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Comparative behaviour of nano ZnO, bulk ZnO and ionic Zinc in marine environment and effect upon biological target organisms such as primary producers (marine algae *Tetraselmis suecica* and *Phaeodactylum tricornutum*) and filter feeder (Mediterranean mussels *Mytilus galloprovincialis*) chronically exposed

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ABSTRACT

Zinc oxide nanoparticles (ZnO NPs), one among the most widely used metal oxide nanomaterias, has unique properties, such as optoelectronic property, UV emission, transparent conductivity, piezoelectricity, UV absorption and reflection, those make ZnO NPs to be currently used in a broad range of products. Such widespread and expanding production and use increase the potential for their release into the environment, in US, Euorpe, and China. With the vast releasing of ZnO NPs, the ecotoxic risk had drawn much research interest in the last decades. The aquatic ecosystem is the final destination of the released ZnO NPs and the coastal seawaters takes into the most of discharge.

At least three distinct mechanisms of the toxic action of ZnO NPs, released toxic Zn^{2+} effect, surface interactions with media may produce toxic substance were report, and photo-induced toxicity, were reported.

Based on the published literatures reviewing, several research points on ecotoxicity of ZnO NPs towards algae and mussel in marine environment were still unexplored and some represent aims of this study.

- ZnO NPs could have a specific behavior and effect in diverse marine seawaters. To this aim the full characterization of ZnO NP dissolution, aggregation, and sedimentation once dispersed into a standard Artificial Sea Water (ASW) and a Reconstituted Sea Water (RSW) from East China Sea was performed.
- The ecotoxicity of ZnO NPs upon a green alga and a diatom would be different. To assess the diverse toxicity upon different algae, growth inhibition algal assays were performed. Additionally, to verify the hypothesis about the role of nano size in the overall toxicity, ZnO bulk, and Zn salt toxicity were assessed as well.
- ZnO NP toxicity towards mussels under a chronic exposure could be shown by Zn bioaccumulation, tissue damages, and transcription of apoptosis and antioxidation-related genes. The hypothesis that nano size plays an important role in its toxicity was also considered together with the all previous ones for mussel *M. galloprovincialis*.

The obtained results answered to the all hypotheses. Behaviors of ZnO NPs and bulk in

different artificial seawater were observed indicating that the primary size of ZnO in suspensions could affect the aggregating rate in higher concentration. The behaviors of ZnO NPs dispersed into ASW and RSW have similar changing trends with some slight differences. Comparative toxicity of ZnO NPs, bulk, and Zn salt towards marine algae indicated that the nano size plays a key role in the overall ZnO toxicity. Zn bioaccumulation and histological damages were observed for mussel exposed to ZnO NPs, bulk, and Zn salt. Pristine ZnO particle size influences the overall toxicity and the rank was represented by three levels of injury (in gill, digestive gland, and gonad): low for bulk; medium for NPs and high for salts. Ecotoxicity of ZnO NPs in mussel revealed by transcription of apoptosis and antioxidation-related genes indicated that active response to ZnO NP exposure which induced DNA damage and oxidant injury contributing to the overall toxicity. In conclusion, zinc oxide nanoparticles induced ecotoxicolgical stress to two algae and a mussel in marine environment that were related to the ROS-induced property.

0 PREFACE

0.1 Review on the production and application of Zinc oxide nanoparticles

Zinc oxide nanoparticles (ZnO NPs), one among the most widely used metal oxide nanomaterias, has a wurtzite crystal structure contributing to its unique optoelectronic properties (Wang, 2004). Besides, many other characteristics such as UV emission, transparent conductivity, and piezoelectricity make ZnO NPs particularly attractive for electronic sensor, solar voltaics, and transducer applications (Ma et al., 2013). It is also a very effective photo catalyst material with excellent properties of UV absorption and reflection (Hoffmann et al., 1995). Those properties make ZnO NPs to be currently used in a broad range of products including plastics, ceramics, rubber, lubricants, paints, foods (source of Zn nutrient), batteries, fire retardants, personal care products, medical disinfection, etc. (Porter, 1991; Mitchnick et al., 1999; Battez et al., 2008; Padmavathy & Vijayaraghavan, 2008; Wilkie & Morgan, 2009; Ma et al., 2013). Consequently a huge quantity of ZnO NPs was produced per year for industrial use (Klingshirn, 2007).

Such widespread and expanding production and use increase the potential for their release into the environment. Keller et al. (2014) estimated that ZnO together with titanium dioxide, represent 94 % of Engineered Nano Material (ENMs) released into the environment from the use of personal care products in US. Particularly, an amount of 3700 mt ZnO ENMs flowing into water system each year was estimated (Keller et al., 2013). Gottschalk et al. (2009) reported the modeled environmental concentration of nano ZnO in surface water (0.010 µg/L in Euorpe, 0.001 µg/L in U.S.), sediment (2.90 µg/L in Euorpe, 0.51 µg/L in U.S.), and soil (0.093 µg/L in Euorpe, 0.050 µg/L in U.S.). On the other side, with the rapid economic development, China become the largest nanomaterial market in the Pacific-Asia (Gao et al., 2013). It was also estimated that about 36000 kt of ZnO NPs could be discharged by wastewater and dust deposition into China's aquatic environments only in 2017 (Gao et al., 2013).

0.2 Review on the ecotoxicological risk of release of ZnO NPs to the environment

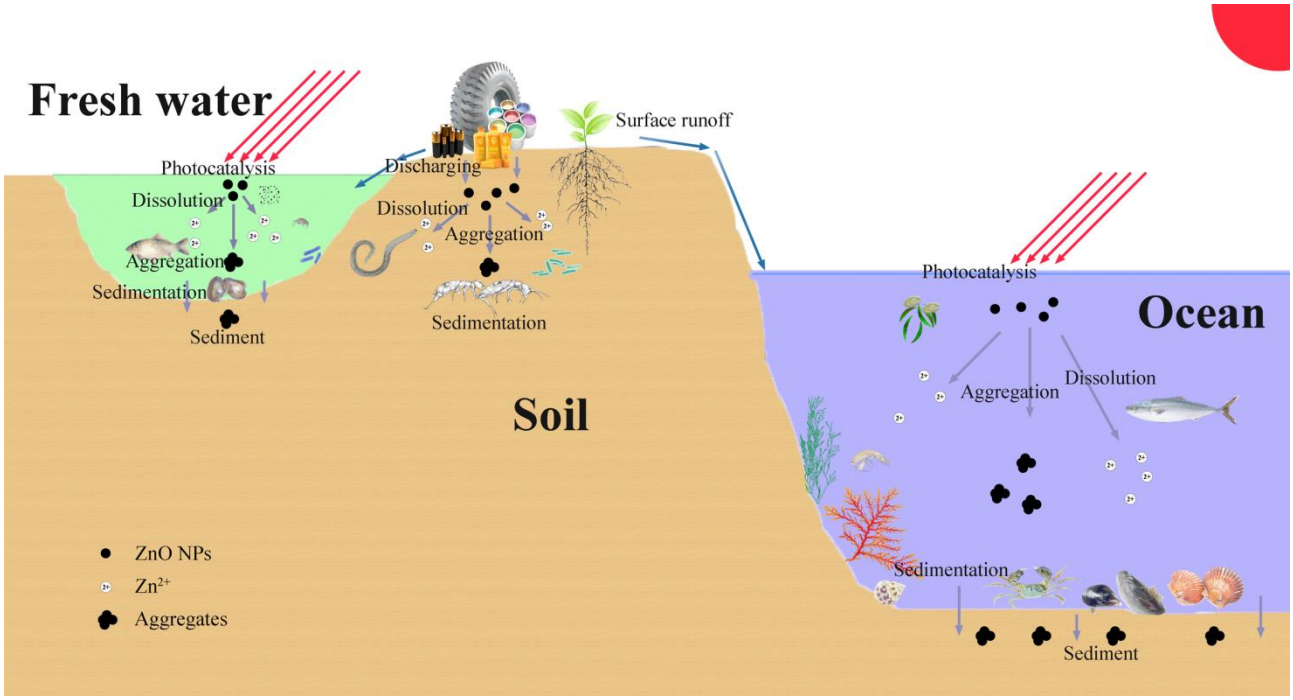

Fig. 0.2-1 ZnO NPs fate and effect in the environment.

With the vast releasing of ZnO NPs discharged from widespread and expanding production, the ecotoxic risk had drawn much research interest in the last decades. Fig. 0.2-1 shows the fate of released ZnO NPs and effect to environmental organisms of different taxa in soil and aquatic ecosystem. Rainfall and surface runoff are the main power to move ZnO from the soil to the ocean. Dissolving into water, aggregating to be bulk and sink into sediment, and combining with organic object into the transformation of ecosystem could be the main three paths of the released ZnO NPs. The discharged ionic Zn, ZnO aggregates, and other types of compound covered ZnO directly and indirectly interacted with organism in soil, freshwater and seawater.

Excluding landfill, soil takes the most amounts of released ZnO NPs (Keller & Lazareva, 2013; Keller et al., 2013; Keller et al., 2014). A relatively broad range of species, such as, plants: radish, rape, ryegrass, lettuce, corn, cucumber (Lin & Xing, 2007), zucchini (Stampoulis et al., 2009), garden cress, broad bean (Manzo et al., 2011), and wheat (Du et al., 2011), and invertebrates: nematode (Ma et al., 2009; Wang et al., 2009; Khare et al., 2011; Ma et al., 2011), earthworm (Hu et al., 2010; Li et al., 2011), soil arthropod (Manzo et al., 2011), isopod (Pipan-Tkalec et al., 2010), have been investigated under soil exposure. Both discharged zinc ions and particles-dependent effect were reported to contribute to the toxicity of ZnO NPs at 2000 mg/L towards plants by seed germination inhibition and root elongation termination (Lin & Xing, 2007). However, Stampoulis et

al. (2009) did not observe any ecotoxic effect of both ZnO NPs and bulk at 1000 mg/L to zucchini. Manzo et al. (2011) reported the root elongation inhibition to garden cress and genotoxicity to *Vicia faba* caused by ZnO NPs. Also, Du et al. (2011) reported the reduced biomass of wheat and the Zn uptake indicating the discharged ionic Zn contributed the overall toxicity of ZnO NPs. Effects of lethality, behavior, reproduction, and transgene expression of adult nematode caused by ZnO NPs at several hundred mg/L represented relatively low toxicity (Ma et al., 2009), however, ZnO NPs showed highly toxicity to larval nematode (Wang et al., 2009), that the whole toxicity was based on Zn ions from dissolution. Comparing of LC50s of two different size ZnO NPs indicated that the initial size of ZnO contributed the toxicity towards nematode (Khare et al., 2011). Manzo et al. (2011) reported the difference of ecotoxicity of ZnO NPs towards two arthropods: 100% mortality in *Heterocypris incongruens* and no effects on the reproduction of *Folsomia candida*, indicating particle-dependent effect is the base of toxicity. However, Pipan-Tkalec et al. (2010) found that ZnO NPs dissolution is responsible for Zn bioaccumulation in isopod. The aquatic ecosystem is the final destination of pollutants and therefore river, lake, in particular seawaters, could be subject to ZnO NPs pollution coming through water movement.

In freshwater, many different organisms were utilized for ecotoxicological assessment of ZnO NPs potential impact: algae (Franklin et al., 2007; Aruoja et al., 2009; Aravantinou et al., 2015; Bhuvaneshwari et al., 2015), crustaceans (Heinlaan et al., 2008; Wiench et al., 2009; Zhu et al., 2009b; Blinova et al., 2010), mollusks (Ali et al., 2012), fishes (Zhu et al., 2008; Zhu et al., 2009a; Bai et al., 2010; Johnston et al., 2010; Yu et al., 2011; Hao & Chen, 2012; Bessemer et al., 2015; Suganthi et al., 2015), and amphibian (Nations et al., 2011a; Nations et al., 2011b). Aruoja et al. (2009) and Franklin et al. (2007) reported that dissolution of ZnO NPs played a role in causing toxicity upon microalgae *Pseudokirchneriella subcapitata*. Aravantinou et al. (2015) observed the growth rate inhibition of the freshwater algae *Chlorococcum* sp. and *Scenedesmus rubescens* under ZnO NPs exposure and suggested that the toxic effect is related also to the culture medium type. In addition, Bhuvaneshwari et al. (2015) reported that the toxicity of ZnO NPs upon the freshwater alga *Scenedesmus obliquus* was mainly related to the initial size and concentrations of NPs, the illumination conditions and dissolution. Heinlaan et al. (2008) and Blinova et al. (2010) reported the lethal concentration of ZnO NPs towards freshwater crustaceans *Daphnia magna* and *Thamnocephalus platyurus* suggesting the toxicity was dependent on ionic Zn. However, Wiench et

al. (2009) suggested that the toxicity towards *D. magna* was independent of primary size of NPs, coating, aggregation, culture medium or the pretreatment of NPs suspensions. Ali et al. (2012) reported the genotoxicity in digestive gland cells of freshwater snail *Lymnaea luteola* exposed to ZnO NPs attributed to oxidative stress. Zhu et al. (2008), Zhu et al. (2009a), and (Bai et al., 2010) focused on the embryo hatching rate of zebrafish exposed to ZnO NPs indicating dissolved Zn^{2+} and ZnO aggregates contributed to the toxicity. However, Yu et al. (2011) suggested that the aggregation and sedimentation of ZnO NPs inhibited the toxic effect. Hao & Chen (2012) reported the toxicity of ZnO NPs towards carp *Cyprinus carpio* on the changes of activity of antioxidative enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) and lipid peroxidation (LPO). The exposure of ZnO NPs caused significant decreasing of enzymes activities and increasing of LPO indicating oxidative stress generation. Johnston et al. (2010) detected limited ZnO NPs uptake in zebrafish tissues suggesting limited invading ability directly to organisms. Bessemer et al. (2015) evidenced oxidative and cellular stress in gill of a freshwater teleost fish *Catostomus commersonii* exposed to ZnO NPs that leaded gill neuroepithelial cells activation and then caused a hypoxic response of the whole adult fish body. Suganthi et al. (2015) reported decreased immune cells in freshwater fish *Oreochromis mossambicus* blood caused by acute exposure of ZnO NPs. Limited studies reported ZnO NP toxicity to amphibians. Nations et al. (2011a); Nations et al. (2011b) reported developmental abnormalities, high mortality, and metamorphosis inhibition in *Xenopus laevis* by ZnO NPs exposure.

Since the destination of ZnO is eventually the coastal seawaters, marine organisms were largely used in the evaluation effects as well: many studies are about marine algae (Brayner et al., 2010; Miao et al., 2010; Miller et al., 2010; Wong et al., 2010; Aravantinou et al., 2015; Suman et al., 2015), marine amphipod (Fabrega et al., 2012), marine crustaceans (Wong et al., 2010; Manzo et al., 2013), marine bivalves (Montes et al., 2012; Trevisan et al., 2014a; Trevisan et al., 2014b), marine fish (Wong et al., 2010). Brayner et al. (2010) reported that the photosynthesis of cyanobacteria *Anabaena flos-aquae* and lethality rate of microalgae *Euglena gracilis euglenoid* were affected by ZnO NPs. Miao et al. (2010) suggested that the dissolved ionic Zn was the only determinant of ZnO NPs toxicity to the marine diatom *Thalassiosira pseudonana*. The growth assay of four marine algae *Skeletonema marioni*, *T. pseudonana*, *Dunaliella tertiolecta*, and *Isochrysis galbana* under ZnO NPs exposure were performed by Miller et al. (2010) indicating the uptake of

Zn^{2+} dissolved from NPs contributed the toxicity. Wong et al. (2010) performed the growth assay of two marine diatoms *Skeletonema costatum* and *T. pseudonana* exposed to ZnO NPs suggesting free Zinc ions from the dissolution of ZnO could be contributed to the toxicity. Aravantinou et al. (2015) examined the growth rate of two marine species *D. tertiolecta* and *T. suesica* exposed ZnO NPs and found that the toxic effect attributed on algal species, exposure time and concentrition of NPs, and primarily the type of culture medium for algae. Suman et al. (2015) tested the level of several molecular biomarkers in marine algae *Chlorella vulgaris* exposed to ZnO NPs and suggested high level of Zn^{2+} from dissolution of NPs contributed to the dose-dependent toxicty. However, on marine amphipod, Fabrega et al. (2012) suggested the toxicity of ZnO NPs can not only contributed to the ionic Zn from dissolution. But still, Wong et al. (2010) performed the mortality assay of the crustaceans *Tigriopus japonicus* and *Elasmopus rapax* exposed to ZnO NPs suggesting that free Zinc ions could contribute to the toxicity. In addition, Manzo et al. (2013) reported for sea urchin *Paracentrotus lividus* exposed to ZnO NPs, that the fertilization and early development of embryos were affected not only by free Zn ions but that also the interactions between ZnO aggregates and sea urchin/seawater play a role in the toxicity. For marine bivalves *M. galloprovincialis,* Montes et al. (2012) applied a observation of invaded ZnO NPs in mussel tissues by scanning electron microscope and suggested that ZnO NPs remained in the mussel body indicating a biotransformation could be real. The gill was suggested to be the first organ in Pacific oysters *Crassostrea gigas* (Trevisan et al., 2014a) and brown mussels *Perna perna* (Trevisan et al., 2014b) to be attacked by ZnO NPs. Wong et al. (2010) detected the protein level of three molecular biomarkers SOD, metallothionein (MT), and heat shock protein 70 (HSP70) of the medaka fish *Oryzias melastigma* exposed to ZnO NPs suggesting dissolution and the oxidative stress generation as major contributors to the toxicity.

In addition, also the effect about bacteria were well investigated (Reddy et al., 2007; Huang et al., 2008; Jones et al., 2008; Applerot et al., 2009; Aruoja et al., 2009; Jin et al., 2009; Liu et al., 2009; Feris et al., 2010; Li et al., 2011; Premanathan et al., 2011; Raghupathi et al., 2011; Xie et al., 2011; Ambika & Sundrarajan, 2015; Read et al., 2015). Growth inhibition and cell viability of bacteria population exposed to ZnO NPs were always the endpoints in the above studies. They focused on the bacteriostasis of NPs and compared the difference among different particles sizes.

In the main of the all above studies, the toxic action of ZnO NPs was potentially

attributed to, at least three distinct mechanisms:

First, ZnO NPs release toxic Zn^{2+} into exposure media. The dissolution process usually involves the following reactions (Yamabi & Imai, 2002; Ma et al., 2013):

$$ZnO(s) + H_2O(l) \rightleftharpoons Zn(OH)_2(s)$$

$$Zn(OH)_2(s) \rightleftharpoons Zn(OH)^+(aq) + OH^-(aq)$$

$$Zn(OH)^+(aq) \rightleftharpoons Zn^{2+}(aq) + OH^-(aq)$$

The dissolubility plays an important role in the toxic effect of ZnO NPs dispersed into aqueous media. The physico-chemical properties (particle size and surface area) and the environmental parameters (pH, temperature, and organic matter) of the exposure media can largely affect the dissolution of ZnO NPs. Generally, the smaller is the particle size the larger is the surface area, resulting in ZnO NPs greater dissolution respect to ZnO powder (ZnO bulk). Size-dependent dissolution is one of the mechanisms of higher toxicity of NPs than bulk.

Second, surface interactions with media may produce toxic substances hydroxyl radicals (OH) and reactive oxygen species (ROS). Besides particle dissolution toxicity, ROS-mediated toxicity induced by NPs and hydroxyl radicals contribute the overall toxicity.

Third, photo-induced toxicity associated with its photocatalytic property may be another important mechanism of toxicity.

0.3 Research prospects of assessment of ZnO NPs global ecotoxic risk

Based on the research contents of reported studies, several aspects are still not sufficiently explored and therefore should be further investigated.

First, the behavior of ZnO NPs in the different exposure media (pure water, natural fresh water, acid/alkali treated fresh water, artificial sea water, natural sea water, etc.) along the exposure time. The measurement of particle size aggregation, sedimentation, dissolubility, etc., is the former step to define the influence of medium physicochemical properties on the ZnO NP toxicity.

Second, the different role of the two main component of the toxic effect: particle-induced toxicity and dissolved ion toxicity should be differentiated by upgraded testing tools and techniques. There is now no very efficient approach to directly determine particle-induced toxicity, instead of a comparing method that test the toxic difference between nano scale particles and normal size powders (Lin & Xing, 2008; Manzo et al., 2011; Fabrega et al., 2012). Also, Inductively Coupled

Plasma-Mass Spectrometry (ICP-MS) is the most popular method to measure the dissolved ionic Zn which is not the most sensitive method recently and could gradually be replaced by other techniques such as the Scanned Stripping Chrono Potentiometry (SSCP) technique (Merdzan, 2014). A new high efficient approach is highly expected to differentiate the particle-induced toxicity and dissolved ions toxicity.

Third, the realistic exposure in environment by chronic assessment should focus on low concentration dose. In the sub lethal long term exposure, endpoints such as Zn accumulation and modification of relevant genes transcription and protein production should be assessed. As well, the toxicity of ZnO NPs via food chain could be the next hotspot.

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1 STATE OF THE ART

1.1 Characterization of ZnO NPs and bulk in marine environment

Once released into marine environment, ZnO NPs will interact with each other and some organic/inorgnic compounds (Rocha et al., 2015). The properties of NPs, such as its nano size, pariticle shape, chemical composition, and surface charge, and the properties of medium, such as pH, temperature, ionic strength, plays an key role on ZnO NPs behaviour (Fabrega et al., 2012; Rocha et al., 2015). Several aspects of ZnO NPs characters changed in marine environment that had been well documented (Miao et al., 2007; Brayner et al., 2010; Miao et al., 2010; Miller et al., 2010; Ma et al., 2013; Rocha et al., 2015). They are progressive and simultaneous happenning in seawater.

Dissolution is a step proceeded rapidly once ZnO NPs suspended into seawater. Miller et al. (2010) measured the concentration of dissolved Zn ion using Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) technique suggesting an equilibrium value approximately 3 mg Zn/L was reached for the initial ZnO concentration 10 mg Zn/L and most Zn (approximately 70%) dissolved for all lower concentrations with in 12 h. However, the dissolution was still going on after 4 days for the high concentration. Miao et al. (2010) performed the dissolution test with Graphite Furnace Atomic Absorption Spectrophotometer (GFAAS) technique resulting no consistent trend was observed in the different experimental conditions. However, a around 5% dissolution for NPs and 2.5% for bulk were reported for high concentration susuoensions (> 80 mg Zn/L) (Wong et al., 2010). Manzo et al. (2013b) summarized all abovementioned data resulting that the average solubility of ZnO NPs in seawater is around 5 mg Zn/L.

Aggregation, at the same time, was rapidly occurred since the very beginning of dispersing in seawater medium. Miller et al. (2010) reported the diameter of NPs increased from initial 250-300 nm (10 mg Zn/L) to approximately 450 nm within 30 min using Dynamic Light Scattering (DLS) technique and suggested rapid aggregation of larger aggregates depends on the low surface/volume ratio. Miao et al. (2010) reported an increasing to 8500 nm after 7 days dispersed into ASW with also DLS. Also, Manzo et al. (2013b) reported similar trends that high

concentration pariticles aggregating much more than low concentration for both ZnO NPs and bulk in ASW and bulk aggregates size increased to 3000 nm after 250 min dispersing is larger than NPs (2600 nm).

Sedimentation would occur with aggregation due to gravity in ASW. Manzo et al. (2013b) observed a clear sedimentation of large aggregates for 100 mg Zn/L NPs and bulk. Low concentration ZnO particles showed very slower sedimentation rate than high ones. In the same time, NPs showed slower sedimentation rate than bulk. Miller et al. (2010) reported similar results. This could be due to the density difference between NPs and bulk.

1.2 The importance of primary producers marine algae in ecosystem and ecotoxicolgical studies of ZnO NPs

Phytoplankton is the autotrophic component of the plankton community and a key factor of oceans, seas and freshwater basins ecosystems. High growth rate, high surface-to-volume ratio (high uptake rate), easy handling in laboratory conditions (Castro-Bugallo et al., 2014), make marine microalgae be an effective tool to test toxicity of contaminants such as antibiotics (Seoane et al., 2014), metals (Wang & Zheng, 2008; Angel et al., 2015), nano-materials (Kadar et al., 2012; Clément et al., 2013; Castro-Bugallo et al., 2014). Additionally, as diverse algae taxa respond differently to chemical toxicants, it is mandatory to conduct tests on a different species representing different classes.

Published literatures documented different nanomaterials toxicity towards vast algae species. Green algae could be the most popular object to investigate ecotoxicity of nanomaterials, such as nano TiO_2, ZnO, Al_2O_3, SiO_2, CuO, C_{60}, Carbon Nanotube, etc. (Baun et al., 2008b; Blaise et al., 2008; Van Hoecke et al., 2008; Wang et al., 2008; Aruoja et al., 2009; Hall et al., 2009; Ji et al., 2011; Lee & An, 2013; Manzo et al., 2013a; Fu et al., 2015). Also Diatom and other algae were utilized to assess the ecotoxic effect of nano metal xoide, metal particles, etc. (Brayner et al., 2009; Wong et al., 2010; Peng et al., 2011; Dahoumane et al., 2012a, b; Manusadžianas et al., 2012; Clément et al., 2013; Fu et al., 2015; Li et al., 2015; Yung et al., 2015).

Among them, most algae are marine species indicated that nanomaterials are released into marine ecosystem arousing many ecotoxicity studies on these common phytoplanktons in recent

years (Özkoc & Taylan, 2010; Wong et al., 2010; Peng et al., 2011; Kadar et al., 2012; Manzo et al., 2013a; Aravantinou et al., 2015; Suman et al., 2015).

Among these investigated nanomaterials, ZnO NPs is currently used in widespread and expanding production that increases the heat of the studies on its ecotoxicity (Ma et al., 2013). A broad range of marine algae became the research organism objects on it (Brayner et al., 2010; Miao et al., 2010; Miller et al., 2010; Wong et al., 2010; Manzo et al., 2013a; Aravantinou et al., 2015; Suman et al., 2015). However, marine green microalgae *Tetraselmis suecica* and diatom *Phaeodactylum tricornutum* which had not been reported on ZnO NPs ecotoxicity were selected as test organisms to respond to ZnO NPs exposure.

P. tricornutum is a widespread pennate diatom, with low silica content and distinct cell wall (i.e. frustule) structures which is essentially composed of organic compounds, particularly sulfated glucomannan (Tesson et al., 2009), has been described in three different morphotypes (i.e. the ovoid, fusiform and triradiate forms), whose occurrence in culture seems to depend on strains as well as environmental conditions (Francius et al., 2008).

T. suecica is an elliptical microalga of the class Chlorophyceae (Prasinophyceae) generally used as the diets of zooplankton, bivalve molluscs and crustacean larvae. The characteristic cell wall (theca) is composed of coalesced rigid carbohydrate scales (Lee et al., 2013) and the typical four flagella are covered by double layer of scales.

1.3 The role of filter feeder Mediterranean mussels *Mytilus galloprovincialis* in marine pollution survey

Bivalves, like mussels *Mytilus* spp., are filter-feeders, widely distributed, and with a long-life span and represent a good choice for the study of marine environmental pollution (Sheir et al., 2013; Balbi et al., 2014; Hu et al., 2014; Cremonte et al., 2015). *M. galloprovincialis*, cultured in China for commercial interest (Lazo & Pita, 2012; FAO, 2016), was instead largely utilized in several countries to investigate biological responses to toxicants and environmental stress (Da Ros et al., 2000; Barmo et al., 2013; Estevez-Calvar et al., 2013; Balbi et al., 2014) and also to assess NPs toxicity (Canesi et al., 2010; Gomes et al., 2011; Hanna et al., 2013; Balbi et al., 2014; Gomes et al., 2014).

In this bivalve NPs uptake can occur by ingestion through the digestive tract (Roberts et al., 2007; Baun et al., 2008a; Gagné et al., 2008; Ward & Kach, 2009) and through the large respiratory surface of the gills, as demonstrated for SiO_2 NPs in the congener species *Mytilus edulis* (Köhler & Riisgård, 1982). The gills and the digestive gland are, therefore, particularly relevant as target organs for nanotoxicological studies (Baun et al., 2008a; D'Agata et al., 2014).

However, it was recently reported that NPs could accumulate with increasing time exposure in the digestive gland (Gomes et al., 2011; 2012; 2013; 2014). This indicates that although the gills are the first target of NPs, the digestive gland is the main tissue for their storage (Ringwood et al., 2010b). These particles could induce oxidative stress in mussel gills and digestive gland, and promote several abnormalities in cellular function which can also trigger major changes in gene transcription (Fabbri et al., 2008).

1.4 Ecotoxicity assessments on algae and mussels

On algae assay, growth inhibition is the most popular approach of ecotoxicity assessment (Aruoja et al., 2009; Ji et al., 2011; Peng et al., 2011; Kadar et al., 2012; Manzo et al., 2013a; Aravantinou et al., 2015; Li et al., 2015; Schiavo et al., 2016). Some approaches involved in oxidentive stress induced by nanoparticles, such as, measurement of related enzyme activity (SOD, CAT, LDH, GSH, etc.)(Li et al., 2015; Suman et al., 2015), ROS generation (H_2O_2, O^{2-}, and OH) (Jagadeesh et al., 2015; Li et al., 2015), and lipid peroxidation (LPO) (Kadar et al., 2012; Jagadeesh et al., 2015; Suman et al., 2015). Besides, observation of nanoparticle invading in cells directly showed the damage from nanoparticles (Gong et al., 2011; Peng et al., 2011; Bhuvaneshwari et al., 2015; Li et al., 2015). In addition, contents of chlorophyll (Gong et al., 2011), observation of DNA damage (Schiavo et al., 2016), extracellular protein content (Jagadeesh et al., 2015), and Zn content per algae surface (Aravantinou et al., 2015) were reported on ecotoxicity assessment.

For mussels, many indexes were reported on nanoparticles toxicity. Enzymatic concentration/activity could be measured in most studies, such as, GSR, GST, CAT, GPX, Se-GPX SOD, MT, and GSSG (Canesi et al., 2010; Tedesco et al., 2010; Gomes et al., 2011; Gomes et al., 2012; Barmo et al., 2013; Gomes et al., 2014). Lysosomal membrane stability, lysosomal lipofuscin content, lysosomal Neutral Lipid content, and LPO were reported as well (Canesi et al., 2010; Gomes et al., 2012). Gomes et al. (2013) reported the genotoxic comet assay on *M.*

galloprovincialis. Wang et al. (2014) reported total hemocyte counting, ROS and Lysosomal content and Barmo et al. (2013) reported some related genes expression, such as *GST*, *CAT*, *Mytilin B*, *Myticin B*, *defensin*, *lysozyme* and *MgC1q*, involved in oxidative stress and immune reponse. Beside of these molecular approaches, observation on tissue damages (Kádár et al., 2010; Barmo et al., 2013; Hu et al., 2014; Trevisan et al., 2014; Vale et al., 2014; Cid et al., 2015), electrophoresis proteomic separations of gill proteins (Tedesco et al., 2008; Tedesco et al., 2010), embryotoxicity (Ringwood et al., 2010a), survival rate (Mwangi et al., 2012), and metal bioaccumulation in tissues (García-Negrete et al., 2013; Hu et al., 2014; Trevisan et al., 2014) were reported as well on nanomaterials toxicity.

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2 AIMS OF THE STUDY

Based on the published literatures reviewing, several research points on ecotoxicity of ZnO NPs towards algae and mussel in marine environment were still unexplored and some represent aims of this study.

