percentage contribution of cells in classes 2, 3 and 4. A clear and statistically significant increase in GDI values was found in the digestive gland and gill cells of the Cu-exposed group when compared to the control mussels.

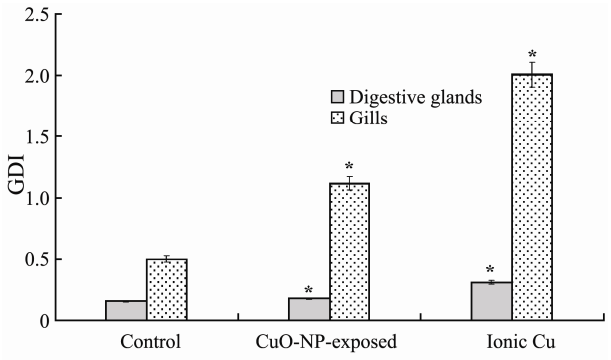

Fig.3 Genetic damage index (GDI) in the gills and digestive gland of mussels *M. trossulus* after being exposed to CuO-NPs and ionic Cu^{2+} for 7 d. Values are means ± S.D. N = 3. $n=15$. Bars with asterisk denote significant difference from control (* $P \leq 0.05$).

However, our results indicated that the CuO-NP treatment produced a remarkable effect, increasing the comet parameter (%DNAt) and GDI significantly only in mussel gill cells.

Marine bivalves are often used to monitor aquatic pollution (Moore, 2006), so it is imperative to study the bioavailability and the toxic mechanisms of CuO-NPs in the far-eastern mussel *M. trossulus*.

In the present study, the data obtained indicated that the nanoparticles of CuO were transferred from the water column to mussel tissues and that mussels can accumulate the suspended CuO-NPs from the surrounding water. Additionally, our results indicated that cells of the digestive gland were more prone to accumulation following CuO-NPs when compared to the gill cells. It is interesting to note that CuO-NPs accumulated more in the digestive gland, while ionic copper accumulated more in the gills. This was likely because in our experimental conditions, CuO-NPs were present in seawater mainly as agglomerates that were taken up by the digestive system. Possible reasons for these results may include the insolubility of CuO-NPs and the copper transportation system via Cu homeostasis in the organism. In the genus *Mytilidae*, CuO-NPs are less likely to be accumulated via gills, as nanoparticles are usually too large to be transported via the trans-membrane metal ion channels (Shaw and Handy 2011). During a short-term exposure, most of the metal oxide nanoparticles suspended in seawater are present in the form of aggregates, as in the present experimental conditions, and NP aggregates are taken up mainly by the digestive system, while the gills are more sensitive to dissolved metals (Gomes *et al*., 2013). The NPs likely followed the same uptake routes as suspended food particles, which are trapped in the mucus and transported toward the digestive gland by cilial action (Beninger *et al*., 1991). A recent study showed that mussels more efficiently capture and ingest NPs that are incorporated into agglomerates when compared to those freely suspended (Ward and Kach, 2009).

McCarthy *et al*. (2013) and Al-Sid-Cheikh *et al*. (2013) observed a similar retention of Ag-NPs in the digestive gland of bivalve mollusks. The increased accumulation was also reported in the digestive glands of *M. edulis* exposed to gold-citrate NPs (Tedesco *et al*., 2010). Other researchers (Gomes *et al*., 2012; Garcia-Negrete *et al*., 2013) found that the copper concentration was higher in the digestive gland for CuO-NPs in comparison with dissolved copper in mussels exposed to both copper forms.

The present study assessed the genotoxic effect of CuO-NPs and dissolved copper in the target tissues of mussels. The *in vivo* results clearly demonstrated that copper present as either CuO-NP or ionic form was genotoxic for mussels. The particle sizes advertised by the manufacturer were found to differ significantly from the DLS measured size in seawater. However, this aggregation did not appear to significantly impact the DNA

responses.

The data obtained for the DNA damage of *M. trossulus* in this work are in agreement with those previously described in the literature for other organisms, including human and mammal cell lines (Ahamed *et al*., 2010; Song *et al*., 2012; Semisch *et al*., 2014), rainbow trout *Oncorhynchus mykiss* (Isani *et al*., 2013), the bivalve mollusk *Mytilus galloprovincialis* (Gomes *et al*., 2013) and the bacterium *E. coli* (Bondarenko *et al*., 2012).

The exposure to copper in the different forms tested produced dissimilar responses regarding DNA damage endpoints. Copper given in soluble form was more toxic than the copper of the same amount given as CuO-NPs. The CuO-NP treatments produced insignificant effects on the DNA integrity in the digestive gland cells when compared to the control treatments. However, a severe level of DNA damage in the gill cells was recorded after the CuO-NP treatment.

In interpreting our results, it should be noted that the digestive gland tissues were more prone to accumulation following CuO-NPs when compared to the gill tissues, while gill cells were more sensitive to CuO-NP toxicity. It is clear that different tissues have different sensitivities to genotoxicants, likely due to differential mechanisms of defense and DNA repair. Additionally, the differences in the genotoxic effect of gill cells compared to digestive gland cells may be associated with cellular differences (membrane composition, number of lysosomes and mitochondria) due to their origin or functions.

Our data suggested that in the gill cells of the mussel *M. trossulus*, the DNA and DNA repair system may represent a significant target for *in vivo* exposure to both copper forms.

