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Cell and tissue level responses in mussels *Mytilus galloprovincialis* dietarily exposed to PVP/PEI coated Ag nanoparticles at two seasons

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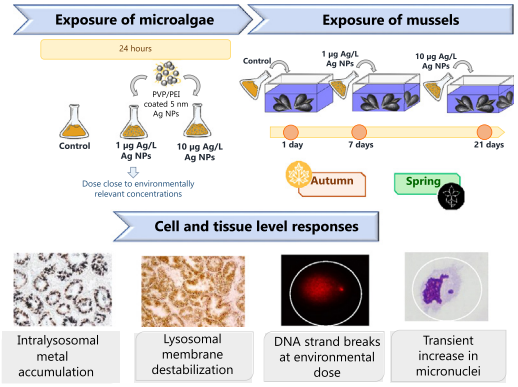
HIGHLIGHTS

- Cell/tissue responses assessed in mussels dietarily exposed to Ag NPs in two seasons.
- Ag accumulation was higher in autumn compared to spring.
- Intralysosomal metal accumulation and reduction of LMS was similar in both seasons.
- DNA strand breaks increased after dietary exposure to 1 and 10 μg/L AgNPs with respect to controls.
- Micronuclei increased transiently suggesting activation of DNA repair mechanisms.

GRAPHICAL ABSTRACT



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ABSTRACT

Silver nanoparticles (Ag NPs) are present in numerous consumer products due to their antimicrobial and other unique properties, thus concerns about their potential input into aquatic ecosystems are increasing. Toxicity of Ag NPs in waterborne exposed aquatic organisms has been widely investigated, but studies assessing the potential toxic effects caused after ingestion through the food web, especially at low realistic concentrations, remain scarce. Moreover, it is not well known whether season may influence toxic effects of Ag NPs. The main objective of this study was to determine cell and tissue level responses in mussels *Mytilus galloprovincialis* dietarily exposed to poly-N-vinyl-2-pirrolidone/polyethyleneimine (PVP/PEI) coated 5 nm Ag NPs for 1, 7 and 21 days both in autumn and spring. Mussels were fed every day with microalgae *Isochrysis galbana* exposed for 24 h to a low dose (1 μg Ag/L Ag NPs) in spring and to a higher dose (10 μg Ag/L Ag NPs) in spring and autumn. Mussels fed with microalgae exposed to the high dose accumulated Ag significantly after 21 days in both seasons, higher levels being measured in autumn compared to spring. Intralysosomal metal accumulation measured in mussel digestive gland and time- and dose-dependent reduction of mussels health status was similar in both seasons. DNA strand breaks increased significantly in hemocytes at both exposure doses along the 21 days in spring and micronuclei frequency showed an increasing trend after 1 and 7 days of exposure to 1 μg Ag/L Ag NPs in spring and to 10 μg Ag/L in both seasons. Values decreased after 21 days of exposure in all the cases. In conclusion, PVP/PEI coated 5 nm Ag NPs ingested through the food web were significantly accumulated in mussel tissues and caused adverse cell and tissue level effects both in autumn and in spring.

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1. Introduction

The input of anthropogenic contaminants to natural water systems has the potential to affect the health status of aquatic organisms altering ecosystem structure and function. In this sense, molecular and cellular biomarkers were proposed as sensitive early warning tools to assess the quality of aquatic environments (Cajaraville et al., 2000; Viarengo et al., 2007). The integrated assessment of biomarkers of exposure (*e.g.* metallothionein levels, intralysosomal metal accumulation or peroxisome proliferation) together with effect biomarkers (*e.g.* genotoxicity, lysosomal membrane stability and tissue damage) has been widely applied in sentinel mussels for marine pollution monitoring (UNEP, 2004; ICES, 2016; Zorita et al., 2007a; Garmendia et al., 2011;) and in laboratory exposure scenarios for the assessment of biological effects caused by different pollutants (Zorita et al., 2007b; Ruiz et al., 2014; Banni et al., 2017). In the case of novel emerging pollutants, biomarkers have been successfully applied as a sensitive tool for evaluating the effects and mechanisms of action of different nanomaterials in aquatic invertebrates, especially in mussels (Canesi et al., 2014, 2019; Rocha et al., 2016; Jimeno-Romero et al., 2017a, 2017b, 2019).

Different nanomaterials, such as carbon nanotubes and silver nanoparticles (Ag NPs), have been identified as potentially present in waste (Marcoux et al., 2013). In fact, the release of Ag NPs into the aquatic environment is expected to rise (Giese et al., 2018) since their commercial and scientific applications are increasing due to their unique antimicrobial, catalytic and optical properties (Fabrega et al., 2011; Zhang et al., 2016; McGuillicuddy et al., 2017). The availability of methods for the proper detection and measurement of engineered NPs is a key aspect for understanding NPs fate and behaviour in the environment (António et al., 2015), but their quantification in complex natural matrices such as seawater, soils, sediments or tissues is still challenging (Von der Kammer et al., 2012; Sikder et al., 2017). In that sense, the potential adverse effects of Ag NPs in aquatic organisms at environmentally relevant concentrations are still little known. Li et al. (2016) reported concentrations from 0.7 to 11.1 ng/L Ag NPs in effluents of waste water treatment plants in Germany. Nevertheless, predictions for aquatic environments based on modelling studies estimated Ag NP concentrations to reach 140 ng/L in rivers of Europe (Blaser et al., 2008; Gottschalk et al., 2009; Tiede et al., 2009; Dumont et al., 2015; Giese et al., 2018) and 40 μg/L in Taiwanese waters (Chio et al., 2012).

As bivalve molluscs have been identified as a key target group to assess NPs toxicity in the marine environment, they are widely studied (Moore, 2006; Canesi et al., 2012, 2019; Corsi et al., 2014). In mussels, the main organ for NP accumulation is the digestive gland but NPs cellular fate and effects differ depending on the NP type and on experimental conditions (Canesi et al., 2012, 2019; Rocha et al., 2015; Canesi and Corsi, 2016). The digestive tubules of the digestive gland of mussels are composed of digestive and basophilic cells and under normal physiological conditions, the digestive cells outnumber basophilic cells (Cajaraville et al., 1990; Garmendia et al., 2011). However, under different stress situations, including exposure to pollutants such as metal-bearing NPs, the relative occurrence of basophilic cells increases (Rocha et al., 2016; Jimeno-Romero et al., 2017a, 2019). The endolysosomal system of digestive cells, due to its role in intracellular digestion of food particles, represents the main subcellular target for metallic NPs in bivalves (Moore, 2006; Canesi et al., 2012; Katsumiti et al., 2014; Rocha et al., 2015). The low pH (≈5.0) in lysosomes may favour the dissolution of metallic NPs and released free ions, together with the remaining NPs, could induce the hypersynthesis of lysosomal acid phosphatase enzyme and the generation of reactive oxygen species (ROS), finally attacking lysosomal membranes (Katsumiti et al., 2014; Katsumiti and Cajaraville, 2019). In fact, lysosomal membrane destabilization has been already measured in the digestive gland of mussels waterborne exposed to Ag NPs (Jimeno-Romero et al., 2017a) or to other metal based NPs (Balbi et al., 2014; Jimeno-Romero et al., 2017b, 2019).

Moreover, NPs can be potentially translocated from the digestive system to the hemolymph and to circulating hemocytes (Canesi and Corsi, 2016). These cells are responsible for bivalve's immune defense (Cajaraville and Pal, 1995) and are relevant targets for metal-bearing NP toxicity (Canesi et al., 2010; Ciacci et al., 2012; Katsumiti et al., 2014, 2015, 2018). In fact, alterations in the phagocytic activity, reduction of cell viability, stimulation of lysosomal enzyme release, increase of ROS production as well as induction of lysosomal membrane destabilization and DNA damage have been widely reported in bivalve hemocytes after *in vitro* exposure to different metallic NPs (Canesi et al., 2010; Ciacci et al., 2012; Katsumiti et al., 2014, 2015, 2018; Katsumiti and Cajaraville, 2019). DNA damage is a stress index usually considered of great importance to define the physiological status of organisms (Cajaraville et al., 2000; Viarengo et al., 2007). DNA damage in mussel hemocytes exposed to metallic NPs is frequently assessed by the comet assay (Gomes et al., 2013; Katsumiti et al., 2014, 2015, 2018). However, the combination of the comet assay and cytogenotoxic assays such as the micronuclei test is considered a more realistic approach to assess the genotoxic effects of NPs in bivalves (Canesi et al., 2014; Rocha et al., 2014) since the comet assay allows determination of reversible DNA strand breaks whereas the micronuclei test identifies chromosomal damage induced by both clastogenic (DNA breakage) or aneugenic (abnormal segregation) effects (Bolognesi and Fenech, 2012).

The application of a battery of biomarkers has helped to understand the effects of Ag NPs on marine bivalves exposed *in vivo* through water (Ringwood et al., 2010; Buffet et al., 2013, 2014; Gomes et al., 2013, 2014; McCarthy et al., 2013; Bebianno et al., 2015; Jimeno-Romero et al., 2017a), but there is limited information about the transfer of engineered NPs through the food web (Tangaa et al., 2016). In fact, apart from our previous studies (Duroudier et al., 2019a, 2019b, 2019c), there are few other works assessing the potential toxic effects of Ag NPs ingested through the diet in marine bivalves (Buffet et al., 2013). In *Scrobicularia plana* clams, even if Ag bioaccumulation was higher in waterborne exposed clams than in dietarily exposed ones, activity of biotransformation and antioxidant enzymes was more affected compared to controls after dietary exposure than after exposure through water to lactate stabilized 40 nm Ag NPs. The dietary exposure of *Mytilus galloprovincialis* mussels to PVP/PEI coated 5 nm Ag NPs caused Ag accumulation in adults, affected spawning success in females and induced abnormal embryo development in their offspring (Duroudier et al., 2019b). Further, the dietary exposure to the same Ag NPs affected the transcriptome and proteome of the digestive gland of mussels in the two studied seasons, autumn and spring (Duroudier et al., 2019a, 2019c).

