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BIVALVES COPING WITH ENVIRONMENTAL CHANGES: NANOPARTICLES AS A NEW POTENTIAL STRESSOR IN COASTAL ECOSYSTEMS

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Abstract

In the marine coastal environment, the organisms are subjected to continuous pressures of different origin. Among these, there are changes in environmental parameters, such as temperature, pH, dissolved oxygen, salinity, and the discharge of pollutants that are potentially harmful to biocoenoses. In fact, in the last century, the increasing impact of these environmental variations has been mainly related to anthropogenic activities. Although these changes are often perceived to be distant (e.g., those regarding climate change) or minimal (e.g., presence of pollutants), their impacts on the marine environment are already evident and likely to worsen with time. Even today, there are many emerging anthropogenic contaminants that are released into the environment daily. But the information about their behavior in different matrices (air, water, sediment), as well as their interactions and effects in natural populations, is very scarce. A wide group of emerging contaminants is represented by nanoparticles (NPs). NPs are used extensively in a variety of emerging technologies and commercial products, including biomedicine, pharmaceuticals and personal care products, renewable energies, and electronic devices. Consequently, NPs can enter into environment. In particular, NPs may enter marine ecosystems either directly through aerial deposition, effluents, dumping and run-off or indirectly, e.g. via river systems. Currently, no data are available regarding NPs occurrence in the marine environment, as a consequence of the difficulty to detect and quantify NPs in complex matrices. Due to lacking analytical tools to evaluate effective NP concentrations in aquatic environments, only predicted environmental concentrations (PECs) are available in literature.

Three of the widely used NPs in common products are: zinc oxide (nZnO), titanium dioxide ($nTiO_2$) and C_{60} fullerene (FC_{60}). The toxicity of nZnO, $nTiO_2$ and FC_{60} NPs has been reported for different taxa of bacteria, algae, plants, and aquatic and terrestrial invertebrates and vertebrates. Nevertheless, studies concerning the potential toxicity of these NPs to marine species are still lacking. The marine clam *Ruditapes philippinarum*, previously used in a number of ecotoxicological studies, has been chosen as model organism in this PhD research, taking into account that bivalves are considered one of the most suitable target group to investigate NP toxicity. However, information concerning the effects of NPs to this species are lacking. Due to its filter-feeding and infaunal habits, Manila clams may be more susceptible to the effects of NPs, given that in seawater NPs tend to aggregate, adsorb to particulate matter, settle to the bottom and accumulate in sediments.

The aim of the PhD thesis was to enlarge and elucidate the possible mechanisms of action and toxicity of these three NPs to the clam *R. philippinarum*. To reach these purposes, medium-term (7 days) exposures to three NPs (nZnO, $nTiO_2$, FC_{60}) were carried out. Many cellular and biochemical biomarkers in haemolymph, gills and digestive gland of clams have been measured in the

perspective of a multi-biomarker approach. In this study, we chose to test low concentrations (1 and 10 µg/L) of NPs that were in the range of PEC values. Moreover, in the two metal oxide experiments the related contaminants were considered: i) $ZnCl_2$ (10 µg/L) was used to investigate possible contributions of Zn^{2+} release to nZnO toxicity, and ii) bulk TiO_2 ($bTiO_2$, 10 µg/L) was used to understand the potential differing action of metal oxide compared with the respective NP.

Wild organisms are generally exposed to mixtures of different chemicals, therefore the combined effects of a mixture of all three NPs have also been investigated. To assess mixture effects, clams were exposed for 7 days to i) 1 µg/L nZnO ii) 1 µg/L $nTiO_2$ iii) 1 µg/L FC_{60} fullerene and iv) all three NPs as a mixture. Further, in this experiment the redox proteomic approach was adopted in combination with the multi-biomarker approach.

In addition, combined effects of NP mixture and changing environmental parameters were addressed to obtain information about possible variations in clam susceptibility to NPs under a global change scenario. Salinity is one of the dominant environmental factors controlling species distribution and influencing physiological processes in marine organisms. Among predicted changes in environmental parameters, there is an increasing concern about future alterations in seawater salinity values, mainly in estuarine and coastal areas, which will affect the performance of native and invasive species. Moreover, salinity is one of the abiotic parameter that can change the behavior of NPs. In this context, to gain a better insight into the potential environmental impacts, also the native species *Ruditapes decussatus* was used for a comparison.

In all the experiments, NPs tested were measured in clam gills and digestive gland to assess possible bioaccumulation.

To get further insight into $nTiO_2$ effects at cell level, an *in vitro* approach was used and phagocytic activity was assessed in clam haemocytes exposed to $nTiO_2$ (0, 1 and 10 µg/mL). The findings confirmed the ability of $nTiO_2$ to decrease immune functions and to enter into cells.

The results of all these experiments (single and combined factors) suggested that NPs modulated various biomarker responses and showed different sub-lethal effects.

The experiments on single NPs revealed significantly higher stress conditions in tissues of treated clams with major effects in gills and digestive gland. The target tissues responded differently to the three NPs, and among them, $nTiO_2$ appeared to exert more detrimental effects in all tissues considered. This result could be determined by the metal *per se*, and by the $nTiO_2$ characteristics, that, for example, could facilitate the entry into cells. Oxidative stress was confirmed to be the main mechanism of action of the three NPs investigated, as reported in literature for all NPs. The comparisons between the two metal oxide NP and their related contaminants highlighted that NPs were more toxic and this depend on NP specific features. In all clams exposed to the three NPs, at the end of exposure the

Zn, Ti and FC_{60} contents showed an increase in treated clams compared to controls, in both NP concentrations tested.

The exposure to NP mixture represents a novel approach that can provide better insight into the NP impacts under environmentally realistic conditions. Respect to single NP treatments, all findings indicated higher oxidative stress in act during the exposure to the mixture, with damage to proteins, lipids and DNA. The digestive gland was the tissue more affected by NP mixture toxicity. In all obtained results, additive effects were observed in the mixture respect to single NP exposures. The observed additive action of the NP mixture could open a new research to understand better the various mechanisms of NP toxicity. Zn, Ti and FC_{60} contents highlighted a bioaccumulation as single NPs and also as a mixture in both gill and digestive gland tissues.