- ZnO NPs could have a specific behavior and effect in diverse marine seawaters as other nanomaterials described before. To this aim the full characterization of ZnO NP dissolution, aggregation, and sedimentation once dispersed into a standard Artificial Sea Water (ASW) and a Reconstituted Sea Water (RSW) from East China Sea was performed.
- The ecotoxicity of ZnO NPs upon a green alga and a diatom would be different. To assess the diverse toxicity upon different algae, growth inhibition algal assays were performed. Additionally, to verify the hypothesis about the role of nano size in the overall toxicity, ZnO bulk, and Zn salt toxicity were assessed as well.
- ZnO NP toxicity towards mussels under a chronic exposure could be shown by Zn bioaccumulation, tissue damages, and transcription of apoptosis and antioxidation-related genes. The hypothesis that nano size plays an important role in its toxicity was also considered together with the all previous ones for mussel *M. galloprovincialis*.

2.1 Behaviors of ZnO NPs and bulk in different artificial seawater

To characterize the behaviour of ZnO NPs and bulk dispersed into two salinities seawater, artificial standard seawater (ASTM, 1998) (salinity 35‰, pH 8.00, 0.22μm filtered) and natural seawater from East China Sea (salinity 25‰, pH 7.90, 0.22μm filtered), aggregation size, sedimentation, and dissolubility of particles were oberved and tested using dynamic light scattering technique (DLS), UV-vis spectrophotometer, and centrifugal ultrafiltration combined with inductively coupled plasma-mass spectrometry (ICP-MS) (Jenner et al., 1990).

2.2 Comparative toxicity of ZnO NPs, bulk, and Zn salt towards marine algae *T. suecica* and *P. tricornutum*

To focus the ecotoxicological effect of ZnO NPs and bulk towards the green alga *T.*

suecica and diatom *P. tricornutum*, the aims are to establish the main toxicity parameters and to compare the sensitivities of both algae to ZnO particles. For each compound No Observed Effect Concentration (NOEC), 1, 10, and 50% Effect Concentration (EC1, EC10, and EC50) were defined to provide biological criteria for the implementation of water quality standards to protect marine organisms.

2.3 Zn bioaccumulation and histological damages of *M. galloprovincialis* exposed to ZnO NPs, bulk, and Zn salt

The histological alterations on 6 organs in *M. galloprovincialis* exposed to ZnO NPs suspensions to characterize the tissue damages were observed. In addition, tissue Zn uptake was measured to reveal the fate of Zn in mussel and give a full understanding of ZnO NPs toxicity with the histological observation. A microwave digestion system (MARS Xpress, CEM, USA) and an atomic absorption spectrometer (AAS; AA240 Duo, Varian, USA) (Massányi et al., 2004; Kramárová et al., 2005; Gasparik et al., 2012) were used.

2.4 Ecotoxicity of ZnO nanoparticles in *M. galloprovincialis* revealed by transcription of apoptosis and antioxidation-related genes

The aim of this work is to investigate the changes in digestive gland transcription levels of key genes, DNA repair enzymes genes *p53*, *PDRP*, antioxidant enzymes genes *superoxide dismutase* (*SOD*), *glutathione transferase* (*GST*), and *catalase* (*CAT*), of *M. galloprovincialis* along four weeks exposure to ZnO NPs and bulk dispersed in RSW, using qRT-PCR.

2.5 Zinc causes oxidative damages in digestive gland in mussel *M. galloprovincialis* revealed by transcription of related genes

The aim of this work is to investigate the changes in digestive gland transcription levels of key genes, DNA repair enzymes genes *p53*, *PDRP*, antioxidant enzymes genes *superoxide dismutase* (*SOD*), *glutathione transferase* (*GST*), and *catalase* (*CAT*), of *M. galloprovincialis* along four weeks exposure to Zn salt dissolved in RSW, using qRT-PCR.

2.6 References

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3 RESULTS AND DISCUSSION

3.1 Behaviors of ZnO NPs and bulk in different artificial seawater

To investigate the ecotoxicity of ZnO NPs on algae and mussels, the evaluation of the ZnO NP behaviours of dispersed in the testing media is a necessary step to understand how ZnO particles interact with organism.

Two artificial seawater were utilized as ecotoxicological testing media: in this research the standard Artificial Sea Water (ASW) (ASTM, 1998) for algae and a Reconstituted Sea Water (RSW) from East China Sea for mussels.

Once released into these media, ZnO NPs, due to their reactive nature, rapidly interact each other and with available inorganic compounds (Rocha et al., 2015).

ZnO particle behaviour in the testing medium largely depends both on particle properties, such as size, shape, surface charge; and on medium properties, such as pH, temperature, ionic strength, play key role on (Fabrega et al., 2012; Rocha et al., 2015). Based on the design of the ecotoxicological investigation, the aggregation described by size changing, the sedimentation induced by gravity, and dissolution described by ionic Zn release, were performed.

3.2 Materials and methods

3.2.1 Chemicals

Bare ZnO NPs (cod. 544906, particle size < 100 nm, surface area 15-25 m^2/g) was purchased from Sigma-Aldrich. (1) Bulk ZnO powder (particle size < 200 nm, surface area 4.9-6.8 m^2/g, purity > 99.9%) was purchased from Galeno S.r.l., Italy. (2) Bulk ZnO powder (Code ZK249038, particle size 150−200 nm, purity > 99.9%) was purchased from Quer Biotech Co., Ltd (Hefei, China). Baysalt crystals (Code Q/XWL 101−2006) were purchased from Shuilifang S&T Ltd. (Xiamen, China).

3.2.2 Particle dispersions

(1) Approach of particles dispersions in standard Artificial Sea Water (ASW) (ASTM,

1998):

Stock suspensions of testing materials (ZnO NPs and bulk) were prepared with ASW (pH 8.0, 0.22 μm filtered) to the final concentration of 100 mg Zn/L. The ZnO suspensions were dispersed by bath-sonication for 30 minutes at 50 W (Elma Transsonic Digital S). Test suspensions, at three concentrations 1, 5, and 10 mg Zn/L, were prepared by diluting the stock suspension with ASW.

(2) Approach of particles dispersions in Reconstituted Sea Water (RSW) from East China Sea:

Stock suspensions of ZnO NPs and ZnO bulk powder were prepared with (RSW). East China Sea baysalt crystals extracted from natural seawater were dissolved in pure water (MilliQ) to salinity 25 ‰ (pH 7.90) which is the salinity level of mussel sampling sea area (Shen et al., 2009) and filtered in 0.22 μm, to the final concentration of 100 mg Zn/L. The ZnO suspensions were dispersed by bath-sonication in the same abovementioned conditions. Test suspensions were prepared by diluting the stock suspension at 10 and 100 mg Zn/L with RSW (Manzo et al., 2013a).

3.2.3 Measurement of the size of the aggregates

After sonication treatment, an aliquot of ZnO NPs and bulk suspension, at concentration of different sea water (ASW: 1, 5, and 10 mg Zn/L; RSW: 10 and 100 mg Zn/L) respectively was put in a disposable polystyrene cuvette to determine particle size with dynamic light scattering technique (DLS) using Zetasizer Nano ZS Malvern instrument. The instrument employs a 4 mW He-Ne laser at wavelength 632.8 nm and the measurement angle of 173 °with a Non-Invasive Back Scatter technology (NIBS). Measurements were made at 25 ℃. The measurements were run in triplicate and the results shown are the average of the runs. The samples were monitored for 4 days (ASW) and 90 min (RSW). Every day a new aliquot of sample was used to measure the aggregation size with DLS for ASW only.

3.2.4 Sedimentation measurement in ASW and RSW

As same as the condition of size measurement, an aliquot of ZnO NPs and bulk suspension, at concentration of 1, 10, and 100 mg Zn/L respectively was put in a disposable polystyrene cuvette to measure the sedimentation using a UV-vis spectrophotometer (at 468 nm) for

24 h.

3.2.5 Dissolution in ASW and RSW

The dissolved Zn concentrations of ZnO NPs and bulk suspensions in 100 and 10 mg Zn/L dispersed into low salinity RSW were measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) described by Navarro et al. (2008). Samples after ultrafiltration (2 nm) have been acidified with HNO_3 (1%) and diluted 1:1000 for Zn analysis. Dissolved Zn (ZnO particle or other Zn complex size < 2 nm) in the four suspensions were daily measured for 3 days.

3.3 Results and discussion

3.3.1 ZnO particle aggregation

(1) In ASW:

ZnO NPs in aqueous media tends to aggregate indeed, particularly in saltwater with increasing of salinity, that ionic strength reduces the negativity of electrophoretic mobility of the particles to encourage agglomeration (Batley et al., 2013). In order to monitored the stability of NPs and their rapid tendency to aggregate, after dispersion of ZnO NPs and bulk in ASW and sonication treatment for 30 minutes, agglomeration phenomenon was observed for the first 120 minutes and subsequently, once a day for four days (Fig. 3.3.1-1 and 2).

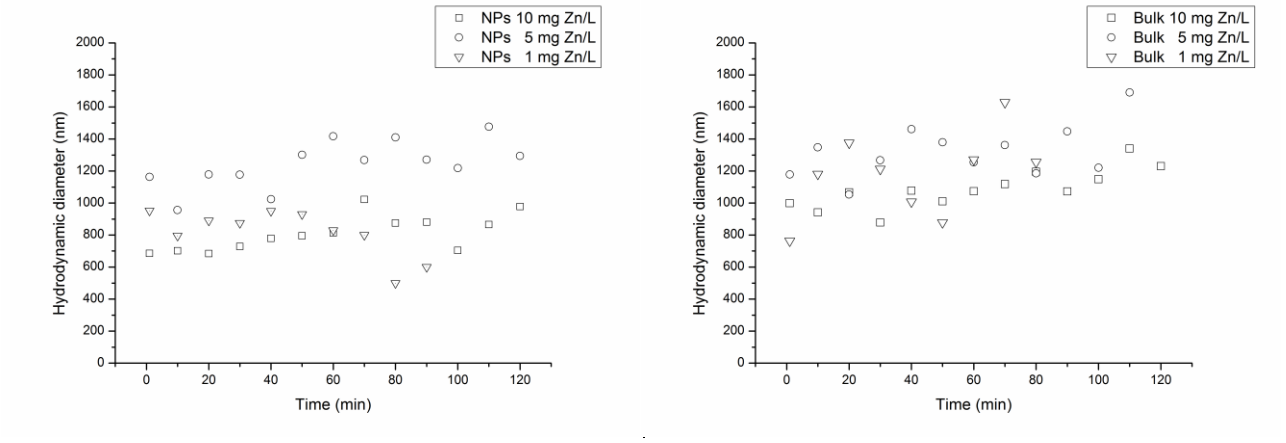

Fig. 3.3.1-1 Hydrodynamic diameters of ZnO NPs and bulk in ASW suspension at three concentrations (10, 5 and 1 mg Zn/L) within two hours.

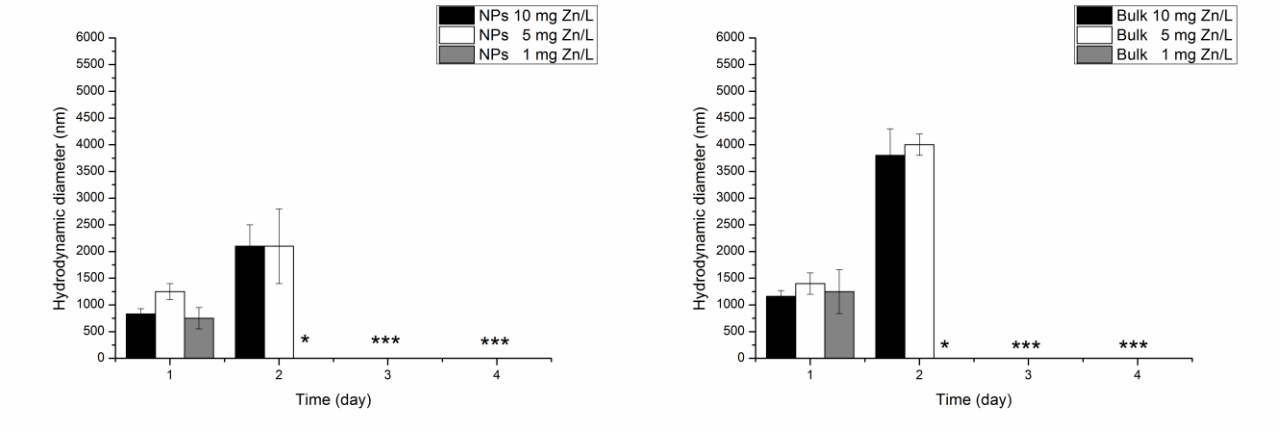

Fig. 3.3.1-2 Hydrodynamic diameters of ZnO NPs and bulk in ASW suspension at three concentrations (10, 5 and 1 mg Zn/L) within four days. * Samples not suitable for DLS analysis.

The analysis of the DLS measurement data of the samples highlights that large aggregate were present for NPs and bulk for all the concentrations but ZnO NPs aggregates were smaller than bulk for all concentration as shown in Table 3.3.1-1.

Table 3.3.1-1 PDI and hydrodynamic diameter of NPs and bulk in ASW suspensions at four concentrations (100, 10, 5, and 1 mg Zn/L) in the initial stage of dispersal after sonication treatment.

mg/L	NPs		Bulk	
	PDI	Hydrodynamic diameter (nm)	PDI	Hydrodynamic diameter (nm)
100	0.3 ± 0.05	1500 ± 300	0.8 ± 0.1	1900 ± 180
10	0.48 ± 0.07	850 ± 100	0.56 ± 0.06	1150 ± 100
5	0.72 ± 0.09	1250 ± 150	0.76 ± 0.09	1400 ± 200
1	0.74 ± 0.03	900 ± 80	0.59 ± 0.17	1250 ± 400

In the next 24 hours the hydrodynamic diameter of ZnO aggregate increased doubling their size. From third day of observation the size was greater than 6 microns (Fig. 3.3.1-1 and 2) than the sample was not suitable for DLS measurements. This observation highlights that the ZnO NPs colloidal suspension should be always freshly prepared and sonicated prior to each experiment in order to minimize the effects of particles aggregation. The Polydispersity Index (PDI) that describes the width of the particle size distribution was in the range of 0.3-0.8 as shown in Table 3.3.1-1. Those values indicated that the sample has a very broad size distribution.

The hydrodynamic diameters of ZnO NPs and bulk particles were already approaching the microns range soon after dispersion in ASW, and showed a clear, although slow, tendency to further aggregate in the next few hours (Table 3.3.1-1 and Fig. 3.3.1-1). In general, bulk ZnO had larger aggregate sizes than NPs. It is worth to note that these results seem to be rather independent of the pristine size of ZnO particles and of dispersion methods. In fact, previous studies report analogous values of aggregate sizes and aggregation trends for ZnO dispersions in seawater although prepared from nano ZnO having pristine size much smaller than the one used in the present study and from different dispersion methods (Fairbairn et al., 2011; Yung et al., 2015). The reported results also show that, in our operating conditions, the aggregate size was only modestly influenced by the initial particle concentration. The average aggregate size of both nano and bulk ZnO first increased from 1 to 5 mg Zn/L then decreased at 10 mg Zn/L and finally showed the largest size at 100 mg Zn/L. However, the oscillation of bulk ZnO particle size between 1 and 10 mg Zn/L was well included in the measurement variability, therefore test suspensions of bulk ZnO were basically characterized by an average aggregate size of around 1400 ± 490 nm. On the other hand, at concentration so high as 100 mg Zn/L the large aggregate size may be due to an increase in particle collision frequency which enhances aggregation (Keller et al., 2010). According to the aggregation trend shown by this particles (Fig. 3.3.1-1) 24 hours after the preparation of test suspensions the average aggregate size was nearly doubled for nano ZnO and almost multiplied by four in case of bulk ZnO (Fig. 3.3.1-2). After 48 hours, ZnO particles in low concentration 1 mg Zn/L were undetectable and the size of which in high concentration 5 and 10 mg Zn/L were too large (> 6 μm) to suitable for DLS. The absence of particles (both nano and bulk) could be addressed to the proceeding of the dissolution phenomenon. The water solubility of ZnO in fact, ranges from 1.6 mg Zn/L to 5 mg Zn/L (PROSPEcT, 2009). The high pH and high ionic strength conditions of the seawater further increase this solubility and highlight some differences between the NPs and bulk forms (Miao et al., 2010; Miller et al., 2010; Wong et al., 2010; Peng et al., 2011; Yung et al., 2015). However, the interaction between particles and algal cells could retard dissolution and promote homo aggregation of ZnO particles (Soldo et al., 2005; Miao et al., 2007; Navarro et al., 2008), that has been observed in this report.

(2) In RSW:

ZnO NPs once in RSW undergo to different physic-chemical processes that modify their pristine characteristics and therefore their availability/reactivity.

ZnO particles dispersed in RSW (low salinity) undergo to complex physicochemical transformations: first, particles stick to each other in order to minimize the repulsive hydrophobic interactions with water, forming micron sized aggregate. DLS data recorded soon after the dispersion preparation illustrate this aggregation behavior and show that the average size of bulk ZnO particles were rather unaffected by the initial solid loading (Fig. 3.3.1-3B). On the contrary, aggregates formed by ZnO NPs showed constant size at low concentration whereas highly concentrated dispersions showed a marked trend to increase (Fig. 3.3.1-3A). The aggregate size increasing becomes evident in the next 48 hours when all the samples were characterized by the presence of very large particles (> 6000 nm) (Table 3.3.1-2).

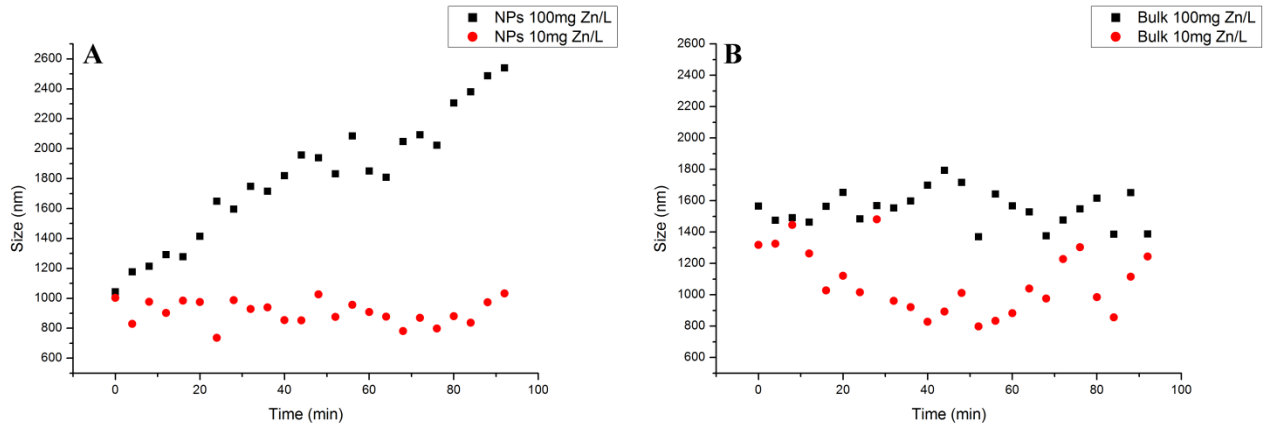

Fig. 3.3.1-3 Hydrodynamic diameters of ZnO NPs and bulk in RSW suspension at two concentrations (100 and 10 mg Zn/L) within about 90 min.

Table 3.3.1-2 PDI and hydrodynamic diameter of ZnO NPs and bulk in RSW suspensions at two concentrations (100 and 10 mg Zn/L) for 72 hours

Suspension	Time (h)	Size (nm)	PdI	Z-potential (mv)
100 mg Zn/L NPs	0	2320±520	0.44±0.119	-0.92
	24	>6000nm	1	-2.49
	48	>6000nm	1	-5.28
	72	>6000	NA	NA
10 mg Zn/L NPs	0	950±160	0.53±0.14	-6.35
	24	1570±200	0.76±0.13	-10.4
	48	>6000	NA	NA
	72	>6000	NA	NA
100 mg Zn/L Bulk	0	1560±100	0.5±0.07	-10.7

	24	3150±220*	0.51±0.11	-10.2
	48	>6000	1	-7.06
	72	>6000	NA	NA
10 mg Zn/L Bulk	0	1000±200	0.63±0.08	-10.8
	24	>6000nm*	1	NA
	48	>6000	1	NA
	72	>6000	1	NA

3.3.2 ZnO particle sedimentation

(1) In ASW:

Manzo et al. (2013b) reported the sedimentation of ZnO NPs and bulk dispersed in ASW (Fig. 3.3.2-1). They observed a clear sedimentation for both ZnO aggregates and the suspended ZnO concentration decreased by almost 30% after 2 h at high concentration (100 mg Zn/L). At each concentration, bulk showed higher sedimentation rate than NPs after 2h. Manzo et al. (2013b) suggested that a main difference between NPs and bulk aggregates is the density which is lower for the nanomaterial.

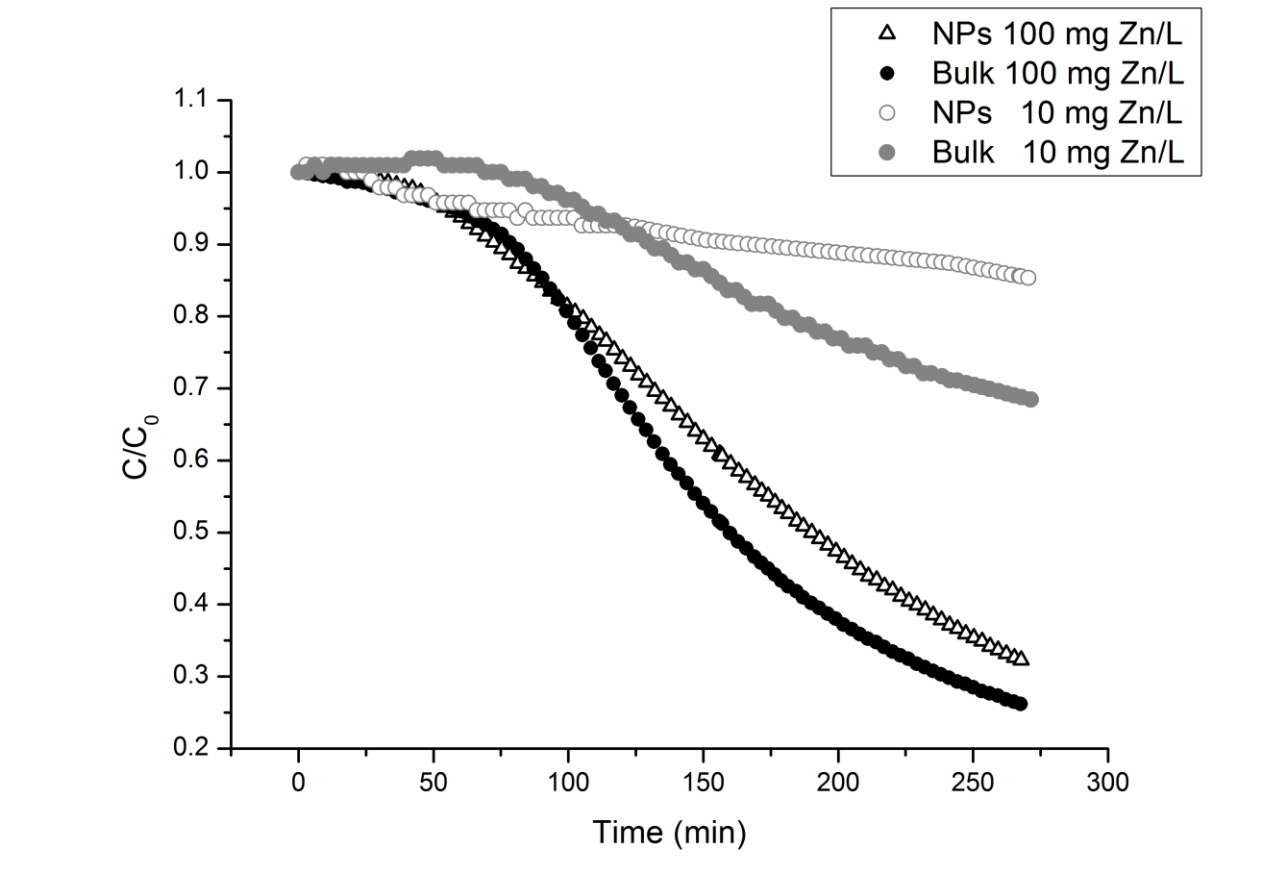

Fig. 3.3.2-1 Sedimentation measurement of ZnO NPs and bulk in ASW at the concentration 100 and 10 mg Zn/L within

about 5 h.

(2) In RSW:

Large particles suspended in RSW have a tendency to settle out of the liquid phase due to gravity. The sedimentation curves recorded showed indeed that the particle concentration in the water column was decreased by more than 80% and 70% already after 8 hours for ZnO NPs and bulk suspensions at 100 mg/L respectively (Fig. 3.3.2-2).

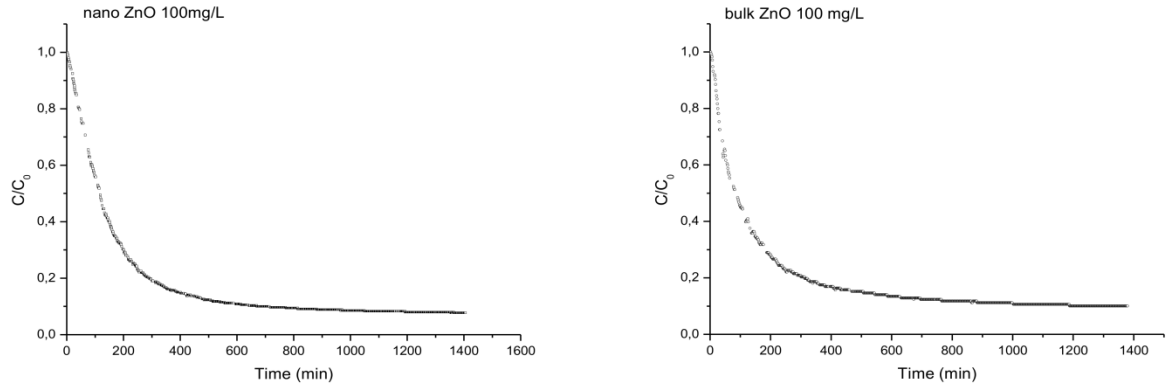

Fig. 3.3.2-2 Sedimentation measurement of ZnO NPs and bulk in RSW suspension at the concentration 100 mg Zn/L within 24h.

3.3.3 ZnO particle dissolution

(1) In ASW:

Dissolution is the other important transformation that occurs to ZnO particle in aqueous media. A dissolution kinetic curve of ZnO NPs and bulk dispersed in ASW was shown in Fig. 3.3.3-1. The curve indicated that a very obvious tend in both NPs and bulk suspension which is 70% dissolution occurred in first few minutes and kept this stable status to the end of measurement.

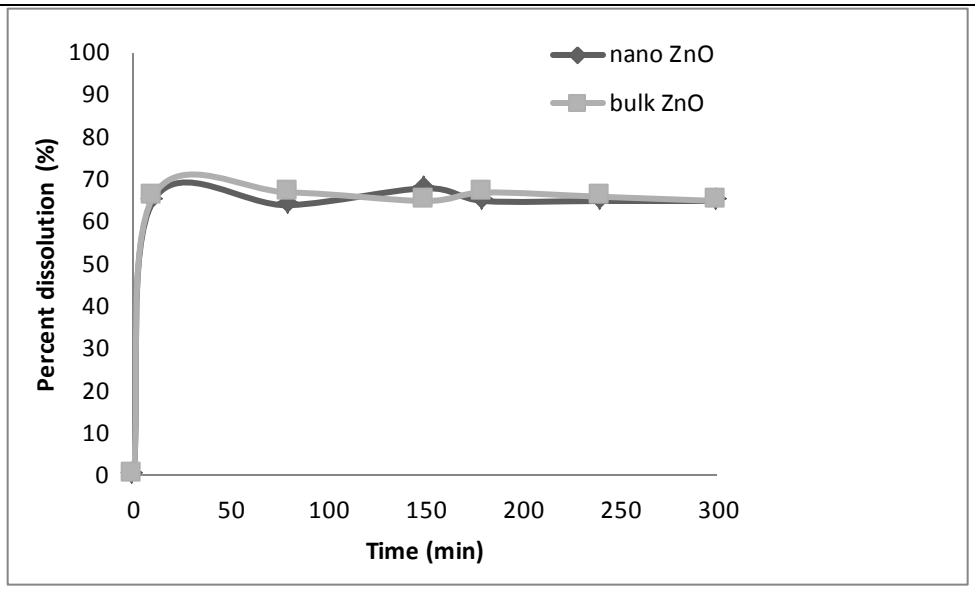

Fig. 3.3.3-1 Dissolution kinetic curve of ZnO NPs and bulk dispersed in ASW at 10 mg Zn/L within 5 h.

(2) In RSW:

The analysis of ionic zinc released in the seawater from NPs and bulk dispersions shows a little displacement in the average Zn concentrations only as a function of the initial solid loadings, i.e. between 100 and 10 mg Zn/L (Fig. 3.3.3-2). In the main, an average Zn concentration around 5 mg/L was found already after 24 hours and afterwards without significant variations ($p > 0.05$). Interestingly, this result is similar to a previous one obtained for ZnO NPs dispersed into an artificial seawater with different salinity and pH (ASW at salinity 35 ‰, pH 8.00, 0.22 µm filtered) with respect to the natural seawater used in the present study (Manzo et al., 2013b).

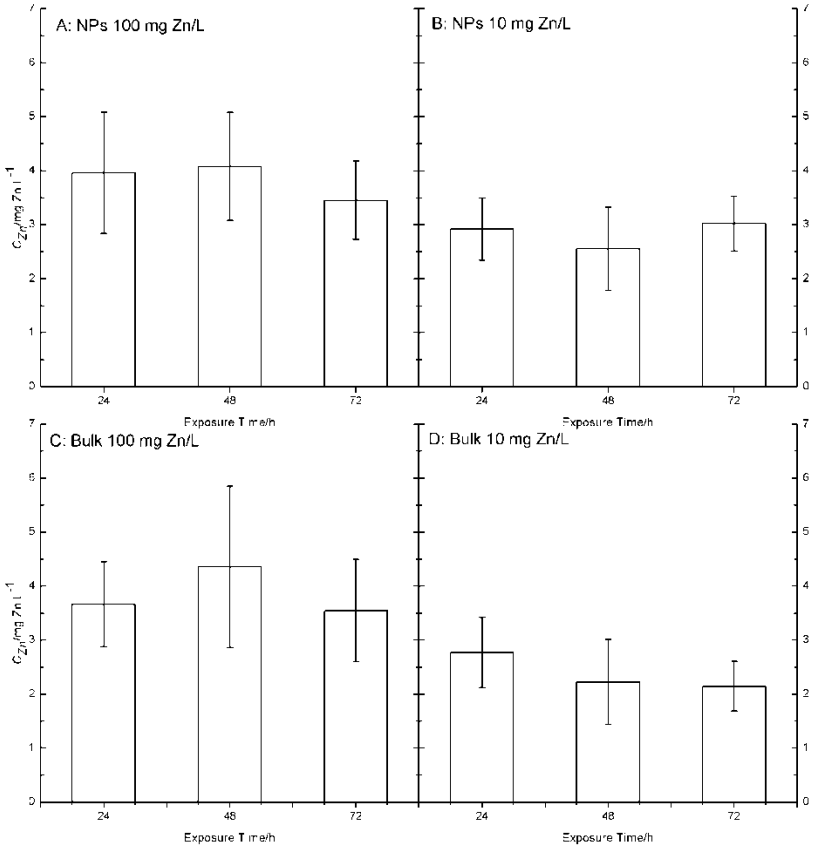

Fig. 3.3.3-2 Bar graphs of the dissolved Zn (Φ < 2 nm) of ZnO NPs (A and B) and bulk (C and D) suspensions in 100 and 10 mg Zn/L measured by ICP-MS.

