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Genotoxic Testing of Titanium Dioxide Nanoparticles in Far Eastern Mussels, *Mytilus Trossulus*

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Abstract: Manufactured nanoparticles (NP) have caused extreme concern about their ecotoxicological effects on the marine systems. In this study, we investigated the biological effects (oxidative stress and genotoxic response) of TiO_2-NP at predicted environmental relevant concentrations (0.2 mg/l and 1 mg/l) on marine mussel *Mytilus trossulus* a dominant member of the far eastern coastal community. The results of the experiment revealed that TiO_2-NP when suspended in seawater, formed agglomerates ranging from 400 nm to several µm in diameter. However, TiO_2-NP caused obviously oxidative damage on the mussel as evidenced by the significant elevated levels of malondialdehyde (MDA) in the gill and digestive gland. The genotoxic potential of TiO_2-NP was assessed by comet assay, which detect primary DNA damage. The gill and digestive gland cells showed significantly enhanced DNA damage for both concentrations of TiO_2-NP compared to the control group. These results propose that the TiO_2-NP are entering the marine coastal waters can cause genotoxic effect on mollusks and comet assay can be successfully applied as an effective tool for risk assessment of NP on the marine invertebrates. The findings of this study demonstrate that the aggregation of TiO_2-NP does not reduced of NP ecotoxicity, but only changes the biological responses.

Keywords: TiO_2-NP, bivalve, DNA comet assay, nanotoxicity.

INTRODUCTION

In recent years, the worlds nanotechnology market is growing constantly, with NP being produced for diverse applications in industry and consumer products. NP possesses unique physic-chemical characteristics because of their small size, electronic charges, dissolution properties and large surface-to-mass ratio. Among the different types of NP titanium dioxide, NP are the most produced; they are used mostly in sunscreens, paints, cosmetics, drugs, additive food, personal care and other commercial products (Skocaj et al., 2011).

Large-scale production and use are likely to result in the release of TiO_2-NP-based products into the environment, making them some of the emerging classes of marine ecosystems contaminants. It is estimated that up to 7500000 tons of TiO_2-NP can enter the coastal waters in the near future by NP-consisted products degradation (Owen, Deplege, 2005; Farrokhpay et al., 2010). The measured concentrations of TiO_2 in raw sewage water range from 181 to 1233 µg/l

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(Westerhoff et al., 2011). The predicted environmental concentrations of these particles were reported as 16 or 24,5 µg/l in water (Mueller, Nowak, 2008; Tiede et al., 2009). Thus, recent estimates show that anthropogenic inputs of TiO_2-NP in marine environment could be dramatically higher than the concentration of naturally derived titanium (Doyle et al., 2016). Although environmental behavior of TiO_2-NP is difficult to predict, it has been noted the filter-feeders marine organisms are capable of accumulating these NP, and could potentially transfer it via the marine food web (Galloway et al., 2010; Barmo et al., 2013; Wang et al., 2017).

The behavior of NP in aqueous media suggests that they are capable of affecting a wide range of organisms, from the smallest particle-attached bacterium to a filter-feeding bivalve (Matranga, Corsi et al., 2012; Baker et al., 2014; Haynes et al., 2017). The task of understanding the impact of NP on marine ecosystems is the imperative challenge for future risk assessments in the marine environment, also taking into account that bioaccumulation and biomagnifications via trophic chains may have a serious impact on human health.

As a result, during the last decade, the potential impact of TiO_2-NP on aquatic ecosystems has become a topic of interest for ecotoxicologists (Canesi et al., 2012; Matranga, Corsi, 2012; Baker et al., 2014; Abdel-Latif et al., 2020). There is emerging evidence of TiO_2-NP toxicity (Xia et al., 2017) which makes it imperative to understand the likelihood of exposure. Previous studies have demonstrated that biological processes and physiological functions such as fertilization, larval development, immune responses, metabolism, growth and survival of various marine organisms could be affected by TiO_2-NP (Canesi et al., 2012; Matranga, Corsi, 2012; Gambardella et al., 2013; Baker et al., 2014; Xia et al., 2017). Despite the rapid emerging literature on the effects of TiO_2-NP on marine organisms, comprehension of the mechanisms underlying NP toxicity in invertebrate species requires further clarification. Thus in our study we used sublethal concentration, based on previous studies (Canesi et al., 2010; Zhu et al., 2011; Doyle et al., 2015; Gornati et al., 2016; Hou et al., 2019).