The study of interactions between different salinity (18-28-38 psu) and NP mixture exposure confirmed the NP toxicity. Overall, at all salinity values tested various changes were shown, depending on the tissues, the biomarkers and the species considered. Moreover, the comparison between the two species did not show a clear pattern of response. Although more in-depth evaluation is needed, it has to be noted that under NP exposure at the three salinities tested, the number of responses significantly varied respect to controls and was slightly higher in *R. decussatus* than in *R. philippinarum*.

All PhD thesis results confirmed the toxicity of these three emerging environmental pollutants and a real potential risk for marine bivalves, even at the low concentrations tested.

Riassunto

A livello dell'ambiente marino costiero, gli organismi sono soggetti a continue pressioni di diversa natura. Tra queste, valutate potenzialmente dannose per le biocenosi, si possono considerare le variazioni dei parametri ambientali, quali la temperatura, il pH, l'ossigeno disciolto, la salinità, e il rilascio di inquinanti. Dal secolo scorso, il crescente impatto di questi cambiamenti ambientali è stato causato principalmente dalle attività antropiche. Anche se questi cambiamenti sono spesso percepiti come distanti (per esempio, quelli relativi ai cambiamenti climatici) o minimi (ad esempio, la presenza di sostanze inquinanti), il loro impatto sull'ambiente marino è già evidente. Quotidianamente vengono rilasciati in ambiente nuovi inquinanti di origine antropica, definiti emergenti. Le informazioni sul loro comportamento nei diversi comparti ambientali (aria, acqua, sedimenti), le loro interazioni e gli effetti sulle popolazioni naturali sono scarse.

Un vasto gruppo di contaminanti emergenti è rappresentato dalle nanoparticelle (NP). Le NP sono utilizzate ampiamente in varie tecnologie emergenti e prodotti commerciali, tra cui la biomedicina, i farmaci, i prodotti per la cura e l'igiene personale, le energie rinnovabili e i dispositivi elettronici. Di conseguenza le NP possono essere rilasciate nell'ambiente. In particolare, le NP possono entrare negli ecosistemi marini sia direttamente, attraverso la deposizione aerea, gli scarichi e gli effluenti, sia indirettamente, ad esempio, tramite la rete fluviale. Attualmente, non sono disponibili dati riguardanti le concentrazioni analitiche delle NP a livello dell'ambiente marino; questa è una conseguenza della difficoltà di rilevare e quantificare le NP in matrici complesse. A causa della mancanza di strumentazioni e protocolli efficaci per misurare le loro concentrazioni negli ambienti acquatici, in letteratura sono disponibili dati sulle concentrazioni ambientali previste delle NP (*Predicted Environmental Concentrations*, PEC).

Tre delle NP maggiormente utilizzate in prodotti di largo consumo sono: l'ossido di zinco (nZnO), il biossido di titanio ($nTiO_2$) e il fullerene C_{60} (FC_{60}). La tossicità di queste tre NP è stata riportata per diversi taxa, come i batteri, le alghe, le piante, gli invertebrati acquatici e terrestri e i vertebrati. Tuttavia, gli studi riguardanti la potenziale tossicità di queste NP nelle specie marine sono ancora molto pochi. La vongola filippina, *Ruditapes philippinarum*, è stata scelta come organismo modello in questa tesi di dottorato, in quanto ampiamente utilizzata in molteplici studi ecotossicologici. Inoltre, i bivalvi sono considerati uno dei target più idonei per lo studio della tossicità delle NP, anche se le informazioni riguardanti gli effetti delle NP proprio in questa specie sono carenti. Essendo un organismo filtratore che vive nel sedimento, la vongola, rispetto ad altre specie, potrebbe essere più sensibile agli effetti delle NP, visto che le NP in acqua di mare tendono ad aggregare e di conseguenza a depositarsi sul fondo e accumularsi nei sedimenti.

Lo scopo di questa tesi di dottorato è quello di aumentare le informazioni e chiarire i possibili meccanismi d'azione e la tossicità di queste tre NP nella

vongola filippina. Per raggiungere questo obiettivo, sono state allestite diverse esposizioni in laboratorio alle tre NP (nZnO, $nTiO_2$, FC_{60}) della durata di 7 giorni. Vari biomarker cellulari e biochimici sono stati misurati a livello dell'emolinfa, delle branchie e della ghiandola digestiva nella prospettiva di un approccio multi-biomarker. In questo studio, sono state scelte basse concentrazioni (1 e 10 µg/L) di NP simili ai valori di PEC. Inoltre, nei due esperimenti riguardanti le NP di ossido di metallo, il cloruro di zinco (10 µg/L) è stato utilizzato per indagare possibili contributi dello ione zinco nella tossicità del nZnO e, invece, la forma bulk del TiO_2 ($bTiO_2$, 10 µg/L) è stata utilizzata per comprendere la potenziale differente azione dell'ossido di metallo rispetto alla corrispondente NP.

In ambiente gli organismi sono generalmente esposti a miscele di diverse sostanze inquinanti, per questo si è deciso di indagare l'effetto combinato di una miscela di tutte e tre le NP considerate. Per valutare gli effetti della miscela di NP, le vongole sono state esposte per 7 giorni a i) 1 µg/L di nZnO, ii) 1 µg/L di $nTiO_2$, iii) 1 µg/L di FC_{60} fullerene e iv) tutte e tre le NP in miscela. In questo esperimento è stata utilizzata la proteomica redox combinata all'approccio multi-biomarker.