The question as to how NPs act at the cellular level has received increasing attention. While the underlying mechanism for its genotoxicity has not been investigated in any marine species, several possible models of action are already recognized for mammals. From our results, and according to studies performed on various animals, we could only formulate a hypothesis on the mechanisms involved in the DNA fragmentation considering the potential adverse effect induced by CuO-NPs. The mechanisms likely to be responsible for nanoparticle-induced genotoxicity fall into two main categories, direct and indirect mechanisms. Direct mechanisms are those imparted by the NPs themselves at the level of the single cell and may be either the result of an interaction between the NPs and DNA and/or its regulatory apparatus (DNA repair system), *i.e*., they come into direct contact with the genetic material causing physical or chemical damage.

The accumulation of DNA damage may occur through either an increase in the number of DNA-damaging events or a decrease in DNA repair. The induction of DNA strand breaks was shown in several cell lines exposed to metals, such as copper (Hayashi *et al*., 2000). CuO-NPs, like other metals, may bind directly to DNA or to DNA repair enzymes, leading to the formation of strand breaks (Garnett and Kallinteri, 2006).

A possible role of genotoxicity has been suggested for DNA repair systems, which are very sensitive targets for metals, due to the ability of metals to displace zinc ions in Zn-finger structures of DNA repair enzymes (Hartwig, 1998).

There is no available information regarding the Cu-induced inactivation of the DNA repair system in mussels; therefore, only assumptions can be made. This ability was not analyzed in the present study, but it could not be excluded because nanoparticles are known to translocate to the cell nucleus in epithelial cells. Supporting evidence for this comes from the investigation by Semisch *et al*. (2014). These researchers showed that the copper derived from CuO-NPs and $CuCl_2$ accumulated in the soluble cytoplasmic and nuclear fractions of the human cell line A549, yielding similar concentrations in the cytoplasm, but the highest concentrations were found in the nucleus in the case of CuO-NPs. The upregulated expression pattern of DNA repair proteins in human lung cells by CuO-NP exposure (Ahamed *et al*., 2010) suggests that these proteins could be involved in the geno- toxic effect of nanoparticles. Furthermore, metals act as DNA-protein crosslinkers, directly participating in complexation of DNA with proteins (Merk and Speit, 1999).

Indirect DNA damage may arise as a result of the induction of intermediates. For example, under oxidative stress, NP exposure results in the excessive generation of reactive oxygen species (ROS) that are responsible for damaging biomolecules including lipids, proteins and DNA. ROS attack on DNA generates a huge range of base and sugar modification products. A number of alterations in DNA (*e.g*., cleavage of DNA, DNA-protein cross links, oxidation of purine and pyrimidine bases, deoxyribose backbone, *etc*.) are due to reactions with ROS (Marnett, 2000). In recent data, oxidative stress is often considered the primary mechanism involved in the genotoxicity of NPs (Singh *et al*., 2009; Song *et al*., 2012; Baker *et al*., 2014). Different ways have been proposed for ROS generation by NPs. NPs can lead to spontaneous ROS generation at their surface owing to their chemical and surface characteristics. Recent evidence showed that CuO-NPs induce oxidative stress (Buffet *et al*., 2011) and a genotoxic response in different animals (Gomes *et al*., 2012; Mwaanga *et al*., 2014) and human cell lines (Ahamed *et al*., 2010).

In marine organisms, there is evidence that the toxicity of copper causes oxidative stress, an increase in antioxidants and DNA damage (Raimundo *et al*., 2010; Schwarz *et al*., 2013).

Unfortunately, it is unclear as to what extent the toxicity of CuO-NPs can be explained by the released Cu or by the particles themselves. According to Bondarenko *et al*. (2012), the particles are endocytosed (a Trojan horse model), and when already inside the cell, their solubilization cannot be controlled by the mechanisms used to regulate the concentration of Cu ions in the cell. The dissolution of CuO-NPs is a key factor triggering the ROS and DNA damage responses in *E. coli*. In the presence of available cellular reductants (*i.e*., ascorbic acid, glutathione), copper may play a catalytic role in the initiation

of free radical reactions under aerobic conditions.

It was recently shown that oxidative stress and the genotoxic effect of CuO-NPs were considered to be mainly attributed to the nanoparticulate form and not to the presence of soluble copper ions (Buffet *et al*., 2011; Gomes *et al*., 2012). In conclusion, the present data demonstrated that CuO-NPs induced genotoxic effects in the marine bivalve mollusk *M. trossulus*, which may be mediated through oxidative stress. The gill cells showed a statistically higher degree of DNA damage, demonstrating an increased sensitivity to potential genotoxic compounds. These data suggested that the DNA stabilizing system in the gills of mussels may represent a significant target for *in vivo* exposure to certain types of NPs in mussels. Our results showed that the detoxified system was insufficient to protect mussel tissues against DNA damage, which was shown in the gill cells of *M. trossulus*.

No acute toxicity was registered for *M. trossulus*, even when the biomarkers of oxidative stress revealed a certain level of oxidative DNA damage that could be interpreted as an early warning signal of damage at higher levels of biological organization. CuO-NP short-time exposure was accompanied by an increase in DNA damage that can lead to the initiation of mutations and malignant transformation in cells.

Our results highlighted the important risk of genotoxic effects of CuO-NPs and showed that genotoxicity assays were a sensitive approach to evaluate the risk of CuO-NP toxicity. This investigation showed the ecological implications of NP release into marine ecosystems. Regulatory agencies and industries need to monitor and regulate NPs.

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