One of the most important effects of anthropogenic pollutants is DNA damage, since increasing of DNA damage level are associated with processes of cancerogenesis and cells death. Thus, the genotoxic potential of TiO2-NP must be exhaustively studied. The genotoxic potential of TiO_2-NP has been relatively well documented in vivo and in vitro studies using different culture of mammal cells (Shukla et al., 2011; Jugan et al., 2012; Chen et al., 2014; Patel et al., 2017; Mottola et al., 2019; Han et al., 2020). Unfortunately, the molecular mechanism revealing the genotoxicity of TiO_2-NP remains poorly understood in marine invertebrates.

Bivalve mollusks are well-known as bioindicators and sentinel species of marine and coastal pollution. Due to mussel filtration activity, its have been considered an important target for NP toxicity in sea water and therefore represent one of a key species to evaluate of potential NP toxicity in marine environmental (Azizi et al., 2018; Saidani et al., 2019; Wang et al., 2019; Roma et al., 2020). In recent years, mussels Mytilus sp. has played a important role in studies detecting of DNA damage and genotoxicity of such engineering NP as Au, Ag and CuO (Tedesco et al., 2010; Gomes et al., 2013; Chelomin et al., 2017; Slobodskova et al., 2019).

The aim of work: increasing knowledge of the genotoxic potential of TiO_2-NP exposure in whole organisms, used a well-established genotoxic assay (the comet assay) on eastern marine bivalve mollusk *Mytilus trossulus*.

MATERIALS AND METHODS

Titanium dioxide NP was obtained from Sigma Aldrich (declared particle size ≤20 nm; 99.5% pure, predominantly anatase structure, uncoated). The NP stock suspension was prepared in bi-distilled water at a concentration of 10 mg/l. To prepare a working suspension (200 and 1000 µg/L), the stock solution was ultrasonicated at a power of 40 W for 20 min and added to experimental tanks. Primary particles size was analyzed by transmission electron microscopy (TEM) on a Carl Zeiss Libra 200 transmission electron microscope at 200 kV. The size distribution of TiO_2 NP suspensions at 1 mg/l were analyzed at 24 h after incubation in filtered sea water by dynamic light scattering (DLS) at ANALYSETTE 22 NanoTec.

Adult *Mytilus trossulus* (4.5-5 cm in shell height) were maintained in Vostok Bay (Peter the Great Gulf, the Sea of Japan, Russia). Mollusks were allowed to acclimate for a minimum of 5 days before experiment. After acclimation all mollusks were divided into three groups: one group without treatment and two groups with treatment (0.2 and 1,0 mg/l) for up to 10 days. During the experimental period, the seawater (T 18–19 °C, pH 8.2 ± 0.1; salinity 32.74 ± 0.34 psu) was changed every 12 h, and NP dosing was repeated at each water change. Aeration (7.5 ± 0.3 mg/L) at the bottom of the tank was used to minimize agglomeration and for subsequent sedimentation of NP. The gills and digestive gland of mollusks were isolated and used in TEM, atomic absorption spectroscopy, TBARS measure and comet assay.

The TBARS were measured by a color reaction with 2-thiobarbituric acid (Buege, Aust, 1978). Measurements were carried out on a UV-2550 spectrophotometer (Shimadzu) with a thermostatic unit.

For comet assay mollusks gills and digestive gland were removed and gently cut in isotonic solution. To this end 50 µl of the cell suspension was added 100 µl of 1% low-melting point agarose (LKB, Sweden). After mixing 50 µl of cell-agarose suspension was added on glass slide coated with 1% agarose and covered with a cover glass. After cover glass removing, slides were placed in lysis chamber for 1 h in fridge. After washing the slides were transferred to an electrophoresis buffer (pH 13) and maintained for 40 min. Electrophoresis was carried out at 2 V/cm for 20 min. Following neutralization, the slides were stained with SYBR Green I. More detail assay described in previous authors work (Slobodskova et al., 2010).