Inoltre, sono stati studiati in un ulteriore esperimento gli effetti combinati della miscela di NP e di un parametro ambientale; per ottenere informazioni su eventuali variazioni della suscettibilità delle vongole alle NP in un possibile scenario di cambiamento globale. La salinità è uno dei fattori ambientali che controlla la distribuzione delle specie e influenza i processi fisiologici negli organismi marini. Tra i cambiamenti previsti dei vari parametri ambientali, vi è una crescente preoccupazione per le future alterazioni nei valori di salinità, soprattutto a livello degli estuari e delle zone costiere, dove potrebbero influenzare la sopravvivenza di specie autoctone e invasive. Inoltre, la salinità è uno dei parametri abiotici in grado di modificare il comportamento delle NP. In questo contesto, per ottenere una migliore comprensione dei potenziali impatti ambientali, è stata impiegata anche la specie autoctona, *Ruditapes decussatus*.

In tutti gli esperimenti, è stato misurato il contenuto delle NP, sia nelle branchie sia nella ghiandola digestiva della vongola, per valutare il loro possibile bioaccumulo.

Inoltre, per avere una visione più completa degli effetti del $nTiO_2$ a livello cellulare, è stato utilizzato anche un approccio *in vitro*. I risultati hanno confermato la capacità del $nTiO_2$ di entrare nelle cellule e di influenzare negativamente parametri legati alla risposta immunitaria.

I risultati di tutti questi esperimenti hanno suggerito che le NP modulino varie risposte degli animali e hanno mostrato diversi effetti sub-letali nei tessuti della vongola.

In particolare, gli esperimenti riguardanti gli effetti delle singole NP hanno mostrato condizioni di stress significativamente maggiori nelle branchie e nella ghiandola digestiva delle vongole. I tessuti analizzati hanno risposto diversamente alle tre NP, e tra loro la $nTiO_2$ ha esercitato maggiori effetti negativi in tutti e tre i

tessuti analizzati. Questo risultato potrebbe essere determinato dal metallo in sé, e dalle caratteristiche del $nTiO_2$, che, per esempio, potrebbero facilitare l'entrata della NP nelle cellule. Lo stress ossidativo è stato confermato essere il principale meccanismo d'azione delle tre NP indagate, come riportato in letteratura. I confronti tra le NP di ossido metallico e i loro contaminanti correlati evidenziano come le NP siano più tossiche e questo dipende dalle caratteristiche specifiche delle NP. Nelle vongole esposte alle tre NP i contenuti di zinco, titanio e FC_{60} hanno mostrato, alla fine dell'esposizione, un aumento nei trattati rispetto ai controlli ad entrambe le concentrazioni testate.

L'esperimento sulla miscela delle NP rappresenta un nuovo approccio in grado di fornire una migliore comprensione degli impatti delle NP in condizioni ambientali più realistiche. Rispetto ai risultati delle singole NP, tutti i dati ottenuti dall'esposizione delle vongole alla miscela indicano un maggiore stress ossidativo in atto, con conseguenti danni osservati alle proteine, ai lipidi e al DNA. La ghiandola digestiva risulta essere il tessuto più colpito dalla tossicità della miscela di NP. Inoltre, per tutti i parametri variati nei trattati rispetto al controllo, sono stati osservati effetti additivi nel trattamento con miscela rispetto a quelli con le singole NP. L'azione additiva riscontrata per la miscela potrebbe aprire nuovi filoni di ricerca utili a comprendere meglio i diversi meccanismi d'azione delle NP. I contenuti di Zn, Ti e FC_{60} quantificati anche in questo esperimento hanno evidenziato un bioaccumulo sia come singole NP, sia come miscela nelle branchie e nelle ghiandole digestive delle vongole esposte rispetto ai controlli.

L'ultimo esperimento, riguardante l'interazione tra diversi valori di salinità (18-28-38 psu) e la miscela delle tre NP, conferma anche a queste condizioni la tossicità delle NP. In generale, a tutti i valori di salinità testati sono stati mostrati vari cambiamenti, in base al tessuto, ai biomarker e alla specie considerata. Il confronto tra le due specie non ha consentito di ricavare per ognuna di esse un chiaro modello di risposta alle diverse combinazioni salinità/assenza-presenza di NP saggiate. Anche se è necessaria una valutazione più approfondita, si è potuto notare tuttavia che il numero di risposte significativamente variate in presenza di miscela di NP era leggermente maggiore in *R. decussatus* rispetto *R. philippinarum* .

Nel complesso, i risultati ottenuti sono in grado di fornire nuovi spunti di discussione negli studi sulla tossicità sulle NP, come pure nella valutazione del rischio, costituito dalle NP come inquinanti ambientali emergenti, negli ecosistemi marini costieri.

Introduction

1.1. Emerging contaminants

Since the middle of the last century, all environmental media (air, water, soil, sediment, biota) were contaminated by a variety of synthetic chemicals, generally characterized by an high environmental persistence (Brubaker et al., 1975; Warren et al., 2003). The degradation of these compounds is, therefore, very slow and the metabolites obtained are very stable and often more toxic than the parental compounds. This category includes numerous classes of substances produced by human activities, with various functions, features and uses. In the last decade, many new substances, recognized as emerging environmental pollutants, have arisen growing concern about their possible environmental effects (Hutchinson et al., 2013).

Across the modern world, synthetic chemicals have become central, for example, to food production, drinking water disinfection, drug discovery, family planning and in a wide range of manufacturing industries (Hutchinson et al., 2013). It is also striking that the pace of chemical discovery is growing rapidly, with the Chemicals Abstracts Service (CAS REGISTRY) reporting in June 2015 more than 100 million chemical substances. Coming after the CAS REGISTRY crossed the 50 million substance registration in only 2009, this second major milestone showed the continued acceleration of synthetic chemical innovation globally (CAS, 2015).

The emerging pollutants are defined as synthetic or naturally occurring chemicals that are not commonly monitored in the environment but which have the potential to enter the environment and cause known or suspected adverse ecological and (or) human health effects. In some cases, release of emerging pollutants to the environment has likely occurred for a long time, but it may not have been recognized until new detection methods were developed. In other cases, synthesis of new chemicals or changes in use and disposal of existing chemicals can create new sources of emerging pollutants (Norman-EU). The emerging pollutants are currently not included in international or national routine monitoring programmes and their fate, behaviour and ecotoxicological effects are often not well understood. They can be released from many and different pollution sources and in direct or indirect way based on the various environmental compartments.