In general, season is not considered in ecotoxicological studies even if seasonal variations in biomarker responses such as lysosomal parameters and DNA damage have been reported in mussels (Pisanelli et al., 2009; Hagger et al., 2010; Nahrgang et al., 2013; Schmidt et al., 2013; Balbi et al., 2017). These changes are driven by the interaction between abiotic factors in the environment, such as food availability, oxygen levels and temperature, and biotic factors, as their reproductive and physiological state (Bayne and Widdows, 1978; Solé et al., 1995; Cancio et al., 1999; Sheehan and Power, 1999).

The objective of the present work was to assess Ag accumulation and cell and tissue level biomarkers in mussels exposed to Ag NPs through the diet using a low dose (1 μg Ag/L Ag NPs) and a higher dose of 10 μg Ag/L Ag NPs in two seasons, autumn and spring. It was hypothesized that cell and tissue level responses to dietary exposure to Ag NPs could differ depending on the season. Thus, this work provides novel knowledge on the effects of dietary exposure to NPs and on the influence of season on bivalve's responses to NPs at cell and tissue levels.

2. Materials and methods

2.1. Obtention and characterization of Ag NPs

Ag NPs were provided as a stable aqueous suspension by Nanogap (O Milladoiro, Galicia, Spain). They were coated with poly N-vinyl-2-pirrolidone/polyethyleneimine (PVP/PEI; 77%:23% at a concentration of 104 g/L in the final dispersion). According to the provider, in distilled water they showed 5.08 ± 2.03 nm average size and + 18.6 ± 7.9 mV zeta potential. Particle size distribution and dissolution of Ag NPs in seawater can be found in Duroudier et al. (2019b).

2.2. Experimental design

The design of the experiment is reported in detail in Duroudier et al. (2019a). Briefly, after the acclimation period, mussels were maintained for the 21 days of duration of the exposure experiment. Mussels in the control tank were fed every day with the microalgae *Isochrysis galbana* and mussels in the treatment tanks were fed every day with microalgae exposed previously for 24 h to 1 μg Ag/L Ag NPs (low dose) in spring or to 10 μg Ag/L Ag NPs (high dose) in autumn and in spring. Thus, microalgae were exposed to Ag NPs every day and then, contaminated microalgae were dosed to mussels every day during 21 days. Under this experimental design, Ag NPs interacted with microalgae cells by entrapping them in a network of heteroaggregates (Schiavo et al., 2017). Microalgae accumulated Ag under exposure to both low and high doses of Ag NPs with bioconcentration factors of 2,49 and 2,12, respectively (Duroudier et al., 2019b).

After 1, 7 and 21 days of exposure, whole soft tissues of 20 mussels per experimental group were obtained for chemical analysis. Hemolymph was extracted for genotoxicity assays from 10 mussels per experimental group and then, digestive glands were dissected out, and stored at −80 °C until processing for lysosomal membrane stability test. Finally, whole soft tissues of 10 mussels per experimental group were fixed in 10% neutral buffered formalin for the assessment of intralysosomal metal accumulation in mussels digestive gland by autometallography as well as for quantification of the volume density of basophilic cells. All samples were analyzed using blind codes.

2.3. Accumulation of Ag in mussel soft tissues

Ag accumulation was determined in soft tissues of 20 individuals (4 pools of 5 individuals each) per treatment and exposure time (1, 7 or 21 days) as described previously (Duroudier et al., 2019a).

2.4. Intralysosomal metal accumulation by autometallography

Intralysosomal accumulation of metals was determined in mussel's digestive tubules (10 mussels per treatment and exposure time) after autometallographic staining of paraffin-embedded tissue sections and quantification of the volume density of black silver deposits, as described previously (Duroudier et al., 2019b).

2.5. Lysosomal membrane stability

Digestive glands of 5 mussels per treatment and exposure time (1, 7 or 21 days) were dissected out, snap frozen in liquid nitrogen and stored at −80 °C. Eight serial sections (10 μm thick) of each frozen digestive gland were cut in a Leica CM 3000 cryotome (Leica Instruments, Wetzlar, Germany) onto successive serial slides and stored at −40 °C until processing. The stability of the lysosomal membrane was based on the time of acid labilization required to produce the maximum staining intensity in digestive cell lysosomes after the detection of *N*-acetylhexosaminidase activity according to UNEP/RAMOGE (1999). Results are expressed as time in minutes (min).

2.6. Cell type composition of the digestive tubules

Histological sections obtained from the same paraffin embedded samples processed in Section 2.4. were stained with hematoxylin-eosin (Gamble and Wilson, 2002). Digestive and basophilic cells were counted in three randomly selected areas with a drawing tube attached to a Leitz Laborlux S (Wetzlar, Germany) light microscope at 40× magnification. Then, changes in the cell-type composition of the digestive tubule epithelium were determined as volume density of basophilic cells (Vv_{BAS}) after applying a stereological point counting procedure (Soto et al., 2002): $Vv_{BAS}=V_{BAS}/(V_{BAS}+V_{DC})$; where V_{BAS} is the volume of basophilic cells and V_{DC} is the volume of digestive cells. Vv_{BAS} is expressed as $\mu m^3/\mu m^3$.

2.7. Genotoxicity in hemocytes

Hemolymph of 10 mussels per treatmet and exposure time (1, 7 or 21 days) was extracted and transferred to a Falcon tube containing 2 mL of 10 mM EDTA/SW for the micronuclei test in both seasons and the comet assay in spring.

2.7.1. Micronuclei test

Hemolymph solution (200 μL) of 8 mussels was cytocentrifuged at 700 rpm for 2 min using a cytocentrifuge (Cytopro® Cytocentrifuge Series 2, ELITechGroup, Utah, United States). Slides were air dried for 20 min and then hemocytes were stained using the Hemacolor® Kit (Merck, Darmstadt, Germany) following manufacturer's instructions. Slides were air dried overnight and then mounted with DPX mounting medium. 1000 randomly selected a granular hemocytes per mussel were observed under the light microscope (Nikon Eclipse *Ni* microscope, Nikon Instruments, Tokyo, Japan) at 100× magnification. Micronucleated cells were classified following the accepted criteria for mussels: well-preserved cell cytoplasm, micronuclei (MN) not touching the main nucleus, similar or weaker staining than the main nucleus and size of MN ≤ 1/3 in comparison to the main nucleus (Venier et al., 1997). Results are reported in ‰ frequencies.

2.7.2. Comet assay

Comet assay was performed in mussel hemocytes according to Raisuddin and Jha (2004) with some modifications reported by Katsumiti et al. (2014). Samples obtained in autumn were accidentally lost and thus only samples obtained in spring were analyzed. Briefly, 100 μL of hemolymph of 10 mussels per experimental group were centrifuged (300 *g* × 10 min at 4 °C) and supernatant was removed. The pellet containing hemocytes was resuspended with 200 μL of 0.5% low melting point agarose. Two drops (100 μL each) of hemocytes suspension were placed on the frosted ends of slides coated with normal melting point agarose (1% in phosphate buffered saline solution). Slides were chilled for 10 min on ice and then immersed in chilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% N-lauroyl-sarcosine sodium salt, 1% Triton X-100, and 10% DMSO, adjusted to pH 10) for 1 h in darkness. Then, slides were washed twice with distilled water and transferred to an electrophoresis tank containing electrophoresis buffer (150 mM NaOH and 300 mM EDTA, pH 13). After 20 min of incubation, electrophoresis was carried out for 30 min (300 mA, 25 V). Finally, samples were immersed in neutralization buffer (0.4 M Tris-HCl buffer, pH 7.5) for 10 min, fixed with methanol (−20 °C) for 3 min and stored at 4 °C until image analysis. For that, samples were stained with 20 μL of ethidium bromide (2 μg/mL in distilled water) and directly observed under an Olympus BX61 fluorescence microscope (Olympus optical Co., Hamburg, Germany). 100 randomly selected cells were analyzed from each slide (50 in each gel from duplicate slides) and scored using the Komet 5.5 image analysis software (Andor Imaging, Liverpool, UK). Results are expressed as percentage (%) of tail DNA.

2.8. Statistical analysis

The statistical package SPSS v.22 (SPSS Inc., IBM Company, Chicago, USA) was used for statistical analysis. Results represented as percentages were subjected to arcsine transformation before statistical analysis, following Sokal and Rholf (1969). The Mann-Whitney's *U* test for pairwise comparisons in autumn and the Kruskal-Wallis test followed by the Dunn's test for multiple comparisons in spring were applied to establish significant differences with respect to controls. Season-dependent differences were established only at the higher exposure dose within each exposure time based on the Kruskal-Wallis test followed by the Dunn's test in all studied endpoints. A significance level of 5% was set globally ($p < .05$).

3. Results

3.1. Accumulation of Ag in mussel soft tissues

Ag was not accumulated after 1 day of dietary exposure to 1 or 10 μg Ag/L Ag NPs in autumn nor in spring (Fig. 1 A,B). After 7 days of dietary exposure to 10 μg Ag/L Ag NPs, Ag was significantly accumulated in spring (Fig. 1 B) and Ag accumulation was significantly higher in spring in comparison to autumn (Fig. 1 A,B). Ag was significantly accumulated after 21 days of dietary exposure to 10 μg Ag/L Ag NPs both in autumn (Fig. 1 A) and in spring (Fig. 1 B). Ag accumulation in mussel soft tissues after 21 days of exposure to 10 μg Ag/L Ag NPs was significantly higher in autumn (0.73 μg Ag/g d.w.) than in spring (0.35 μg Ag/g d.w.) (Fig. 1 A,B).