The DNA comets were visualized and registered using a scanning fluorescence microscope (Carl Zeiss, AxioImager A1) equipped with a digital camera AxioCam MRc. Digital images were processed using a V 1.2.2. CASP program that can be used to make calculations of various parameters of comets indicating the DNA damage level. The percentage of DNA in the comet tail (% DNAt) was determined for each comet. A total of 100 nuclei (n) from each replicates (N=15) 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 (<5% DNA in tail); Class 1, (5%–20% DNA in tail); Class 2, (20%- 40% DNA in tail); Class 3 (40%–75% DNA in tail) and Class 4 (>75% DNA in tail).

For Titanium determination gill and digestive gland samples were removed from 15 mussels for each treatment. The presence of titanium in samples was quantified by atomic absorption spectrophotometry (AAS) on a Shimadzu-6800F device. Data is expressed as µg Ti /g dry weight. Each sample was analyzed in triplicate.

Used laboratory equipment is regularly calibrated and verified to eliminate measurement errors. All data obtained in this study were thoroughly checked for outliers and then statistics and pots were calculated

and drown, respectively. The results of the experiment were processed in the MS Excel and Statistica 10 software packages. For "DNA content in the tail of the comet" dates nonparametric Kruskal-Wallis ANOVA followed by pair-wise Mann-Whitney tests were performed. Cu concentrations in gills and digestive gland were compared with the control by a Mann-Whitney test. A difference at $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

For traditional chemical contaminants, toxicity is related to mass concentration. In case of engineering NP, bioavailability and toxicity will be affected by mass, but also by primary particle size, particle numbers, the degree of aggregation of the NP and particle size distribution in the exposure environment. In our study, we observed a fast aggregation of TiO_2-NP in natural seawater and sedimentation it onto the bottom of the exposure tanks. Data examining the physicochemical behavior of TiO_2-NP immersed in natural seawater showed the formation of agglomerates. TEM-analysis showed average primary particle size as 25nm. It also highlighted the strong tendency of TiO_2-NP to aggregate (Fig. 1A). In fact, DLS analysis showed that TiO_2-NP suspension in seawater formed agglomerates ranging from 400 nm to several μm in diameter (Fig. 1B).

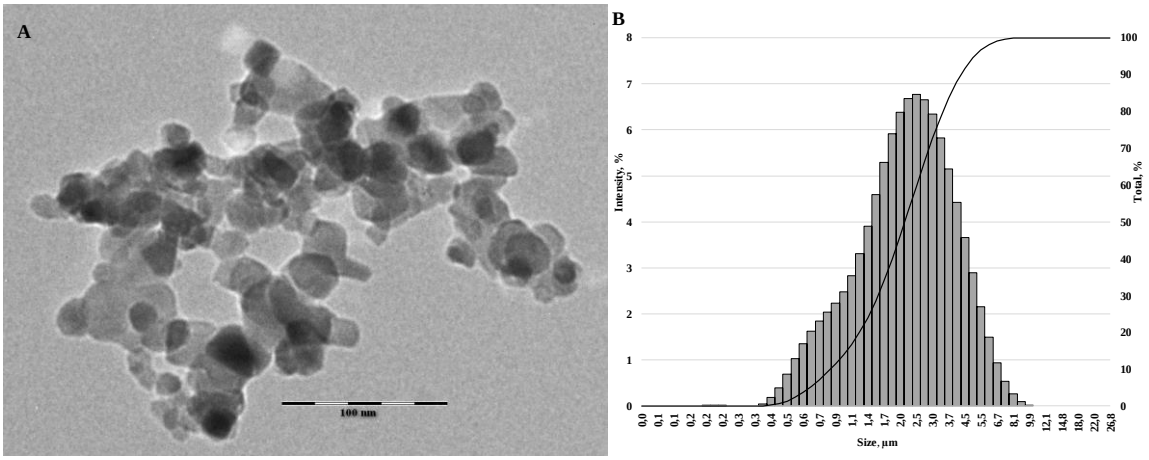

Fig. 1. Transmission electron microscopic (TEM) image (A) and DLS size distribution of TiO2-NP (B) showing the aggregation pattern of TiO2-NP in seawater (24 h after sonication). Scale bar is 100 nm.