It is very important to study the effects of these changes in wildlife and at different levels of biological organization (Geissen et al., 2015). In this regard, researches could contribute to promote new environmental protection campaigns and strategies to preserve biodiversity and habitats. Thanks to several recent studies, some new pollutants, that are produced and released from human activities in many Countries, have been recognized as priority contaminants to be monitored and controlled. The European Union has inserted three emerging pollutants (the steroidal hormones, 17-β-estradiol and 17-α-ethynilestradiol, and

the anti-inflammatory diclofenac) in the Water Framework Directive (European Directive 2011/0429/COD).

In particular, the development of sensitive analytical monitoring methods has shown the potential for synthetic and natural chemicals to enter marine ecosystems as a result of human activities, in some cases being linked with adverse health impacts on marine species or seafood supplies. Protecting marine ecosystems and food resources from the adverse effects of chemical contaminants remains an important goal, reflecting one key aspect of the socio-economic value of the coastal zones (Martìnez et al., 2007) and oceans (Costanza, 1999). The Millennium Ecosystem Assessment (2005) noted the important impact of marine pollution and subsequently there have been other estimates of economic losses resulting from marine pollution. For example, the environmental losses in Spain because of marine pollution from the Prestige oil spill were estimated to be 574 million Euros (Loureiro et al., 2009). Additionally, Cai and Li (2011) reported that the economic losses from marine pollution adjacent to the Pearl River estuary, China, were 5,040 million US dollars per year (accounting for 16.5% of the total economic value of the marine ecosystem). Moreover, the 2010 oil spill from the Deepwater Horizon disaster currently has total estimated costs of $37.2 billion (BBC, 2012), although others suggest costs could be up to $63 billions (Wall Street Journal, 2010).

The emerging pollutants are categorized into more than 20 classes related to their origins and characteristics (Norman-EU). The prominent classes are: pharmaceuticals (urban, stock farming), pesticides, plastics, wood preservation, industrial chemicals and nanoparticles (NPs). In light of the potential impact of these substances on aquatic life and human health researches at multiple levels are urgently needed to fill the lack of knowledge regarding environmental behaviour, toxicity and mode of action.

1.2. Nanoparticles

NPs are particles in the 1- to 100-nm size range, they can be composed of many different base materials and have different shapes. Based on NP composition, the main types are: carbon NPs with a spherical- (e.g., C_{60} fullerene) or nanotube- (e.g., single wall or multiple wall) shape; and metallic and metal oxide NPs (e. g., copper oxide, zinc oxide, silver and gold NPs) (Farrè et al., 2009).

Particles in the nanometer size range occur both in nature and as a result of direct (i.e. industrial production) and indirect (e.g. traffic, incinerators, combustion processes in industries, domestic heating) anthropogenic activity. The natural sources of NPs include ash (volcanic eruptions and forest fires), desert dusts, aerosols and metal oxide particles. Some plants synthesize NPs that are used to reduce metal uptake in contaminated soils, and anaerobic bacteria may use them in respiration (Handy et al., 2008; Bernhardt et al., 2010; Camatini, 2013).

The NP and nanotechnology field is a fast-growing research niche (Ostiguy et al., 2008). Nanotechnology is a collective term that implies the capacity to work with

materials at a nanometre scale, the engineered NPs. Nanotechnology thus has potential applications in a wide range of sectors, from energy (production, catalysis, storage), materials (lubricants, abrasives, paints, tires, and sportswear), electronics (chips and screens), optics, and remediation (pollution absorption, water filtering and disinfection), to food (additives and packaging), agriculture, cosmetics (skin lotions and sun screens), and medicine (diagnostics and drug delivery). This huge range of applications reflects the diversity of materials that are being or will be used (Handy et al., 2008; Rana and Kalaichelvan, 2013; Exbrayat et al., 2015).

NP properties differ compared with those of the parent compounds because about 40–50% of the atoms in NPs are on the surface, resulting in greater reactivity than bulk materials. The decreasing size causes their surface effects to become more significant, due to an increase in the volume fraction of surface atoms, which determines in some instances their special properties (Farrè et al., 2009). Taking the advantages of these singular properties in order to develop new products is the main purpose of nanotechnology, and that is why it is regarded as "the next industrial revolution" (Lane, 2002; Matranga and Corsi, 2012).

ENM	Worldwide (t/year) Median and 25/75 percentile	Europe (t/year) Median and 25/75 percentile	US (t/year) (Hendren et al. 2011) Range	Switzerland (t/year) (Schmid and Riediker 2008) In brackets values extrapolated to Europe
TiO_2	3,000 (550–5,500)	550 (55–3,000)	7,800–38,000	435 (38,000)[a]
ZnO	550 (55–550)	55 (5.5–28,000)		70 (6,100)
SiO_2	5,500 (55–55,000)	5,500 (55–55,000)		75 (6,500)
FeO_x	55 (5.5–5,500)	550 (30–5,500)		365 (32,000)
AlO_x	55 (55–5,500)	550 (0.55–500)		0.005 (0.4)
CeO_x	55 (5.5–550)	55 (0.55–2,800)	35–700	
CNT	300 (55–550)	550 (180–550)	55–1,101	1 (87)
Fullerenes	0.6 (0.6–5.5)	0.6 (0.6–5.5)	2–80	
Ag	55 (5.5–550)	5.5 (0.6–55)	2.8–20	3.1 (270)
Quantum dots (QDs)	0.6 (0.6–5.5)	0.6 (0.6–5.5)		

Fig. 1. Production/utilization quantities of ten NPs in the world and in Europe (in t/year), estimation data reported in Piccinno et al. (2012).