3.4 Comparisons and Conclusions

To respect the behavior of other nanoparticles dispersed in natural seawater repored by Garner & Keller (2014), that aggregation and sedimentation have similar time scales for most nanoparticles in general in seawater and dissolution is highly dependent on nanomaterials composition, for example, nano Ag, Al_2O_3, CuO, and NiO will dissolve over days to weeks but only hours to days for ZnO NPs, results in this case indicated similar and more detailed conclusion.

The analysis of the size measurement data of NPs and bulk in ASW highlights that large aggregate were present for NPs and bulk for all the concentrations but ZnO NPs aggregates were smaller than bulk for all concentrations. In RSW, aggregates formed by ZnO NPs showed constant size at low concentration (10 mg Zn/L) whereas highly concentrated dispersions (100 mg Zn/L) showed a marked trend to increase. Hence, the primary size of ZnO in suspensions could affect the aggregating rate in higher concentration. To combine the previous conclusion (Manzo et al., 2013b)

and the sedimentation data obtained in this case indicated that the sedimentation occurred since in the first minutes to few hours and that was not related to ZnO particles size. The analysis of ionic zinc released in the seawater from NPs and bulk dispersions shows a little displacement in the average Zn concentrations only as a function of the initial solid loadings. An average Zn concentration around 5 mg/L was found already after 24 hours and afterwards without significant variations ($p > 0.05$). This result is similar to a previous one obtained for ZnO NPs dispersed into an artificial seawater with different salinity and pH (ASW at salinity 35 ‰, pH 8.00, 0.22 μm filtered) with respect to the natural seawater used in the present study (Manzo et al., 2013b).

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Yung MM, Wong SW, Kwok KW, Liu F, Leung Y, Chan W, Li X, Djurišić A, Leung KM (2015) Salinity-dependent toxicities of zinc oxide nanoparticles to the marine diatom *Thalassiosira pseudonana*. Aquat Toxicol 165:31-40.

3.6 Comparative toxicity of ZnO NPs, bulk, and Zn salt towards marine algae *T. suecica* and *P. tricornutum*

ZnO NPs is currently used in a broad range of products including plastics, ceramics, rubber, lubricants, paints, foods (source of Zn nutrient), batteries, fire retardants, personal care products, etc. (Ma et al., 2013). Such widespread and expanding production and use increase the potential for their release into the environment. Keller et al. (2014) estimated that ZnO together with titanium dioxide, represent 94% of engineered nanomaterial (ENMs). Gottschalk et al. (2009) reported the modeled environmental concentration of ZnO NPs in surface water (0.010 µg/L in Europe, 0.001 µg/L in U.S.). If current production and subsequent release quantities were to increase 100-fold, ZnO would raise greatest concern since all studies indicate ZnO is toxic at some concentration to all species tested (Franklin et al., 2007; Blinova et al., 2010; Miller et al., 2010; Li et al., 2013). It is clear than the effects and the behavior of ZnO NPs in the marine environment would need to be monitored closely.

Particle-induced effect was suggested as one mechanisms of toxic action of ZnO NPs in previous studies (Ma et al., 2013). The nanoparticles agglomeration/aggregation plays an important role in determining reactivity, toxicity, fate, transport, and risk in the environment indeed has been implicated as a mitigating factor in the transport, cellular level interactions, and fate of NPs in the environment (Hotze et al., 2010).

Phytoplankton is the autotrophic component of the plankton community and a key factor of oceans, seas and freshwater basins ecosystems. High growth rate, high surface-to-volume ratio (high uptake rate), easy handling in laboratory conditions (Castro-Bugallo et al., 2014), make marine microalgae be an effective tool to test toxicity of contaminants such as antibiotics (Seoane et al., 2014), metals (Wang & Zheng, 2008; Angel et al., 2015), nano-materials (Kadar et al., 2012; Clément et al., 2013; Castro-Bugallo et al., 2014). Additionally, as diverse algae taxa respond differently to chemical toxicants, it is mandatory to conduct tests on a different species representing different classes.

Marine green microalgae *T. suecica* and diatom *P. tricornutum* were selected as test organisms to respond to ZnO NPs exposure.

P. tricornutum is a widespread pennate diatom, with low silica content and distinct cell

wall (i.e. frustule) structures which is essentially composed of organic compounds, particularly sulfated glucomannan (Tesson et al., 2009), has been described in three different morphotypes (i.e. the ovoid, fusiform and triradiate forms), whose occurrence in culture seems to depend on strains as well as environmental conditions (Francius et al., 2008).

T. suecica is an elliptical microalga of the class Chlorophyceae (Prasinophyceae) generally used as the diets of zooplankton, bivalve molluscs and crustacean larvae. The characteristic cell wall (theca) is composed of coalesced rigid carbohydrate scales (Lee et al., 2013) and the typical four flagella are covered by double layer of scales.

It worth to note that, as previously observed, ZnO NP aggregation and sedimentation will occur in first few hours in seawater suspensions (Manzo et al., 2013a). Therefore it is ecologically relevant to study the interactions of these ZnO aggregates with very diverse classes of algae (i.e. green algae and diatoms) with peculiar differences in size, shape, cell wall composition and motility. In this study we focused on the ecotoxicological effect of ZnO NPs and bulk towards the green alga *T. suecica* and diatom *P. tricornutum* with the aim to establish the main toxicity parameters and to compare the sensitivities of both algae to ZnO particles.

For each compound No Observed Effect Concentration (NOEC), 1, 10, and 50% Effect Concentration (EC1, EC10, and EC50) were defined to provide biological criteria for the implementation of water quality standards to protect marine organisms.

3.7 Materials and methods

3.7.1. Chemicals

Bare ZnO NPs (cod. 544906, particle size <100 nm, surface area 15-25 m^2/g) and $ZnSO_4$ (cod. 204986, purity 99.999%) were purchased from Sigma-Aldrich. ZnO bulk powder (particle size < 200 nm, surface area 4.9-6.8 m^2/g, purity >99.9%) was purchased from Galeno S.r.l., Italy.

3.7.2. Organisms

T. suecica (Prasinophyceae: Chlorodendrales) and *P. tricornutum* (Bacillariophyceae: Naviculales) (CriAcq Laboratory, Naples, Italy) algae, were maintained in sterilized standard medium (Guillard, 1975) made with artificial standard seawater (pH 8.00, 0.22 μm filtered) (ASTM, 1998). To provide inoculant for experiments, microalgae were incubated under cool continuous

white fluorescent lights (about 58 µmol photons m^{-2} s^{-1}) at 24 ± 1 ℃ with aeration for 5-7 days until log phase growth prevailed. Cell density was measured by hemacytometer.

3.7.3. Particle dispersions

Stock suspensions and solution of testing materials (ZnO NPs, ZnO bulk, and $ZnSO_4$) were prepared with Artificial Sea Water (ASW) to the final concentration of 100 mg Zn/L. The ZnO suspensions were dispersed by bath-sonication for 30 minutes at 50 W (Elma Transsonic Digital S). Test suspensions, at concentrations ranging between 0.1 and 10 mg Zn/L, were prepared by diluting the stock suspension with ASW and sonicated once again. Before the addition of micronutrients and algae each test suspension was briefly vortexed.

3.7.4. FIB observation methods

The algal cells were preliminarily analyzed by an optical microscope (ZEISS Axioskop 50) for observing the possible damage and its extent. High resolution morphological characterization was made by FEI Dual Beam Quanta 200 3D which integrates a high focused ion beam (FIB). FIB operates with a finely focused beam of gallium ions accelerated at 30 keV which scans over the surface at low beam currents taking care not to damage sample.

Before FIB observations algal cells were fixed as described in Li et al. (2015). After 72 h of exposure algal cells were centrifuged (4000 rpm, 10 min) then the samples were fixed with 3% gluteraldehyde solution in 4 ℃ for 2 h. The samples were then washed with 0.1 M PBS (pH 7.8) by centrifugation (4000 rpm, 10 min) three times. Algal cells were fixed with 1% osmium tetra oxide for 2 h in 4 ℃, and 0.1 M PBS (pH 7.8) was added to wash the cells by centrifugation (3800 rpm, 10 min) three times. The control and treated (10 mg Zn/L) cells were coated on a thin glass slide, air dried and observed under the FIB.

3.7.5. Algal growth inhibition test and data analysis

Algal bioassays were performed according to our previous research (Manzo et al., 2013a) slightly modified. Test plates were kept in the growth chamber with continuous light for 3 days. The growth inhibition was expressed with respect to the control. The concentrations of the testing suspensions and solution were modified to 10, 5, 3, 1, 0.5, and 0.1 mg Zn/L for ZnO NPs, bulk, and $ZnSO_4$. The EC1, EC10, and EC50 were calculated using the Linear Interpolation Method

(Inhibition Concentration procedure, ICp) (Norberg-King, 1993). NOEC was determined by Dunnett's test (US-EPA, 1989).

3.8 Results and discussion

3.8.1 Effects of ZnO particles upon algae

T. suecica:

During the three days of exposure to ZnO NPs, the parameters of algae inhibition were recorded every 24 h and similarly bulk ZnO and $ZnSO_4$ trials were set up as comparing experiments. The overall toxic effect after three days of *T. suecica* exposure to investigated chemicals (Fig. 3.8.1-1) indicated that, at the same Zn amount, ZnO NPs was more toxic than bulk ZnO and than $ZnSO_4$, suggesting that the dominant cause of the algal growth inhibition by ZnO NPs was not only related to ion release. Our previous study upon marine algae *Dunaliella tertiolecta* (Manzo et al., 2013a) also reported a higher toxicity of ZnO NPs respect to bulk. Similar toxicity trend was also observed for *Chlorella sp* (Ji et al., 2011).

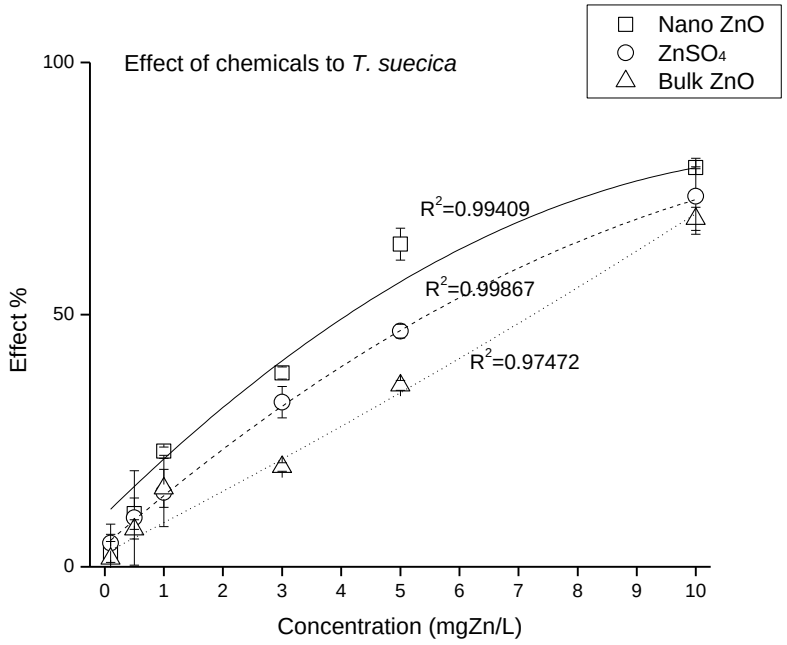

Fig. 3.8.1-1 Toxic effects on *T. suecica* cells, together with the corresponding regression fit curves: nano ZnO toxic effects (diamond); $ZnSO_4$ toxic effects (triangle); bulk ZnO toxic effects (square). The best fit function of toxicity data was sigmoid growth function. 50% effect level is represented.

The ecotoxicological parameters (NOEC, EC1, EC10, and EC50), at each exposure time point, reported in Table 3.8.1-1 showed that ZnO NPs was more toxic than bulk at all tested concentrations and zinc salts played a middling role between ZnO NPs and bulk.

Table 3.8.1-1 NOEC, LOEC, EC1, EC10, EC20 and EC50 evaluated for each tested chemicals (nano ZnO, bulk ZnO and $ZnSO_4$) to *T. suecica* at each exposure time point.

Exposure time	Chemicals	NOEC (mg Zn/L)	EC1 (mg Zn/L)	EC10 (mg Zn/L)	EC50 (mg Zn/L)
24h	Nano ZnO	0.1	0.01 [0.004, 0.06]	0.07 [0.05, 1.13]	4.09 [3.78, 4.59]
	$ZnSO_4$	0.1	0.01 [0.004, 0.02]	0.06 [0.04, 0.15]	0.69 [0.42, 3.20]
	BulkZnO	1.0	0.01 [0.007, 0.46]	0.39 [0.07, 1.67]	4.55 [4.17, 4.98]
48h	Nano ZnO	< 0.1	0.06 [0.005, 0.008]	0.06 [0.05, 0.09]	4.28 [3.98, 4.46]
	$ZnSO_4$	< 0.1	0.01 [0.004, 0.006]	0.05 [0.04, 0.06]	5.97 [5.17, 6.46]
	BulkZnO	1.0	0.04 [0.02, 0.25]	1.32 [0.74, 1.60]	8.17 [7.53, 8.71]
72h	Nano ZnO	0.1	0.04 [0.01, 0.18]	0.47 [0.11, 0.63]	3.91 [3.66, 4.14]
	$ZnSO_4$	0.5	0.02 [0.01, 0.24]	0.53 [0.08, 1.29]	5.61 [4.93, 6.23]
	BulkZnO	0.5	0.06 [0.01, 0.20]	0.66 [0.32, 0.99]	7.12 [6.65, 7.46]

In particular for ZnO NPs in all three days exposure time (72 h), NOEC was recorded at the lowest setting concentration (0.1 mg Zn/L). The value resulted lower than that obtained for ZnO bulk (NOEC 0.5 mg Zn/L). ZnO NPs EC1, EC10, and EC50 of were respectively recorded at 0.04 [0.01, 0.18] mg Zn/L, 0.47 [0.11, 0.63] mg Zn/L, and 3.91 [3.66, 4.14] mg Zn/L, which likewise were lower than those of bulk (0.06 [0.01, 0.20] mg Zn/L, 0.66 [0.32, 0.99] mg Zn/L, and EC50 = 7.12 [6.65, 7.46] mg Zn/L).

However *T. suecica* on the basis of these values resulted less sensitive than *D. tertiolecta* to ZnO (EC50 = 1.94 [0.78–2.31] mg Zn/L) as recorded in previous work (Manzo et al., 2013a) while very low EC50 value were found upon microalgae *Pseudokirchneriella subcapitata* (EC50 = 0.042 mg Zn/L) (Aruoja et al., 2009) and *Thalassiosira pseudonana* (EC50 = 0.82 mg Zn/L, leading to the decrease of cell division rates by 50%) (Peng et al., 2011).

With the aim to evaluate the relation between algae exposure and growth effect all these

parameters were recorded also in early testing phases (24 and 48 h, Table 3.8.1-1). At the beginning of the exposure (24 h), the parameter values for ZnO NPs were higher than those recorded at followed phases (48 and 72 h). Respect to ZnO NPs, bulk ZnO and $ZnSO_4$ showed increasing trends for EC10 and EC50 values along with exposure time that could represent a different action of these particles upon the algal population: ZnO NPs effectively affected algal population from the rising phase (48 h) of growth curve in the most.

The observations by optical microscope (Fig. 3.8.1-2) and by FIB (Fig. 3.8.1-3) of *T. suecica* exposed to ZnO (both nano and bulk) showed how ZnO aggregates tend to gather mainly around algae flagella area (Fig. 3.8.1-2). This phenomenon increased with particle concentration and exposure time and resulted in algae injury due to motility hindrance in culture media. Recently, some evidences of toxic mechanism due to the direct interaction of NP aggregates and algae cell wall, provoking the generation of "holes" was reported (Li et al., 2015). However, in our case, the size for both the ZnO particles was very large, and the main aggregation sites, as reported above, were flagella (Fig. 3.8.1-3).

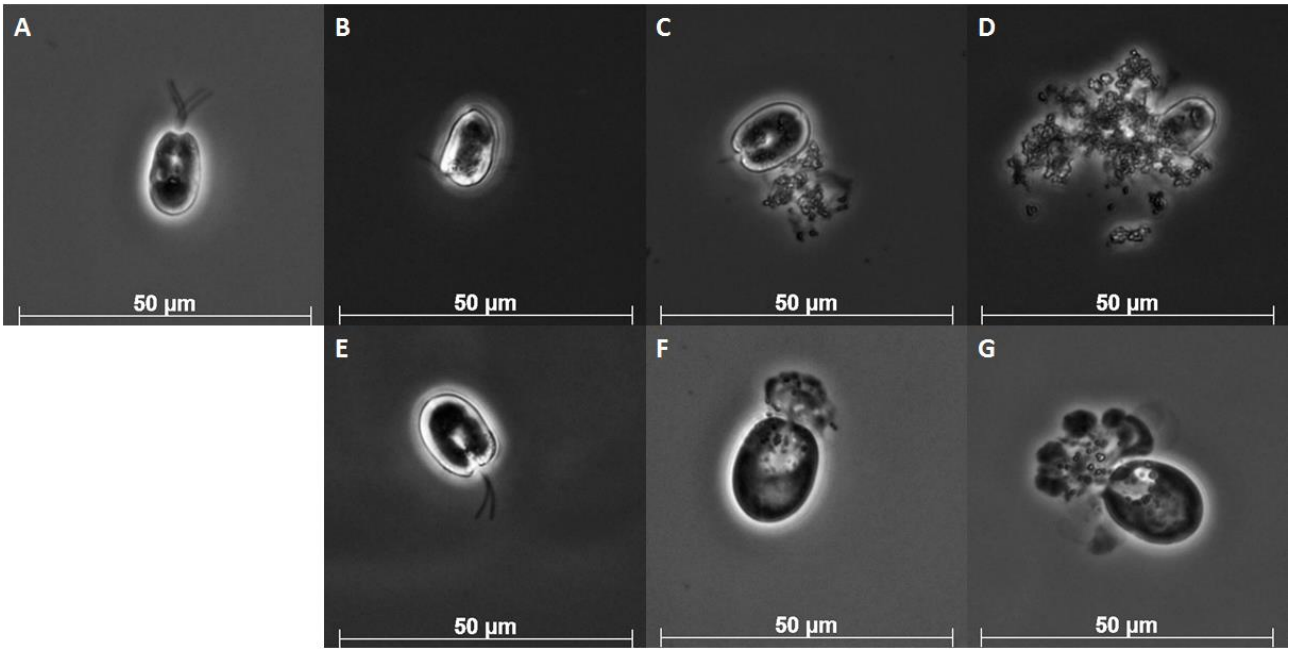

Fig. 3.8.1-2 Behavior of *T. suecica* with aggregates of zinc oxide in 72 h, A: control, B: 0.1 mg Zn/L nano, C: 5 mg Zn/L nano, D: 10 mg Zn/L nano, E: 0.1 mg Zn/L bulk, F: 5 mg Zn/L bulk and G: 10 mg Zn/L bulk. The flagella showed more easily aggregates than cells walls.

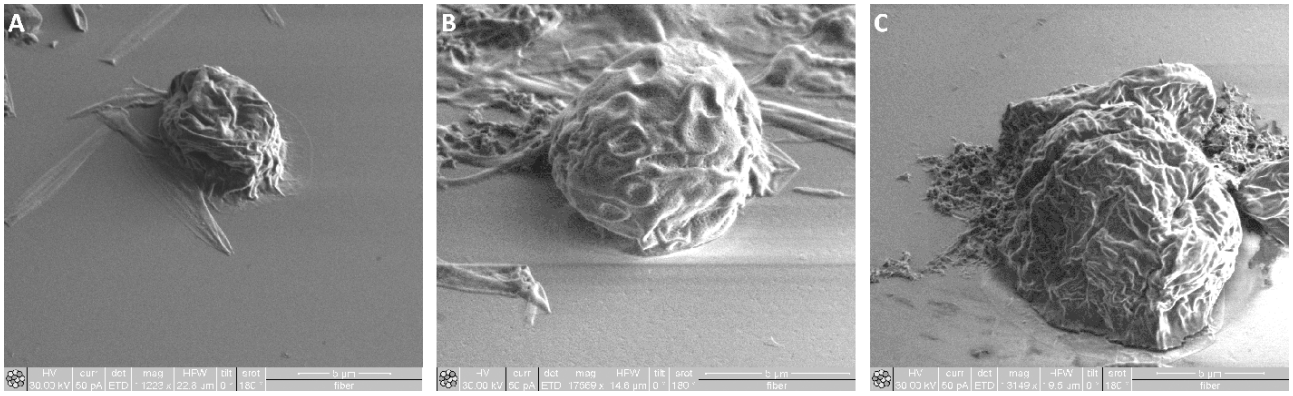

Fig. 3.8.1-3 Observation of *T. suecica* interacting with aggregates (A: control, B: in nano ZnO exposure for 72 h, C: in bulk ZnO exposure for 72 h) by FIB. Aggregation occurred around flagella area with both particles.

P. tricornutum:

As for *T. suecica* during three days exposure to ZnO NPs, the parameters of *P. tricornutum* inhibition were recorded every 24 h and bulk ZnO and $ZnSO_4$ trials were set up as comparing experiment as well. The overall toxic effect after three days of exposure to the investigated chemicals indicated a clear higher toxic effect of ZnO NPs respect to zinc salt and bulk ZnO (Fig. 3.8.1-4). Dose response curve obtained for $ZnSO_4$ was almost overlapped with ZnO bulk one especially below 5 mg Zn/L.

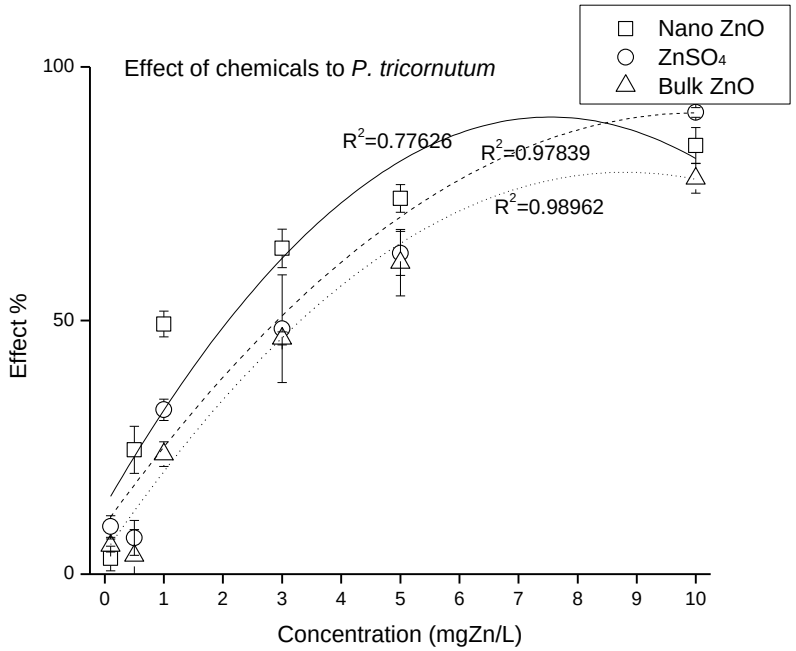

Fig. 3.8.1-4 Toxic effects on *P. tricornutum* cells, together with the corresponding regression fit curves: nano ZnO toxic effects (diamond); $ZnSO_4$ toxic effects (triangle); bulk ZnO toxic effects (square). The best fit function of toxicity data

was sigmoid growth function. 50% effect level is represented.

The ecotoxicological parameters (NOEC, EC1, EC10, and EC50), at each exposure time point, reported in Table 3.8.1-2 showed that ZnO NPs was more toxic than bulk at all tested concentrations.

Table 3.8.1-2 NOEC, LOEC, EC1, EC10, EC20 and EC50 evaluated for each tested chemicals (nano ZnO, bulk ZnO and $ZnSO_4$) to *P. tricornutum* at each exposure time point.

Exposure time	Chemicals	NOEC (mg Zn/L)	EC1 (mg Zn/L)	EC10 (mg Zn/L)	EC50 (mg Zn/L)
24h	Nano ZnO	1.0	0.01 [0.004, 0.52]	0.08 [0.04, 0.73]	2.57 [1.93, 3.23]
	$ZnSO_4$	0.5	0.06 [0.01, 0.16]	0.36 [0.05, 0.67]	3.60 [2.17, 5.49]
	BulkZnO	0.5	0.01 [0.004, 0.21]	0.06 [0.03, 0.57]	1.12 [0.89, 1.57]
48h	Nano ZnO	< 0.1	0.006 [0.004, 0.01]	0.06 [0.04, 0.33]	1.41 [0.97, 3.40]
	$ZnSO_4$	0.1	0.01 [0.01, 0.02]	0.14 [0.09, 0.23]	2.34 [2.05, 2.60]
	BulkZnO	0.5	0.05 [0.01, 0.35]	0.59 [0.09, 0.90]	3.32 [2.89, 3.79]
72h	Nano ZnO	0.1	0.03 [0.01, 0.12]	0.23 [0.14, 0.31]	1.09 [0.96, 1.57]
	$ZnSO_4$	0.1	0.01 [0.01, 0.02]	0.54 [0.09, 0.61]	3.22 [2.48, 3.94]
	BulkZnO	0.5	0.02 [0.01, 0.52]	0.64 [0.52, 0.75]	3.47 [3.06, 3.91]

In particular for ZnO NPs in 72 h, NOEC was recorded at the lowest concentration 0.1 mg Zn/Lwhich resulted lower than that obtained for bulk ZnO (NOEC 0.5 mg Zn/L).

Also for this alga the relation between exposure time and growth effect was investigated by evaluating the main ecotoxicological parameters along each testing day (24 and 48 h, Table 3.8.1-2). After 24 h the toxic effect of bulk ZnO (EC10 and EC50: 0.06 mg/L and 1.12 mg Zn/L, respectively) was higher than ZnO NPs (EC10 and EC50: 0.08 mg Zn/L and 2.57 mg Zn/L, respectively). Along with increasing exposure time the effect of chemicals turned into that higher ZnO NPs toxicity. It could be supposed that the toxic effect in the first phase was related to the larger size of bulk ZnO aggregates that rapidly settled down on the bottom of the wells where the immobile algae lay, which were reported in our previous work (Manzo et al., 2013b), exerting there the toxic action. Instead, the action of ZnO NPs aggregates became evident only in the second day

of exposure when aggregation and sedimentation processes were completed.

Although in this study *P. tricornutum* was very sensitive to ZnO particles especially at the beginning of exposure, some authors (Peng et al., 2011) reported this alga as the less sensitive to ZnO nanoparticles suspensions respect to *Chaetoceros gracilis* (EC50 not reported) and *Thalassiosira pseudonana* (EC50 = 0.82 mg Zn/L).

The observations at optical microscope (Fig. 3.8.1-5) and at FIB (Fig. 3.8.1-6) provided supportive evidences to the interaction between ZnO particles (both NPs and bulk) and algae. The morphology and the lacking of motility of *P. tricornutum* algae let a large hetero aggregation along the cells that rapidly were completely wrapped around. An increasing trend of the process in dependence of exposure time and ZnO concentration (Fig. 3.8.1-5 and 6) could be observed. In comparison to *T. suecica* (Fig. 3.8.1-2), serious aggregation occurred between ZnO particles and diatom cells (Fig. 3.8.1-5).

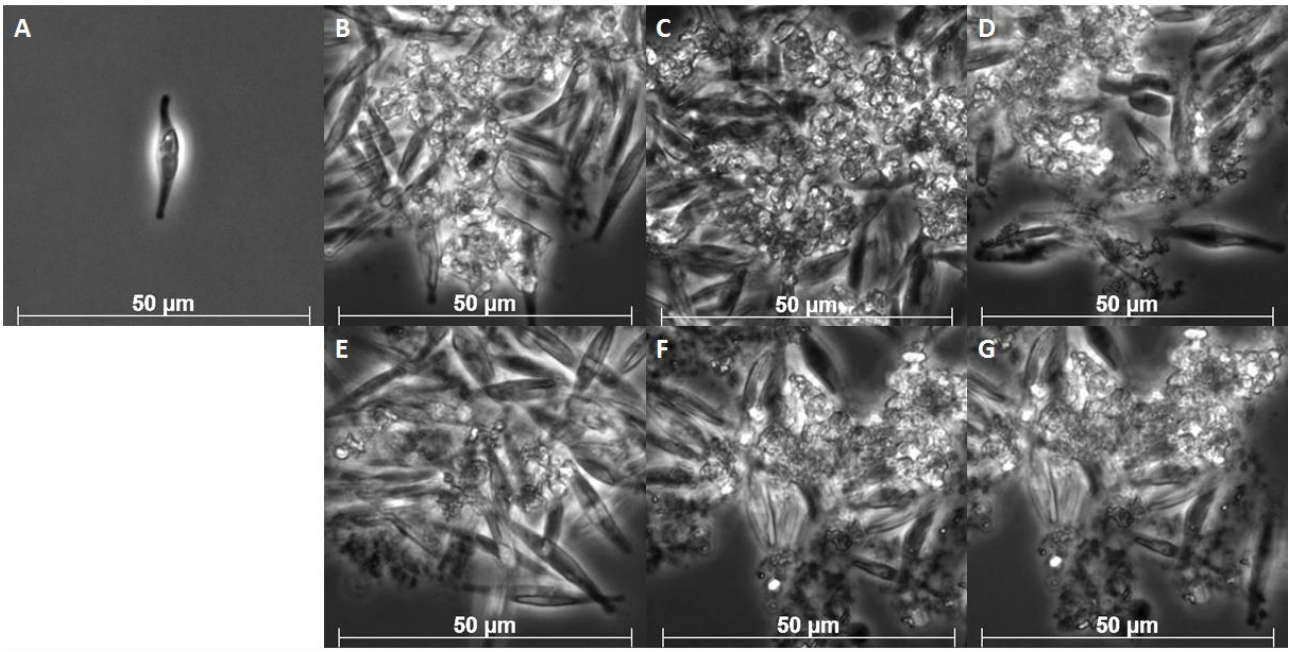

Fig. 3.8.1-5 Behavior of *P. tricornutum* with aggregates of zinc oxide in 72 h, A: control, B: 0.1 mg Zn/L nano, C: 5 mg Zn/L nano, D: 10 mg Zn/L nano, E: 0.1 mg Zn/L bulk, F: 5 mg Zn/L bulk and G: 10 mg Zn/L bulk. Algae adsorb the zinc oxide aggregates to be clumps along with increasing of concentrations and exposure time.