3.2. Intralysosomal metal accumulation by autometallography

Results of the volume density of BSDs in lysosomes of digestive cells indicated that in both seasons intralysosomal metal accumulation was significantly higher after the dietary exposure to 10 μg Ag/L Ag NPs in comparison to non-exposed mussels (Fig. 2 A,B). No significant differences in intralysosomal metal accumulation were observed between seasons (Fig. 2 A,B).

3.3. Alterations in lysosomal membrane stability

A significant reduction of lysosomal membrane stability was measured in both seasons after the dietary exposure of mussels to 10 μg Ag/L Ag NPs (Fig. 3 A,B). Both in autumn and in spring, lysosomal membrane stability was significantly reduced in exposed mussels compared to non-exposed ones at all exposure times (Fig. 3 A,B). A dose-dependent response was observed in spring, although differences with respect to controls at 1 μg Ag/L Ag NPs were not significant (Fig. 3 B). Mussels lysosomal membrane stability was similarly affected in both seasons (Fig. 3 A, B).

3.4. Cell type composition of the digestive tubules

The volume density of basophilic cells of mussels digestive gland epithelium was not altered after the dietary exposure to 10 μg Ag/L Ag NPs in autumn (Fig. 4 A) and 1 μg Ag/L or 10 μg Ag/L Ag NPs in spring (Fig. 4 B) for 1, 7 and 21 days. In non-exposed mussels, the volume density of basophilic cells was significantly higher in spring compared to autumn at day 1 (Fig. 4 A,B).

3.5. Genotoxicity in hemocytes

3.5.1. Micronuclei test

In autumn, micronuclei frequency increased after 1, 7 and 21 days of dietary exposure to 10 μg Ag/L Ag NPs but differences with respect to controls were significant only at day 1 (Table 1). In spring, the increase in micronuclei frequency was dose-dependent after 1 and 7 days of dietary exposure being this increase significant with respect to controls after the dietary exposure to 10 μg Ag/L Ag NPs for 7 days (Table 1). The response to 10 μg Ag/L Ag NPs was similar in both seasons showing an increasing trend in micronuclei frequency along the 21 days of exposure with respect to controls, but a decrease in micronuclei levels at day 21 compared to days 1 and 7 (Table 1). Season-dependent differences were not observed in the micronuclei frequency within the same exposure time.

3.5.2. Comet assay

The nuclei of hemocyte cells showed strand breaks in DNA after dietary exposure of mussels both to 1 and 10 μg Ag/L of Ag NPs at all exposure times in spring (Fig. 5). The quantification of these strand breaks in DNA revealed a significant increase of DNA damage with respect to controls after the dietary exposure to 1 or 10 μg Ag/L Ag NPs at all exposure times (Fig. 6). DNA strand breaks were also significantly higher after the dietary exposure to 10 μg Ag/L Ag NPs compared to 1 μg Ag/L Ag NPs at all exposure times (Fig. 6).

4. Discussion

Marine environments are likely to be the ultimate sink for any NP, where organisms can be exposed through water, diet or both. It has been widely reported that waterborne Ag NPs affect to cellular

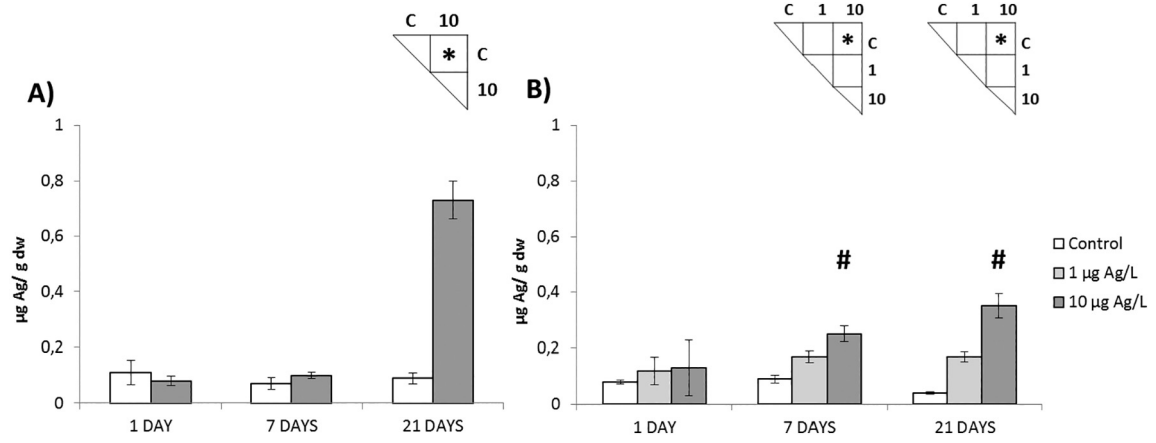

Fig. 1. Bioaccumulation of Ag in mussels (μg Ag/g d.w.) exposed through the diet for 1, 7 and 21 days to A) 10 μg Ag/L Ag NPs in autumn and to B) 1 μg Ag/L or 10 μg Ag/L of Ag NPs in spring. Values are given as means ± S.D. Mean values belong to 4 pools of 5 individuals per exposure group. Significant differences with respect to controls based on the Mann-Whitney's U test in autumn and the Dunn's test in spring are shown in the upper triangular matrices ($p < .05$). Significant differences ($p < .05$) between seasons within the same day are indicated with #.

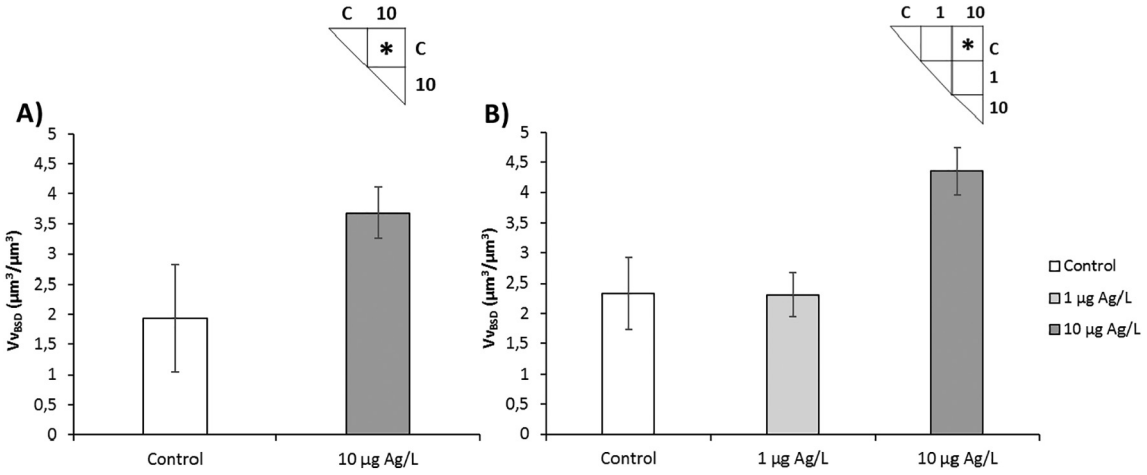

Fig. 2. Intralysosomal metal accumulation as volume density of BSDs (Vv_{BSD}; $\mu m^3/\mu m^3$) in mussel digestive cells after 21 days of dietary exposure to A) 10 µg Ag/L of Ag NPs in autumn and to B) 1 µg Ag/L or to 10 µg Ag/L of Ag NPs in spring. Values are given as means ± S.D. Significant differences with respect to controls based on the Mann-Whitney's U test in autumn and the Dunn's test in spring are shown by asterisks ($p < .05$).

mechanisms involved in nano-internalization, induce DNA damage and alter the protein expression, the antioxidant cellular defense as well as the immune response in marine invertebrates (reviewed in Magesky and Pelletier, 2018 and Canesi et al., 2019). In the present study, cell and tissue level responses in mussels *M. galloprovincialis* dietarily exposed to PVP/PEI coated 5 nm Ag NPs at a dose close to environmentally relevant concentrations and at a higher dose were assessed in spring and autumn.

It has been shown that marine bivalves exposed to different types of Ag NPs usually accumulate low concentrations of Ag in their tissues (Buffet et al., 2013, 2014; Jimeno-Romero et al., 2017a) similar as in the present study. Although an increasing trend of Ag accumulation was observed after the dietary exposure to 1 µg Ag/L Ag NPs in spring, this accumulation was not statistically significant. On the other hand, Ag was significantly accumulated after 7 days of dietary exposure to 10 µg Ag/L Ag NPs in spring, being Ag accumulation higher in spring compared to autumn. Mussel soft tissues significantly accumulated Ag after 21 days of dietary exposure to 10 µg Ag/L Ag NPs in both seasons, but higher levels of Ag (around 2-fold) accumulated in autumn than in spring. These differences in mussel metal concentrations can arise from changes in the physiology of animals dependent on the season, rather than from changes in conditions of metal exposure (Mubiana et al., 2005) since the latter were similar regarding temperature, food ration and salinity in the two seasons in the present work. The effect of seasonal development of gonadic tissues on whole body weight in bivalves has been shown to biologically dilute the total burden of different metals such as Cu, Zn, Ag, Cd, Cu and Pb, thus resulting in lower concentrations of metals during the gametogenesis period (Regoli and Orlando, 1994; Páez-Osuna et al., 1995; Fattorini et al., 2008; Lanceleur et al., 2011). Hence, the lower concentration of Ag measured in mussel soft tissues in spring could be linked to increased body weight related to the development of gametes, that took place in spring (Duroudier et al., 2019a). In order to avoid variability in metal accumulation due to variations in soft-body weight related to the season, the measurement of the metal/shell-weight index could be useful for future studies (Soto et al., 1995).