The increasing and widespread use of NPs in different fields was projected to result in a $1.5 trillion of profit by 2015 (Nel et al., 2006). Moreover, in the Lux Research (2007) it was reported that new emerging nanotechnology applications will affect nearly every type of manufactured product through the middle of the next decade, becoming incorporated into 15% of global manufacturing output (CDC 24/7).

Not much is known so far about the amounts of engineered NPs that are produced in industries (Fig. 1). The production and usage in various fields of different types of manufactured NPs, is estimated to grow to over half a million tons by 2020, this would lead to their release in substantial amounts in the environment, including the aquatic compartment (Canesi et al., 2015). Among NPs, three of the

widely used in common products are: zinc oxide (nZnO), titanium dioxide ($nTiO_2$) and C_{60} fullerene NPs (FC_{60}) (Fig. 2).

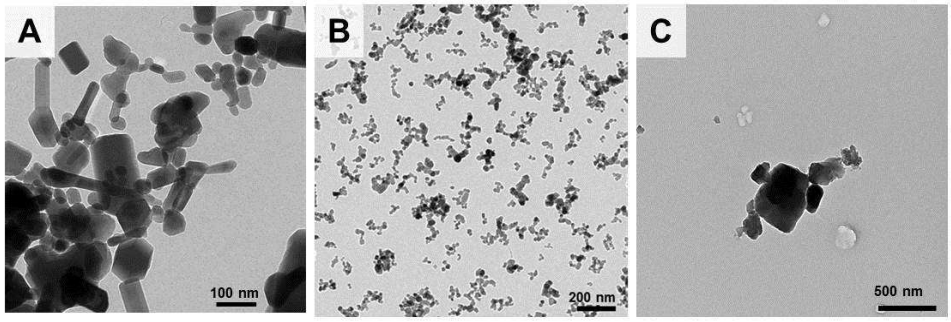

Fig. 2. Transmission electron microscope images of nZnO (A), $nTiO_2$ (B) and FC_{60} (C).

1.2.1. Zinc oxide NPs

ZnO is a widely used metal oxide NP that has a zincite crystal structure that contributes to its unique optoelectric properties (Wang, 2004). nZnO is a wide band-gap semiconductor (Eg=3.37 eV) with a large excitation binding energy (60 eV) and exhibits near UV emission, transparent conductivity (providing for clear coatings on transparent surfaces), and piezoelectricity, which make it particularly attractive for electronic sensors, solar voltaics, and transducer applications. It is also a very effective photocatalyst and has been shown to be effective in a range of environmental control technologies, from remediation of environmental pollutants to medical disinfection (Hoffmann et al., 1995). nZnO is currently used in products including plastics, ceramics, glass, cement, rubber, lubricants, paints, pigments, foods (source of Zn nutrient), batteries and fire retardants. In addition, nZnO are common constituents of personal care products including cosmetics and sunscreens due to their excellent UV absorption and reflective properties. The global production of NPs for sunscreen products alone was estimated to be approximately 1,000 tons during 2003/2004, consisting principally of $nTiO_2$ and nZnO (Borm et al., 2006). Environmental levels of nZnO are expected to increase continually given the widespread application of these NPs (Ma et al., 2013).

1.2.2. Titanium dioxide NPs

TiO_2 is a mineral that can exist in three crystalline forms, known as rutile, anatase, and brookite (Reyes-Coronado et al., 2008). Anatase phase exhibits the highest photocatalytic activity and because of that it is used in catalysis and photocatalysis applications. Rutile is known as a white pigment providing opacity to paints, papers, inks, and consumer products such as toothpaste. In cosmetic products, rutile phase is used as a pigment and thickener, and it is used in plastics and other applications for its ultraviolet (UV) light absorbing properties (Mueller and Nowack, 2008). Anatase and brookite are used as electrodes in dye-sensitized solar cells (Jiang et al., 2002). Such properties have led to use $nTiO_2$ for a wide variety of applications, including self-cleaning surface coatings, light-emitting

diodes, solar cells, disinfectant sprays, sporting goods, water treatment agents and topical sunscreens (Menard et al., 2011). Moreover, $nTiO_2$ enhance the penetration of vitamins and anti-oxidants into the skin and make the product more aesthetically pleasing (Minetto et al., 2014).

Commercial production of $nTiO_2$ between 2006 and 2010 has been estimated at 5,000 metric tons per year, and approximately 2.5 million metric tons are foreseen by 2025 (Robichaud et al., 2009). In Shi et al. (2013) a yearly four million tons consumption was estimated worldwide.

$nTiO_2$ is by far the most extensively studied metal oxide NP in ecotoxicology studies (Kahru and Dubourguier, 2010; Rocha et al., 2015). One of the reasons for the large amount of ecotoxicity data on $nTiO_2$ is the adoption of this NP by a variety of industries; $nTiO_2$ was among the first NPs made readily commercially available to a wide variety of research activities.

1.2.3. C_{60} fullerene NPs

Fullerenes, first discovered by Kroto et al. (1985), are carbon allotropes similar in structure to graphene but rolled up to form closed-cage, hollow spheres. FC_{60}, also known as buckminsterfullerene or buckyball, is a nano-sized carbon allotrope which has important physical properties that depend on the icosahedral structure and elemental composition, such as thermal stability, conductivity, adsorption and catalytic capacity (Wang et al., 2014). The FC_{60} is a remarkably stable compound consisting of 60 carbon atoms with 60 vertices and 32 faces, 12 of which are pentagonal and 20 hexagonal. Each carbon atom is placed at a vertex and each atom has the valences satisfied by two single bonds and one double bond. It has a diameter of approximately 0.7 nm and a molecular weight of 720 g/mol (Nielsen et al., 2008). Thirty carbon double bonds are present in the structure, to which free radicals can easily be added. FC_{60} has therefore also been described as a ‘‘radical sponge”. Fullerenes often aggregate into larger particles, so in reality they may exist as crystals much larger than 100 nm (Aschberger et al., 2010).