There is often assuming that aggregation will facilitate sinking and reducing of NP bioavailability, but it is also likely, that is some cases, primary particles and small aggregates may be more bioavailable to mussels and other benthic filter-feeders. Furthermore, an increase in size allows NP to be captured and ingested by both filter-feeding and bottom-feeding organisms (Ward, Koch, 2009; Canesi et al., 2010; Chelomin et al., 2017).

Under the conditions of our experiment the mussels *Mytilus trossulus* rapidly accumulated TiO_2-NP through filtering activity and primarily stored the NP in the digestive gland. Moreover, TEM showed that TiO_2-NP were incorporated into the digestive gland tissues of the exposed mussels (Fig. 2), confirming data from the literature (Canesi et al., 2014; D`Agata et al., 2014). The uptake of Ti into *M. trossulus* defined as Ti tissue concentrations appeared to be exposure cases (Fig.3). The results indicate that TiO_2 NP mainly accumulated in the digestive gland cells. This finding agrees with the Portuguese investigators (Gomez et al., 2013) who reported that aggregated metal NP are largely assimilated by the digestive gland of *M. galloprovincialis*, while the gills are more susceptible to dissolved metal forms.

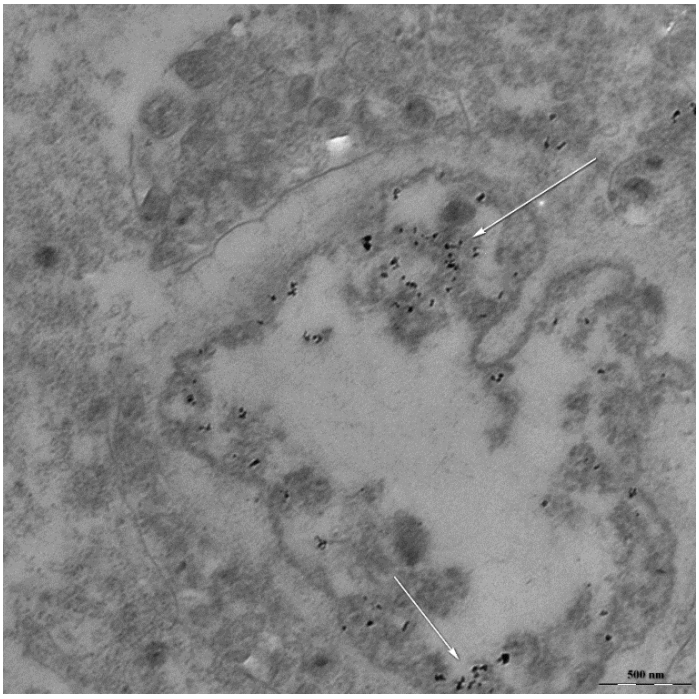

Fig. 2. Transmission electron microscopic observations for location of TiO2-NP in digestive gland of mussels after exposure to 1mg/l TiO2-NP for 10 days. Arrows point to a TiO2-NP. Scale bar is 500 nm.