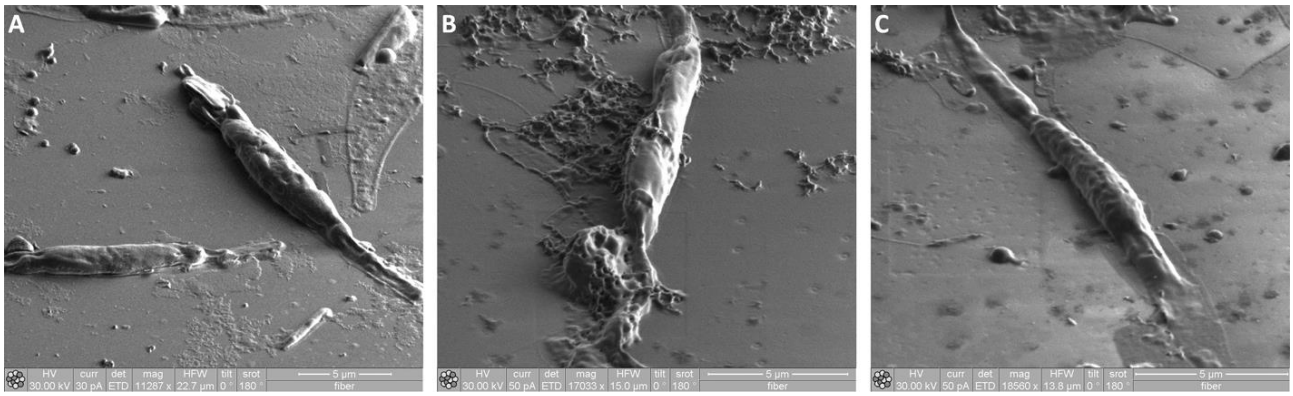

Fig. 3.8.1-6 Observation of *P. tricornutum* interacting with aggregates (A: control, B: in nano ZnO exposure for 72 h, C: in bulk ZnO exposure for 72 h) by FIB. Aggregation occurred wrapping the whole cell body with both particles.

3.8.2 A comparison between the effects upon the two algae

A comparison of the effects upon *P. tricornutum* and *T. suecica* along exposure times (24, 48, 72 h) in relation to hydrodynamic diameters of ZnO NPs and bulk particles was performed (Fig. 3.8.2-1) for thoroughly characterizing the diverse observed sensitivity of algae (Fig. 3.8.1-1 and 4, Table 3.8.1-1 and 2).

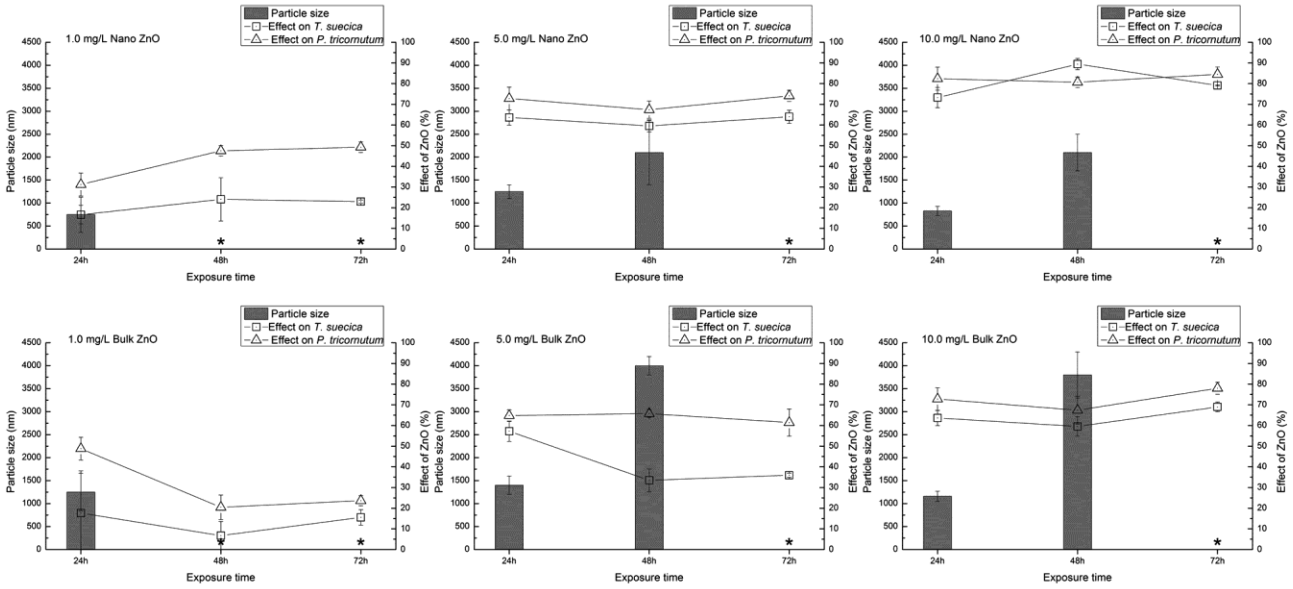

Fig. 3.8.2-1 A graph panel of toxic effect on both algal cells and hydrodynamic diameters of ZnO particles in ASW at three exposure phase points 24 h, 48 h, and 72 h orderly. The exposed chemicals were reported as Zn concentration.* Samples not suitable for DLS analysis

Results suggested a clear effect on algae replication capability linked to the physic-chemical state of the nanoparticles in the medium, during the exposure time. In addition to

the toxicity mechanisms described for ZnO as bulk and nano materials in general (Miao et al., 2010; Miller et al., 2010). A close interference of the aggregates with algal cells likely related to a peculiar reactivity or to a mechanical entrapment and or wrapping of the cells in the culture media was figure out (Ji et al., 2011).

The effect curves for ZnO NPs of both algae showed different trends and the diatom population always kept higher effect than green alga. It is worth to note that *T. suecica* at 1 mg Zn/L and 5 mg Zn/L showed constant effect trend around 20% in the first case and 60% in the second one while at highest concentration the effect were around 80% with a spike at 48 h (90%). *P. tricornutum* instead showed an increasing trend with time at the lowest concentrations, reaching the 50% of effect, while at the next concentrations the effect were more or less stable during the time (around 70% for 5 mg Zn/L and > 80% for 10 mg Zn/L). At all concentrations increasing size of ZnO NPs particle was measured (Fig. 1 and 2). In particular, at 1 mg Zn/L just after 48 h there are particles with dimensions upper than 6 microns (not detectable by DLS), while at 24 h particle lesser than 500 nm were observed. For 5 and 10 mg Zn/L ZnO NPs concentrations a slower increasing aggregation process was observable, with particle around 2 microns at 48 hours and > 6 microns at 72 hours, although with a different size at 24 h (> 1200 nm for 5 mg Zn/L and > 700 nm for 10 mg Zn/L). It could speculated that the toxic effect was related to the agglomeration process, in fact when the agglomeration proceed fast (i.e. at 1 mg Zn/L) the toxic effect were more evident, due probably to a durable and effective interaction solely exerted upon immobile algae on the bottom of the wells. When the aggregation turned more slowly (i.e. smaller aggregates at 48 h), the effect trend became more similar for the two algae although the diatom always registered the highest effects. This is because the aggregates could hardly interact with motile algae (swimming in the test media).

Also for bulk ZnO generally the effect curves showed different trend with the time for the two algae. In particular at 1 mg Zn/L *T. suecica* showed always effect less than 20% while a decreasing trend starting from 60% down to 35% for 5 mg Zn/L and a slowly increasing trend starting from 60% up to 70% at 10 mg Zn/L were evaluable.

P. tricornutum showed a decreasing toxicity trend starting from 50% down to 30% at 1 mg Zn/L while a quite constant response around values > 70% was registered at 5 mg Zn/L and a slowly increasing effects from 70% to 80% were obtained at 10 mg Zn/L.

The aggregation trend for bulk ZnO was similar to that described for ZnO NPs but with higher aggregate sizes (i.e. agglomerates). For bulk the homo aggregation process resulted more efficient than hetero aggregation, and these could be the reason why the final effects were lower. Additionally at concentration < 5 mg Zn/L the different actions upon the two algae were more evident due to the less probability that aggregates/agglomerates could interact with motile algae respect to settled ones. At 10 mg Zn/L the numbers of particles are sufficient to exert a similar toxic action for both algae.

Regarding homo aggregation of ZnO and aggregation between algal cells and aggregates during exposure, the algae, stimulated by ZnO particle, could promote this process, producing some compounds such as citrate, cysteine or carbonate (Mafunéet al., 2000). It was also reported that in some cases this phenomenon reduce the toxicity of some chemicals (Soldo et al., 2005; Miao et al., 2007; Navarro et al., 2008). Based on the observation upon algae morphology (Fig. 3.8.1-2, 3, 5, and 6) we can observe that the higher suface area-volume ratio of *P. tricornutum* respect to *T. suecica* represent a very important factor because the interaction between ZnO particles and algae mainly happened on the cell surface. The toxicity reflected the difference in contact time and in contact area between algae and aggregates. In addition motility should be a key factor in the algae different sensitivity. Motility made algal cells largely distributed in culture medium reducing than the contact time with aggregates differently by the immobile diatoms. Consequently, shading effects could lead to a reduction in the light availability to entrapped cells thus inhibiting their growth (Wang et al., 2008; Aruoja et al., 2009; Gong et al., 2011).

3.9 Conclusions

Comparative toxicity of ZnO NPs, bulk, and Zn salt towards greenmicroalga *T. suecica* and diatom *P. tricornutum* has been detected in this study, aiming to understand if the presence of NPs themselves induces any additional toxic effect to those already attributed to the released metal ions. We have found that the nano size plays a key role in the overall ZnO toxicity. EC50s had been detected at 3.91 [3.66, 4.14] mg Zn/L for NPs, 5.61 [4.93, 6.23] mg Zn/L for $ZnSO_4$ and 7.12 [6.65, 7.46] mg Zn/L for bulk ZnO towards green microalgae and 1.09 [0.96, 1.57] mg Zn/L for NPs, 3.22 [2.48, 3.94] mg Zn/L for $ZnSO_4$ and 3.47 [3.06, 3.91] mg Zn/L for bulk ZnO towards diatom. Distinct inhibition effect difference on both algae indicated that diatom appears more sensitive than

T. suecica because of the difference of the contact time by motility, contact area by surface-to-volume ratio and available light by shading effects. Additional, effect of low concentration had been recorded that EC10 values were under 1 mg Zn/L for all chemicals towards *P. tricornutum* and NOEC were detected at the lowest concentration for NPs to both algae. Inhibition at low concentration is worth to be notice for exposure risk in environment.

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3.11 Zn bioaccumulation and histological damages of *M. galloprovincialis* exposed to ZnO NPs, bulk, and Zn salt

As nanomaterials (NMs) are used extensively in a variety of emerging technologies and commercial products so they can be largely be released in the environment. Fate and transport models indicate that they can reach the marine environment and can have an ecological impact.

In particular the increasing use of ZnO NPs, their modeled release into the aquatic environment and the toxic effects to the aquatic organisms are well documented (Peng et al., 2011; Keller et al., 2013; Ma et al., 2013; Manzo et al., 2013a; Keller et al., 2014).

However the NP ecotoxicological assessment was still uncompleted also because it is necessary to take into account several factors, including physicochemical properties of NPs, seawater parameters, interactions with both physical and chemical factors and organisms' physiology and ecology.

Bivalves are largely used as bioindicators to monitor the health of an aquatic environment, either fresh or sea water. They are extremely useful as they are sessile which means they are closely representative of the environment where they are sampled or placed (caging), and because they are filter feeding, they expose their gills and internal tissues to bioaccumulation also of contaminants. Common mussels such as *M. edulis* and the similar species Mediterranean mussel *M. galloprovincialis* were also largely used as indicators of marine contamination since 1970s, (Golberg, 1975; Phillips, 1976) also for their worldwide distribution. Due to their peculiar characteristics, in recent years, *M. galloprovincialis* was more and more employed to investigate biological response to toxicants and then also NPs, in laboratorial condition (Bebianno & Serafim, 1998; Da Ros et al., 2000; Barmo et al., 2013; Estevez-Calvar et al., 2013; Balbi et al., 2014; Bebianno et al., 2015).

The mechanism of ZnO NPs toxicity was summarized in three aspects (Ma et al., 2013): ionic Zn dissolved from NPs, particle-induced effects, and NP photocatalytic activity, which were well documented on various organisms, bacteria (Sawai et al., 1998; Brunner et al., 2006; Zhang et al., 2007; Applerot et al., 2009; Xie et al., 2011), fungus (Lipovsky et al., 2011), plants (Lin & Xing, 2008), microalgae(Manzo et al., 2011), and amphipod (Fabrega et al., 2012).

However the ZnO NP toxic effects upon marine bivalve *M. galloprovincialis*,

physiological processes, such as respiration, accumulation of Zn, growth, and survival are still very scarce (Montes et al., 2012; Hanna et al., 2013). Despite the effects at histological level had been well documented of bivalvia, crustacea, fish, and mammal exposed to other nanomaterials, heavy metals salts, and organic contaminants (Sunila, 1988; Aarab et al., 2004; Aarab et al., 2006; Federici et al., 2007; Griffitt et al., 2007; Griffitt et al., 2009; Kádár et al., 2010; Sheir et al., 2010; Al Kaddissi et al., 2011; Abdelhalim & Jarrar, 2012; Barmo et al., 2013; Sheir et al., 2013; Balbi et al., 2014; Hariharan et al., 2014; Hu et al., 2014; Trevisan et al., 2014; Vale et al., 2014; Cid et al., 2015) only rare records reported the effects at histological level in mussels (Kádár et al., 2010; Barmo et al., 2013; Hu et al., 2014) (Table 3.11-1). About investigated tissue, gill, digestive gland, and gonad were frequently used in bivalvia. For fish and rat, gill, intestine, and liver were the most investigated organs, haemocytes were used to assess the toxicity as well (Kádár et al., 2010; Barmo et al., 2013).

Table 3.11-1 Histological alterations of exposed species

Experimental species	Exposure toxicants	Histological alterations comparing with control *Observed organs* Gill	Digestive gland	Gonad	References
		Mantle	Adductor muscle	Others	
Bivalvia					
M. edulis	North Sea oil, alkylphenols and PAHs	–	–	Size, precocious degree, degeneration of spermatic cysts/ovarian follicles; morphologic change of connective tissue	(Aarab et al., 2004)
M. edulis	SO_4^{2-}, Cd^{2+}, Cu^{2+}, PbO, Co^{2+}, Fe^{3+}, Ag^{+}, PCB, DDT and dieldrin	Morphologic change and inflammation of gill filaments	–	–	(Sunila, 1988)
M. edulis	BPA, DAP and PBDEs	–	–	Morphologic change of ovocytes; Size, number of ovarian follicles/spermatic cysts; filling degree of gametocytes in ovarian follicles/spermatic cysts;	(Aarab et al., 2006)
M. edulis	Cd^{2+}	Morphologic change and necrosis of gill filaments	Necrosis of tubules	Necrosis and inflammation of ovarian follicles/spermatic cysts	(Sheir et al., 2013)
M. edulis	CuO NPs	Aberrant brown cells	–	–	(Hu et al., 2014)
M. edulis	$HgCl_2$	Morphologic change and necrosis of gill filaments; morphologic change and number of haemolymph vessel;	Nuclear morphologic change and necrosis of epithelial cells; necrosis of connective tissue Morphologic change of fiber texture; morphologic change and necrosis of connective tissue	–	(Sheir et al., 2010)
M.	TiO_2 NPs	–	Nanoparticles direct invasion	Haemocyte: nuclear	(Barmo et al.,

galloprovincialis				abnormalities	2013)
M. galloprovincialis	Cd^{2+}	–	–	D-larva: shell indentations and protruding mantle	(Balbi et al., 2014)
M. sp.	Fe NPs and Fe^{3+}	Morphologic change of epithelial cells	–	Haemocyte: size and number	(Kádár et al., 2010)
Perna viridis	Pb^{2+}	Morphologic change of gill filaments; damage of epithelium	Morphologic change and inflammation of fiber texture; necrosis and inflammation in connective tissue; loss of muscular integrity	–	(Hariharan et al., 2014)
Crassostrea gigas	ZnO NPs	Morphologic change and nanoparticles direct invasion in mitochondria of gills filaments	–	–	(Trevisan et al., 2014)
Corbicula fluminea	Nanodiamond	–	Degeneration of digestive cells; morphologic change of digestive epithelia	–	(Cid et al., 2015)
C. fluminea	Cd^{2+} and TiO2 NPs	–	Inflammation	–	(Vale et al., 2014)
Crustacea					
Procambarus clarkii	$UO_2(NO3)_2$	–	–	Hepatopancreas tubules: Pathologic change of epithelium; degeneration of tubules	(Al Kaddissi et al., 2011)
Fish					
Danio rerio	TiO_2 NPs, Ag NPs, Ag^+, Cu NPs and Cu^{2+}	Gill filament width change	–	–	(Griffitt et al., 2009)
D. rerio	Cu NPs and Cu^{2+}	Proliferation of epithelial cells and edema of gill filaments	–	–	(Griffitt et al., 2007)
Oncorhynchus mykiss	TiO_2 NPs	Pathologic and morphologic change of gill filaments	Pathologic and morphologic change of intestinal villi	Liver: Pathologic change of hepatocytes; apoptotic bodies appeared; Brain: necrotic cell bodies appeared	(Federici et al., 2007)
Mammal					
Rattus norvegicus	Au NPs	–	–	Liver: Pathologic change, degeneration, inflammation in hepatocytes	(Abdelhalim & Jarrar, 2012)

However it is really informative the performance of different tissue injuries for the understanding the effect pathway in the exposed organisms (Federici et al., 2007; Abdelhalim & Jarrar, 2012; Barmo et al., 2013; Cid et al., 2015).

Many different histological alterations were observed in gill filaments of bivalvia animals exposed to, nano-metal oxides, and ionic metals (Sunila, 1988; Kádár et al., 2010; Sheir et al., 2010; Sheir et al., 2013; Hariharan et al., 2014; Trevisan et al., 2014) such as: fusion and cilia erosion, chronic inflammation, proliferation, severe loss, and shrinkage of epithelial cells, edema, absence mitochondrial cristae, endocytic vesicles containing electron-dense particles, swollen mitochondria and lumen, highly vesciculated cytoplasmic space, disrupted mitochondria, total loss of gill architectures, and damaged interlamellar junctions. In digestive gland, degenerating digestive cells, increased vacuolation, unclear nuclei of eroded digestive epithelia, necrotic tubules, necrotic connective tissue, accompanied with irregular and dilated lumina were directly observed (Sheir et

al., 2010; Barmo et al., 2013; Sheir et al., 2013; Vale et al., 2014; Cid et al., 2015). The gonad was mainly investigated in the case of heavy metals and organic pollutants effects and was generally reported precocious developmental, enlarged and degenerating ovarian follicles; very loosen connective tissue, shrunken and empty oocytes in ovarian follicles, and empty spermatic cysts with few spermatozoa (Aarab et al., 2004; Aarab et al., 2006; Sheir et al., 2013). The loss of fibrous structure and muscular integrity, necrosis, hydropic change (swelling), decrease in extracellular spaces, and inflammatory responses and necrosis of connective tissue, vacuolization between the muscle bundles, and finally splitting of muscle fibers were observed in adductor muscle exposed to heavy metal (Sheir et al., 2010; Hariharan et al., 2014). Hu et al. (2014) reported that brown cells were found along the mantle margin and lining sinuses in CuO nanoparticles exposed mussel. No records have been found for characterising pleopod injury in bivalvia.

Generally, together with the histological response, metals accumulation had been investigated as well to explain the toxicity of metals or metal oxides (Montes et al., 2012; Hanna et al., 2013). Hanna et al. (2013) determined the accumulation of Zn in *M. galloprovincialis* long term exposed to ZnO NPs showeing that the mussel gonad accumulated Zn efficiently in lower exposure concentration more than higher ones. Montes et al. (2012) detected an amount of four times of Zn accumulation in the whole mussel *M. galloprovincialis* respect to un-exposed sample. Fate and effect of metal-based nanoparticles had been well investigated in other marine oragnisms as well, e.g., the bivalve mollusk *Scrobicularia plana* and the annelid polychaete *Hediste diversicolor* (Mouneyrac et al., 2014).

In this section, the histological alterations on 6 organs in *M. galloprovincialis* exposed to ZnO NPs suspensions were observed to characterize the tissue damages.

To a comparative purpose ZnO bulk and Zn ions were tested as well, in order to also evidence a peculiar effect linked to the pristine ZnO size and to the ionic zinc alone.

In addition, tissue Zn uptake was measured to reveal the fate of Zn in mussel and give a full understanding of ZnO NPs toxicity with the histological observation.

3.12 Materials and methods

3.12.1 Chemicals

Bare ZnO nanopowder (Code 544906, particle size < 100 nm, surface area 15-25 m^2/g) and $ZnSO_4 \cdot 7H_2O$ crystals (Code 204986, 99.999% trace metals basis) were purchased from Sigma-Aldrich Co. LLC. (USA). Bulk ZnO powder (Code ZK249038, particle size 150−200 nm, purity > 99.9%) was purchased from Quer Biotech Co., Ltd (Hefei, China). Baysalt crystals (Code Q/XWL 101-2006) were purchased from Shuilifang S&T Ltd. (Xiamen, China).

3.12.2 Particle dispersions

Stock suspensions of nano and bulk ZnO powder were prepared with Reconstructed Sea Water (RSW, East China baysalt crystals dissolved in pure water; salinity 25 ‰, pH 7.90, 0.22 μm filtered) to the final concentration of 1g Zn/L. The ZnO suspensions were dispersed by bath-sonication for 30 min at 50 W (Yuhao ultrasonic cleaner, YH-200DH, Shanghai, China). Test suspensions of concentrations 0.01, 0.1, 1, 10 and 100mg Zn/L were prepared by diluting the stock suspension with RSW. Zn salt solution was prepared in the same five graded concentration without sonication.

3.12.3 Animals and exposure experiment

Wild adult specimens of *M. galloprovincialis* (shell length 4-5 cm) were collected from coastal rocks (30°41′ N, 122°27′ E; Zhoushan, Zhejiang Province) in the East China Sea. The site does not belong to a national park or a protected sea area or a relevant regulatory body concerned with wildlife protection or a private owner. The mussels were further acclimatised to aerated ASW in an aquarium for 7 days at 23℃ (1 L/animal) (Scarlato, 1981; Ye et al., 2011) with feeding and water changing (3.5 dyas intervals; 1 mL per 50 L water of Phytoplex^{TM} phytoplankton feed, Kent Marine Inc., Acworth, GA, USA) (Tedesco et al., 2010).

Mussels were then treated with ZnO NPs and bulk counterpart in 0 (control), 0.01, 0.1, 1, 10, and 100 mg Zn/L concentration, respectively, for four weeks. In each exposure tank, 25 mussel individuals were exposed to 5 L medium with aerating, feeding and water changing (twice per week). On the 1st, 2nd, 3rd, 7th, 14th, 21st, and 28th day, 3 individuals per tank were sampled, and fresh tissue of 6 organs (gill, digestive gland, gonad, adductor muscle, mantle, and pleopod) were used to make paraffin section and to perform the measurement of Zn accumulation.

3.12.4 Measurement and analysis of tissue Zn accumulation

A 500 mg portion of fresh tissues was digested in 10 mL HNO_3 (69%) using a microwave digestion system (MARS Xpress, CEM, USA). The vessels were heated from room temperature to 120 ℃ in 5 min, and to 150 ℃ in 3 min and held for 5 min, then to 180 ℃ in 3 min and held for 10 min; were cooled down to room temperature before they were opened. After cooling the resulting solutions were diluted to 25 mL with deionized water. Zn concentrations were measured by an atomic absorption spectrometer (AAS; AA240 Duo, Varian, USA) in a graphite furnace (Massányi et al., 2004; Kramárová et al., 2005; Gasparik et al., 2012). The concentrations are expressed on wet-weight basis in μg/g. A relative Zn uptake rate (R) is used to express the efficiency of Zn accumulation in each tissue with an equation:

$$R = \frac{C_E - C_K}{C_K} \times 100\% \quad \text{(a)}$$

In the equation, C_E means Zn concentration of exposed sample and C_K means Zn concentration of control sample. All data are expressed as the mean ± SD of the different biological samples specified.

3.12.5 Histological observation

Fresh tissues were processed following the histological procedures described by Martoja & Martoja-Pierson (1967). The samples were fixed overnight in a Bouin's solution and stored in a 70% ethanol solution; washed for 24h in deionized water and then dehydrated through a series of graded ethanol solutions (70–100%) and xylene for intermediate impregnation. After immersion in paraffin, sections of 5–7 μm were cut using a microtome (Kedee, China) and mounted on glass slidesand stained with hematoxyline and eosin (H&E). Histological observation was performed by an optical microscope (Olympus, Japan) and an image system (Shineso, China).

3.13 Results and discussion

3.13.1 Zn bioaccumulation

The Zn concentrations of 6 organs in mussel exposed to 3 chemicals in 5 concentrations at 7 exposure time points were measured and the relative Zn uptake rates were calculated using equation (a) to express the efficiency of Zn accumulation. The relative Zn uptake rates in the middle exposure concentration 1 mg Zn/L were given in Fig. 3.13.1-1 which shows the trends of Zn

accumulation in different tissues along with exposure time. Curves of digestive gland and gonad represented higher amplification than other organs along with time. Zn ion showed the highest uptake rate and NPs was in somewhere between ion and bulk for each organ.

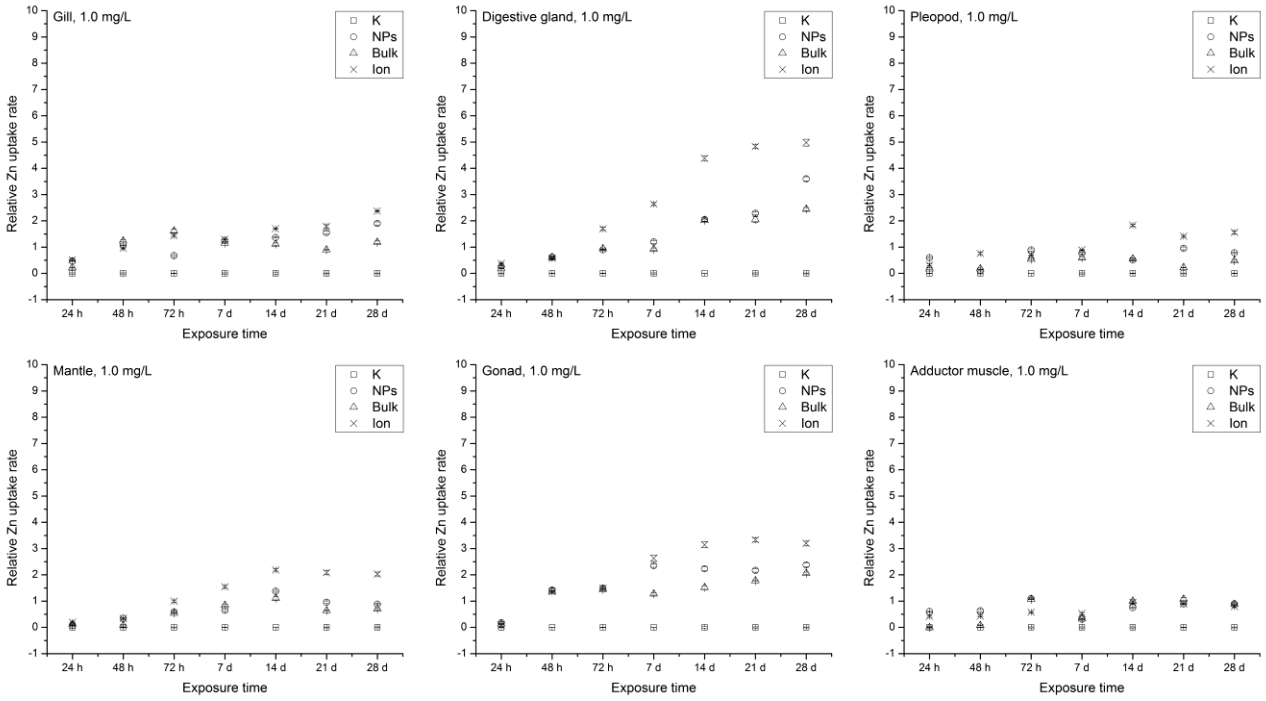

Fig. 3.13.1-1 The relative Zn uptake rates in 6 organs exposed in 1mg Zn/L ZnO suspensions and salt solution.

To compare the Zn uptakes among all tissues, the percents of relative Zn uptake rate of each tissue in whole exposed individual were drawn in Fig. 3.13.1-2. At low exposure concentrations (0.01 and 0.1 mg Zn/L), no evident trend was found. At the three higher concentrations (1.0, 10.0 and 100.0 mg Zn/L), the digestive glands represented increasing Zn uptake rates along with exposure time, gills showed a trend similar to digestive glands in the beginning three days of exposure (24-72 h) and evidenced a stable Zn uptake rates in the long exposure phases (7-28 d). Other tissues represent relative stable trends on Zn uptake rate along with exposure time.

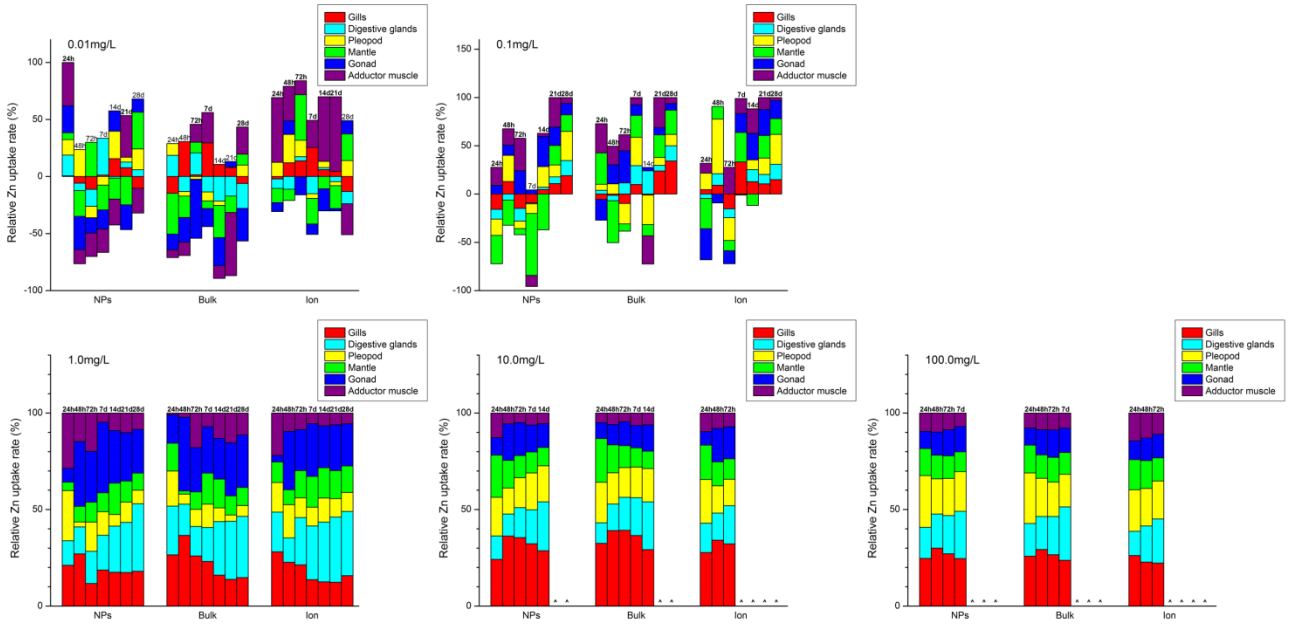

Fig. 3.13.1-2 The percents of relative Zn uptake rate of each tissue exposed in ZnO suspensions and salt solutions in all concentrations. ^ means all mussel individuals dead in the experimental group.