It is an elementary component in many modern manufactured products, indeed, FC_{60} is included in drug delivery systems, medical imaging, antitumor agents, microelectronics, solar panels, cosmetics and fuels. It is one of the most ubiquitous NPs, generally present in polluted air as a result of fuel combustion. In the USA, their industrial production was estimated between 2 and 81 tons, in 2011 (Al-Subiai et al., 2012; Sanchis et al., 2015).

1.3. NPs as emerging contaminants

The high amount of NP production and use worldwide can lead to inevitable release into environment of various NPs (Corsi et al., 2014). From different sources and via various routes, released NPs can reach marine ecosystems (Matranga and Corsi, 2012). In this regard, coastal environments are considered the ultimate sink for NPs (Corsi et al., 2014), as well as for most contaminants.

NPs can enter marine habitats either directly (through aerial deposition, effluents, dumping and run-off) or indirectly, e.g., via river discharge (Baker et al., 2014).
The main sources of NPs released into marine ecosystems are summarized as follows:

- personal care products (cosmetics and sunscreens);
- sewage and river discharge;
- anti-fouling applications in paints on vessel hulls;
- maritime traffic;
- atmospheric deposition.

Currently, no data are available regarding NP occurrence in the marine environment, as a consequence of the difficulty to detect and quantify NPs in complex matrices. Due to lacking analytical tools to evaluate effective NP concentrations in aquatic environments, only predicted environmental concentrations (PECs) are available in literature, also regarding nZnO, $nTiO_2$ and FC_{60}. Estimates of nZnO concentrations in the UK environments indicated less than 100 µg/L in water (Boxall et al., 2007). Another study reported nZnO PECs of 10 ng/L in natural surface water and 430 ng/L in treated wastewater in Europe (Gottschalk et al., 2009). nZnO PEC values of 76 µg/L in water were also reported by Ferreira da Silva et al. (2011). In Europe, PECs of $nTiO_2$ in water were in the range of 0.012-0.057 µg/L (Gottschalk et al., 2009). In Switzerland water samples, the PEC values were 0.7-16 µg/L (Mueller and Nowak, 2008). Ferreira da Silva et al. (2011) reported $nTiO_2$ PECs in water samples in the range of 0.7-24.5 µg/L. Moreover, PECs of FC_{60} in waste water treatment plants were estimated to be in the range of few ng/L to about 100 µg/L (Gottschalk et al., 2009). In Ferreira da Silva et al. (2011), FC_{60} PECs in water in the range of 0.31-5 µg/L have been also reported.
Studies regarding the behaviour of NPs in aquatic environments are lacking. In particular, interactions among NPs under various environmental conditions should be defined to predict their fate in aquatic environments and to estimate exposure scenarios and potential ecotoxicity (Corsi et al., 2014). Nevertheless, the complexity of the seawater environment could further change the behaviour of NPs compared to freshwater ecosystems (Keller et al., 2010). Seawater has a more pronounced effect on the surface charge of NPs causing more particle collisions and, consequently, more aggregation/agglomeration and thus sedimentation (Klaine et al., 2008; Keller et al., 2010). Furthermore, bioturbation and resuspension of sediments can lead to an increase in NP concentration at the sediment-water interface, promoting particle exchange between the sediment and the water column (Rocha et al., 2015).
Although evolved to deal with natural NPs and their fluctuations over millennia, it is not known how organisms will cope with high discharges of anthropogenic NPs into the environment. Currently, there are no safe guidelines regarding the release of NPs into fresh or salt water. Only Commissions are established to discuss about the regulation of some categories of NPs, with reference, for example, to the

Regulation (EC) (1223/2009) of the European Parliament and of the Council on cosmetic products in Europe and to the International Council of Nanotechnology in USA.
The development of nanotechnologies and nano-enabled products is continuously progressing, but the paucity of information and data regarding their potential impacts on the marine environment pose serious questions about the risks of exposure for wildlife (Colvin, 2003; Rocha et al., 2015).

1.4. The potential environmental impact of NPs

With the increased presence of NPs in commercial products, a growing public debate is emerging on whether the environmental and social costs of nanotechnology outweigh its many benefits (Colvin, 2003).
NP small dimensions, high surface/volume ratio and tendency to aggregate are indicative of a high potential toxicity in organisms. These characteristics increase chemical and biological reactivity of NPs, and their ability to penetrate into tissues and cells (Moore, 2006).
Moreover, it has been suggested that NP binding to other marine pollutants (metals and organics) to form nanoparticle-toxicant complexes promotes pollutant uptake and accumulation into the organisms. This behaviour in NPs may exacerbate the toxicity of other environmental contaminants, such as PAHs (Polycyclic Aromatic Hydrocarbons), constituents of paints and PPCPs (Pharmaceuticals and Personal Care Products). These pollutants and NPs together may act synergistically or have a new mechanism of action (Handy et al., 2008).
In human toxicology, concerning the atmospheric NP pollution, the US Environmental Protection Agency has attributed 60,000 deaths per year to the inhalation of atmospheric NPs; and there is evidence for direct transfer into the brain (Oberdörster et al., 2004; Raloff, 2003).
Despite these evidence, data available in the literature concerning the effects of NPs, not only to humans, but much more to marine species are still scarce. In the latter case, they are mainly related to in *in vitro* studies or based on the evaluation of acute NP toxicity, with unrealistic concentrations.
It is known that the uptake of NPs by filtration or ingestion is likely to be the major route in aquatic organisms. At the cellular level, most internalisation of NPs will occur via endocytosis. Endocytotic pathways into cells can either lead to the endosomal and lysosomal compartments (conventional endocytosis) or else via cell-surface lipid raft associated domains known as caveolae which avoids the degradative fate of material entering the endosomal/lysosomal system (Fig. 3) (Moore, 2006; Rocha et al., 2015).
Most of the currently available ecotoxicological data regarding NPs are limited to species used in regulatory testing or freshwater species (Matranga and Corsi, 2012). It has been demonstrated that NPs are accumulated in the soft tissues of marine organisms and determine adverse effects to different organs. In particular, NPs cause oxidative stress, and damage to membranes, genetic material,

reproductive organs (decreased fertility) and embryonic development (decreased growth rate, developmental anomalies and mortality). The NP toxicity depends on species, concentration and tissue considered, as well as the type of NPs (Bour et al., 2015; Grillo et al., 2015; Rocha et al., 2015).