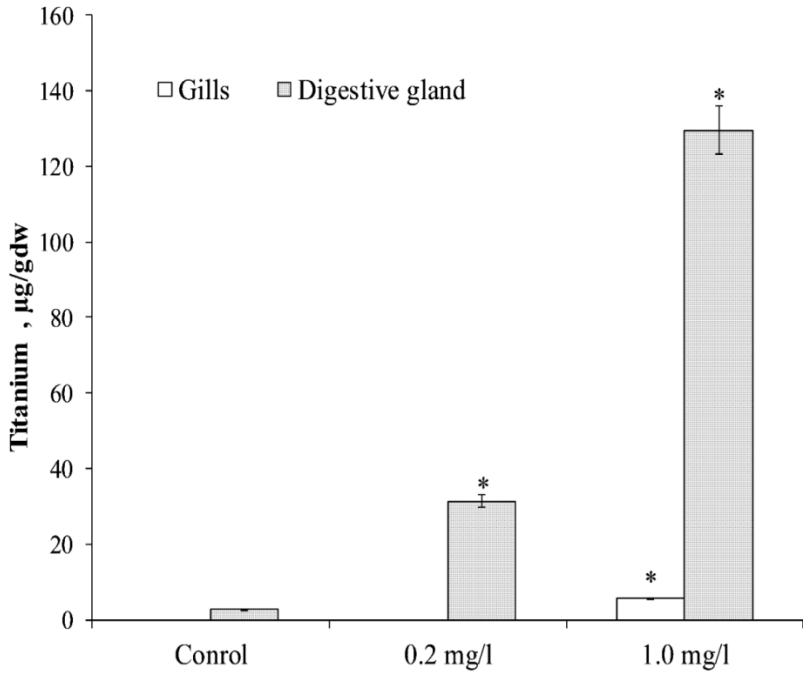

Fig. 3. Titanium levels in gills and digestive gland of mussels treated with different concentrations of TiO2-NP. Values are mean ± SD (N=15). Significant differences from controls are indicated by * (ρ<0.05)

In the exposure experiments measurable concentrations of Ti were found in the gills of mussels only at 1,0 mg/l after 10 days exposure; tissues sampled at 0.2 mg/l had no detectable concentrations of Ti. In accordance with previous studies (Canesi et al., 2014; Doyle et al., 2015), our results confirm the differentiated accumulation of NP in mussel tissues, in particular, the digestive gland accumulated more NP in

comparison with gills in our experiments. Despite the fact that the main function of the digestive gland is digestion, it is also the main place for the accumulation and detoxification of various xenobiotics of organic and inorganic nature, including metal oxide nanoparticles (Chelomin et al., 2017). At the same time, tissue such as gills that are more secretory may be less susceptible to a NP aggregates effect and more sensitive to dissolved NP constituents. However, as the TEM measurements of TiO_2-NP revealed larger aggregates in digestive gland (Fig.1), it is interesting that there appears gills can't "trap" this form of NP. It is possible high part of aggregates are ignored by gills epithelium, then transported to digestive gland. Our results and an earlier study on NP uptakes support this hypothesis (Canesi et al., 2012, D'Agata et al., 2014., Chelomin et al., 2017).

Our results support generally the findings of other researches, which reported that bivalves, effectively capture a TiO_2-NP (Canesi et al., 2012; D'Agata et al., 2014; Gornati et al., 2016). Moreover, it is likely that these NP were captured and ingested by the mussels because they were directly in an aggregated form (Canesi et al., 2012). Furthermore, the presence of NP in tissue after the mussel's treatment probably indicate the organism's inability to eliminate the NP, thus causing their bioaccumulation and toxicity. The bioaccumulation of Ti, recorded by AAS suggests that digestive gland and gills can be considered the main targets of TiO_2-NP mussels exposure. We have thus focused further experiments on these two organs.

Exposure to toxic substances can produce a wide range of adverse health effects. One of the toxic effects of metal NP oxide is lipid peroxidation. TBARS levels were measured in the tissues of control and exposed mollusks as an indication of lipid peroxidation potentially induced by reactive oxygen substances (ROS) production. The results showed that the content of malondialdehyde (MDA) in the digestive gland and gills of exposed mussels was near 2.0 times higher than that in control mussels (Fig. 4A).

The increased ROS production and its peroxidation of unsaturated fatty acids one of the most common oxidative stress reactions under the influence of different xenobiotics. Therefore, the increase in the MDA levels has been considered as a marker of oxidative damage in mollusks after TiO_2-NP exposure (Zhu et al., 2011; Girardello et al., 2016). The increased MDA level were observed over our experiment, may suggest the antioxidant capacity was overwhelmed by the enhanced ROS production induced by TiO_2-NP exposure. In agreement with our results Xia with colleagues (Xia et al. 2017) also reported that the MDA contents showed a significant increase in the marine scallop *Chlamys farreri* following exposure to relevant concentration of TiO_2 NP.

The comet assay was used to evaluate a DNA damage in gill and digestive gland cells from unexposed and exposed mussels. A visual analysis of comets microphotographs after electrophoresis clearly showed that the DNA molecules from the gill and digestive gland cells of the control mussels did not have destructive changes and formed a symmetrical bright nucleus with a small halo. In contrast, the DNA of the mussels exposed to TiO_2-NP formed a distinct 'comet' after electrophoresis due to the degradation and migration of genomic DNA.