The main organ for accumulation of NPs in bivalves is the digestive gland (Canesi and Corsi, 2016). In fact, in a previous study (Duroudier et al., 2019a) using the same experimental design as in the present one, presence of Ag NPs was assessed in mussel tissue sections using a hyperspectral imaging system and Ag in nano form was found mainly in digestive tubule cells and lumen in both seasons, clearly indicating that transfer of Ag NPs from microalgae to mussels took place. Thus, intralysosomal metal accumulation was measured in this organ. Several studies have already reported a significant intralysosomal metal accumulation in digestive cells of mussels waterborne exposed to different types of metal-containing NPs (Jimeno-Romero et al., 2017a, 2017b, 2019). Waterborne exposure of mussels to different sized Ag NPs provoked a dose-dependent increase in intralysosomal metal accumulation (Jimeno-Romero et al., 2017a). Mussels and zebrafish dietarily exposed

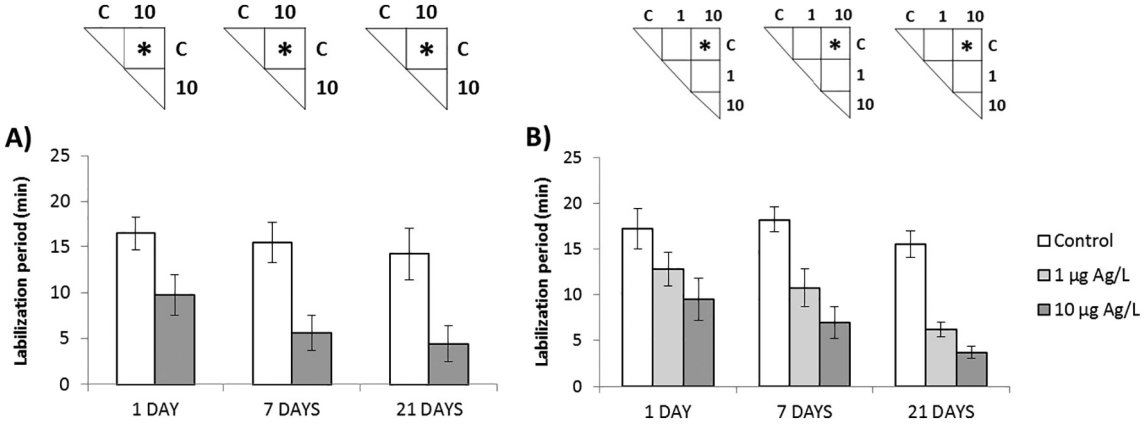

Fig. 3. Labilization period (min) of lysosomal membrane in mussels digestive cells after 1, 7 and 21 days of dietary exposure to A) 10 µg Ag/L Ag NPs in autumn and to B) 1 µg Ag/L or 10 µg Ag/L of Ag NPs in spring. Values are given as means ± S.D. Significant differences with respect to controls based on the Mann-Whitney's U test in autumn and the Dunn's test in spring are shown by asterisks ($p < .05$).

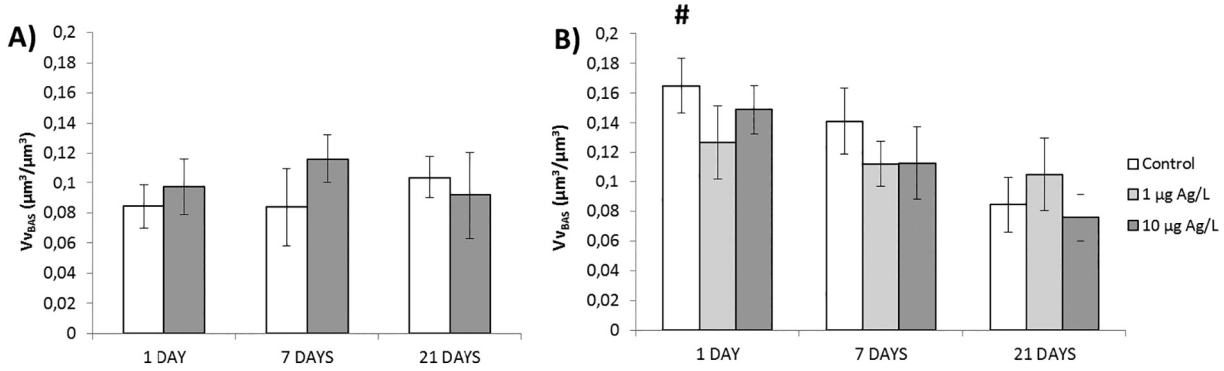

Fig. 4. Volume density of basophilic cells (Vv_{BAS}; $\mu m^3/\mu m^3$) in the digestive tubules of mussels exposed through the diet to A) 10 µg Ag/L Ag NPs in autumn and to B) 1 µg Ag/L or 10 µg Ag/L of Ag NPs in spring for 1, 7 and 21 days. Values are given as means ± S.E. No significant differences with respect to controls were observed. Significant differences ($p < .05$) between seasons within the same day are indicated with #.

to the same Ag NPs studied in the present work, showed a dose-dependent increase in intralysosomal metal accumulation in digestive cells of mussels in spring (Duroudier et al., 2019b) and in liver and intestine of zebrafish (Lacave et al., 2017). In the present work, a significant intralysosomal metal accumulation occurred in mussels digestive cells dietarily exposed to 10 µg Ag/L Ag NPs for 21 days in both seasons, but not for the 1 µg Ag/L Ag NPs dose,suggesting that the exposure concentration or time were not high or long enough to cause a significant response, similar to results of ICP-MS. Additionally, the few BSDs quantified in control digestive cell lysosomes could be due to the high levels of Cu measured in wild mussels inhabiting the same estuary (Besada et al., 2011) since autometallography is not metal-specific and BSDs do not reflect only Ag levels (Marigómez et al., 2002). However, the significant higher volume density of BSDs determined in the digestive tubules of mussels exposed to 10 µg Ag/L Ag NPs, as well as results measured by ICP-MS, indicate that observed BSDs reflected the presence of Ag, in either dissolved or particulate form.

In mussels, lysosomal perturbations such as the destabilization of the lysosomal membrane are considered early indicators of adverse effects provoked by an array of different factors, including exposure to pollutants (Cajaraville et al., 2000; Moore et al., 2006). Damage to lysosomal membranes can cause the release of acid hydrolases into the cytosol, possibly leading to a more severe damage and to cell death (Viarengo et al., 2007). Destabilization of the lysosomal membrane has been widely reported in digestive cells of mussels exposed to metal-containing NPs such as TiO_2 NPs (Barmo et al., 2013; Balbi et al., 2014), Au NPs (Jimeno-Romero et al., 2017b), CdS QDs (Jimeno-Romero et al., 2019) as well as Ag NPs (Jimeno-Romero et al., 2017a). In accordance with the reported studies, in the present work the dietary exposure of mussels to the high dose of PVP/PEI coated 5 nm Ag NPs for 1, 7 and 21 days provoked a time-dependent decrease in lysosomal membrane stability in both seasons, suggesting a general stress response in dietarily exposed mussels that could be also related to the measured intralysosomal metal accumulation in digestive tubules. Even if not statistically significant, a decreasing trend in lysosomal membrane stability was also observed after the dietary exposure of mussels to the low dose, indicating that low concentrations of Ag NPs could also affect mussel general health status.

Further, the cell type composition of the digestive gland epithelium in molluscs can be altered due to environmental factors or to the exposure to pollutants (Cajaraville et al., 1990; Garmendia et al., 2011; Bignell et al., 2012). Thus, the increase in the relative proportion of basophilic cells in mussel digestive tubules as a result of digestive cell loss and basophilic cell hypertrophy is also considered a general stress condition (Cajaraville et al., 1990; Garmendia et al., 2011). According to previous studies, waterborne exposure of mussels to different types of metal-containing NPs affected the cell type composition of the digestive gland epithelium increasing the proportion of basophilic cells (Rocha et al., 2016; Jimeno-Romero et al., 2017a, 2019). After 1 and 21 days of exposure, a higher basophilic cell volume density was measured in mussels waterborne exposed to two differently sized Ag NPs, as well as to ionic Ag and bulk Ag (Jimeno-Romero et al., 2017a). However, in the present study, cell type composition in the digestive gland of mussels dietarily exposed to Ag NPs was not altered along the exposure period nor in autumn nor in spring. This disagreement could be related to differences in the exposure route, exposure concentrations and/or NP characteristics such as surface coating and size.

NPs can induce indirectly DNA damage by oxidative stress or can directly interact with DNA due to their small size and high surface area (Singh et al., 2009). Among the methods developed for detecting DNA damage, the measurement of DNA strand breaks by the comet assay and the chromosomal DNA damage by the micronucleus test are the most used techniques in bivalves (Mitchelmore and Chipman, 1998; Lee and Steinert, 2003; Bolognesi and Hayashi, 2011). In this sense, the application of the comet assay in several *in vitro* (Katsumiti et al., 2014, 2015, 2018; Volland et al., 2018) and *in vivo* investigations (Gomes et al., 2013; Buffet et al., 2014; Mouneyrac et al., 2014; Rocha et al., 2014) revealed that exposure to different types of metal-based NPs, including Ag NPs, induced DNA damage in bivalves. Maltose stabilized Ag NPs produced DNA damage at 1.25 and 2.5 mg Ag/L in mussel hemocytes according to *in vitro* studies (Katsumiti et al., 2015). Waterborne exposure to 10 µg /L Ag NPs increased the percentage of tail DNA with the exposure time in mussel hemocytes (Gomes et al., 2013) and induced a higher genotoxicity in the digestive gland of *S. plana* clams than soluble Ag after 21 days of exposure (Buffet et al., 2014). In agreement with these waterborne exposure studies, in the present work the

Table 1
Micronuclei frequency (‰) in hemocytes of mussels exposed through the diet to 10 µg Ag/L Ag NPs in autumn and to 1 µg Ag/L or 10 µg Ag/L of Ag NPs in spring. Values are given as means ± S.E. Significant differences with respect to controls based on the Mann-Whitney's *U* test in autumn and the Dunn's test in spring are shown by asterisks ($p < .05$).