3.13.2 Relationship of Zn uptake in different tissues

An equation (a) was used to calculate the relative Zn uptake rate (*R*) for describing Zn accumulation difference among tissues and exposure time. Zn is rich in marine bivalve animals (George & Pirie, 1980; Wang & Fisher, 1996), such as the Mediterranean mussel in the present study, of which the concentrations are different among different organs (Regoli, 1998). The aim of comparing the Zn uptake rate in different tissues is to verify the tissue sensitivity on Zn accumulation in this study.

But also to know in which organ the major NP effect could be waited, due to the Zn locally accumulated. The concentrations are expressed on wet-weight basis which could better image the Zn distribution in live mussel organs than dry-weight (Sager & Cofield, 1984).

The relative Zn uptake rates in 6 different tissues were represented by 1 mg Zn/L concentration in Fig. 3.13.1-1 (the rest of all data were shown in Appendix 1). As the comparing objects, *R* values of control group (K) were set as the standard line with zero percent. On the basis of the control group, *R* values went a growing trend along with exposure time in all tissues, in particular digestive gland, which were bringed into correspondence with the longest stay time and largest area for ZnO exposure. The digestive gland is the required organ for measuring pollutants

uptake (Regoli, 1998; Gomes et al., 2012; Mouneyrac et al., 2014; Gornati et al., 2015). In the present resarch, gonad was also verified to have high Zn uptaking ability with a peak number (about 3.5) at 21d exposure time that the *R* value was approximately 5.0 in digestive gland at the last exposure timepoint. The anatomical position of gonad is very close to digestive gland that ZnO staying in digestive gland for long time might to affect the exposure extent in gonad. Gill and mantle represented more uptake extent than pleopod and adductor muscle due to larger contact area with suspensions. However, in the beginning of exposure phase (0-72 h), *R* of gill, digestive gland, and gonad were the same level with the value almost 2.0, which indicated gill seems uptaking Zn with a capacity limit in the end three weeks being different with digestive gland and gonad. Ionic Zn showed the highest uptake rate and NPs was in somewhere between ion and bulk for each organ (Fig. 3.13.1-1).

Actually, 1 mg Zn/L ZnO can dissolve at all into sea water which had been proved before (Manzo et al., 2013b). Digestive gland showed higher uptake rate on ionic Zn than dissolved Zn from NPs and bulk (Fig. 3.13.1-1) indicating the presence of a mechanism capable to limit the ionic Zn access in the tissue. The interaction between ZnO and live mussel might affect Zn uptaking.

The percents of *R* values among all tissues were shown in Fig. 3.13.1-2 to compare the tissue difference on Zn uptake. No obvious pattern was found in the low exposure concentrations (0.01 and 0.1 mg/L) because *R* values were well included in the measurement variability. At 1 mg Zn/L concentration, gill showed gradually decline trends to three exposures which were not continued in higher concentrations. Instead, digestive gland showed gradually increasing trends of %*R* along with exposure time in all higher exposure concentrations (Appendix 1). This suggest that digestive tract could hold the ZnO during the exposure time and that it is the final sink to store Zn. Gonad showed similar increasing changes of %*R* values during the four weeks: the anatomical position near to digestive gland and its own capacity to hold Zn can explain this result. Obviously, the higher %*R* values were measured for digestive gland, gonad, and gill (Fig. 3.13.1-2). For the other organs, no clear trends have been found and the low percents of *R* values indicated that they are not the target tissues to investigate the Zn uptake.

3.13.3 Histologic observation

The microscopy observation indicated that NPs can induce varying degrees of histologic

injure in each organ and very obvious histologic alteration was observated in gill, digestive gland, and Gonad. Fig. 3.13.3-1 showed the observation of tissue injury in 6 organs exposed in 10 mg Zn/L ZnO suspensions and salts for 72 hours that a full observation can be taken in this situation representing the injury occurred in mussel tissue. Because tissue exposed into the lower concentrations (0.01-1.0 mg Zn/L) were not shown obvious observable injury by microscrope and that were too much in the highest concentration. In Fig. 3.13.3-1 Gill, the distances between adjacent gill filaments were measured. In a same length standard, 250-260 for control (A), 340-410 for bulk (B), 440-490 for NPs (C), and 330-350 for Zn ion (D) indicated that NPs represented the highest effect among all. With respect to control, edema (red arrows) were found in all three exposed gill filaments and Zn salts caused swollen cavity (green arrows) and damaged interlamellar junctions in Fig. 3.13.3-1 Gill. For Digestive gland observation, necrotic tubules with tissue off (red arrows), necrotic connective tissue with irregular and dilated lumina (green arrows) were observed in all exposed tissues, especially severely by salts. Eight photos were exhibited in Fig. 3.13.3-1 Gonad for showing damages in both of ovarian follicle (left) and spermatic cyst (right). Disrupted oocytes in ovarian follicles (red arrows) and very loosen connective tissue (green arrows) were observed in femal individuals exposed to all chemicals (B, C, and D in Fig. 3.13.3-1 Gonad). Broken and almost empty ovarian follicles were found for NPs and Zn ion exposed mussels, respectively. In male individuals, empty spermatic cysts with few spermatozoa (red arrows) and very loosen connective tissue (green arrows) were observed for all three exposed chemicals. In the observation of adductor muscle, loss of fibrous structure and muscular integrity and vacuolization between the muscle bundles (red arrows) were found in NPs (C) and, in particular, ion (D). Very loosen connective tissue (green arrows) were observed as well. Loosen epithelium (red arrows) were found along the margin of mantle and pleopod. Few lacunas (green arrows) were observed in ion exposed mantle (D) and NPs and ion exposed pleopod (C and D).

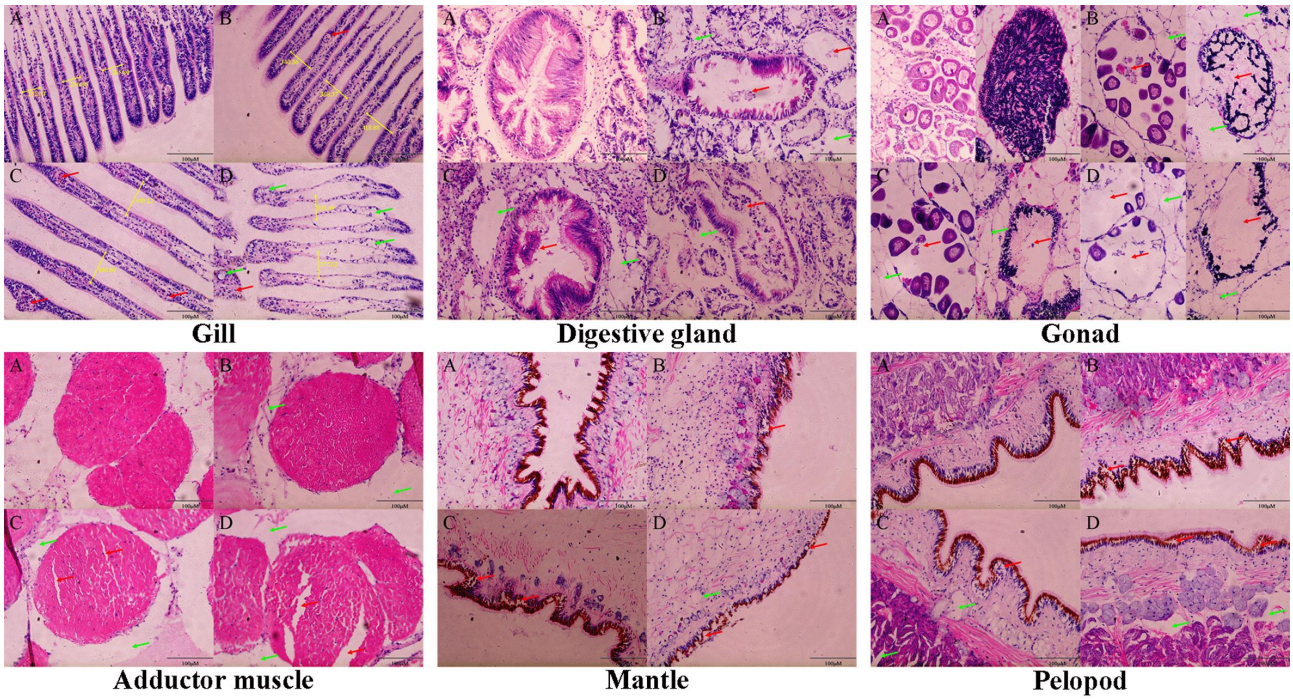

Fig. 3.13.3-1 Observation of tissue injury in 6 organs exposed in 10 mg Zn/L ZnO suspensions and salts for 72 hours. A: Control group, B: ZnO bulk, C: ZnO NPs, D: Zn salt; Gonad: left part is female and right part is male.

3.13.4 Difference of tissue damage between tissues, chemicals

The main representative pictures of tissues injury were reported in Fig. 3.13.2-1. The distance between adjacent gills filaments were utilized to compare the difference among ZnO NPs, ZnO bulk and Zn ions and control individuals (Griffitt et al., 2007; Griffitt et al., 2009). NPs showed the highest effect respect to zinc ions and ZnO bulk. Also, more edemas were observed in NPs exposed gill than in the others. However, swollen cavities were found only in gill samples exposed to Zn salts. Various degree of injury occurred in interlamellar junctions for all treated samples.

Effects similar to those evidenced in this study where previously reported in gills of *M. edulis* exposed to environmental pollutants (Sunila, 1988; Sheir et al., 2013), though erosion or necrosis was never evidenced. Gill damages were also observed in nano-Fe exposed mussel *Mytilus* sp. (Kádár et al., 2010) although not similar to those observed in the present research.

In digestive gland section, necrotic tubules with tissue off and necrotic connective tissues with irregular and dilated lumina were observed in all exposed tissues, especially severe in the case of salt exposure, in which necrosis is the main sign of injury occurred in digestive gland. Similarly, Sheir et al. (2010); Sheir et al. (2013) observed necrotic tubules and necrotic connective tissue in

the digestive gland of exposed *M. edulis*; inflammatory response, such as tubule lumen widening and thickness of the epithelium reducing, were observed in the digestive tubules of the fresh water bivalve *C. fluminea* exposed to Cd and TiO_2 NPs (Vale et al., 2014); and degenerating digestive cells, increased vacuolation, thinness of the digestive epithelia, and accompanied with irregular and dilated lumina were observed in *C. fluminea* exposed to diamond NPs (Cid et al., 2015). Digestive tract holds the pollutants for the longest time in all mussel organs and seems the final sink in exposed individuals where ZnO and Zn salt can stay in enough to lead the maximum injury. Therefore, necrosis occurred in tubules and connective tissues without clear differences in the injury levels among three exposed chemicals.

With superiority in Zn uptake rate, the gonad plays a quite important role in histological response to ZnO and ions. Both in ovarian follicle and spermatic cyst loosen connective tissues were observed in all chemicals exposed mussels. Disrupted, broken, even almost empty ovarian follicles and empty spermatic cysts with few spermatozoa occurred in female and male mussels, respectively, in particular when exposed to Zn salts. Aarab et al. (2004) reported larger, precocious developmental, even degenerating ovarian follicles and very loosen connective tissues with numerous haemocytes in female blue mussel *M. edulis* exposed to Sea oil for long term and similar trends were found in male individuals as well. Additional, *M. edulis* also was found to represent empty, atretic ovocytes and empty spermatic cysts with few spermatozoa (Aarab et al., 2006). Loss of gametes in both ovarian follicles and spermatic cysts with loosen connective tissues could be considered as the most evident histological response to environmental stress.

For exposed adductor muscle, mantle, and pleopod, there is no that much clear histological changes respect to the unexposed organisms as reported for other three organs. But, still, loss of fibrous structure and muscular integrity and vacuolization between the muscle bundles were found in NPs and, in particular, in ions exposure; loosen epithelium were found along the margin of mantle and pleopod; few lacuna were observed in salts exposed mantle and pleopod. Those had been reported in previous researches (Sheir et al., 2010; Balbi et al., 2014; Hariharan et al., 2014) in mussels, however, Hu et al. (2014) reported also brown cells along the mantle margin and lining sinuses which were not observed in the present study. In summary, toxicity caused by NPs, bulk, and salts represented various degree, low by bulk < medium by NPs < high by salts, of injury in gill, digestive gland, and gonad, not in others.

3.14 Conclusions

In this section, the histological alterations were observed on 6 organs in mussel *M. galloprovincialis* exposed to ZnO NPs, bulk suspensions and Zn salts. Tissue Zn uptake was measured to reveal the fate of Zn in tissues as well. With comparing of histological observation and Zn accumulation in different tissues exopsed three chosen chemicals, NPs toxicity were represented on tissue injury and Zn uptake which indicated the initial size effect contributing to the overall toxicity.

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3.16 Ecotoxicity of ZnO nanoparticles in *M. galloprovincialis* revealed by transcription of apoptosis and antioxidation-related genes

With the rapid economic development, China becomes the largest nanomaterial market in the Asia–Pacific (Gao et al., 2013). In particular, the possible use of zinc oxide nanoparticles (ZnO NPs) in several productive fields such as: medical plastic, ship antifouling paints (Han, 2011; Yue, 2014), solar cells (Luo, 2013), antimicrobial agents (Shi, 2015), food additives (Liu, 2014), photocatalyst for pollution abatement (Gao et al., 2007; Gu et al., 2007) was largely investigated. This increasing use is consequently leading to a concurrent release increment into the environment where toxic effects to the aquatic organisms could be exerted (Peng et al., 2011; Keller et al., 2013; Ma et al., 2013; Manzo et al., 2013; Keller et al., 2014). It was estimated that about 36,000 kt of ZnO NPs could be discharged by wastewater and dust deposition into China's aquatic environments only in 2017 (Gao et al., 2013).

Although in the last years the toxicity towards some marine organisms (Wong et al., 2010; Peng et al., 2011; Hanna et al., 2013; Keller et al., 2013; Ma et al., 2013; Manzo et al., 2013; Keller et al., 2014) was investigated, unfortunately, there is still a lack of information about the possible adverse effects especially toward organisms chronically exposed.

Moreover, as the fate and the behavior of NPs in marine environment largely depend on the seawater characteristics that may promote NP aggregation/agglomeration/dissolution processes, the extrapolation of ZnO toxicity results to organisms of South East China is valid to a limited degree.

Bivalves, like mussels *Mytilus* spp., are filter-feeders, widely distributed, and with a long-life span and represent a good choice for the study of marine environmental pollution (Sheir et al., 2013; Balbi et al., 2014; Hu et al., 2014; Cremonte et al., 2015). *Mytilus galloprovincialis*, cultured in China for commercial interest (Lazo & Pita, 2012; FAO, 2016), was largely utilized in several countries to investigate biological responses to toxicants and environmental stress (Da Ros et al., 2000; Barmo et al., 2013; Estevez-Calvar et al., 2013; Balbi et al., 2014) and also to assess NPs toxicity (Canesi et al., 2010; Gomes et al., 2011; Hanna et al., 2013; Balbi et al., 2014; Gomes et al., 2014b).

In this bivalve NPs uptake can occur by ingestion through the digestive tract (Roberts et

al., 2007; Baun et al., 2008; Gagné et al., 2008; Ward & Kach, 2009) and through the large respiratory surface of the gills, as demonstrated for SiO_2 NPs in the congener species *Mytilus edulis* (Köhler & Riisgård, 1982). The gills and the digestive gland are, therefore, particularly relevant as target organs for nanotoxicological studies (Baun et al., 2008; D'Agata et al., 2014). In particular, the digestive gland is the main tissue for their storage (Ringwood et al., 2010), where NPs could accumulate with increasing time exposure (Gomes et al., 2011; Gomes et al., 2012; Gomes et al., 2013; Gomes et al., 2014b).

Once in the tissue, the particles could induce oxidative stress and promote several abnormalities in cellular function which can also trigger major changes in gene transcription (Fabbri et al., 2008).

It was previously reported that ZnO induce reactive oxygen species (ROS) generation and subsequent oxidative stress, which leads to damaged DNA, lipids, and proteins and potentially to cell death as reported for several organisms (Diamond et al., 2002; Adams et al., 2006; Lipovsky et al., 2011; Ma et al., 2011; Schiavo et al., 2016). Regarding *M. galloprovincialis* exposed to ZnO, effects upon the individual performance (Hanna et al., 2013), immunomodulation and on the energy budget in mussel body (Muller et al., 2014) were mainly reported, while studies about oxidative stress response were, to the best of our knowledge, not still available.

The oxidative stress is a common pathway of toxicity induced by pollutants (Winston & Di Giulio, 1991; Regoli, 1998). Organisms have adapted various stress response pathways, which play pivotal roles dealing with environmental insult (Gupta et al., 2010). These pathways (e.g. Fig. 3.16-1) include antioxidant enzymes such as superoxide dismutase (SOD), glutathione transferase (GST), catalase (CAT), to remove ROS (Zelko et al., 2002); and DNA repair enzymes such as p53 and PDRP involved in the pathway between ROS and DNA damages to minimize the impacts of genotoxicity such as DNA lesions, mutation accumulation, and chromosomal aberrations (Brierley & Martin, 2013). Accordingly different biomarkers were used to evaluate ROS-mediated NPs injury also in mussels (Table 3.16-1). On the other hand, it was showed that, in the ecotoxicological assessment, quantitative reverse-transcription polymerase chain reaction (qRT-PCR) grants an accurate mRNA transcription quantification useful for gene expression profile assessment in biological samples exposed to stressors, providing at the same time, higher sensitivity (Huggett et al., 2005; Hellemans et al., 2007; Schmittgen & Livak, 2008).

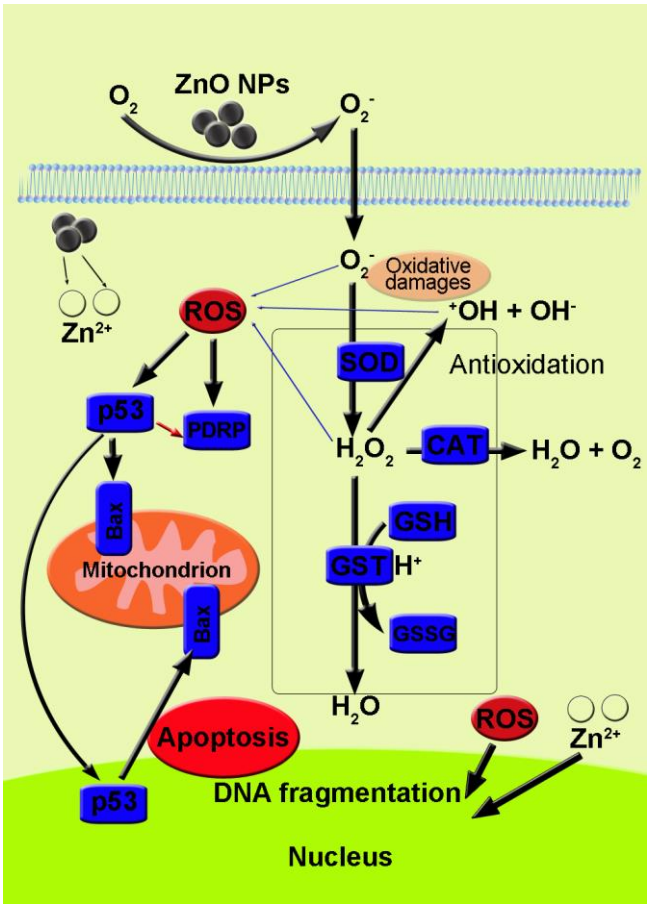

Fig. 3.16-1 The signaling pathway of ZnO NPs toxic mechanism in viable cell involved in apoptosis and antioxidation, cited from Estevez-Calvar et al. (2013) and Gomes et al. (2011).

Table 3.16-1 Biomarkers in three marine mussels (*M. galloprovincialis*, *M. edulis* and *Perna viridis*) exposed to various chemicals or environmental stress

Experimental species	Exposure toxicant/ treatment	Biomarker and response to toxic exposure			Reference
		Enzymatic concentration/activity	Gene expression	Others	
M. galloprovincialis	TiO_2 NPs	GSR, GST, CAT, GPX, and Se-GPX	*GST, CAT, Mytilin B, Myticin B, defensin, lysozyme* and *MgC1q*		(Barmo et al., 2013)
	Cd	MT			(Bebianno & Serafim, 1998)
	Polluted environment	SOD and CAT			(Da Ros et al., 2000)
	CuO NPs	SOD, CAT, GPX, and MT		lipid peroxidation (LPO)	(Gomes et al., 2012)
	Polluted waters	Glutathione, Glyoxalase I and II, GST, Se-GSR, Se-GPx, CAT, SOD			(Regoli, 1998)

	CuO and Ag NPs			Genotoxic comet assay	(Gomes et al., 2013)
	Ag NPs	SOD, CAT and GPX			(Gomes et al., 2014b)
	Nano carbon black, C_{60} fullerene, Nano-TiO_2 and Nano-SiO_2	CAT and GST		Lysosomal membrane stability, lysosomal lipofuscin content, and lysosomal Neutral Lipid content	(Canesi et al., 2010)
	UV		*p53*, *PDRP*, *Bcl-2*, *Bax*, *BI-1*, and *Dff-A*		(Estevez-Calvar et al., 2013)
	CuO NPs	SOD, CAT, and GPX			(Gomes et al., 2011)
M. edulis	Au NPs	Oxidized glutathione			(Tedesco et al., 2010b)
P. viridis	TiO_2 NPs and hypoxia			Total hemocyte counting, ROS and Lysosomal content	(Wang et al., 2014)

Gene expression profiling has been used extensively in toxicological studies to determine the impacts of biotic and abiotic stressors to understand the function of differential gene activity under the challenges of environmental toxicants (Burnett et al., 2007; Altshuler et al., 2011). These DNA damage-responsive genes and antioxidant enzymes genes have not been yet documented in *M. galloprovincialis*. The toxicity of ZnO NPs to mussel could provoke gene transcription level modification also in dependence of its size.

Therefore, the aim of this work was to investigate the changes in digestive gland transcription levels of key genes, *p53*, *PDRP*, *SOD*, *CAT*, and *GST*, of *M. galloprovincialis* along four weeks exposure to ZnO NPs and ZnO bulk dispersed in East China Sea water.

A full physic-chemical characterization of ZnO NPs and ZnO bulk dispersed in East China Sea water in order to understand how the particles can be available for marine organisms was also performed.

3.17 Materials and methods

3.17.1 Chemicals

Bare ZnO nanopowder (Code 544906, particle size < 100 nm, surface area 15−25 m^2/g) was purchased from Sigma-Aldrich Co. LLC. (USA). Bulk ZnO powder (Code ZK249038, particle size 150−200 nm, purity > 99.9%) was purchased from Quer Biotech Co., Ltd (Hefei, China). Baysalt crystals (Code Q/XWL 101−2006) were purchased from Shuilifang S&T Ltd. (Xiamen, China).

3.17.2 Particle dispersions

Stock suspensions of ZnO NPs and ZnO bulk powder were prepared with Reconstituted Sea Water (RSW). East China Sea baysalt crystals extracted from natural seawater were dissolved in pure water (MilliQ) to salinity 25 ‰ (pH 7.90) which is the salinity level of mussel sampling sea area (Shen et al., 2009) and filtered in 0.22 μm, to the final concentration of 1g Zn/L. The ZnO suspensions were dispersed by bath-sonication for 30 min at 50 W (Yuhao ultrasonic cleaner, YH-200DH, Shanghai, China). Test suspensions were prepared by diluting the stock suspension at 0.01, 0.1, 1, 10, and 100 mg Zn/L with RSW (Manzo et al., 2013).

3.17.3 Animals and exposure experiment

Wild adult specimens of M. galloprovincialis (shell length 4−5 cm) were collected from coastal rocks (30 °41′ N, 122°27′ E; Zhoushan, Zhejiang Province) in the East China Sea. The site does not belong to a national park or a protected sea area or a relevant regulatory body concerned with wildlife protection or a private owner. The mussels were further acclimatized to aerated RSW in an aquarium for 7 days at 23℃ (1 L/animal) (Scarlato, 1981; Ye et al., 2011) with feeding and water changing (3.5 days intervals; 1ml per 50L water of Phytoplex^{TM} phytoplankton feed, Kent Marine Inc., Acworth, GA, USA) (Tedesco et al., 2010a).

Mussels were exposed to ZnO NP and bulk suspensions in 0 (control), 0.01, 0.1, 1, 10, and 100 mg Zn/L concentration, respectively, for four weeks. In each exposure tank, 25 mussel individuals were exposed to 5 L medium with aerating, feeding and water changing (twice per week). On the 1st, 2nd, 3rd, 7th, 14th, 21st, and 28th day, 3 individuals per tank were sampled, and then a part of fresh tissue of digestive gland was used to extract total RNA immediately after dissection.

3.17.4 RNA isolation and qRT-PCR

Homogenization of 50mg tissues was performed by using Liquid Nitrogen Method and then the total RNA were extracted from homogenize tissues using E.Z.N.A.^{TM} Total RNA Kit II (Omega Bio-tek, Inc.). RNA integritywas assessed with an electrophoresis system (Liuyi-Bio Co., Ltd). RNA concentration was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher, Inc.). First strand complementary DNA (cDNA) synthesis was performed by RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Inc.). The quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was performed by SYBR® Premix Ex Taq^{TM} II Kit (Takara, Inc.) and using a Applied Biosystems 7500 Real-Time PCR System (ABI, Inc.). The standard cycling conditions were: 94 ℃ for 1 min, followed by 40 cycles of 10 s at 95 ℃, 45 s at 60 ℃. A melting curve of PCR products from 55 to 94 ℃ was also performed to rule out the presence of artefacts and the β-actin was used as the internal standard (Wang et al., 2013b). All primers were shown in Table 3.17.4-1.

Table 3.17.4-1 Primer of qRT-PCR for relevent genes

Primers	Sequence	Reference
p53-F	CTAGGTAGACGGGCAGTAGAAGTT	(Estevez-Calvar et al., 2013)
p53-R	GCCTCCTGGTGTTACTGTAGTGAT	
PDRP-F	CTGCCAAAGAAAGCTACAAAGAAG	
PDRP-R	CCTTTGACAATGGATTGAGGTT	
GSTα-F	ATCAGGAGGCTGCCAAAGTA	(Wang et al., 2013a)
GSTα-R	CTACAGCCAACAGGCACTCA	
CAT-F	AACCGAGAAACTCACCTGAAGGATCC	(Dondero et al., 2006)
CAT-R	ACCTTGGTCAGTCTTGAAGTGGAAT	
SOD-F	AGGCGCAATCCATTTGTTAC	(Wang et al., 2013b)
SOD-R	CATGCCTTGTGTGAGCATCT	
β-actin-F	TGTAACAAACTGGGACGATA	
β-actin-R	AGCATGAGGAAGGGCATAAC	

3.17.5 Data analysis

The relative gene expression level was analyzed by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). The gene expression in unexposed individuals was considered as the control and used forcomparison with various treated samples. All data are expressed as the mean ± SD of the different biological samples specified and were analyzed by one-way ANOVA with SPSS v13.0 software. The differences were considered statistically significant at $p \leq 0.05$. The LC50

(concentration for 50% mortality effect) was calculated using the Linear Interpolation Method (Inhibition Concentration procedure, ICp) (Norberg-King, 1993).

3.18 Results and discussion

ZnO NPs once in seawater undergo to different physic-chemical processes that modify their pristine characteristics and therefore their availability/reactivity. Consequently the mussel exposure, particularly if prolonged, implies several biological stress responses which can also trigger the modification of gene transcription (Fabbri et al., 2008) and drive to the death.

Most of the assays using bivalves were performed along short exposure time and then the long-term effects of ZnO in the bivalves deserve further attention (Rocha et al., 2015). In particular only few studies reported data about *M. galloprovincialis* exposed to ZnO NPs longer than two weeks (Hanna et al., 2013; Muller et al., 2014).

This study report for *M. galloprovincialis* along 28 days, the evaluation of transcription level of *p53* and *PDPR*, key genes involved in DNA damage/repair and *GSTα*, *CAT*, *SOD* genes associated with oxidative stress, together with physic-chemical characterization of ZnO NPs and ZnO bulk dispersed in the reconstituted East China Sea water.

3.18.1 Transcription of genes

In the pathways (Fig. 3.16-1) of organisms respond to environmental insult, the antioxidant enzymes SOD, GST, and CAT, were utilized to remove ROS (Zelko et al., 2002); while DNA repair enzymes p53 and PDRP were involved in the pathway between ROS and DNA damages to minimize possible genotoxic effects such as DNA lesions, mutation accumulation, and chromosomal aberrations (Brierley & Martin, 2013).

The relative expression of DNA damage-responsive genes, *p53* and *PDPR*, measured in the fresh digestive gland of mussels along to four weeks ZnO NPs and bulk exposure were showed respectively in Fig. 3.18.1-1 and 2. The relative expression of the three antioxidant enzymes genes *GSTα*, *CAT*, and *SOD* measured in the same abovementioned conditions were represented in Fig. 3.18.1-3, 4, and 5.

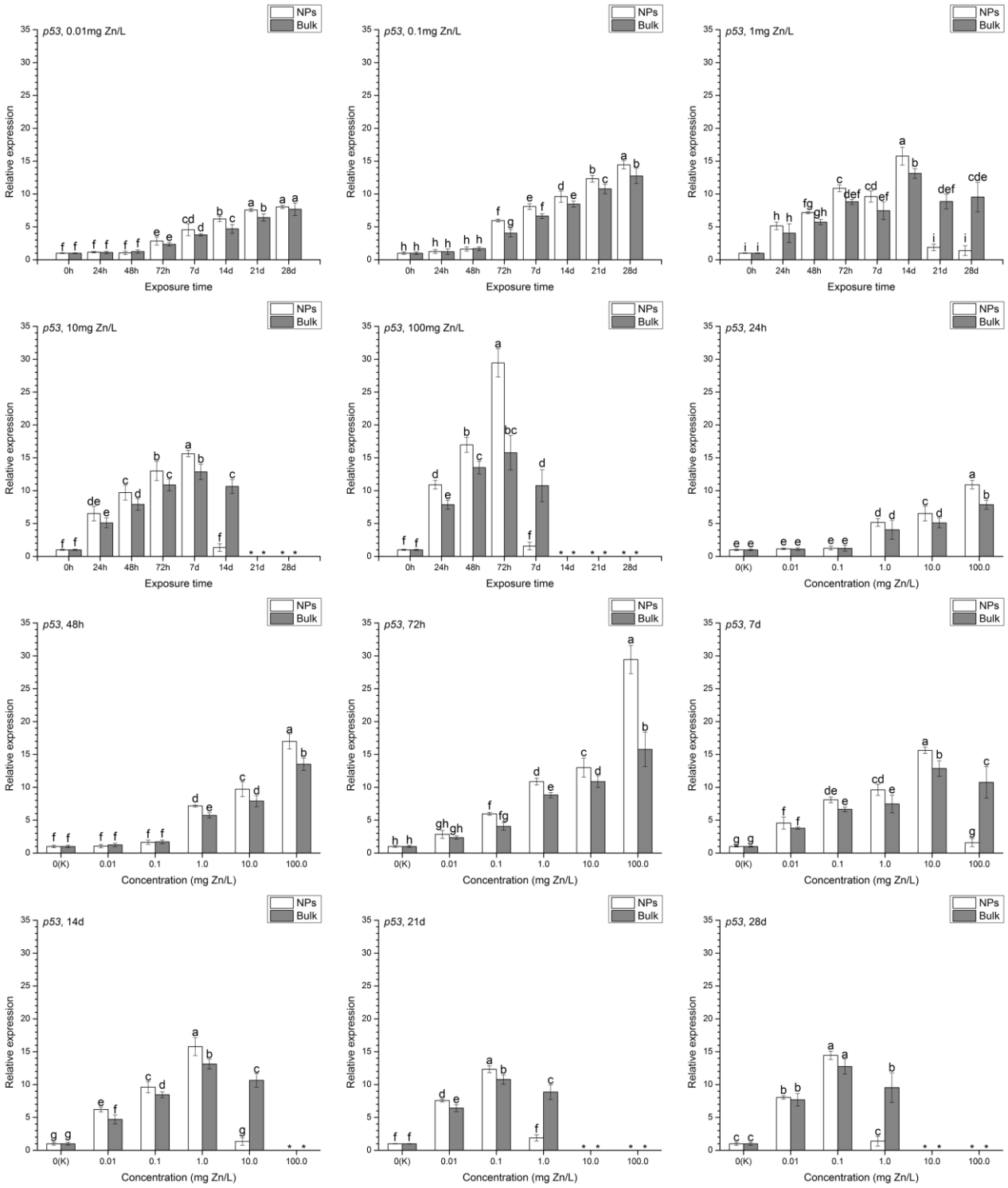

Fig. 3.18.1-1 Effects of ZnO NPs and Bulk on gene *p53* transcription in mussel digestive gland. Gene transcription was determined by qRT-PCR as described in methods. Relative expression was calculated with respect to control mussels. Data are the mean ± SD obtained from at least 3 independent RNA samples in triplicate. The differences were considered statistically significant at $p \leq 0.05$.* denotes the all individualswere dead in the group.