The fig. 4B shows a quantitative parameter of the obtained comets: mean percentage of DNA in the comet tail. An estimation of this parameter showed that in cells of gills and digestive gland of TiO_2-exposed mussels the DNA damage was significantly higher than in the control mussels.

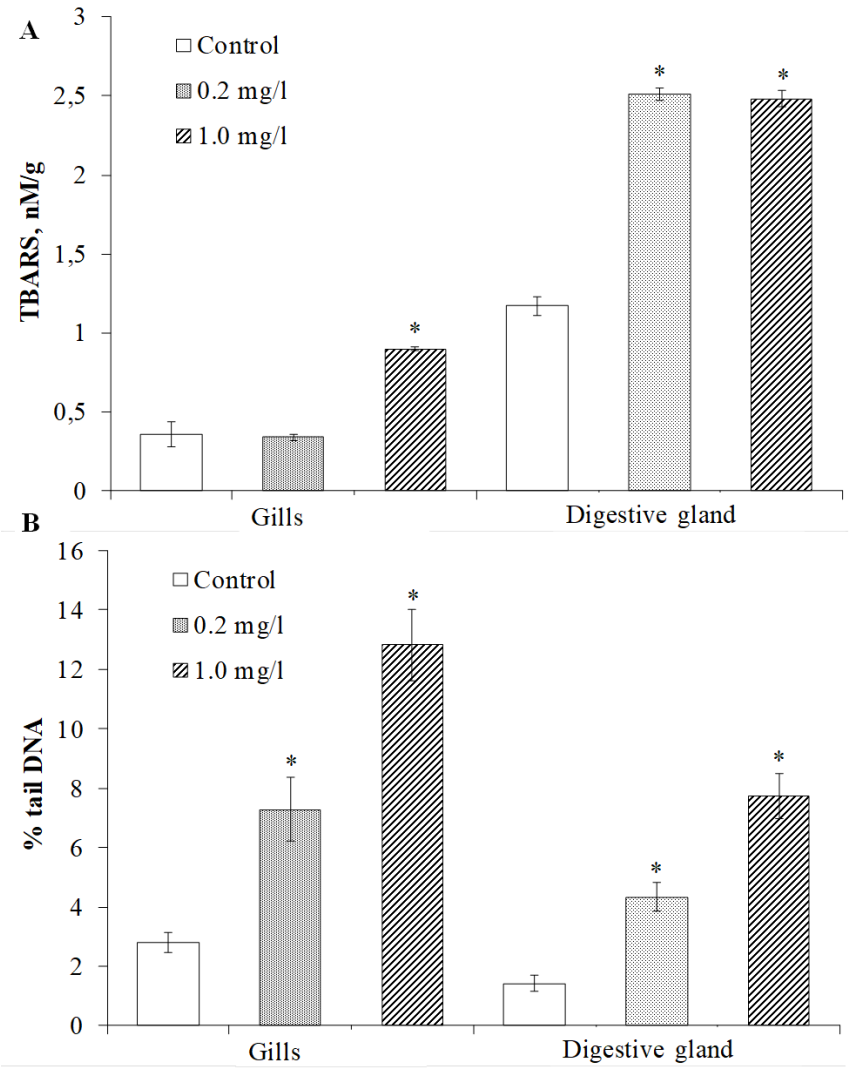

Fig. 4 A. Changes of TBARS (MDA) contents in the gills and digestive gland of mussels treated by TiO2-NP for 10 days. B. Effects of TiO2-NP on DNA strand breakage in mussel gill and digestive gland cells using comet assay. Error bars represent standard deviations (N=15). Asterisk indicates differences between the control and treatments (ρ<0.05).

The fist classification of comets was used by Collins group (1995). In this article, the percentage of DNA in the tail region was used to classify the grade of damage in different mussels group as per the Mitchelmore and Chipman classification (1998). Fig. 5 shows the distribution of cells according to the five classes of DNA damage. Fig. 5 shows the distribution of cells according to the five classes of DNA damage. The mussels tissue cell data is 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 the digestive gland cells (Fig. 5) 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 (> 2 %) of the control gill cells (Fig. 5) exhibited damage (C2 class) that is, probably associated to some DNA strand breaks occurring with different stage of cell cycle or environmental stress.