	Autumn			Spring		
	1 DAY	7 DAYS	21 DAYS	1 DAY	7 DAYS	21 DAYS
Control	0.13 ± 0.13	0.75 ± 0.31	0.25 ± 0.16	0.75 ± 0.37	0.375 ± 0.18	0.875 ± 0.40
1 µg Ag/L Ag NPs	–	–	–	1.85 ± 0.85	1.0 ± 0.27	1.28 ± 0.44
10 µg Ag/L Ag NPs	1.63 ± 0.42 *	1.75 ± 0.70	0.75 ± 0.36	2.0 ± 0.71	2.5 ± 0.5 *	1.25 ± 0.36

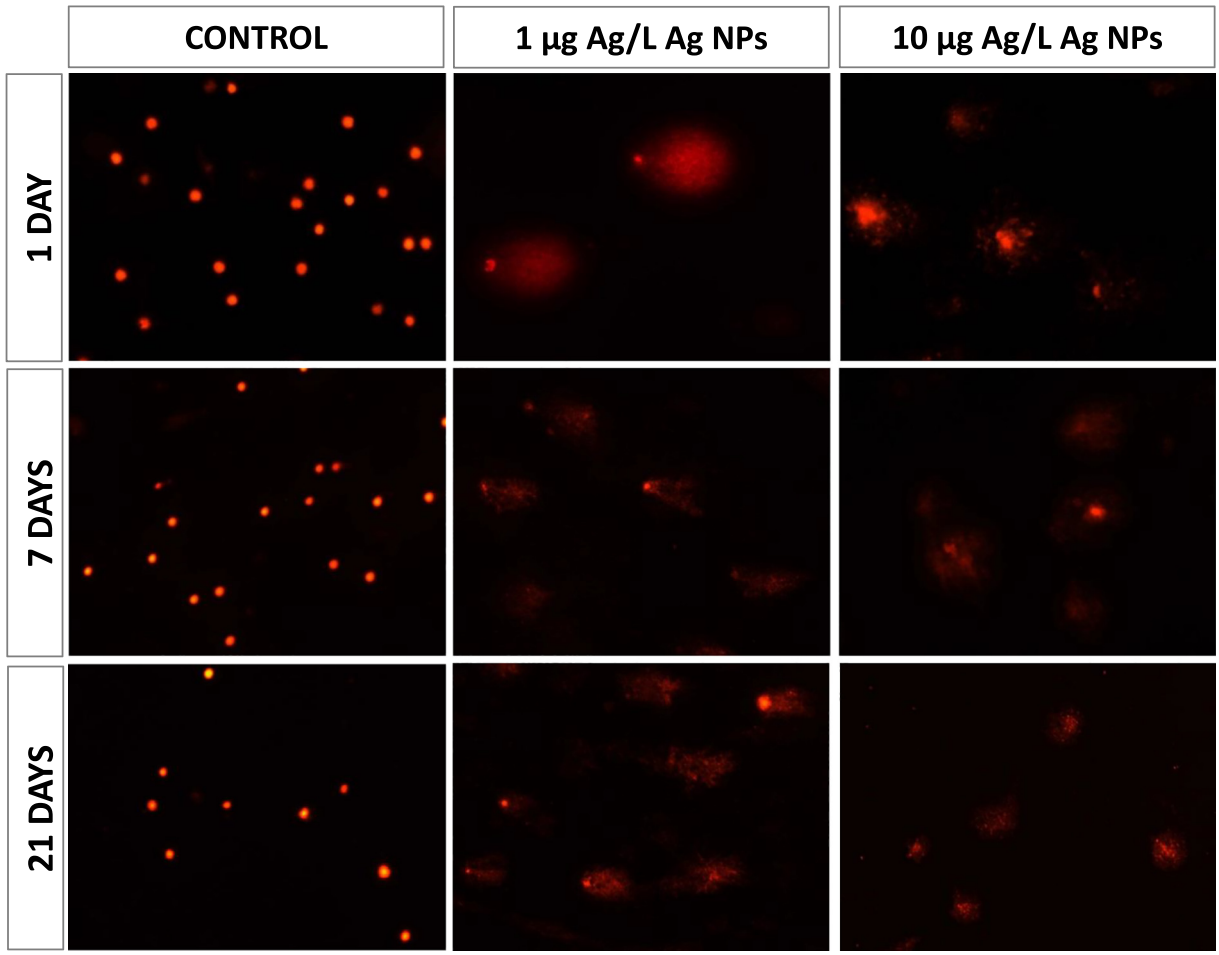

Fig. 5. Micrographs showing strand breaks observed in hemocytes of mussels exposed to 1 µg Ag/L or 10 µg Ag/L Ag NPs for 1, 7 and 21 days in spring.

dietary exposure of mussels to Ag NPs in spring provoked a significant dose-dependent increase of DNA damage in exposed mussels, even at the low dose approaching environmentally realistic concentrations at all exposure times.

Additionally, an increasing trend in micronuclei frequency was also observed after 1 and 7 days of dietary exposure to 10 µg Ag/L Ag NPs both in autumn and in spring, similar to that reported after 4 days of exposure to TiO_2 NPs (Rocco et al., 2015) or 21 days of exposure to CuO NPs (Ruiz et al., 2015). However, after 21 days of dietary exposure to the same dose, micronuclei frequency values decreased with respect to levels recorded at days 1 and 7 in both seasons. Micronuclei are formed when the levels of double strand breaks in DNA exceed the repair capacity of dividing cells (Luzhna et al., 2013) and chromosomal DNA damage occurs as a result of either chromosome breakage or chromosome mis-segregation during mitosis (Bolognesi and Fenech, 2012). Thus, the decrease of micronuclei frequencies could suggest the elimination of damaged cells by apoptosis (Luzhna et al., 2013) or the activation of DNA repair mechanisms. The activation of the p53 tumor suppressor gene has been described as responsible for arresting the cell cycle and activating transcription of genes that mediate DNA repair, thus preventing the conversion of damage to mutation (Singh et al., 2009). In fact, mussels exposed to 0.01 mg/L ZnO NPs for 28 days showed an up-regulation of p53 already after 72 h of exposure (Li et al., 2018).

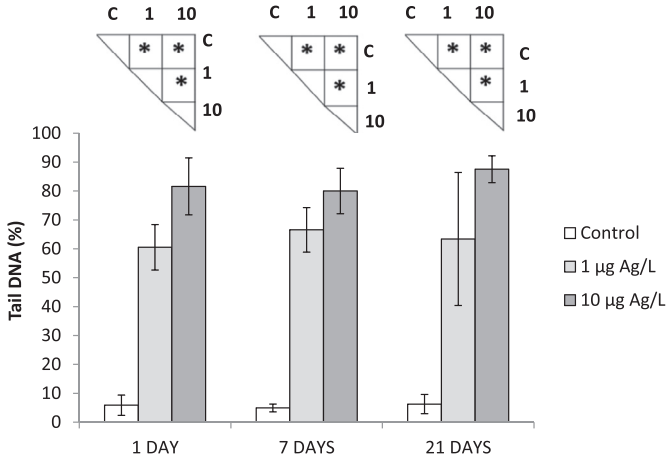

Fig. 6. DNA damage (% tail DNA) in hemocytes of mussels exposed through the diet to 1 µg Ag/L or 10 µg Ag/L Ag NPs in spring according to the comet assay. Values are given as means ± S.D. Significant differences between pairs of means are shown in the upper triangular matrices based on the Dunn's test ($p < .05$).

In the present work, genotoxicity assessed by the comet assay was the most sensitive biomarker of those tested. In fact, this assay is considered more sensitive than other available methods for the assessment of genotoxic effects since DNA strand breaks form very quickly and allow the detection of early genotoxic responses (Mitchelmore and Chipman, 1998; Frenzilli et al., 2009). But, interestingly, the comet assay and the micronuclei test give complementary information that is crucial to understand the time course of genotoxic effects caused by pollutants (Frenzilli et al., 2009) such as NPs. Even if several works have reported seasonal variations in genotoxic biomarkers (Pisanelli et al., 2009; Schmidt et al., 2013) as well as in effect biomarkers such as lysosomal parameters (Hagger et al., 2010; Nahrgang et al., 2013; Balbi et al.,

2017), in the present study, season did not influence on the selected cellular biomarkers after the dietary exposure to Ag NPs. Changes in water temperature and salinity related to season have been described as natural environmental factors responsible for alterations in lysosomal membrane stability and baseline micronuclei frequency values in mussels (Regoli, 1992; Domouhtsidou and Dimitriadis, 2001; Bolognesi and Hayashi, 2011). The seasonal gamete developmental cycle is also known to affect biomarker responses (Bayne and Widdows, 1978; Solé et al., 1995; Cancio et al., 1999; Sheehan and Power, 1999). In this work, temperature, salinity, food ration and other exposure conditions were similar in the two seasons whereas gamete developmental stage differed (early gametogenesis in autumn *versus* advanced gametogenesis in spring) (Duroudier et al., 2019c). In spite of this, selected cellular biomarkers responded similarly to the dietary Ag NP exposure in the two studied seasons, underlining their reliability as effect assessment tools in Nanotoxicology. This is in contrast to responses at transcriptome and proteome levels, which showed a clear season-dependent pattern after dietary Ag NP exposure (Duroudier et al., 2019a, 2019c).

5. Conclusions

The transfer of PVP/PEI coated 5 nm Ag NPs from microalgae to mussels caused significant cellular responses in mussels which were comparable to those reported in waterborne exposure studies to Ag NPs. Ag was significantly accumulated in mussels after 21 days of exposure to the high dose of Ag NPs through the diet both in autumn and in spring. Although higher levels of Ag were accumulated in autumn in comparison to spring, intralysosomal metal accumulation in digestive cells in comparison to controls was similar in both seasons. Lysosomal membrane stability decreased in a dose- and time-dependent manner showing a general stress response in mussels in both seasons. Additionally, Ag NPs ingested through the diet caused genotoxic effects in mussel hemocytes even at the low dose approaching environmentally realistic concentrations. On the other hand, the increase in micronuclei frequency was transitory in both seasons, suggesting the activation of DNA repair mechanisms. Overall, PVP/PEI coated 5 nm Ag NPs ingested through the food web caused similar cellular effects in mussels both in autumn and in spring, suggesting that selected cellular biomarkers are stable enough to be applied in sentinel mussels at different seasons.