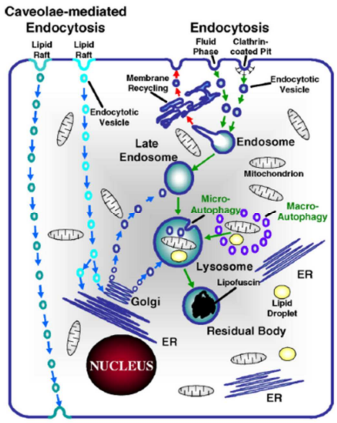

Fig. 3. Pathways for endocytosis in the cell which could be exploited by manufactured nanoparticles. Endocytosis via clathrin-coated pits (receptor mediated) or uncoated pits (fluid phase) transfers materials to the lysosomal degradative compartment, while caveolar endocytosis can result in translocation to the endoplasmic reticulum (ER), Golgi or through the cell by trancytosis (Moore, 2006).

It is very important to get more information on the potential toxicity and mechanism of action of NPs in marine species, mainly in those from coastal areas that are the major collector of these and other contaminants (Matranga and Corsi, 2012).

1.5. Bivalves as model species to study NP toxicity

Coastal areas are complex and highly changing environments at the interface between freshwater and marine aquatic ecosystems. Contaminants are frequently detected in these areas and represent a potential threat to marine organisms, especially bivalves (Renault, 2015).

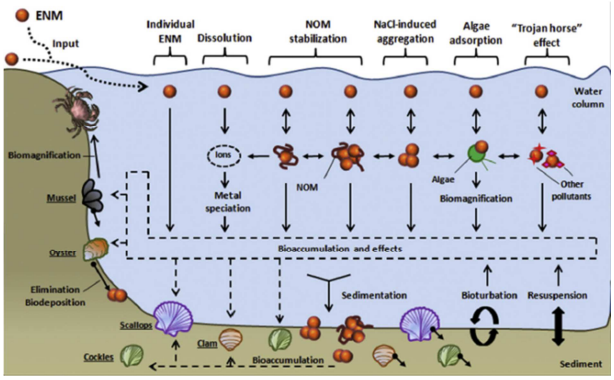

Fig. 4. Scheme illustrating the potential behaviour and fate of NPs in the aquatic environment and associated biological processes involving bivalve molluscs.

Once released into the environment, NPs will interact with each other and with their surrounding environment (Wiesner et al., 2009). In Fig. 4 the possible interactions of NPs in the aquatic environment are reported.

Moore (2006) first proposed that suspension-feeding invertebrates, bivalve molluscs in particular, may represent an unique target group for NP toxicology. These organisms have highly developed processes for the cellular internalization of nano- and micro-scale particles, endocytosis and phagocytosis, respectively, that are integral to key physiological functions such as intracellular digestion and cellular immunity. Bivalves can filter large volumes of water, processing microalgae, bacteria, sediments, particulates, and natural NPs, potentially accumulating different chemicals in their tissues. These organisms have been long recognized as valuable indicators of pollution, and extensive background information is now available on their biological responses to a wide range of both inorganic and organic chemicals (Dagnino et al., 2007; Moore et al., 2006).

In bivalve species the major effects of NP toxicity were detected in digestive gland and haemolymph. In particular, several of the available studies confirm that the digestive gland is the main organ for NPs accumulation (Rocha et al., 2015). Among physiological processes possibly disturbed by pollutants, the immune system is likely to be one of the more sensitive (Fournier et al., 2000). Among marine species, the mussel *Mytilus sp.* has been the most used bivalve species to assess NP toxicity.

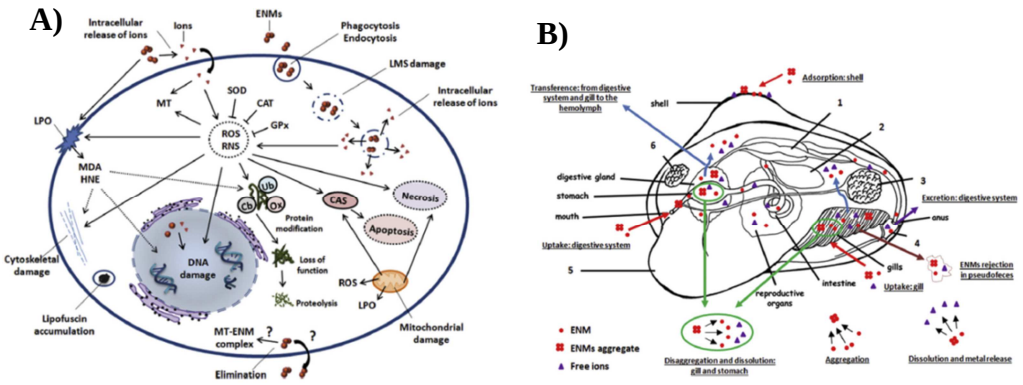

Fig. 5. A) General scheme illustrating the mode of action of NPs in bivalve molluscs. **B**) General scheme illustrating the potential toxicokinetics of NPs in bivalve molluscs (e.g. clams). Red arrow: Uptake; Brown arrow: rejection in pseudofeces; Green arrow: Desaggregation and dissolution; Blue arrow: Transfer in tissues; Purple arrow: Excretion; 1: Heart; 2: Kidney; 3: Posterior shell muscle; 4: Siphon out (excurrent); 5: Foot; 6. Anterior shell muscle.
(Rocha et al., 2015)

In a recent review (Rocha et al., 2015), the authors tried to describe the mode of action of NPs in bivalve cells (Fig. 5A). Overall, the data indicate that dissolution and release of ions from the metal oxide particles, oxidative stress and cell injury in proteins and membrane, and DNA damage are the major modes of action of NPs in bivalves. The best developed paradigm to explain most of the cytotoxic effects exerted by NPs in bivalves is directly or indirectly mediated by reactive

oxygen species (ROS) and free radicals production (Gomes et al., 2013; Rocha et al., 2015). Moreover, NPs are known to be filtered by the gills, accumulate in the digestive gland, and transferred to the haemolymph through the epithelium of the digestive gland tubules (Fig. 5B)

It is known that the characteristics of seawater increase the aggregation and sedimentation of NPs and consequently their deposition at the sea bottom. Considering that, NPs are a reason of concern for bivalve infaunal species.