Exposure to both TiO2-NP concentrations (0.2 and 1 mg/l) resulted in a statistically significant increase in DNA damage in the

digestive gland and gill cells (Fig. 2B). For mussels exposed to TiO2-NP, the tissue cells showed mid-DNA damage with decreasing level of C0 comet class and increasing levels of C1 and C2 comet classes (Fig. 5). Significantly, elevated DNA damage was observed in the gill cells from the mussels exposed to 1.0 mg/l TiO2-NP. These mollusks had higher frequencies in the number of cells in classes 2 and 3.

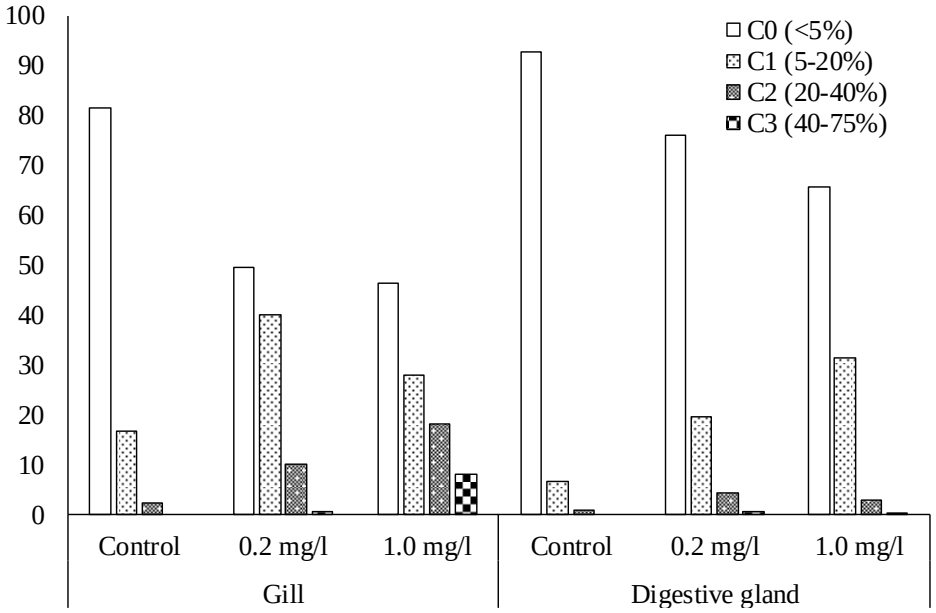

Fig. 5. Induction of DNA strand breaks, represented as percent of comet damage classes in the gill and digestive gland cells of mussels following in 10 days in vivo exposure to TiO_2-NP (N=15; n=1500)

Genotoxical studies on TiO_2-NP are even more limited with only a few reports on the genetic material damage effects of NP on aquatic organisms. It was shown that the presence of TiO_2-NP in the bottom sediments increased DNA damages in the cells of the *Arenicola marina* (Galloway et al., 2010). The exposures of mussels *M. galloprovincialis* to TiO_2-NP induced a significant increase of MN cell frequency in gill tissues (Rocco et al., 2015) and DNA damage in heamocytes (D`Agata et al., 2014). In the blue mussel, *M. edulis* exposed to TiO_2-NP for 96 h the micronucleus frequency increased with increasing NP concentrations (Farkas et al., 2015). The results of both SCGE and MN assays indicated higher level of DNA damage in heamocytes of $nTiO_2$-exposed clams *Ruditapes philippinarum* (Marisa et al., 2018).

Recent studies indicate that TiO_2-NP can cause chromosomal changes in marine fish *Dicentrarchus labrax* (Della Torre et al., 2015) and in *Trachinotus carolinus* (Vignardi et al., 2015), it was noted that the number of micronucleus cells increased by almost six time. Reeves et al. (2008) have shown that TiO_2 NP are potentially genotoxic to fish cells (goldfish skin cells – GFSk-S1) under *in vitro* conditions. In addition, the cytotoxic and genotoxic potential of TiO_2-NP was shown by Vever and Jha (2008) on rainbow trout cells obtained from gonadal tissues (RTG-2 cells).