CRediT authorship contribution statement

Nerea Duroudier: Methodology, Visualization, Formal analysis, Writing - original draft. **Alberto Katsumiti:** Methodology. **Mathilde Mikolaczyk:** Methodology. **Jörg Schäfer:** Supervision, Writing - review & editing. **Eider Bilbao:** Supervision, Writing - review & editing. **Miren P. Cajaraville:** Conceptualization, Resources, Project administration, Funding acquisition, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

António, D.C., Cascio, C., Jakšić, Ž., Jurašin, D., Lyons, D.M., Nogueira, A.J., Rossi, F., Calzolai, L., 2015. Assessing silver nanoparticles behaviour in artificial seawater by mean of AF4 and spICP-MS. Mar. Environ. Res. 111, 162–169.

Balbi, T., Smerilli, A., Fabbri, E., Ciacci, C., Montagna, M., Graselli, E., Brunelli, A., Pojana, G., Marcomimi, A., Gallo, G., Canesi, L., 2014. Co-exposure to n-TiO_2 and Cd^{2+} results in interactive effects on biomarker responses but not in increased toxicity in the marine bivalve *M. galloprovincialis*. Sci. Total Environ. 493, 355–364.

Balbi, T., Fabbri, R., Montagna, M., Camisassi, G., Canesi, L., 2017. Seasonal variability of different biomarkers in mussels (*Mytilus galloprovincialis*) farmed at different sites of the Gulf of La Spezia, Ligurian sea, Italy. Mar. Pollut. Bull. 116, 348–356.

Banni, M., Sforzini, S., Arlt, V.M., Barranguer, A., Dallas, L.J., Oliveri, C., Aminot, Y., Pacchioni, B., Millino, C., Lanfranchi, G., Readman, J.W., Moore, M.N., Viarengo, A., Jha, A.N., 2017. Assessing the impact of benzo[a]pyrene on marine musels: application of a novel targeted low density microarray complementing classical biomarker responses. PLoS One 12 (6), e0178460.

Barmo, C., Ciacci, C., Canonico, B., Fabbri, R., Cortesec, K., Balbi, T., Marcomini, A., Pojana, G., Gallo, G., Canesi, L., 2013. In vivo effects of n-TiO_2 on digestive gland and immune function of the marine bivalve *Mytilus galloprovincialis*. Aquat. Toxicol. 132-133, 9–18.

Bayne, B.L., Widdows, J., 1978. The physiological ecology of two populations of *Mytilus edulis* L. Oecologia 37, 137–162.

Bebianno, M.J., Gonzalez-Rey, M., Gomes, T., Mattos, J.J., Flores-Nunes, F., Bainy, A.C.D., 2015. Is gene transcription in mussel gills altered after exposure to Ag nanoparticles? Environ. Sci. Pollut. Res. 22, 17425–17433.

Besada, V., Andrade, J.M., Schultze, S., González, J.J., 2011. Monitoring of heavy metals in wild mussels (*Mytilus galloprovincialis*) from the Spanish North-Atlantic coast. Continenal Shelf Research 31, 457–465.

Bignell, J., Cajaraville, M.P., Marigómez, I., 2012. Chapter 15: background document: histopathology of mussels (*Mytilus* spp.) for health assessment in biological effects monitoring. Integrated monitoring of chemicals and their effects. In: Davies, I.M., Vethaak, A.D. (Eds.), ICES Cooperative Research Report N. 315, Copenhagen, Denmark, pp. 111–120.

Blaser, S.A., Scheringer, M., Macleod, M., Hungerbühler, K., 2008. Estimation of cumulative aquatic exposure and risk due to silver: contribution of nano-functionalized plastics and textiles. Sci. Total Environ. 390, 396–409.

Bolognesi, C., Fenech, M., 2012. Mussel micronucleus cytome assay. Nat. Protoc. 7, 1125–1137.

Bolognesi, C., Hayashi, M., 2011. Micronucleus assay in aquatic animals. Mutagenesis 26, 205–213.

Buffet, P.E., Pan, J.F., Poirier, L., Amiard- Triquet, C., Amiard, J.C., Gaudin, P., Risso. De Faverney, C., Guibbolini, M., Gilliland, D., Valsami-Jones, E., Mouneyrac, C., 2013. Biochemical and behavioural responses of the endobenthic bivalve *Scrobicularia plana* to silver nanoparticles in seawater and microalgal food. Ecotoxicol. Environ. Saf. 89, 117–124.

Buffet, P.E., Zalouk-Vergnoux, A., Châtel, A., Berthet, B., Métais, I., Perrein-Ettajani, H., Poirier, L., Luna-Acosta, A., Thomas-Guyon, H., Risso-de Faverney, C., Guibbolini, M., Gilliland, D., Valsami-Jones, E., Mouneyrac, C., 2014. A marine mesocosm study on the environmental fate of silver nanoparticles and toxicity effects on two endobenthic species: the ragworm *Hediste diversicolor* and the bivalve mollusc *Scrobicularia plana*. Sci. Total Environ. 470-471, 1151–1159.

Cajaraville, M.P., Pal, S.G., 1995. Morphofunctional study of the haemocytes of the bivalve mollusc *Mytilus galloprovincialis* with emphasis on the endolysosomal compartment. Cell Struct. Funct. 20, 355–367.

Cajaraville, M.P., Díez, G., Marigómez, I.A., Angulo, E., 1990. Responses of basophilic cells of the digestive gland of mussels to petroleum hydrocarbon exposure. Dis. Aquat. Org. 9, 221–228.

Cajaraville, M.P., Bebianno, M.J., Blasco, J., Porte, C., Sarasquete, C., Viarengo, A., 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. Sci. Total Environ. 247, 295–311.

Cancio, I., Ibabe, A., Cajaraville, M.P., 1999. Seasonal variation of peroxisomal enzyme activities and peroxisomal structure in mussels *Mytilus galloprovincialis* and its relationship with the lipid content. Comparative Biochemistry and Physiology, Part C 123, 135–144.

Canesi, L., Corsi, I., 2016. Effects of nanomaterials on marine invertebrates. Sci. Total Environ. 565, 933–940.

Canesi, L., Barmo, C., Fabbri, R., Ciacci, C., Vergani, L., Roch, P., Gallo, G., 2010. In vitro effects of suspensions of selected nanoparticles (C60 fullerene, TiO_2, SiO_2) on *Mytilus* hemocytes. Aquat. Toxicol. 96, 151–158.

Canesi, L., Ciacci, C., Fabbri, R., Marcomini, A., Pojana, G., Gallo, G., 2012. Bivalve molluscs as a unique target group for nanoparticle toxicity. Mar. Environ. Res. 76, 16–21.

Canesi, L., Frenzilli, G., Balbi, T., Bernardeschi, M., Ciacci, C., Corsolini, S., Della Torre, C., Fabbri, R., Faleri, C., Focardi, S., Guidi, P., Kočan, A., Marcomini, A., Mariottini, M., Nigro, M., Pozo-Gallardo, K., Rocco, L., Scarcelli, V., Smerilli, A., Corsi, I., 2014. Interactive effects of n-TiO_2 and 2,3,7,8-TCDD on the marine bivalve *Mytilus galloprovincialis*. Aquat. Toxicol. 153, 53–65.

Canesi, L., Auguste, M., Bebianno, M.J., 2019. Sublethal effects of nanoparticles on aquatic invertebrates: from molecular to organism level. In: J BLASCO, I.C.O.R.S.I. (Ed.), Ecotoxicology of Nanoparticles in Aquatic Systems. Science Publishers CRC Press/Taylor & Francis Group, Boca Raton, London, New York, pp. 62–98.

Chio, C.P., Chen, W.Y., Chou, W.W., Hsieh, N.H., Ling, M.P., Liao, C.M., 2012. Assessing the potential risks to zebrafish posed by environmentally relevant copper and silver nanoparticles. Sci. Total Environ. 420, 111–118.