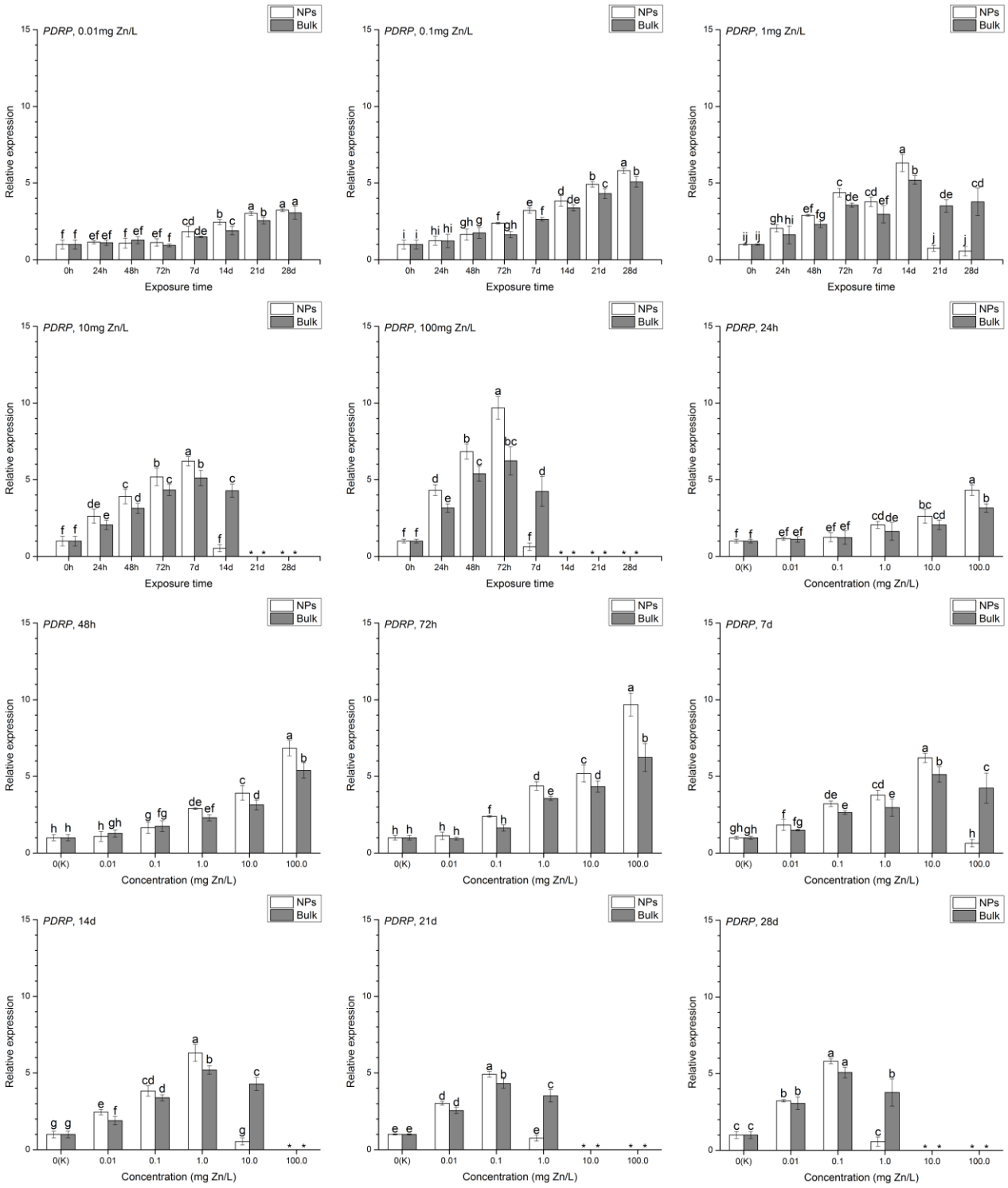

Fig. 3.18.1-2 Effects of ZnO NPs and Bulk on gene *PDRP* transcription in mussel digestive gland. Gene transcription was determined by qRT-PCR as described in methods. Relative expression was calculated with respect to control mussels. Data are the mean ± SD obtained from at least 3 independent RNA samples in triplicate. The differences were considered statistically significant at $p \leq 0.05$.* denotes the all individualswere dead in the group.

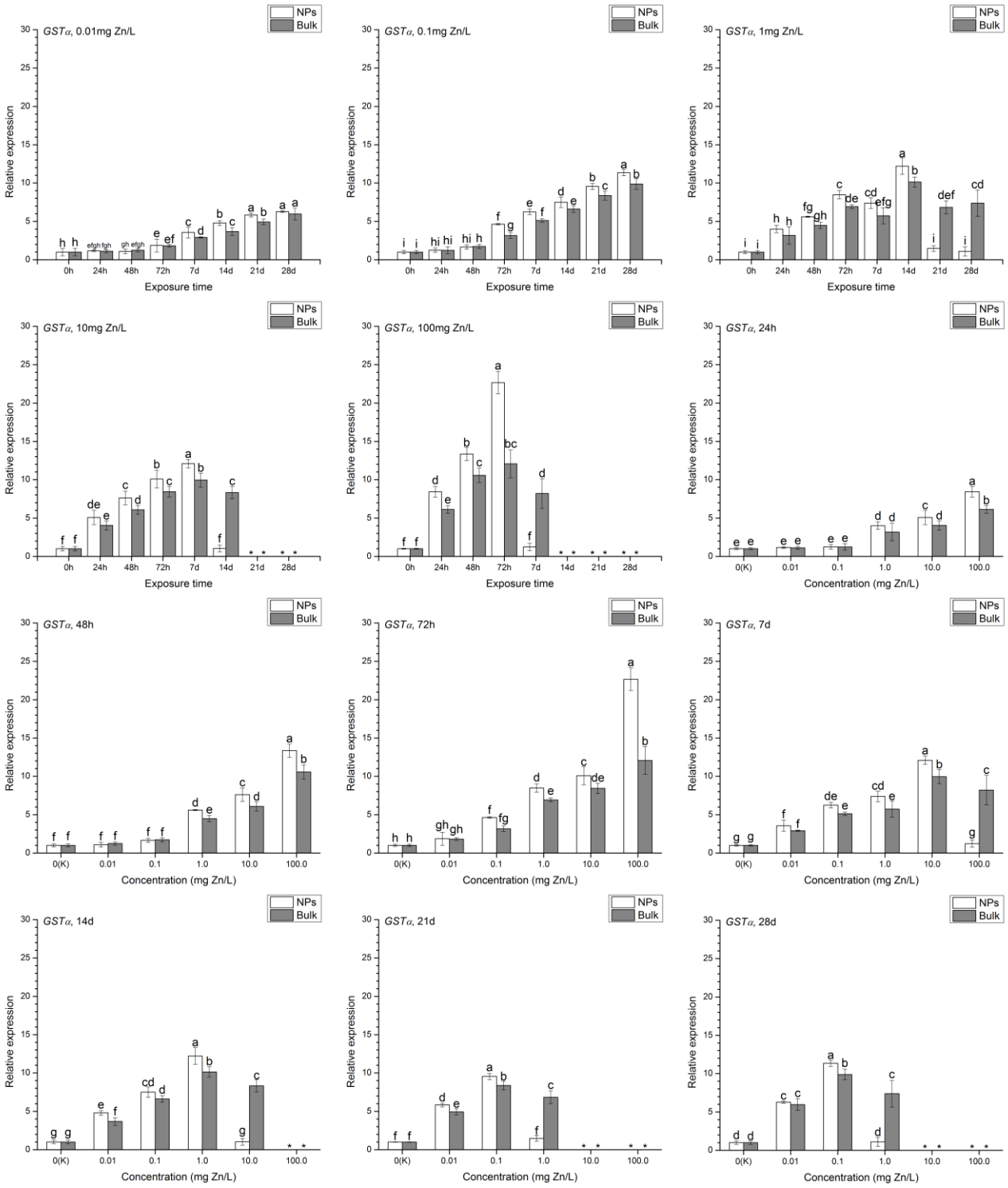

Fig. 3.18.1-3 Effects of ZnO NPs and Bulk on gene *GSTα* transcription in mussel digestive gland. Gene transcription was determined by qRT-PCR as described in methods. Relative expression was calculated with respect to control mussels. Data are the mean ± SD obtained from at least 3 independent RNA samples in triplicate. The differences were considered statistically significant at $p \leq 0.05$.* denotes the all individualswere dead in the group.

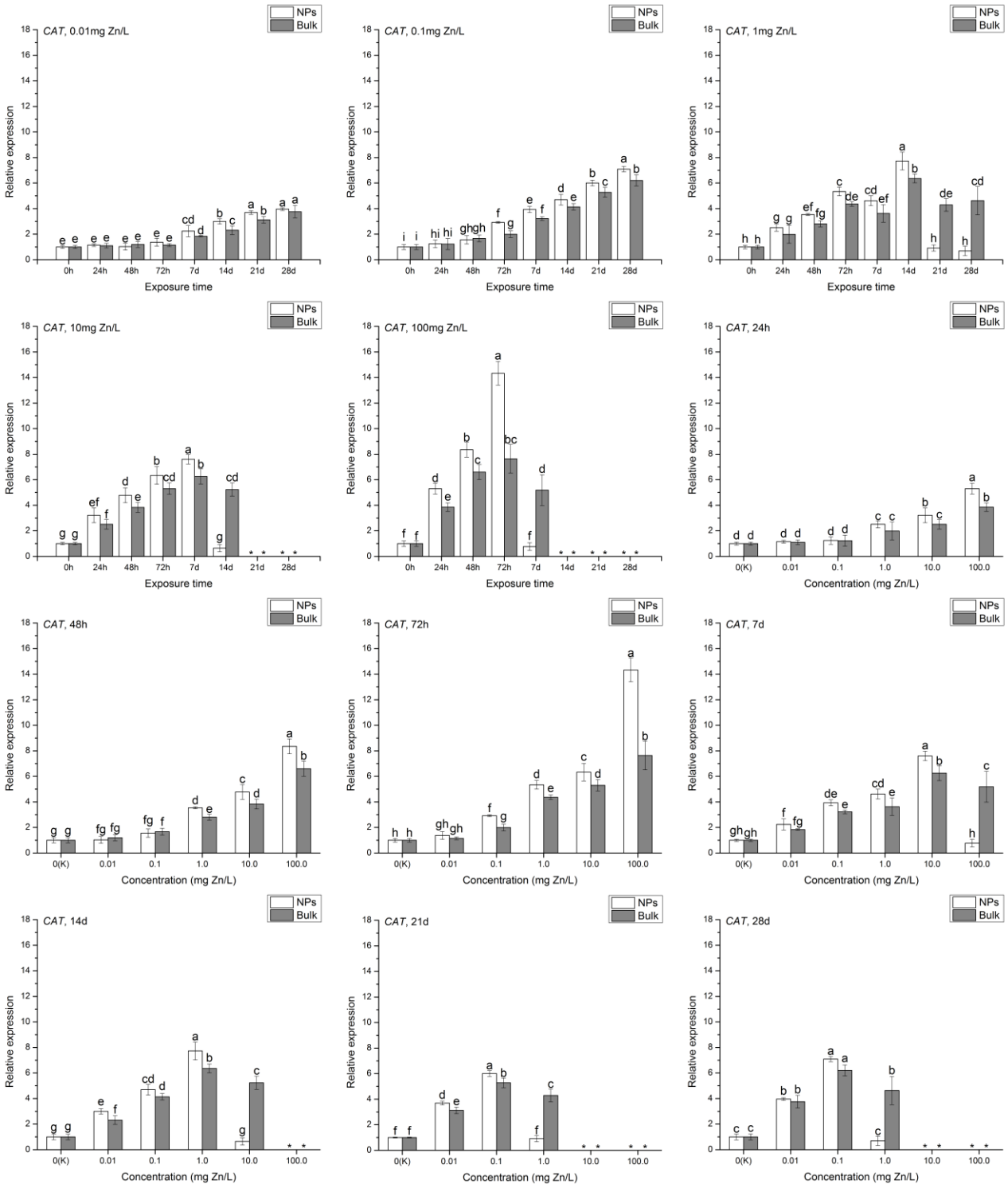

Fig. 3.18.1-4 Effects of ZnO NPs and Bulk on gene *CAT* transcription in mussel digestive gland. Gene transcription was determined by qRT-PCR as described in methods. Relative expression was calculated with respect to control mussels. Data are the mean ± SD obtained from at least 3 independent RNA samples in triplicate. The differences were considered statistically significant at $p \leq 0.05$. * denotes the all individualswere dead in the group.

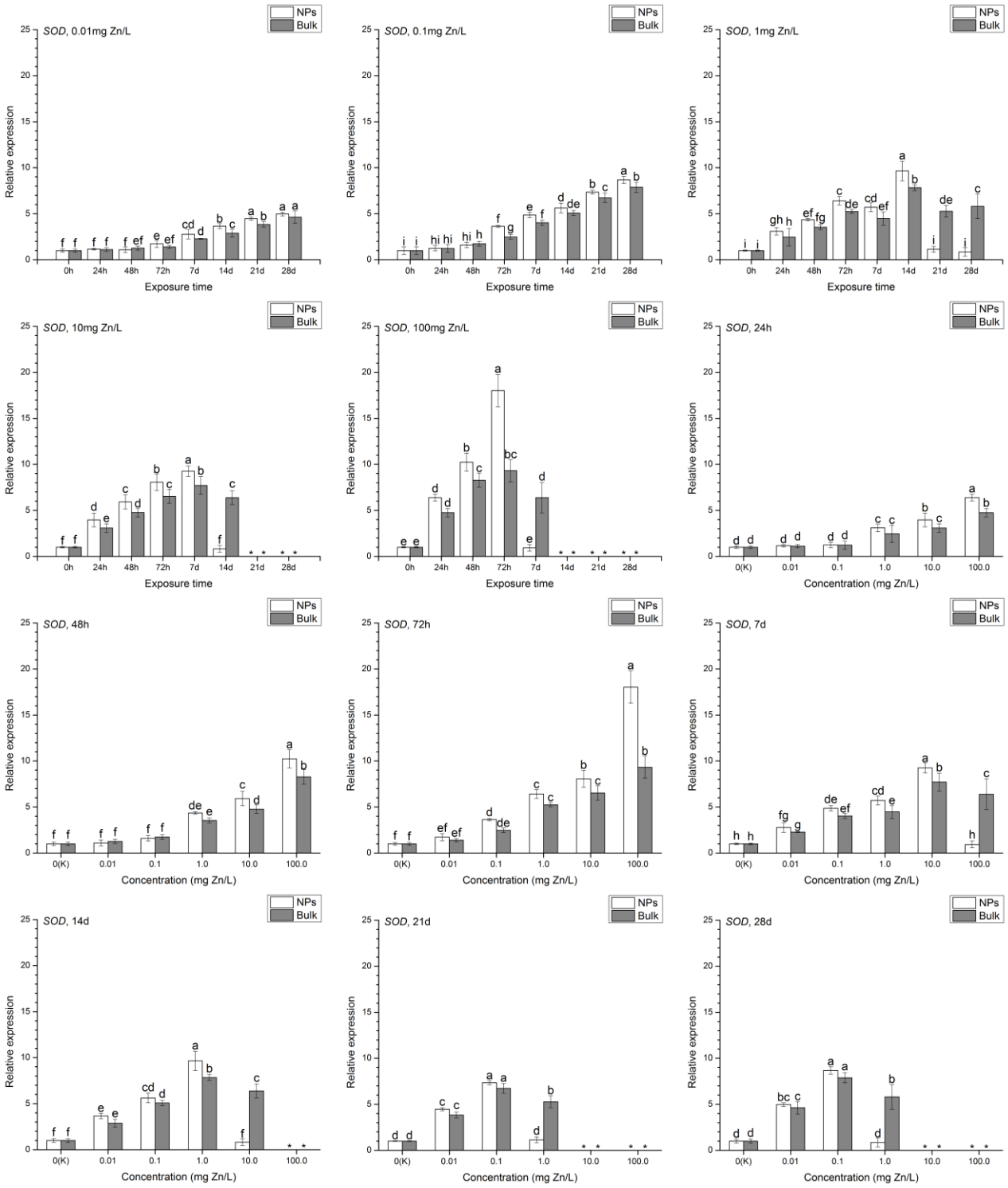

Fig. 3.18.1-5 Effects of ZnO NPs and Bulk on gene *SOD* transcription in mussel digestive gland. Gene transcription was determined by qRT-PCR as described in methods. Relative expression was calculated with respect to control mussels. Data are the mean ± SD obtained from at least 3 independent RNA samples in triplicate. The differences were considered statistically significant at $p \leq 0.05$. * denotes the all individualswere dead in the group.

DNA damage-responsive genes (*p53* and *PDPR*) transcription profile:

The first significant difference ($p \leq 0.05$) of *p53* expression with respect to the control occurred only after 72h of exposureat the lowest concentrations (0.01 and 0.1 mg Zn/L) while at higher concentrations (1−100 mg Zn/L) since the beginning of the exposure time, a significant increased expression were detected (Fig. 3.18.1-1). At the first two tested concentrations the *p53* gene expression increased in dependence of the exposure time until the end (28 Day) with a maximum expression level of about 15 fold the control. At 1 mg Zn/L this level could be observed after only 14 days followed by a sharp decrease in the following time exposure (21 and 28 days), and then by the death of the organisms. A similar pattern could be observed after 7 days exposure at 10 mg Zn/L and after 72 h at 100 mg Zn/L, but in both cases, at the respective following time exposure, first a decrease of response and then the death of the organisms could be observable (21 d and 14 d for 10 mg Zn/L and 100 mg Zn/L, respectively).

Therefore it seemed that up to 1 mg Zn/L the organisms can activate the maximum response in a time span linearly dependent on the concentration. Additionally, it was worthy to note that, at 100 mg Zn/L (72 h) there was an *p53* overexpression, in fact the level measured was about twice the maximum level measured at the lower concentration independently by the exposure time.

The comparison between NPs and bulk showed significant difference ($p \leq 0.05$) depending on dose and exposure time, with NPs higher (of about 25 %) than bulkin most phases. The opposite situation was represented at the end of exposure time, in particular after the maximum *p53* expression when the drop was evident for NPs but not for bulk. Also for bulk exposure the dramatic death effect could be observable with the above reported scheme for NPs.

The ZnO exposure has induced *PDRP* expression response as well. In Fig. 3.18.1-2, the relative expression represented a very similar trend as that of *p53*, although the expressions are not at the same level. Diversely from *p53* the first significant difference ($p \leq 0.05$) occurred at 7 d for 0.01 mg Zn/L and at 48 h for 0.1 mg Zn/L this indicates that *PDRP* response was less sensitive at the lowest exposure concentration than *p53*. In the range 1−100 mg Zn/L, gene expression levels showed a rising trend from the beginning of exposure up to a peak and then, in particular for NPs, they went down. The highest relative expression (approximately 6 fold the control) occurred at 28 d in 0.1 mg Zn/L, at 14 d in 1 mg Zn/L, and 7 d in 10 mg Zn/L. However, this pattern common for low concentrations was not evident at 100 mg Zn/L: in this case, the first level of gene expression was 4 fold the control and it went up to almost 10 fold the control at 72 h. The special

overexpression for high concentration exposure was also found in the assay for *p53*. Additional, the difference in gene expression between NPs and bulk was very evident in Fig. 3.18.1-2. Significant differences ($p \leq 0.05$) between NPs and bulk were found since the day 7 in the lowest concentration exposure, the 72 h in 0.1 and 1 mg Zn/L, the 48 h in 10 mg Zn/L, and 24 h in 100 mg Zn/L, with a constant difference level, except at the end of exposure in the high concentration.

Few studies evaluated the *p53* gene expression in mussels as response to insult exposure evidencing modification of regular expression. Estevez-Calvar et al. (2013) reported the increasing *p53* gene expression in *M. galloprovincialis* hemocytes since the beginning of UV exposure, up to 25 times the control and, and a drop off after 48 hours. Banni et al. (2009) evaluated the *p53* gene expression in *Mytius* spp. digestive gland exposed to two different organic compounds and reported two different relative expression, one higher (1.5 fold) and one lower (1/4 fold) the control, after approximately 24 hours and a similar level of control samples after 50 hours. Our study did not evidence early increment of expression after the first hours of exposure (Estevez-Calvar et al., 2013) and, although the exposure to concentration > 1 mg Zn/L induced significant relative expression of *p53* gene after the first 24 h (Banni et al., 2009), however, no adapting trend in the subsequent exposure time could be found.

Similarly, *PDRP* expression in our study, showed a continued rising trend in the first 48 h differently to Estevez-Calvar et al. (2013) who reported a very high expression level for *PDRP* at the beginning of exposure that turned down to normal level (control group) at 48 h. The trend of DNA damage-responsive genes during the first 48 hours ZnO exposure indicated the activity of the DNA repair process in the mussel. The signal pathway of the two DNA damage-responsive genes (Fig. 3.16-1) shows how ROS directly induce *p53* and *PDRP* gene expression. ZnO exposure activated the process of DNA damage and repair at the same time in this report. The difference between ZnO NPs and bulk effects could be attributable to different ROS amount production in the cell as response to different particle size exposure (Avalos et al., 2014).

Antioxidant enzymes genes (*GSTα*, *CAT*, and *SOD*) transcription profile:

The gene expression of the three antioxidant enzymes showed a common increasing transcription level along with exposure time (28 days) and chemicals (ZnO NPs and bulk) concentrations (Fig. 3.18.1-3, 4, and 5).

In the digestive gland of mussel exposed to ZnO NPs, the *GSTα* gene transcription linearly increased along with time for concentration below 1 mg Zn/L. At 1 mg Zn/L, instead, the relative expression of *GSTα* showed a peak (approximately 12 fold the control) at 14 d, that, at 21 d and 28 d, turned down to level not significantly differentfrom the control ($p > 0.05$). At the two higher concentrations (10 and 100 mg Zn/L) the same pattern of expression was observable. The peak of expression, measured at 7 d for 10 mg Zn/L and at 72 h for 100 mg Zn/L, was followed by a sharp decrease of expression in the next exposure times that, differently from the lower concentrations, culminated with the death of exposed mussels at 21 d and 14 d respectively. It is worth to note that the maximum gene relative expression (12 fold the control) occurred at 28 d in 0.1 mg Zn/L, at 14 d in 1.0 mg Zn/L, and at 7 d in 10.0 mg Zn/L, while at 100 mg Zn/L the maximum level was reached at 48 h and at 72 h an overexpression (approximately 22 fold the control) was registered.

In the case of ZnO bulk exposure, data suggested that gene transcription was less affected at the most exposure situations ($p \leq 0.05$, in Fig. 3.18.1-3) respect to ZnO NP exposure. In the range of 0.01−0.1 mg Zn/L concentrations, an increasing trend along with exposure time similar to NPs was observable. At 1−100 mg Zn/L only a slight decline had been found at the end of exposure for bulk ZnO in comparison to the sharp decrease registered for ZnO NPs. This different decline level made gene expression induced by bulk significant higher than NPs at the end of exposure time ($p \leq 0.05$).

Although GSTα protein is a key enzyme that catalyzes H_2O_2 to H_2O which is one common path to reduce ROS (H_2O_2) (Gomes et al., 2011; Estevez-Calvar et al., 2013), different GSTα production as response to NP exposure was reported in literature: some works (Canesi et al., 2010; Pan et al., 2012; Buffet et al., 2013) reported an increased GST protein activity in clam (*Scrobicularia plana)* and mussel (*M. galloprovincialis*) exposed to several nanomaterials; while others (Renault et al., 2008) observed the repression of *GST* gene in gill cells of benthic bivalve *Corbicula fluminea* exposed to Au NPs. Saddick et al. (2015) measured in fish brain exposed in ZnO NPs for 15 days a slight increment or decrement of *GST* transcription in dependence to concentration.

However to the best of our knowledge, *GSTα* trends similar to this study were not described before.

The transcription profile of the two antioxidant enzymes genes *CAT* and *SOD* in the 28 days exposure to ZnO NPs and ZnO bulk were showed in Fig. 3.18.1-4 and 5. Their trends were similar to *GST*α one although at different level of relative expression. In particular, thehighest relative expression at concentrations lower than 100 mg Zn/L was about 8 times the control for *CAT* and 10 for *SOD*, while an overexpression level of 14 (*CAT*) and approximately 18 (*SOD*) in 100 mg Zn/L was obtained. The first significant differences appeared for *CAT* at 7 d, 0.01 mg Zn/L and at 48 h, 0.1 mg Zn/L for both ZnO; while for *SOD* at 72 h, 0.01 mg Zn/L for NPs; 48 h, 0.1 mg Zn/L for bulk differently from *GST*α that occurred at 72 h, 0.01 mg Zn/L for both ZnO; 48 h, 0.1 mg Zn/L for bulk. This pattern indicated that *CAT* is less involved than other two genes at low Zn concentrations probably because while, SOD, as key enzyme in the first step of antioxidation, catalyzes O_2^- (ROS) to H_2O_2, CAT works on reducing the volume of H_2O_2 and then downstream the antioxidation induced by ZnO exposure.

Studies focused on CAT and SOD enzyme activity of bivalve exposed to nano materials (Tedesco et al., 2008; Canesi et al., 2010; Buffet et al., 2011; Buffet et al., 2012; Gomes et al., 2012; Buffet et al., 2013; Buffet et al., 2014; Gomes et al., 2014b; Vale et al., 2014) generally indicated an increased enzyme activities. In addition, limited studies reported data about *SOD and CAT* gene expression: Renault et al. (2008) reported in gill cells of benthic bivalve *Corbicula fluminea* exposed to Au NPs, besides the *GST* repression, the *SOD* gene induction and left *CAT* expression unaltered, also an over expression of *CAT* in visceral mass that indicated the need to consider also other organs, such as digestive gland. In addition, Barmo et al. (2013) reported for digestive gland cells of *M. galloprovincialis* exposed to TiO_2 NPs for 96 h, a *GST*π and *CAT* significant repression at low concentration and *GST*π and *CAT* not significant adaption, at high concentration. Although the repression occurredat low exposed concentrations was not observable in our study, the adaption phenomenon seemed to represent a kind of self-protective mechanism in exposed mussel similar to that described in our case.

3.18.2 Mortality effect

The mortality curves of mussel exposed to different concentration of ZnO NPs and bulk for 28 days were reported in Fig. 3.18.2-1. Both graphs showed, starting from 72h, increasing values along with the exposure time together with Zn increment. From 7d the effect exceeded 50%

and adifferent level of lethality could be observed in dependence of particle pristine size, with NPs always more toxic than bulk (see LC50s). The highest difference was evident after 28 d when NPs resulted three times more toxic than bulk (LC50 = 0.78 [0.64, 1.00] and 2.62 [1.00, 4.00] respectively). The 100% effect occurred at 14 d in the 100 mg Zn/L and at 21 d in 10 mg Zn/L.

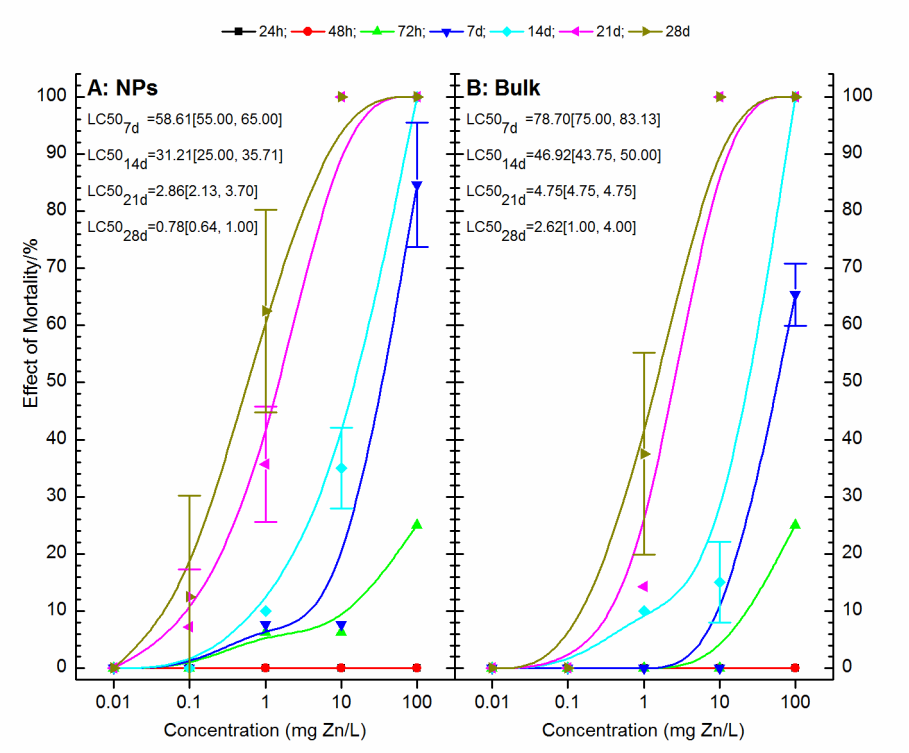

Fig. 3.18.2-1 Mortality effect of mussel exposed to ZnO NPs (A) and bulk (B) suspensions for 28 days. LC50s of mortality effect were calculated for 7d, 14d, 21d, and 28d. LC50s were undetectable for the first three days of exposure.

No mortality was observed for the clam *Scrobicularia plana* exposed to 10 µg Cu/L (CuO NPs) for 21 days (Buffet et al., 2013) and to 10 µg Ag/L (Ag NPs) for 21 days (Buffet et al., 2014); for the mussel *M. galloprovincialis*in 10 µg Cu/L (CuO NPs) exposure for 15 days (Gomes et al., 2014a), and for the *Macoma balthica* in 150−200 µg/g AgO and CuO NP exposure for 35 days (Dai et al., 2013). However, Muller et al. (2014) performed long term (100 days) *M. galloprovincialis*exposure to ZnO NPs reporting very low mortality rate 1.66×10^{-3} 1/d for concentrations < 2 mg Zn/L: a toxic effect lower than in the present study, probably because of the different experimental design adopted.