In recent time, studies on NP genotoxicity on marine organisms are limited, the description of NP genotoxicity mechanisms is generally based on experimental data that were obtained on mammals. From our results, and based on the findings from studies performed on various animals, we could only formulate a hypothesis on the mechanisms involved in the DNA damage considering the potential

adverse effect induced by TiO_2-NP. The mechanisms likely to be responsible for NP-induced genotoxicity fall into main categories, direct and indirect mechanisms.

The studies conducted until now detected TiO_2-NP inside lysosomes (indicating an entry by phagocytosis or endocytosis), but TiO_2-NP were also detected freely in cytoplasm, mitochondria and nucleous showing that these NP are also able to pass through the membrane by diffusion (Shukla et al., 2011; Wang et al., 2017). The cytoplasmic compartmentation of TiO_2-NP supports the hypothesis that is an indirect mechanism mediating DNA damage. On the other hand, since engineering TiO_2-NP can enter into nucleus (Wang et al., 2017; Patel et al., 2017), a direct interaction between them and DNA can lead to physical damage to the genetic material. Li et al. (2010) reported the presence of TiO_2-NP in the liver DNA of mice after TiO_2-NP intraperitoneal expose. The authors showed that Ti can bounded DNA nucleotides, that altered the conformation of the DNA and caused DNA cleavage (Li et al., 2010). Alternatively, DNA damage can occur through indirect mechanism, which there is no physical interaction with DNA molecule, but with proteins involved in the process of replication or DNA damage repair (Jugan et al., 2012). Reeves et al. (2008) showed that repair mechanisms are not able to repair a significant increase of DNA damage after NP exposure.

Many studies using different experimental models indicate that oxidative stress may be the main mechanism of TiO_2-NP genotoxicity in aquatic organisms (Reeves et al., 2008; Canesi et al., 2014; D`Agata et al., 2014; Girardello et al., 2016; Marisa et al., 2018). Among the properties of TiO_2-NP, which make them interesting include their ability to absorb UV radiation and generate ROS. Unlike most of the metallic NP (such as ZnO-NP, CuO-NP and AgO-NP) that release metal ions, which are cytotoxic, the solubility of TiO_2-NP is extremely low in aqueous solutions and its cytotoxicity pathway were proposed via photocatalysis and surface ROS (Reeves et al., 2008; Miller et al., 2012; Haynes et al., 2017).It was shown that free radicals can be generated at the TiO_2-NP surface when their aqueous dispersions are strong and nonselective oxidants that can damage a range of biomolecules, such as membrane lipids, proteins and DNA.

For example, a rapid increase ROS and nitric oxide production was observed in *M. galloprovincialis* haemocytes after short-term exposure to NP, including TiO_2 (Canesi et al., 2010). Showed changes in the activity of the antioxidant enzymes (SOD, CAT, GST) and elevated MDA levels in different tissues, suggesting oxidative stress caused by the TiO_2-NP (Barmo et al., 2013; Xia et al., 2017; Huang et al., 2018; Sureda et al., 2018).

In the aquatic environment TiO_2-NP can be sorbed to the gills surface where the UV-activation of complex between metallic NP and gill cells could then participate in a reaction of ligand-to-metal charge transfer , resulted in the gill cells membrane undergoes oxidation. Other potential interactions between TiO_2-NP and gill cells may arise through the diffusion of TiO_2-mediated ROS from NP surface onto the lipid membrane or into the surrounding media, where ROS may damge cells or organic compounds (Miller et al., 2012).

CONCLUSION

In this study, the genotoxicity of TiO_2 NP on target organs of mussel *M.trossulus* was significant for both NP concentrations. Our study demonstrated a tendency in the accumulation of NP in digestive gland of mollusks, despite an intensive aggregation processes of NP in sea water. Nevertheless, at low concentrations of Ti in gills, the DNA damage was also significant,

meaning some undirect mechanisms of DNA damage of NP were involved. The high level of TBARS suggested the oxidative nature of this damage. Our result showed the ecological implications of NP release into marine ecosystems. Regulatory agencies and industries need to monitor and regulate NP.

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CONFLICT OF INTEREST

The authors declare no competing financial interest.

LIFE SCIENCE REPORTING

No life science threat was practiced in this research.

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