Ciacci, C., Canonico, B., Bilaniĉovă, D., Fabbri, R., Cortese, K., Gallo, G., Marcomini, A., Pojana, G., Canesi, L., 2012. Immunomodulation by different types of N-oxides in

the hemocytes of the marine bivalve *Mytilus galloprovincialis*. PLoS One 7 (5), e36937. https://doi.org/10.1371/journal.pone.0036937.
Corsi, I., Cherr, G.N., Lenihan, H.S., Labille, J., Hassellov, M., Canesi, L., Dondero, F., Frenzilli, G., Hristozov, D., Puntes, V., Della Torre, C., Pinsino, A., Libralato, G., Marcomini, A., Sabbioni, E., Matranga, V., 2014. Common strategies and technologies for the ecosafety assessment and design of nanomaterials entering the marine environment. ACS Nano 8, 9694–9709.
Domouhtsidou, G.P., Dimitriadis, V.K., 2001. Lysosomal and lipid alterations in the digestive gland of mussels, *Mytilus galloprovincialis* (L.) as biomarkers of environmental stress. Environ. Pollut. 115, 123–137.
Dumont, E., Johnson, A.C., Keller, V.D.J., Williams, R.J., 2015. Nano silver and nano zinc-oxide in surface waters-exposure estimation for Europe at high spatial and temporal resolution. Environ. Pollut. 196, 341–349.
Duroudier, N., Cardoso, C., Mehennaoui, K., Mikolaczyk, M., Schäfer, J., Gutleb, A.C., Giamberini, L., Bebianno, M.J., Bilbao, E., Cajaraville, M.P., 2019a. Changes in protein expression in mussels *Mytilus galloprovincialis* dietarily exposed to PVP/PEI coated silver nanoparticles at different seasons. Aquat. Toxicol. 210, 56–68.
Duroudier, N., Katsumiti, A., Mikolaczyk, M., Schäfer, J., Bilbao, E., Cajaraville, M.P., 2019b. Dietary exposure of mussels to PVP/PEI coated Ag nanoparticles causes Ag accumulation in adults and abnormal embryo development in their offspring. Sci. Total Environ. 655, 48–60.
Duroudier, N., Markaide, P., Cajaraville, M.P., Bilbao, E., 2019c. Season influences the transcriptomic effects of dietary exposure to PVP/PEI coated Ag nanoparticles on mussels *Mytilus galloprovincialis*. Comparative Biochemistry and Physiology, Part C 222, 19–30.
Fabrega, J., Luoma, S.N., Tyler, C.R., Galloway, T.S., Lead, J.R., 2011. Silver nanoparticles: behaviour and effects in the aquatic environment. Environ. Int. 37, 517–531.
Fattorini, D., Notti, A., Di Mento, R., Cicero, A.M., Gabellini, M., Russo, A., Regoli, F., 2008. Seasonal, spatial and inter-annual variations of trace metals in mussels from the Adriatic sea: a regional gradient for arsenic and implications for monitoring the impact of off-shore activities. Chemosphere 72, 1524–1533.
Frenzilli, G., Nigro, M., Lyons, B.P., 2009. The comet assay for the evaluation of genotoxic impact in aquatic environments. Mutat. Res. 681, 80–92.
Gamble, M., Wilson, I., 2002. The hematoxylins and eosin. In: Brancfort, J.D., Gamble, M. (Eds.), Theory and Practice of Histological Techniques. Churchill Livingstone- Elsevier Science Ltd, London, UK, p. 125.
Garmendia, L., Soto, M., Vicario, U., Kim, Y., Cajaraville, M.P., Marigómez, I., 2011. Application of a battery of biomarkers in mussel digestive gland to assess long-term effects of the Prestige oil spill in Galicia and Bay of Biscay: tissue-level biomarkers and histopathology. J. Environ. Monit. 13, 915–932.
Giese, B., Klaessig, F., Park, B., Kaegi, R., Steinfeldt, M., Wigger, H., von Gleich, A., Gottschalk, F., 2018. Risks, release and concentrations of engineered nanomaterial in the environment. Sci. Rep. 8. https://doi.org/10.1038/s41598-018-19275-4.
Gomes, T., Araújo, O., Pereira, R., Almeida, A.C., Cravo, A., Bebianno, M.J., 2013. Genotoxicity of copper oxide and silver nanoparticles in the mussel *Mytilus galloprovincialis*. Mar. Environ. Res. 84, 51–59.
Gomes, T., Pereira, C.G., Cardoso, C., Sousa, V.S., Ribau Teixeira, M., Pinheiro, J.P., Bebianno, M.J., 2014. Effects of silver nanoparticles exposure in the mussel *Mytilus galloprovincialis*. Mar. Environ. Res. 101, 208–214.
Gottschalk, F., Sonderer, T., Scholz, R.W., Nowack, B., 2009. Modeled environmental concentrations of engineered nanomaterials (TiO_2, ZnO, Ag, CNT, fullerenes) for different regions. Environ. Sci. Technol. 43, 9216–9222.
Hagger, J.A., Lowe, D., Dissanayake, A., Jones, M.B., 2010. The influence of seasonality on biomarker responses in *Mytilus edulis*. Ecotoxicology 19, 953–962.
ICES, 2016. Report of the Working Group on the Biological Effects of Contaminants (WGBEC). 9-13 March 2015. ICES, Bergen, Norway (CM 2015/SSGEPI:02. 66 pp).
Jimeno-Romero, A., Bilbao, E., Izagirre, U., Cajaraville, M.P., Marigómez, I., Soto, M., 2017a. Digestive cell lysosomes as main targets for Ag accumulation and toxicity in marine mussels, *Mytilus galloprovincialis*, exposed to maltose-stabilized Ag nanoparticles of different sizes. Nanotoxicology 11, 168–183.
Jimeno-Romero, A., Izagirre, U., Gilliland, D., Warley, A., Cajaraville, M.P., Marigómez, I., Soto, M., 2017b. Lysosomal responses to different gold forms (nanoparticles, aqueous, bulk) in mussel digestive cells: a trade-off between the toxicity of the capping agent and form, size and exposure concentration. Nanotoxicology 11, 658–670.
Jimeno-Romero, A., Bilbao, E., Valsami-Jones, E., Cajaraville, M.P., Soto, M., Marigómez, I., 2019. Bioaccumulation, tissue and cell distribution, biomarkers and toxicopathic effects of CdS quantum dots in mussels, *Mytilus galloprovincialis*. Ecotoxicol. Environ. Saf. 167, 288–300.
Katsumiti, A., Cajaraville, M.P., 2019. *In vitro* toxicity testing with bivalve mollusc and fish cells for the risk assessment of nanoparticles in the aquatic environment. In: Blasco, J., Corsi, I. (Eds.), Ecotoxicology of Nanoparticles in Aquatic Systems. Science Publishers CRC Press/Taylor & Francis Group, Boca Raton, London, New York, pp. 62–98.
Katsumiti, A., Gilliland, D., Arostegui, I., Cajaraville, M.P., 2014. Cytotoxicity and cellular mechanisms involved in the toxicity of CdS quantum dots in hemocytes and gill cells of the mussel *Mytilus galloprovincialis*. Aquat. Toxicol. 153, 39–52.
Katsumiti, A., Gilliland, D., Arostegui, I., Cajaraville, M.P., 2015. Mechanisms of toxicity of Ag nanoparticles in comparison to bulk and ionic Ag on mussel hemocytes and gill cells. PLoS One 10 (6), e0129039. https://doi.org/10.1371/journal.pone.0129039.
Katsumiti, A., Thorley, A.J., Arostegui, I., Reip, P., Valsami-Jones, E., Tetley, T.D., Cajaraville, M.P., 2018. Cytotoxicity and cellular mechanisms of toxicity of CuO NPs in mussel cells *in vitro* and comparative sensitivity with human cells. Toxicol. in Vitro 48, 146–158.
Lacave, J.M., Fanjul, A., Bilbao, E., Gutierrez, N., Barrio, I., Arostegui, I., Cajaraville, M.P., Orbea, A., 2017. Acute toxicity, bioaccumulation and effects of dietary transfer of silver from brine shrimp exposed to PVP/PEI coated silver nanoparticles to zebrafish. Comparative Biochemistry and Physiology, Part C 199, 69–80.
Lanceleur, L., Schäfer, J., Chiffoleau, J.F., Blanc, G., Auger, D., Renault, S., Baudrimont, M., Audry, S., 2011. Long-term records of cadmium and silver contamination in sediments and oysters from the Gironde fluvial-estuarine continuum - evidence of changing silver sources. Chemosphere 85, 1299–1305.
Lee, R.F., Steinert, S., 2003. Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. Mutat. Res. 544, 43–64.
Li, L., Stoiber, M., Wimmer, A., Xu, Z., Lindenblatt, C., Helmreich, B., Schuster, M., 2016. To what extent can full-scale wastewater treatment plant effluent influence the occurrence of silver-based nanoparticles in surface waters? Environ. Sci. Technol. 50, 6327–6333.
Li, J., Schiavo, S., Xiangli, D., Rametta, G., Miglietta, M.L., Oliviero, M., Changwen, W., Manzo, S., 2018. Early ecotoxic effects of ZnO nanoparticle chronic exposure in *Mytilus galloprovincialis* revealed by transcription of apoptosis and antioxidant-related genes. Ecotoxicology 27, 369–384.
Luzhna, L., Kathiria, P., Kovalchuk, O., 2013. Micronuclei in genotoxicity assessment: from genetics to epigenetics and beyond. Front. Genet. 4, 131–147.
Magesky, A., Pelletier, E., 2018. Cytotoxicity and physiological effects of silver nanoparticles on marine invertebrates. In: Saquib, Q., Faisal, M., Al-Khedhairy, A.A., Alatar, A.A. (Eds.), Cellular and Molecular Toxicology of Nanoparticles. Springer International Publishing, pp. 285–309.
Marcoux, M.A., Matias, M., Olivier, F., Keck, G., 2013. Review and prospect of emerging contaminants in waste -key issues and challenges linked to their presence in waste treatment schemes: general aspects and focus on nanoparticles. Waste Manag. 33, 2147–2156.
Marigómez, I., Soto, M., Cajaraville, M.P., Angulo, E., Giamberini, L., 2002. Cellular and subcellular distribution of metals in molluscs. Microsc. Res. Tech. 56, 358–392.
McCarthy, M., Carroll, D.L., Ringwood, A.H., 2013. Tissue specific response of oysters, *Crassostrea virginica*, to silver nanoparticles. Aquat. Toxicol. 138-139, 123–128.
McGuillicuddy, E., Murray, I., Kavanagh, S., Morrison, L., Fogarty, A., Cormican, M., Dockery, P., Prendergast, M., Rowan, N., Morris, D., 2017. Silver nanoparticles in the environment: sources, detection and ecotoxicology. Sci. Total Environ. 575, 231–246.
Mitchelmore, C.L., Chipman, J.K., 1998. DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. Mutat. Res. 399, 135–147.
Moore, M.N., 2006. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? Environ. Int. 32, 967–976.
Moore, M.N., Allen, J.I., McVeigh, A., 2006. Environmental prognostics: an integrated model supporting lysosomal stress responses as predictive biomarkers of animal health status. Mar. Environ. Res. 61, 278–304.
Mouneyrac, C., Buffet, P.E., Poirier, L., Zalouk-Vergnoux, A., Guibbolini, M., Faverney, C.R., Gilliland, D., Berhanu, D., Dybowska, A., Châtel, A., Perrein-Ettajni, H., Pan, J.F., Thomas-Guyon, H., Reip, P., Valsami-Jones, E., 2014. Fate and effects of metal-based nanoparticles in two marine invertebrates, the bivalve mollusc *Scrobicularia plana* and the annelid polychaete *Hediste diversicolor*. Environ. Sci. Pollut. Res. Int. 21, 7899–7912.
Mubiana, V.K., Qadah, D., Meys, J., Blust, R., 2005. Temporal and spatial trends in heavy metal concentrations in the marine mussel *Mytilus edulis* from the Western Scheldt estuary (the Netherlands). Hydrobiologia 540, 169–180.
Nahrgang, J., Brooks, S.J., Evenset, A., Camus, L., Jonsson, M., Smith, T.J., Lukina, J., Frantzen, M., Giarratano, E., Renaud, P.E., 2013. Seasonal variation in biomarkers in blue mussel (*Mytilus edulis*), Icelandic scallop (*Chlamys islandica*) and Atlantic cod (*Gadus morhua*): implications for environmental monitoring in the Barents Sea. Aquat. Toxicol. 127, 21–35.
Páez-Osuna, F., Frías-Espericueta, M.G., Osuna-López, J.I., 1995. Trace metal concentrations in relation to season and gonadal maturation in the oyster *Crassostrea iridescens*. Mar. Environ. Res. 40, 19–31.
Pisanelli, B., Benedetti, M., Fattorini, D., Regoli, F., 2009. Seasonal and inter-annual variability of DNA integrity in mussels *Mytilus galloprovincialis*: a possible role for natural fluctuations of trace metal concentrations and oxidative biomarkers. Chemosphere 77, 1551–1557.
Raisuddin, S., Jha, A.N., 2004. Relative sensitivity of fish and mammalian cells to sodium arsenate and arsenite as determined by alkaline single-cell gel electrophoresis and cytokinesis-block micronucleus assay. Environmental Molecular Mutagenesis 44, 83–89.
Regoli, F., 1992. Lysosomal responses as a sensitive stress index in biomonitoring heavy metal pollution. Mar. Ecol. Prog. Ser. 84, 63–69.
Regoli, F., Orlando, E., 1994. Seasonal variation of trace metal concentrations in the digestive gland of the mediterranean mussel *Mytilus galloprovincialis*: comparison between a polluted and a non-polluted site. Arch. Environ. Contam. Toxicol. 27, 36–43.
Ringwood, A.H., McCarthy, M., Bates, T.C., Carroll, D.L., 2010. The effects of silver nanoparticles on oyster embryos. Mar. Environ. Res. 69, S49–S51.
Rocco, L., Santonastaso, M., Nigro, M., Mottola, F., Costagliola, D., Bernardeschi, M., Guidi, P., Lucchesi, P., Scarcelli, V., Corsi, I., Stingo, V., Frenzilli, G., 2015. Genomic and chromosomal damage in the marine mussel *Mytilus galloprovincialis*: effects of the combined exposure to titanium dioxide nanoparticles and cadmium chloride. Mar. Environ. Res. 111, 144–148.
Rocha, T.L., Gomes, T., Cardoso, C., Letendre, J., Pinheiro, J.P., Sousa, V.S., Teixeira, M.R., Bebianno, M.J., 2014. Immunocytotoxicity, cytogenotoxicity and genotoxicity of cadmium-based quantum dots in the marine mussel *Mytilus galloprovincialis*. Mar. Environ. Res. 101, 29–37.
Rocha, T.L., Gomes, T., Sousa, V.S., Mestre, N.C., Bebianno, M.J., 2015. Ecotoxicological impact of engineered nanomaterials in bivalve molluscs: an overview. Mar. Environ. Res. 111, 74–88.