3.18.3 Comparison of DNA damage-responsive genes and antioxidant enzymes genes

The DNA damage-responsive genes and antioxidant enzymes genes actively responded to

ZnO exposure. The gene expression trends along with exposure dose and time showedan extremely consistent pattern within each other. The production of ROS has been identified as one of the main causes of NP toxicity. Our results support other studies; it is probably that the ROS formation is strongly induced as this is associated with both a rapid upregulation of the *CAT* and *SOD* gene and a high level of expression of *GST*. The fact that, up to 1 mg Zn /L, gene expression levels subsequently subsides suggests that the antioxidant system is able to neutralize the hydrogen peroxide and other ROS formed by the ZnO NPs and thereby protect the organism (Varela-Valencia et al., 2014). This trend was previously reported as adaptation effect to Ag NPs at the end of exposure (15 days) in *M. galloprovincialis* gill by Bebianno et al. (2015). Based on the results reported here, it is suggested that 1 mg Zn /L, represents a threshold value below which the cell activates defences mechanisms to cope with the related oxidative stress (Davies, 2000; Clauditz et al., 2006).

In the case of concentration higher than 1mg/L, instead, theobserved gene overexpression followed by the sharp decrease to the control level and by death, suggests the possibility for the cell antioxidant system to activate an additional extra expression in the effort of counteract the increased stimulus. This process was eventually insufficient to neutralize ROS deriving effects as DNA damages, alteration of cell metabolism and that drive to cell apoptosis. The general trend evidenced for bulk exposure showed many differences with that deriving from ZnO NP exposure. These differences were attributable to the pristine size of ZnO (Gomes et al., 2014b) and to their peculiar behavior in seawater (Fig. 3.3.1-3): In the main, because dissolution was very similar for the two ZnO, it could not be considered the main or the only process at the base of the oxidative stress and relative activation of gene expression. Moreover the ionic zinc alone showed an increasing pattern of antioxidant gene expression along with concentrations and time exposure (unpublished data).

Therefore it should be hypothesized a toxicity mechanism linked to ZnO pristine nano size that induce ROS production promoting antioxidation and apoptosis involved in the expression of damage-responsive genes (*p53* and *PDPR*) and antioxidant enzymes genes (*GSTα*, *CAT*, and *SOD*).

However, Wang et al. (2014) reported that TiO_2 NPs reduced the ROS production in mussel *Perna viridis* and Gomes et al. (2011) suggested that CuO NPs induced a decrease trend of enzymatic activity of SOD and CAT in *M. galloprovincialis* (Fig. 3.16-1). This contrast suggested

that the toxic mechanism of ZnO NPs to mussel is probably different of TiO_2 NPs and CuO NPs. Nonetheless, some evidence to support the ROS-mediated toxic mechanism of nanomaterials to mussels were reported: Au NPs increased oxidized glutathione (GSSG) in digestive gland of *M. edulis* (Tedesco et al., 2010b) indicating that NPs induced oxidative stress; carbon black, C_{60} fullerene, nano-TiO_2 and nano-SiO_2 (Canesi et al., 2010) increased specific activity of digestive gland CAT and GST in *M. galloprovincialis* along with exposure dose.

3.19 Conclusion

The results of this work showed the ROS-mediated injury on marine invertebrates *M. galloprovincialis* by ZnO NPs using qRT-PCR technique which could be a sensitive approach to reveal ecotoxicity of nanomaterials. The differences of genes transcription and lethality between both ZnO powders answered our hypothesis that all five investigated genes, *p53*, *PDPR*, *GSTα*, *CAT*, and *SOD*, involved in antioxidation and apoptosis represented active response to ZnO NPs exposure that induced DNA damage and oxidant injury contributing to the overall toxicity and which is to the pristine size of ZnO and its behavior in specific seawater.

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3.21 Zinc causes oxidative damages in digestive gland in mussel *M. galloprovincialis* revealed by transcription of related genes

Zinc, an essential micronutrient, is present in unpolluted areas at nanomolar levels and could reach micromolar values in metal-contaminated environments (Garcia et al., 2008; Gowd & Govil, 2008; Voets et al., 2009). Bivalves, being filter feeding, easily take up soluble zinc, mainly by gills and mantle, while the Zn particulate forms are taken up mainly by the digestive organs and stored in the digestive gland (George & Pirie, 1980; Wang, 2001; Cooper et al., 2010). Consequently, Zn in the body of bivalves can be easily related to zinc level in the environment (Rebelo et al., 2003).

At cellular level Zn is a key transition metallic element and takes part in the modulation process of regulatory proteins and cellular activities such as antioxidant defenses (Brocardo et al., 2007; Chasapis et al., 2012; Oteiza, 2012).

However, Formigari et al. (2007) reported that higher level of Zn induces apoptosis and oxidative stress. Zn plays the dual role in oxidant/antioxidant and pro-apoptotic/anti-apoptotic process in live cells.

The increasing use of pesticide, pharmaceuticals, alloy is leading to Zn release into the environment and the ecological hazard of Zn had received much attention in decades (Bonnevie et al., 1993; Morgan & Morgan, 1999; Gan et al., 2000; Lee et al., 2005; Hou et al., 2013; Kun et al., 2015). Heavy metal Zn could be ionic in aquatic environment and accumulated in marine organism.

On the basis of their world-wide distribution (Golberg, 1975; Phillips, 1976) common mussel *Mytilus edulis* and its similar species *Mytilus galloprovincialis* Lamarck, 1819 since 1970s were proposed and used as indicators of marine contamination by trace metals. In recent years, *M. galloprovincialis* was largely used to investigate biological response to toxicants and environmental stress in laboratorial condition (Bebianno & Serafim, 1998; Da Ros et al., 2000; Barmo et al., 2013; Estevez-Calvar et al., 2013; Balbi et al., 2014).

As it was well documented (Leonard et al., 2004; Mithöfer et al., 2004; Matés et al., 2010) heavy metal toxicity is based on ROS-mediated injury. To present many examples of ROS-mediated injury in mussels by various toxicants or stress, biomarkers were reported (Regoli, 1998; Canesi et al., 2010; Tedesco et al., 2010a; Gomes et al., 2012; Wang et al., 2014).

The toxicity of zinc to bivalves has been demonstrated at different levels, such as mortality in adults, embryos and gametes (Nadella et al., 2009; Fathallah et al., 2010), decrease in oxygen consumption and metabolic dysfunction (Devi, 1995), as well as modulation of the antioxidant system and induction of oxidative stress (Geret & Bebianno, 2004; Franco et al., 2006). Several biomarkers of oxidative stress, such as enzymatic concentration/activity, genes expression, lipid peroxidation, DNA damage (comet assay), lysosomal membrane stability, etc., were analyzed in mussels (Table 3.21-1). Many studies reported the gill and/or digestive gland of *M. galloprovincialis* concentration or activity of enzymes which are involved in oxidative stress, apoptosis, and DNA damage/repair such as oxidized glutathione reductase (GSR), glutathione transferase (GST), catalase (CAT), total glutathione peroxidase (GPX) (Canesi et al., 2010; Gomes et al., 2011; Gomes et al., 2012; Barmo et al., 2013; Gomes et al., 2014b).

Table 3.21-1 Biomarkers in three marine mussels (*M. galloprovincialis*, *M. edulis* and *Perna viridis*) under various environmental stress

Experimental species	Biomarkers			Reference
	Enzymatic concentration/activity	Gene expression	Others	
M. galloprovincialis	GSR, GST, CAT, GPX, and Se-GPX	*GST, CAT, Mytilin B, Myticin B, defensin, lysozyme* and *MgC1q*		(Barmo et al., 2013)
	MT			(Bebianno & Serafim, 1998)
	SOD and CAT			(Da Ros et al., 2000)
	SOD, CAT, GPX, and MT		lipid peroxidation (LPO)	(Gomes et al., 2012)
	Glutathione, Glyoxalase I and II, GST, Se-GSR, Se-GPx, CAT, SOD			(Regoli, 1998)
			Genotoxic comet assay	(Gomes et al., 2013)
	SOD, CAT and GPX			(Gomes et al., 2014b)
	CAT and GST		Lysosomal membrane stability, lysosomal lipofuscin content, and lysosomal Neutral Lipid content	(Canesi et al., 2010)
		p53, PDRP, Bcl-2, Bax, BI-1, and *Dff-A*		(Estevez-Calvar et al., 2013)
	SOD, CAT, and GPX			(Gomes et al., 2011)
M. edulis	Oxidized glutathione			(Tedesco et al., 2010a)
P. viridis			Total hemocyte counting, ROS and Lysosomal content	(Wang et al., 2014)

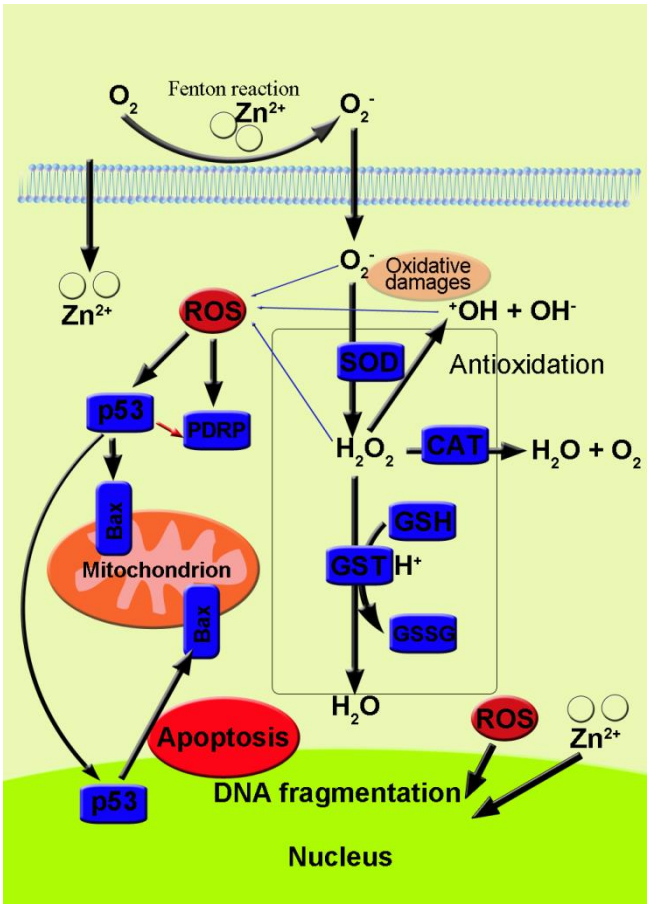

Fig. 3.21-1 The signaling pathway of Zn^{2+} toxic mechanism in viable cell involved in apoptosis and antioxidation, cited from Estevez-Calvar et al. (2013) and Gomes et al. (2011).

Therefore (Fig. 3.21-1) ROS-mediated injury activate antioxidation of cellular response that key enzymes, SOD, CAT, and GST, play important roles to reduce ROS, as well as apoptosis that p53 and PDRP were involved in the pathway between ROS and DNA damages. These DNA damage-responsive genes and antioxidant enzymes genes have not been documented in *M. galloprovincialis*. Thus, it is necessary to investigate the transcript level changes which are more sensitive and direct than protein response for exposure to Zn.

The aim was to investigate the pattern of oxidative stress and DNA damage/repair gene expression in response to prolonged *M. galloprovincialis* exposure (28 days) at different concentration of soluble Zinc. We report the transcription of key genes, *p53*, *PDRP*, *SOD*, *CAT*, and *GST*, involved in DNA damage/repair and antioxidation in this article to complete the whole picture of mechanism of Zn salt toxicity.

3.22 Materials and methods

3.22.1 Chemicals

$ZnSO_4 \cdot 7H_2O$ crystals (Code 204986, 99.999% trace metals basis) were purchased from Sigma-Aldrich Co. LLC. (USA). Baysalt crystals (Code Q/XWL 101-2006) were purchased from Shuilifang S&T Ltd. (Xiamen, China).

3.22.2 Particle dispersions

Stock solution of Zinc salt was prepared with Reconstituted Sea Water (RSW). East China Sea baysalt crystals extracted from natural seawater were dissolved in pure water (MilliQ) to salinity 25 ‰ (pH 7.90) which is the salinity level of mussel sampling sea area (Shen et al., 2009) and filtered in 0.22 μm, to the final concentration of 1g Zn/L. Test suspensions were prepared by diluting the stock solution at 0.01, 0.1, 1, 10, and 100 mg Zn/L with RSW.

3.22.3 Animals and exposure experiment

Wild adult specimens of *M. galloprovincialis* (shell length 4-5 cm) were collected from coastal rocks (30°41′ N, 122°27′ E; Zhoushan, Zhejiang Province) in the East China Sea. The site does not belong to a national park or a protected sea area or a relevant regulatory body concerned with wildlife protection or a private owner. The mussels were further acclimatised to aerated RSW in an aquarium for 7 days at 23℃ (1 L/animal) (Scarlato, 1981; Ye et al., 2011) with feeding and water changing (3.5 dyas intervals; 1 mL per 50 L water of Phytoplex^{TM} phytoplankton feed, Kent Marine Inc., Acworth, GA, USA) (Tedesco et al., 2010b).

Mussels were then treated with Zinc salt solution in 0 (control), 0.01, 0.1, 1, 10, and 100 mg Zn/L concentration, respectively, for four weeks. In each exposure tank, 25 mussel individuals were exposed to 5 L medium with aerating, feeding and water changing (twice per week). On the 1st, 2nd, 3rd, 7th, 14th, 21st, and 28th day, 3 individuals per tank were sampled, and then a part of fresh tissue of digestive gland was used to extract total RNA immediately after dissection.

3.22.4 RNA isolation and qRT-PCR

Homogenization of 50mg tissues was performed by using Liquid Nitrogen Method and then the total RNA were extracted from homogenize tissues using E.Z.N.A.^{TM} Total RNA Kit II (Omega Bio-tek, Inc.). RNA integritywas assessed with an electrophoresis system (Liuyi-Bio Co.,

Ltd). RNA concentration was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher, Inc.). First strand complementary DNA (cDNA) synthesis was performed by RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Inc.). The quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was performed by SYBR® Premix Ex Taq^{TM} II Kit (Takara, Inc.) and using a Applied Biosystems 7500 Real-Time PCR System (ABI, Inc.). The standard cycling conditions were: 94 ℃ for 1 min, followed by 40 cycles of 10 s at 95 ℃, 45 s at 60 ℃. A melting curve of PCR products from 55 to 94 ℃ was also performed to rule out the presence of artefacts and the β-actin was used as the internal standard (Wang et al., 2013). All primers were shown in Table 3.17.4-1.

3.22.5 Data analysis

The relative gene expression level was analyzed by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). The gene expression in unexposed individuals was considered as the control and used forcomparison with various treated samples. All data are expressed as the mean ± SD of the different biological samples specified and were analyzed by one-way ANOVA with SPSS v13.0 software. The differences were considered statistically significant at $p \leq 0.05$. The LC50 (concentration for 50% mortality effect) was calculated using the Linear Interpolation Method (Inhibition Concentration procedure, ICp) (Norberg-King, 1993).

3.23 Results and discussion

3.23.1 Transcription of genes

In the pathways (Fig. 3.21-1) of organisms respond to environmental insult, the antioxidant enzymes SOD, GST, and CAT, were utilized to remove ROS (Zelko et al., 2002); while DNA repair enzymes p53 and PDRP were involved in the pathway between ROS and DNA damages to minimize possible genotoxic effects such as DNA lesions, mutation accumulation, and chromosomal aberrations (Brierley & Martin, 2013).

The relative expression of DNA damage-responsive genes, *p53* and *PDPR*, measured in the fresh digestive gland of mussels along to four weeks Zn salt exposure were showed respectively in Fig. 3.23.1-1 and 2. The relative expression of the three antioxidant enzymes genes *GST*α, *CAT*, and *SOD* measured in the same abovementioned conditions were represented in Fig. 3.23.1-3, 4 and

5.

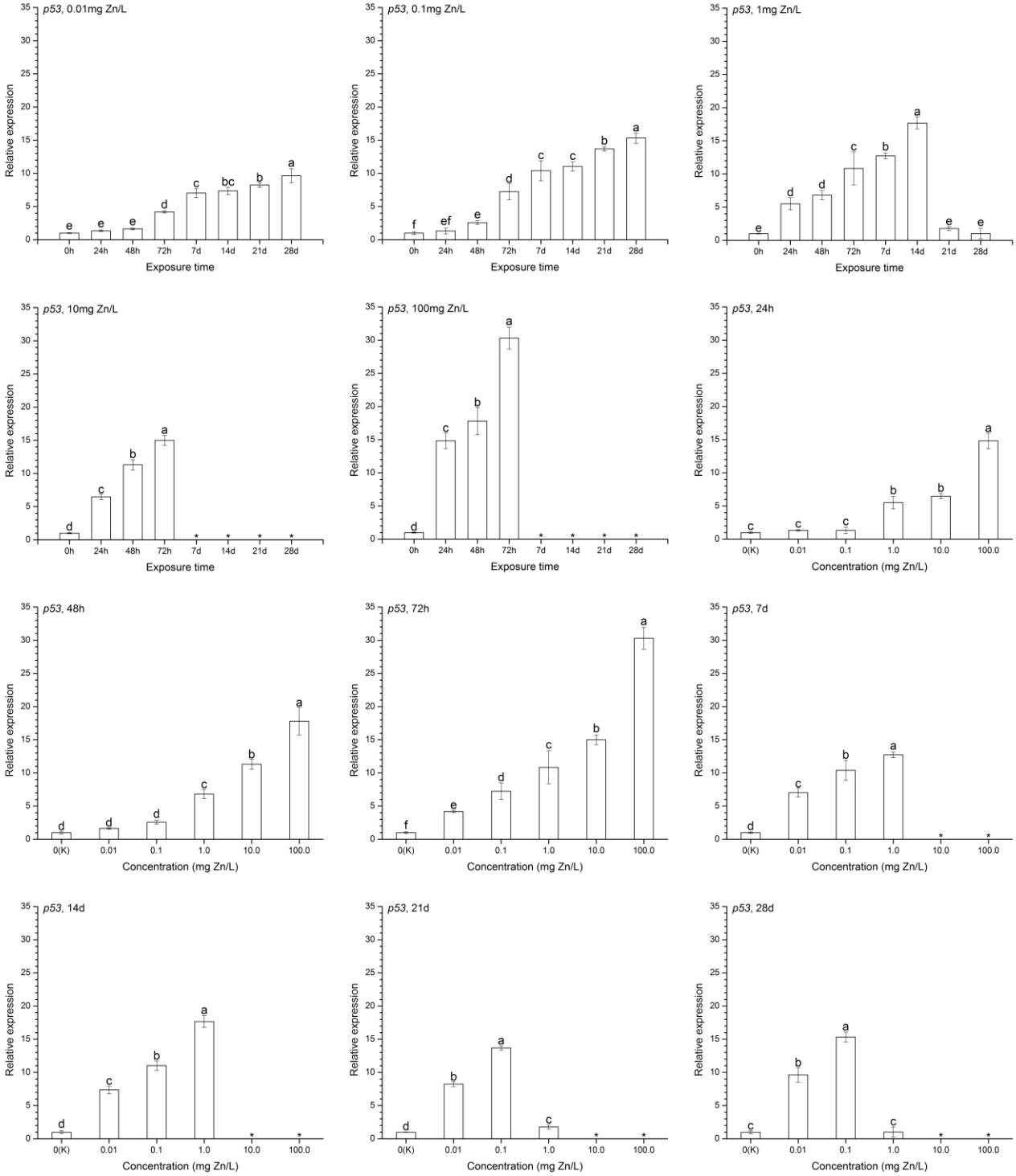

Fig. 3.23.1-1 Effects of Zn salt on gene *p53* transcription in mussel digestive gland. Gene transcription was determined by qRT-PCR as described in methods. Relative expression was calculated with respect to control mussels. Data are the mean ± SD obtained from at least 3 independent RNA samples in triplicate. The differences were considered statistically significant at $p \leq 0.05$.

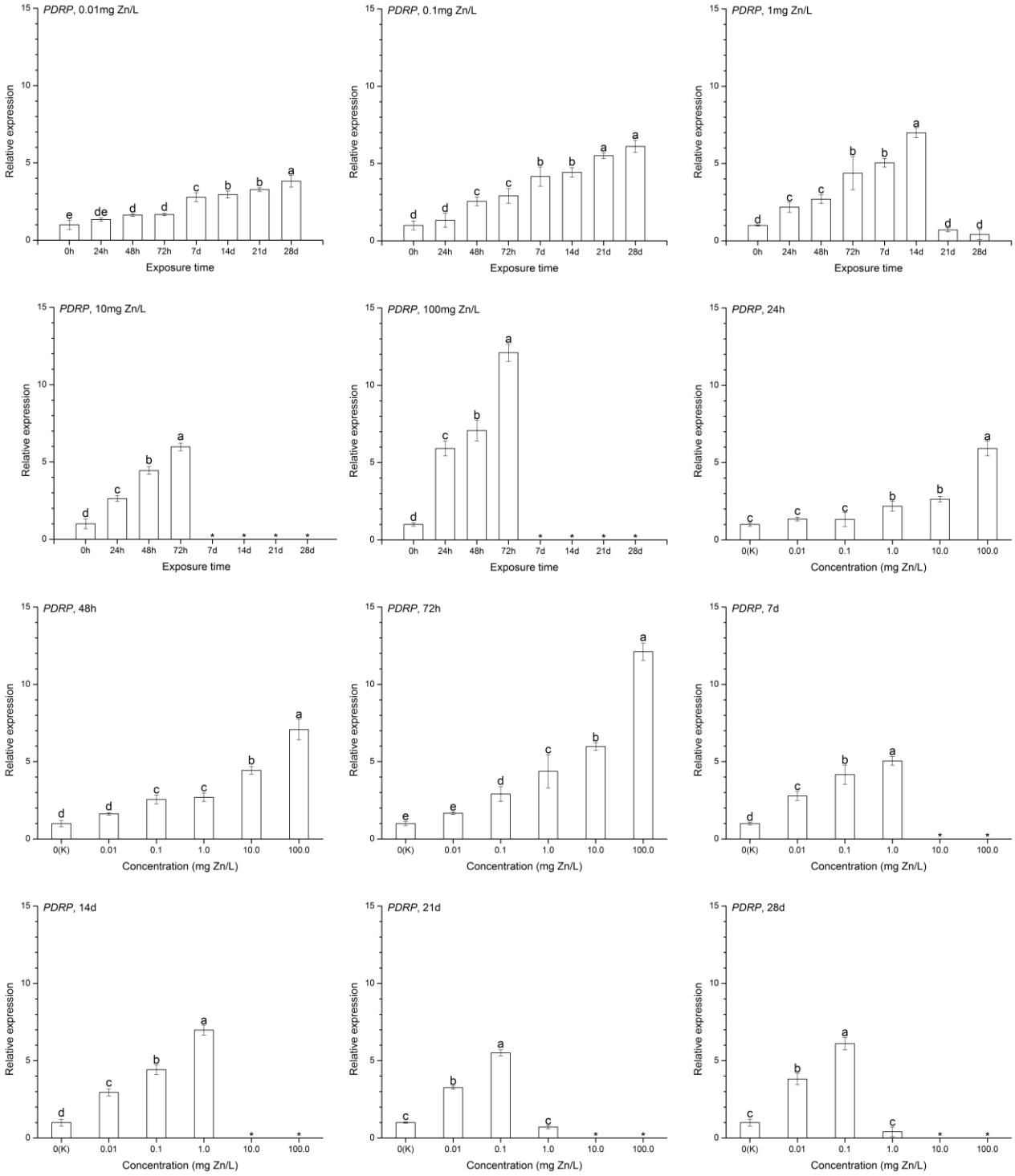

Fig. 3.23.1-2 Effects of Zn salt on gene *PDRP* transcription in mussel digestive gland. Gene transcription was determined by qRT-PCR as described in methods. Relative expression was calculated with respect to control mussels. Data are the mean ± SD obtained from at least 3 independent RNA samples in triplicate. The differences were considered statistically significant at $p \leq 0.05$.

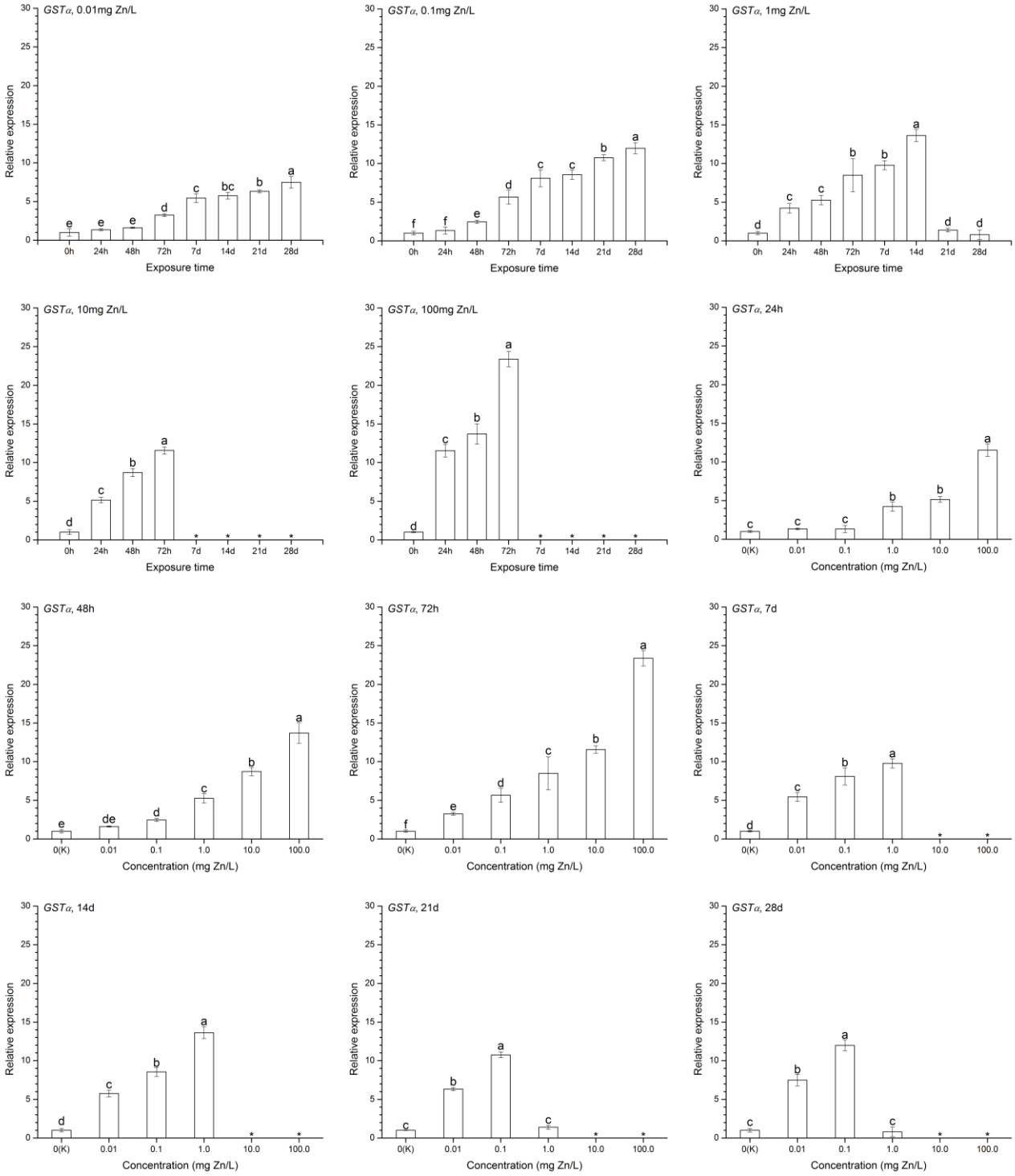

Fig. 3.23.1-3 Effects of Zn salt on gene *GSTα* transcription in mussel digestive gland. Gene transcription was determined by qRT-PCR as described in methods. Relative expression was calculated with respect to control mussels. Data are the mean ± SD obtained from at least 3 independent RNA samples in triplicate. The differences were considered statistically significant at $p \leq 0.05$.

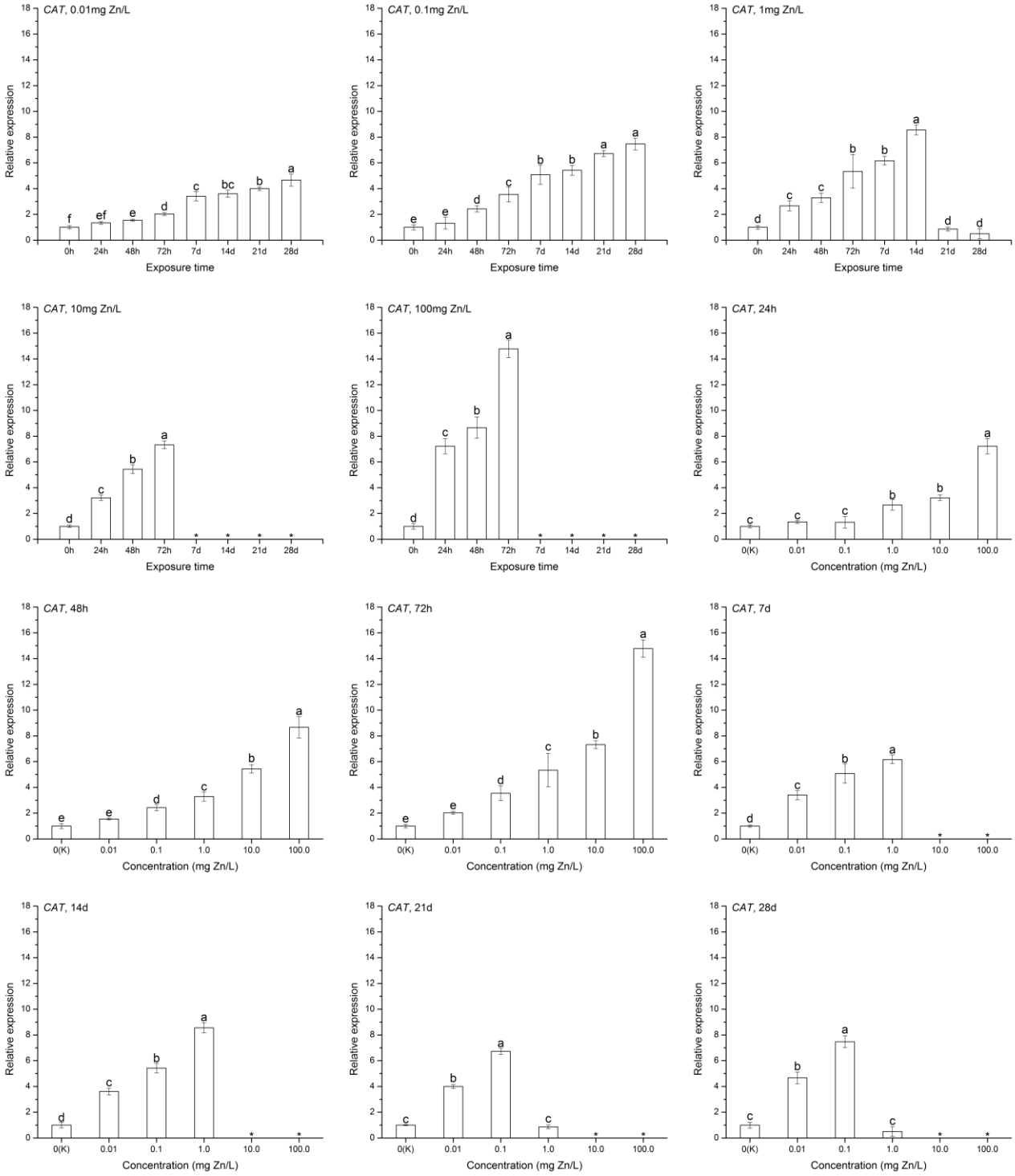

Fig. 3.23.1-4 Effects of Zn salt on gene *CAT* transcription in mussel digestive gland. Gene transcription was determined by qRT-PCR as described in methods. Relative expression was calculated with respect to control mussels. Data are the mean ± SD obtained from at least 3 independent RNA samples in triplicate. The differences were considered statistically significant at $p \leq 0.05$.