Rocha, T.L., Sabóia-Morais, S.M., Bebianno, M.J., 2016. Histopathological assessment and inflammatory response in the digestive gland of marine mussel *Mytilus galloprovincialis* exposed to cadmium-based quantum dots. Aquat. Toxicol. 177, 306–315.

Ruiz, P., Ortiz-Zarragoitia, M., Orbea, A., Vingen, S., Hjelle, A., Baussant, T., Cajaraville, M.P., 2014. Short- and long-term responses and recovery of mussels *Mytilus edulis* exposed to heavy fuel oil no. 6 and styrene. Ecotoxicology 23, 861–879.

Ruiz, P., Katsumiti, A., Nieto, J.A., Bori, J., Reip, P., Orbea, A., Cajaraville, M.P., 2015. Short-term effects on antioxidant enzymes and long-term genotoxic and carcinogenic potential of CuO nanoparticles in mussels. Mar. Environ. Res. 111, 107–120.

Schiavo, S., Duroudier, N., Bilbao, E., Mikolaczyk, M., Schäfer, J., Cajaraville, M.P., Manzo, S., 2017. Effects of PVP/PEI coated and uncoated silver NPs and PVP/PEI coating agent on three species of marine microalgae. Sci. Total Environ. 577, 45–53.

Schmidt, W., Power, E., Quinn, B., 2013. Seasonal variations of biomarker responses in the marine blue mussel (*Mytilus* spp.). Mar. Pollut. Bull. 74, 50–55.

Sheehan, D., Power, A., 1999. Effects of seasonality on xenobiotic and antioxidant defence mechanisms of bivalve molluscs. Comparative Biochemistry and Physiology, Part C 123, 193–199.

Sikder, M., Lead, J.R., Chandler, G.T., Baalousha, M., 2017. A rapid approach for measuring silver nanoparticle concentration and dissolution in seawater by UV-Vis. Sci. Total Environ. 618, 597–607.

Singh, N., Manshian, B., Jenkins, G.J., Griffiths, S.M., Williams, P.M., Maffeis, T.G., Wright, C.J., Doak, S.H., 2009. NanoGenotoxicology: the DNA damaging potential of engineered nanomaterials. Biomaterials 30, 3891–3914.

Sokal, R.R., Rholf, F.J., 1969. Introduction to Biostatistics. 2nd edition. Dover Publications Inc, Mineola, New York.

Solé, M., Porte, C., Albaigés, J., 1995. Seasonal variation in the mixed-function oxigenase system and antioxidant enzymes of the mussel *Mytilus galloprovincialis*. Environ. Toxicol. Chem. 14, 157–164.

Soto, M., Kortabitarte, M., Marigómez, I., 1995. Bioavailable heavy metals in estuarine waters as assessed by metal/shell-weight indices in sentinel mussels *Mytilus galloprovincialis*. Mar. Ecol. Prog. Ser. 125, 127–136.

Soto, M., Bignell, B., Cancio, I., Taylor, M.G., Turner, M., Morgan, A.J., Marigómez, I., 2002. Subcellular distribution of cadmium and its cellular ligands in mussel digestive gland cells as revealed by combined autometallography and X-ray microprobe analysis. Histochem. J. 34, 273–280.

Tangaa, S.R., Selck, H., Winther-Nielsen, M., Khan, F.R., 2016. Trophic transfer of metal-based nanoparticles in aquatic environments: a review and recommendations for future research focus. Environmental Science: Nano 3, 966–981.

Tiede, K., Hassellöv, M., Breitbarth, E., Chaudhry, Q., Boxall, A.B.A., 2009. Considerations for environmental fate and ecotoxicity testing to support environmental risk assessments for engineered nanoparticles. J. Chromatogr. A 1216, 503–509.

UNEP, 2004. UNEP/MAP/MED POL: Guidelines for the Development of Ecological Status and Stress Reduction Indicators for the Mediterranean Region. MAP Technical Reports Series No. 154. UNEP/MAP, Athens, p. 94.

UNEP-RAMOGE, 1999. Manual on the Biomarkers Recommended for the MED POL Biomonitoring Programme. UNEP, Athens.

Venier, P., Maron, S., Canova, S., 1997. Detection of micronuclei in gill cells and haemocytes of mussels exposed to benzo[a]pyrene. Mutation Research-Genetic Toxicology and Environmental Mutagenesis 390, 33–44.

Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E., Koehler, A., 2007. The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. Comparative Biochemistry and Physiology, Part C 146, 281–300.

Volland, M., Hampel, M., Katsumiti, A., Yeste, M.P., Gatica, J.M., Cajaraville, M.P., Blasco, J., 2018. Synthesis methods influence characteristics, behaviour and toxicity of bare CuO NPs compared to bulk CuO and ionic Cu after in vitro exposure of *Ruditapes philippinarum* hemocytes. Aquat. Toxicol. 199, 285–295.

Von der Kammer, F., Ferguson, P.L., Holden, P.A., Masion, A., Rogers, K.R., Klaine, S.J., Koelmans, A.A., Horne, N., Unrine, J.M., 2012. Analysis of engineered nanomaterials in complex matrices (environment and biota): general considerations and conceptual case studies. Environ. Toxicol. Chem. 31, 32–49.

Zhang, C., Hu, Z., Deng, B., 2016. Silver nanoparticles in aquatic environments: physicochemical behavior and antimicrobial mechanisms. Water Res. 88, 403–427.

Zorita, I., Apraiz, I., Ortiz-Zarragoitia, M., Orbea, A., Cancio, I., Soto, M., Marigómez, I., Cajaraville, M.P., 2007a. Assessment of biological effects of environmental pollution along the NW Mediterranean Sea using mussels as sentinel organisms. Environ. Pollut. 148, 236–250.

Zorita, I., Bilbao, E., Schad, A., Cancio, I., Soto, M., Cajaraville, M.P., 2007b. Tissue- and cell-specific expression of metallothionein genes in cadmium- and copper-exposed mussels analyzed by in situ hybridization and RT-PCR. Toxicol. Appl. Pharmacol. 220, 186–196.