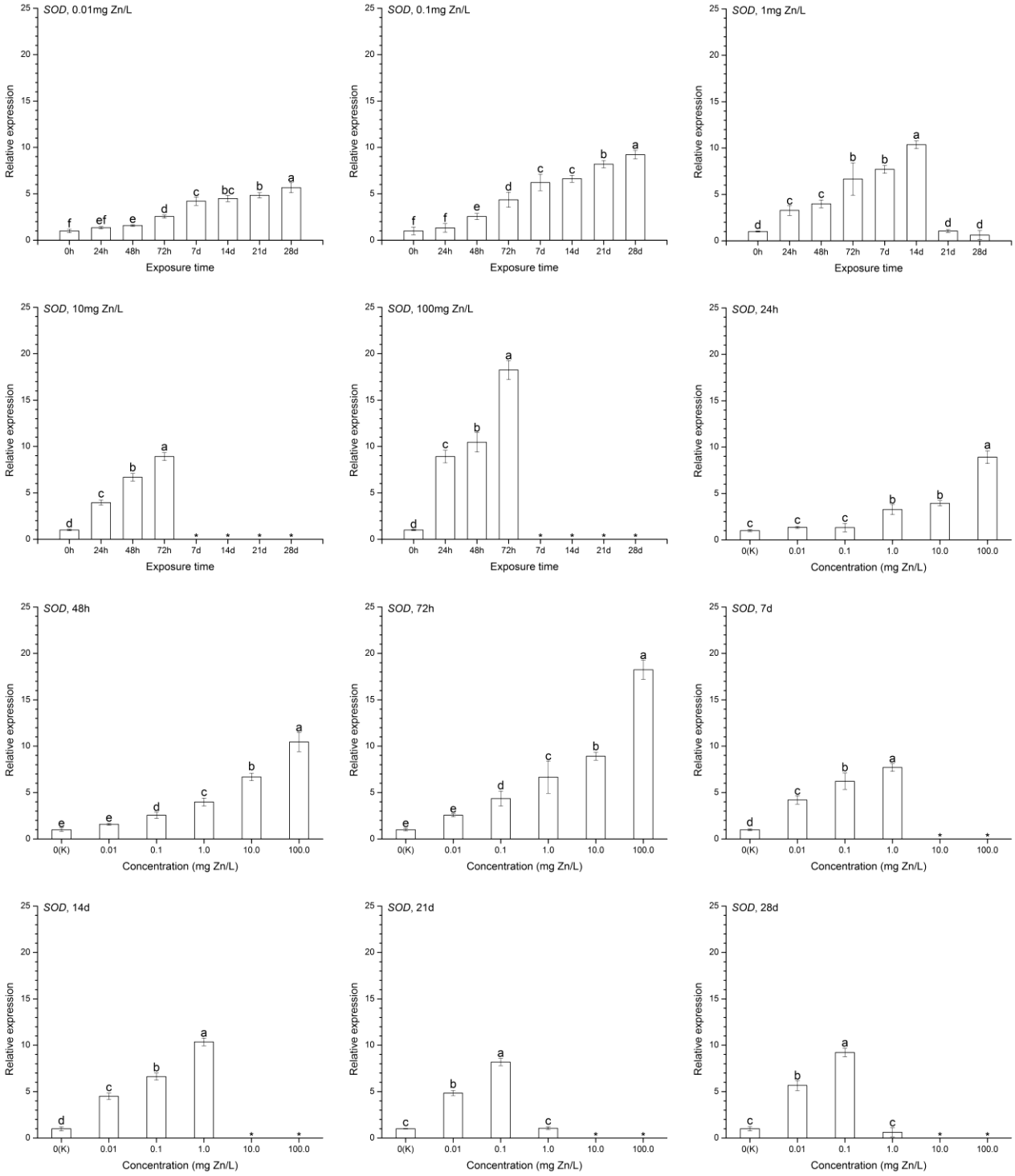

Fig. 3.23.1-5 Effects of Zn salt on gene *SOD* transcription in mussel digestive gland. Gene transcription was determined by qRT-PCR as described in methods. Relative expression was calculated with respect to control mussels. Data are the mean ± SD obtained from at least 3 independent RNA samples in triplicate. The differences were considered statistically significant at $p \leq 0.05$.

DNA damage-responsive genes (*p53* and *PDPR*) transcription profile

The first significant difference ($p \leq 0.05$) of *p53* expression with respect to the control occurred only after 48 h of exposure at concentration 0.1 mg Zn/L while at higher concentrations (1−100 mg Zn/L) since the beginning of the exposure time, a significant increased expression were detected (Fig. 3.23.1-1). At the first two tested concentrations the *p53* gene expression increased in dependence of the exposure time until the end (28 Day) with a maximum expression level of about 15 fold (10 in 0.01 mg Zn/L)the control. At 1 mg Zn/L this level could be observed after only 14 days followed by a sharp decrease in the following time exposure (21 and 28 days), and then by the death of the organisms. However, at both 10 and 100 mg Zn/L, no data were obtained due to death after three days exposure.

Therefore it seemed that up to 1 mg Zn/L the organisms can activate the maximum response in a time span linearly dependent on the concentration. Additionally, it was worthy to note that, at 100 mg Zn/L (72 h) there was an *p53* overexpression, in fact the level measured was about twice the maximum level measured at the lower concentration independently by the exposure time.

The Zn salt exposure has induced *PDRP* expression response as well. In Fig. 3.23.1-2, the relative expression represented a very similar trend as that of *p53*, although the expressions are not at the same level. Diversely from *p53* the first significant difference ($p \leq 0.05$) occurred at 48 h for both 0.01 and 0.1 mg Zn/L this indicates that *PDRP* response was more sensitive at the lowest exposure concentration than *p53*. In the range up to 1 mg Zn/L, gene expression levels showed a rising trend from the beginning of exposure up to a peak and then they went down. The highest relative expression (approximately 6.5 fold the control) occurred at 28 d in 0.1 mg Zn/L, at 14 d in 1 mg Zn/L, and 7 d in 10 mg Zn/L. However, this pattern common for low concentrations was not evident at 100 mg Zn/L: in this case, the first level of gene expression was 6 fold the control and it went up to almost 12 fold the control at 72 h. The special overexpression for high concentration exposure was also found in the assay for *p53*.

Few studies evaluated the *p53* gene expression in mussels as response to insult exposure evidencing modification of regular expression. Estevez-Calvar et al. (2013) reported the increasing *p53* gene expression in *M. galloprovincialis* hemocytes since the beginning of UV exposure, up to 25 times the control and, and a drop off after 48 hours. Banni et al. (2009) evaluated the *p53* gene expression in *Mytius* spp. digestive gland exposed to two different organic compounds and reported two different relative expression, one higher (1.5 fold) and one lower (1/4 fold) the control, after

approximately 24 hours and a similar level of control samples after 50 hours. Our study did not evidence early increment of expression after the first hours of exposure (Estevez-Calvar et al., 2013) and, although the exposure to concentration > 1 mg Zn/L induced significant relative expression of *p53* gene after the first 24 h (Banni et al., 2009), however, no adapting trend in the subsequent exposure time could be found.

Similarly, *PDRP* expression in our study, showed a continued rising trend in the first 48 h differently to Estevez-Calvar et al. (2013) who reported a very high expression level for *PDRP* at the beginning of exposure that turned down to normal level (control group) at 48 h. The trend of DNA damage-responsive genes during the first 48 hours Zn salt exposure indicated the activity of the DNA repair process in the mussel. The signal pathway of the two DNA damage-responsive genes (Fig. 3.21-1) shows how ROS directly induce *p53* and *PDRP* gene expression. Zn salt exposure activated the process of DNA damage and repair at the same time in this report.

Antioxidant enzymes genes (*GSTα*, *CAT*, and *SOD*) transcription profile

The gene expression of the three antioxidant enzymes showed a common increasing transcription level along with exposure time (28 days) and chemical concentrations (Fig. 3.23.1-3, 4, and 5).

In the digestive gland of mussel exposed to Zn salt, the *GSTα* gene transcription linearly increased along with time for concentration below 1 mg Zn/L. At 1 mg Zn/L, instead, the relative expression of *GSTα* showed a peak (approximately 14 fold the control) at 14 d, that, at 21 d and 28 d, turned down to level not significantly differentfrom the control ($p > 0.05$). At the two higher concentrations (10 and 100 mg Zn/L) no data were observed after three days exposure due to death. It is worth to note that the maximum gene relative expression (14 fold the control) occurred at 28 d in 0.1 mg Zn/L, at 14 d in 1.0 mg Zn/L, and at 72 h in 10.0 mg Zn/L, while at 100 mg Zn/L the maximum level was reached at 72 h an overexpression (approximately 24 fold the control) was registered.

Although GSTα protein is a key enzyme that catalyzes H_2O_2 to H_2O which is one common path to reduce ROS (H_2O_2) (Gomes et al., 2011; Estevez-Calvar et al., 2013), different GSTα productions were reported in literatures, such as, some works (Canesi et al., 2010; Pan et al., 2012; Buffet et al., 2013) reported an increased GST protein activity in clam (*Scrobicularia plana)*

and mussel (*M. galloprovincialis*) exposed to several nanomaterials; while others (Renault et al., 2008) observed the repression of *GST* gene in gill cells of benthic bivalve *Corbicula fluminea*. Saddick et al. (2015) measured in fish brain exposed in ZnO for 15 days a slight increment or decrement of *GST* transcription in dependence to concentration.

However to the best of our knowledge, *GSTα* trends similar to this study were not described before.

The transcription profile of the two antioxidant enzymes genes *CAT* and *SOD* in the 28 days exposure to Zn salt were showed in Fig. 3.23.1-4 and 5. Their trends were similar to *GSTα* one although at different level of relative expression. In particular, the highest relative expression at concentrations lower than 100 mg Zn/L was about 8 times the control for *CAT* and 10 for *SOD*, while an overexpression level of 14 (*CAT*) and approximately 18 (*SOD*) in 100 mg Zn/L was obtained. The first significant differences appeared for both *CAT* and *SOD* at 48 h, 0.01–0.1 mg Zn/L.

Studies focused on CAT and SOD enzyme activity of bivalve (Tedesco et al., 2008; Canesi et al., 2010; Buffet et al., 2011; Buffet et al., 2012; Gomes et al., 2012; Buffet et al., 2013; Buffet et al., 2014; Gomes et al., 2014b; Vale et al., 2014) generally indicated an increased enzyme activities. In addition, limited studies reported data about *SOD and CAT* gene expression: Renault et al. (2008) reported in gill cells of benthic bivalve *Corbicula fluminea* exposed to Au, besides the *GST* repression, the *SOD* gene induction and left *CAT* expression unaltered, also an over expression of *CAT* in visceral mass that indicated the need to consider also other organs, such as digestive gland. In addition, Barmo et al. (2013) reported for digestive gland cells of *M. galloprovincialis* a *GSTπ* and *CAT* significant repression at low concentration and *GSTπ* and *CAT* not significant adaption, at high concentration. Although the repression occurredat low exposed concentrations was not observable in our study, the adaption phenomenon seemed to represent a kind of self-protective mechanism in exposed mussel similar to that described in our case.

3.23.2 Mortality effect

The mortality curves of mussel exposed to different concentrations for 28 days were reported in Fig. 3.23.2-1. The graph showed, starting from 72h, increasing value along with the exposure time together with Zn increment. From 7d the effect exceeded 50%. The 100% effect

occurred at 14 d in the 100 mg Zn/L, at 21 d in 10 mg Zn/L and at 28 d in 1 mg Zn/L.

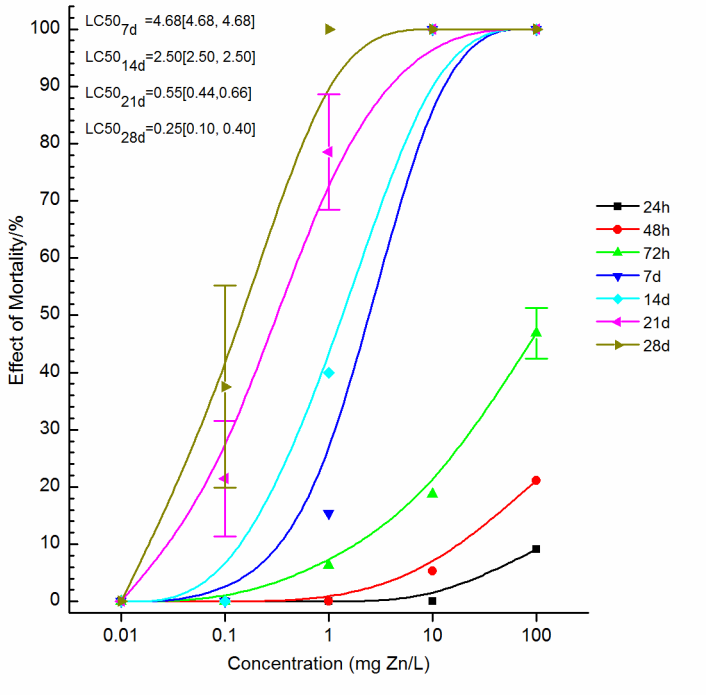

Fig. 3.23.2-1 Mortality effect of mussel exposed to $ZnSO_4$ solution for 28 days. LC50s of mortality effect were calculated for 7d, 14d, 21d, and 28d. LC50s were undetectable for the first three days of exposure.

No mortality was observed for the clam *Scrobicularia plana* exposed to 10 µg Cu/L for 21 days (Buffet et al., 2013) and to 10 µg Ag/L for 21 days (Buffet et al., 2014); for the mussel *M. galloprovincialis*in 10 µg Cu/L exposure for 15 days (Gomes et al., 2014a), and for the *Macoma balthica* in 150−200 µg/g AgO and CuO exposure for 35 days (Dai et al., 2013). However, Muller et al. (2014) performed long term (100 days) *M. galloprovincialis*exposure to ZnO reporting very low mortality rate 1.66×10^{-3} 1/d for concentrations < 2 mg Zn/L: a toxic effect lower than in the present study, probably because of the different experimental design adopted.

3.23.3 Comparison of DNA damage-responsive genes and antioxidant enzymes genes

The DNA damage-responsive genes and antioxidant enzymes genes actively responded to Zn salt exposure. The gene expression trends along with exposure dose and time showed an extremely consistent pattern within each other. The production of ROS has been identified as one of the main causes of ionic toxicity. Our results support other studies; it is probably that the ROS formation is strongly induced as this is associated with both a rapid upregulation of the *CAT* and

SOD gene and a high level of expression of *GST*. The fact that, up to 1 mg Zn /L, gene expression levels subsequently subsides suggests that the antioxidant system is able to neutralize the hydrogen peroxide and other ROS formed by the Zn ion and thereby protect the organism (Varela-Valencia et al., 2014). Few studies focused on *p53* and *PDRP* gene in metal ionic exposure. Chae et al. (2009) reported a decay trend of *p53* expression in Ag^{+}-exposed fish *Oryzias latipes* from the 1st day to the 10th day followed by death that a restrained expression is different of data in this case. Also, Tang et al. (2013) reported similar lightly decreasing trend of *p53* expression in Cd^{2+} exposed *Danio rerio* liver cells. However, similar situations were reported for antioxidant enzymes genes. Kim et al. (2011) reported low concentration (100 μg/L) of Ag^{+}, Cu^{2+}, and Zn^{2+} can induced *SOD* gene expression increasing ($p < 0.05$) in copepod *Tigriopus japonicus* for 96 h that supports the data in this case (Fig. 3.23.1-5). In addition, Won et al. (2012) reported very similar trends of *GST* and *CAT* gene expressions and protein activities in polychaete *Perinereis nuntia* expopsed into Cu^{2+} that an upregulation was occurred at earlier exposure phase (6-24 h) and a decreasing was observed at the end of exposure, particularly *GST* went to the control level ($p > 0.05$). Wan et al. (2009) reported a rapid increasing of *GST* gene expression in disk abalone *Haliotis discus discus* exposed into 50-500 μg/L Cd^{2+} for 12 h while a increasing trend at lower concentrations and a decreasing at higher concentration (went to control level, $p > 0.05$) for both Cu^{2+} and Hg^{2+}. Those showed the similar adaptation effect both in long exposure time and high exposed concentration with respect to this case. Based on the results reported here, it is suggested that 1 mg Zn /L, represents a threshold value below which the cell activates defences mechanisms to cope with the related oxidative stress (Davies, 2000; Clauditz et al., 2006).

In the case of concentration at 1 mg/L, instead, the observed gene overexpression followed by the sharp decrease to the control level, suggests the possibility for the cell antioxidant system to activate an additional extra expression in the effort of counteract the increased stimulus. This process was eventually insufficient to neutralize ROS deriving effects as DNA damages, alteration of cell metabolism and that drive to cell apoptosis. The ionic zinc alone showed an increasing pattern of antioxidant gene expression along with concentrations and time exposure.

3.24 Conclusion

The results of this work represent the ROS-mediated injury on marine invertebrates *M.*

galloprovincialis by Zn salt using qRT-PCR approach. All five genes involved in antioxidation and apoptosis represented positive response on ZnO NPs exposure that induced DNA damage and oxidant injury contributed the total toxicity. In *M. galloprovincialis*, the effects of heavy metal, nanomaterials, UV treatment, and polluted environment stress on different biomarkers have been previously evaluated in different experimental conditions (Bebianno & Serafim, 1998; Regoli, 1998; Da Ros et al., 2000; Canesi et al., 2010; Gomes et al., 2011; Gomes et al., 2012; Barmo et al., 2013; Estevez-Calvar et al., 2013; Gomes et al., 2013; Gomes et al., 2014b). The relevant genes expressions in *M. galloprovincialis* exposed into Zn salt solution in the range of concentration 0.01 to 100 mg Zn/L for four weeks were reported in this paper.

As biomarkers on the basis of gene expression, insufficient literatures had recorded the five genes in *M. galloprovincialis* to respond metal ions (Hoarau et al., 2006; Ciacci et al., 2011). Hoarau et al. (2006) reported that active *GST* expression occurred in *M. galloprovincialis* exposed to Cd. The specific activities and gene expression of the enzymes are related to apoptosis and antioxidation which had been well documented (Gomes et al., 2011; Gomes et al., 2012; Barmo et al., 2013; Estevez-Calvar et al., 2013; Gomes et al., 2014b; Wang et al., 2014). In Fig. 3.21-1, *p53* and *PDRP* are on the upstream of the signaling pathway of ROS-mediated appotosis and *SOD*, *GST* and *CAT* are the key genes in the signaling pathway of ROS-mediated antioxidation (Gomes et al., 2011; Estevez-Calvar et al., 2013; Romero et al., 2015). In this research, all investigated genes represented obvious increasing expression in the exposure of Zn salts that Zn ion could induced ROS-mediated injury. In conclusion, the present data of the investigated genes, *p53*, *PDPR*, *GSTα*, *CAT*, and *SOD* evidenced distinct action of apoptosis and antioxidation occurred in *M. galloprovincialis* exposed to Zn salts. The increase in antioxidant defenses and apoptosis suggested an obvious signaling pathway requesting more studies on cellular signaling in marine invertebrates exposed into stress.

3.25 Reference

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3.26 Glossary

NPs: Nanoparticles; ENMs: Engineered Nanomaterials; ASW: Artificial Sea Water; RSW: Reconstituted Sea Water; NOEC: No Observed Effect Concentration; EC1: 1% Effect Concentration; EC10: 10% Effect Concentration; EC50: 50% Effect Concentration; LC50: 50% Lethal Concentration; ANOVA: Analysis of Variance; Inhibition Concentration procedure, ICp; H&E: Hematoxyline and Eosin; PBS: Phosphate Buffer Saline; FAO: Food and Agriculture Organization; UV: Ultra Violet; UV-vis: Ultraviolet-Visible; ROS: Reactive Oxygen Species; *R*: Zn Uptake Rate.

RNA: Ribonucleic Acid; DNA: Deoxyribonucleic Acid; cDNA: Complementary DNA; qRT-PCR: quantitative Real-Time Polymerase Chain Reaction.

Bax: Bcl-2-associated X protein; Bcl-2: Bcl-2 Integral Membrane Protein; BI-1: Bax inhibitor-1; CAT: Catalase; DFF-A: DNA Fragmentation Factor A; GPx: Glutathione Peroxidase; GSH: Glutathione; GSR: Oxidized Glutathione Reductase; GSSG: Oxidized Glutathione; GST: Glutathione Transferase; HSP70: Heat Shock Protein 70; LDH: Lactate Dehydrogenase; LPO: Lipid Peroxidation; MgC1q: the Complement Factor MgC1q; MT: Metallothionein; p53: p53 Tumor Suppressor-Like Protein; PDRP: DNA damage regulated protein; Se-GPx: Se-Glutathione Peroxidase; Se-GSR: Se-Oxidized Glutathione Reductase; SOD: Superoxide Dismutase.

BPA: Bisphenol A; DAP: Diallyl Phthalate; PAHs: Polycyclic Aromatic Hydrocarbon; PBDEs: Polybrominated Diphenyl Ethers; PCB: Polychlorinated Biphenyl; DDT: Dichlorodiphenyltrichloroethane.

AAS: Atomic Absorption Spectrometer; DLS: Dynamic Light Scattering; FIB: Focused Ion Beam; GFAAS: Graphite Furnace Atomic Absorption Spectrophotometer; ICP-AES: Inductively Coupled Plasma-Atomic Emission Spectrometry; ICP-MS: Inductively Coupled Plasma-Mass Spectrometry; NIBS: Non-Invasive Back Scatter; PDI: Polydispersity Index; SSCP: Scanned Stripping Chrono Potentiometry.

4 GENERAL CONCLUSION

Three hypotheses were presented on ecotoxicity of ZnO NPs towards algae and mussels in marine environment which were still to be explored and became the aims of this study.

First, ZnO NPs could have a specific behavior and effect in diversemarine seawaters as other nanomaterials described before. To this aim the full characterization of ZnO NP dissolution, aggregation, and sedimentation once dispersed into a standard Artificial Sea Water (ASW) and a Reconstituted Sea Water (RSW) from East China Sea was performed.

Second, the ecotoxicity of ZnO NPs upon a green alga and a diatom would be different. To assess the diverse toxicity upon different algae, growth inhibition algal assays were performed. Additionally, to verify the hypothesis about the role of nano size, ZnO NP toxicity for ZnO NPs, bulk, and Zn salt, were assessed as well.

Final, ZnO NP toxicity towards mussel under a chronic exposure would be shown on Zn bioaccumulation, tissue damages, and transcription of apoptosis and antioxidation-related genes. The hypothesis that nano size plays an important role in its toxicity was also considered together with the all previous ones for mussel *M. galloprovincialis*.

The results answered to the all hypotheses. Behaviors of ZnO NPs and bulk in different artificial seawater were observed indicating that the primary size of ZnO in suspensions could affect the aggregating rate in higher concentration. The behaviors of ZnO NPs dispersed into ASW and RSW have similar changing trends with some slight differences: aggregates size in ASW for hours is slightly larger than in RSW; Sedimentation speed in ASW (50%) is slower than in RSW (70%) within 200 min; Solubility is very similar in two seawaters (both in 70%, for 10 mg Zn/L).

Comparative toxicity of ZnO NPs, bulk, and Zn salt towards marine algae *T. suecica* and *P. tricornutum* indicated that the nano size plays a key role in the overall ZnO toxicity.

Zn bioaccumulation and histological damages were observed for mussel exposed to ZnO NPs, bulk, and Zn salt. Pristine ZnO particle size influences the overall toxicity and the rank was represented by three levels of injury (in gill, digestive gland, and gonad): low for bulk; medium for NPs and high for salts. Ecotoxicity of ZnO NPs in mussel revealed by transcription of apoptosis and antioxidation-related genes indicated that active response to ZnO NP exposure which induced DNA

damage and oxidant injury contributing to the overall toxicity. In conclusion, zinc oxide nanoparticles induced ecotoxicolgical stress both to algae and mussels and could exert effect also at realistic environmental concentrations during a chronic exposure. Therefore NPs should be considered as a real risk for marine environment.

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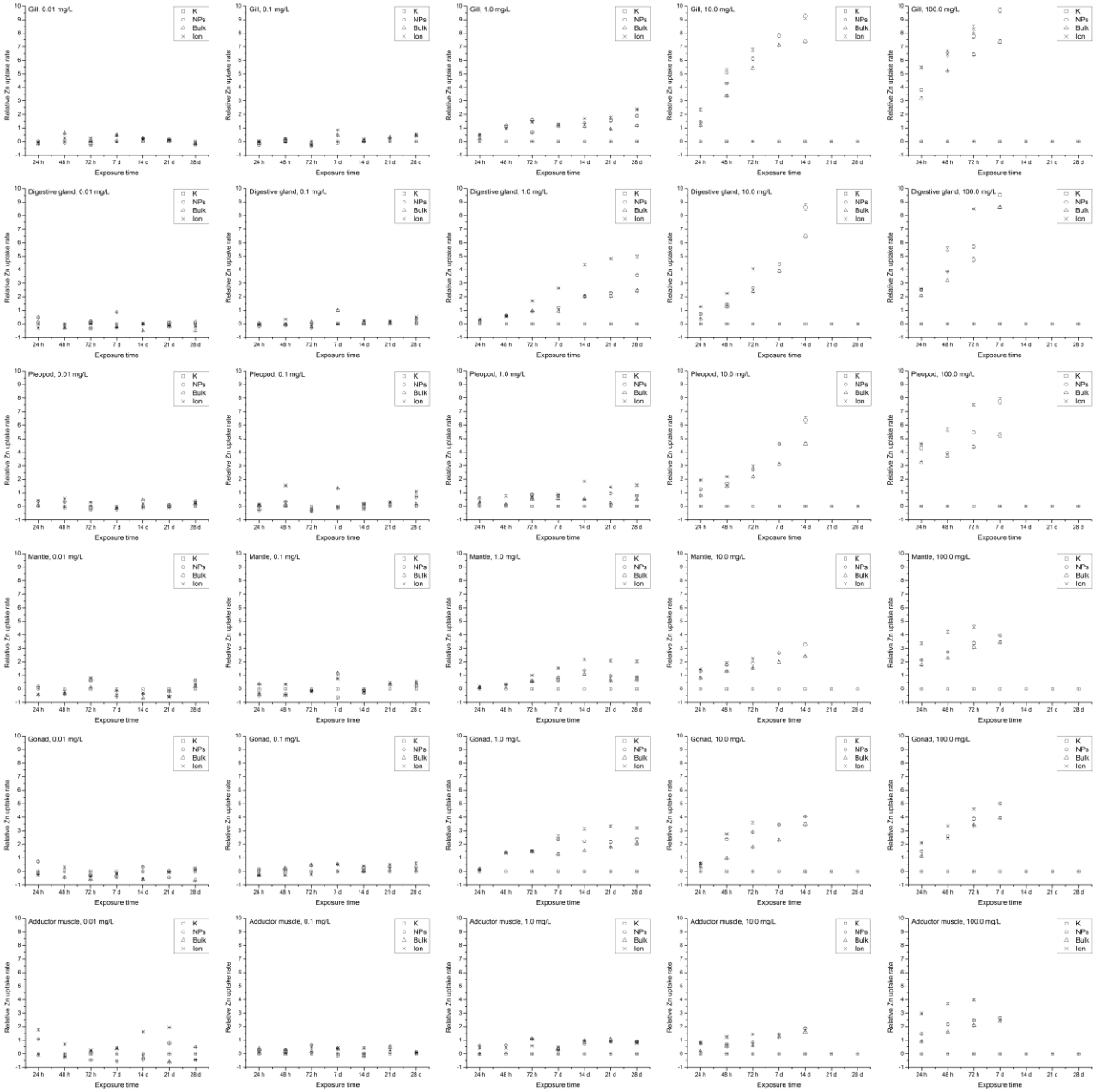
Jiji Li

APPENDIX 1: The relative Zn uptake rates in 6 organs exposed in ZnO suspensions and salt solution.tif



APPENDIX 2: The list of publications in 2013-2016

1. **Li, J.**, S., Schiavo, G., Rametta, M.L., Miglietta, V., La Ferrara, C. Wu & S. Manzo, 2016. Comparative toxicity of nano ZnO, bulk ZnO and Zn salt towards marine algae *Tetraselmis suecica* and *Phaeodactylum tricornutum*. Environmental Science and Pollution Research (Under review).
2. **Li, J.**, S., Schiavo, B., Wu, W., Yao, G., Rametta, M.L., Miglietta, C. Wu & S. Manzo, 2016. Tissues zinc uptake and histological damages of Mediterranean mussel exposed to ZnO nanoparticles. (Submission preparing).
3. **Li, J.**, S., Schiavo, X., Dong, G., Rametta, M.L., Miglietta, C. Wu & S. Manzo, 2016. Active expression of DNA damage-responsive genes and antioxidant enzymes genes in *Mytilus galloprovincialis* exposed to ZnO nanoparticles. (Submission preparing).
4. **Li, J.**, S., Schiavo, X., Dong, C. Wu & S. Manzo, 2016. Active expression of DNA damage-responsive genes and antioxidant enzymes genes in *Mytilus galloprovincialis* exposed to Zn salt. (Submission preparing).
5. **Li, J.**, Y., Ye, C., Wu, B. Guo & Y. Gul, 2014. Genetic diversity and population structure of *Sepiella japonica* (Mollusca: Cephalopoda: Decapoda) inferred by mitochondrial DNA (COI) variations. Biochem. Syst. Ecol. 56:8-15.
6. **Li, J.**, Y., Ye, C., Wu, P., Qi, B. Guo & Y. Chen, 2013. Genetic variation of *Mytilus coruscus* Gould (Bivalvia: Mytilidae) populations in the East China Sea inferred from mtDNA COI gene Sequence. Biochem. Syst. Ecol.50:30-38.
7. Guo, B., Y., Ye, **J., Li**, P., Qi, Z., Lv, A. Guan & C. Wu, 2016. Genetic diversity and population structure of *Sepiella japonica* (Mollusca: Cephalopoda: Decapoda) inferred by 16S rDNA variations. Aquac. Res. (Accepted, in press).
8. Wang, H., P., Qi, B., Guo, **J., Li**, J., He, C. Wu & Y. Gul, 2015. Molecular characterization and expression analysis of a complement component C3 in large yellow croaker (*Larimichthys crocea*). Fish Shellfish Immun. 42:272-279.
9. Wang, W., B., Guo, **J., Li**, P. Qi & C. Wu, 2014. Complete mitochondrial genome of the common cuttlefish *Sepia pharaonis* (Sepioidea, Sepiidae). Mitochondrial DNA 25:198-199.
10. Wang, W., B., Guo, **J., Li**, H., Wang, P., Qi, Z. Lv & C. Wu, 2013. Complete mitochondrial genome of the spineless cuttlefish *Sepiella inermis* (Sepioidea, Sepiidae). Mitochondr. DNA 26:151-152.