1.5.1. The marine clam *Ruditapes philippinarum*

Manila clam *Ruditapes philippinarum* (Adams and Reeve, 1850) is native of the subtropical to low boreal zone of the western Pacific but, due to its high commercial value, fast growth and great adaptability and resistance to a wide range of environmental conditions and stressors, it has been introduced into several parts of the world where it has become permanently established. Different factors may explain the successful spread of the Manila clam, such as its high tolerance to variations of environmental parameters (salinity, temperature, dissolved oxygen), its high capability to adapt to different substratum typologies and its high fitness (early gonadic maturation, high fertility, and its long spawning period in which multiple spawning is possible) (Paesanti and Pellizzato, 2000; Pellizzato and Da Ros, 2005). The first attempts to introduce Manila clam in Italy date from 1983, when a small amount of spat purchased in an English hatchery was sown in the southern basin of the Venice lagoon (Breber, 2002). Some promising results were rapidly achieved and during the 1980s, further sowing attempts were carried out in many Italian transitional sites (in the lagoons of Po delta, Grado and Marano, Lazio, and Sardinia). The great suitability of these environments for Manila clam, both in terms of growth and natural reproduction, soon became evident, especially in the case of the Northern Adriatic transitional systems characterized by shallow waters and high freshwater inputs rich in nutrients promoting high natural productivity. In such areas, the species rapidly spread to all favourable sites and Manila clam harvesting soon became the most economically important fishing activity. Landing data indicate that production peaked at around 64,000 tons in 1999 and the Venice lagoon contribution accounted for up to 60% of Italy's overall production (Boscolo Brusà et al., 2013). These organisms have been long recognised as valuable indicators of pollution, and extensive background information is now available on their biological responses to a wide range of both inorganic and organic pollutants (Dame, 2011). The Manila clams are often used to monitor marine coastal pollution and to understand the potential effects determined by various groups of pollutants in laboratory experiments (Blasco and Puppo, 1999; Ji et al., 2006; Matozzo et al., 2010; Milan et al., 2013; Marisa et al., 2015). Due to its prevalence in lagoon and coastal areas, *R. philippinarum* is considered a model species to assess environmental contamination. As an infaunal filter-feeder, the Manila clam is

particularly exposed to the impact of contaminants having in sediments their ultimate sink, such as NPs.

Regnum: Animalia
Phylum: Mollusca
Classis: Bivalvia
Subclassis: Heterodonta
Ordo: Veneroida
Superfamilia: Veneroidea
Familia: Veneridae
Genus: Ruditapes
Species: *R. philippinarum*
(Adams and Reeve, 1850)

Fig. 6. Specimens of *Ruditapes philippinarum* and its classification.

To study nZnO, $nTiO_2$ and FC_{60} sub-lethal effects and bioaccumulation, we decided to use *R. philippinarum* (Fig. 6) as a model species for many reasons:

- ✓ it is a target species, due to its wide spread in lagoon and coastal areas of the Northern Adriatic, characterized by a lot of anthropogenic impacts, where clams are also economically important, they being fished and farmed;
- ✓ its biological and ecological characteristics are very well known and it is used in studies of different type, even in the laboratory, given that it has a good adaptability to laboratory conditions;
- ✓ in the literature the effects of NPs in this species are lacking;
- ✓ the biomarker approach in this species has been validated in a number of ecotoxicological studies aimed at understanding the molecular and physiological effects of environmental changes (Munari et al., 2011; Matozzo et al., 2012). The use of biomarkers is extremely effective in assessing exposure to environmental stressors, risk conditions being highlighted before the occurrence of irreversible damage, not only at individual but also at population level, driving changes in the entire biocoenoses;
- ✓ recently, Omics Technologies were applied in ecotoxicology and also in clams to explain more in-depth the effects of environmental changes (Chora et al., 2010; Milan et al., 2013).

1.6. Biomarkers in ecotoxicology

The simplest way to assess the pollution status of a specific ecosystem is to carry out chemical analysis of water, soil, biological extracts and other environmental samples. However, given the large number, complexity and sometimes low toxicity threshold of environmental chemicals, chemical analysis alone may not offer meaningful assessment of the pollution status of the studied ecosystem.

Moreover, chemical analysis *per se* offers little insight into the environmental fate or biological threat posed by pollutants. It is now well established that contaminants can cause changes at all levels of biological organization (Fig. 7).

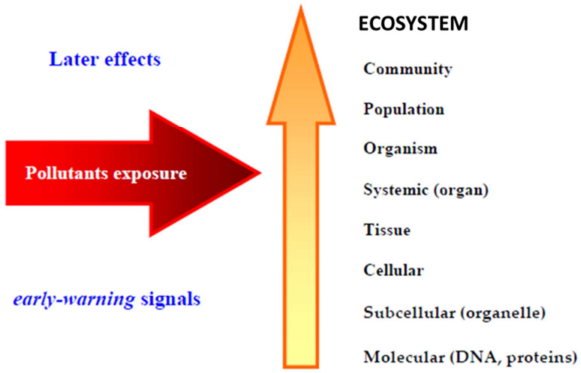

Fig. 7. Schematic representation of the sequential order of responses to pollutant stress within a biological system.

In some cases research still bases on the measurement of chemicals in organisms or measuring changes only at the population level, by which only an indirect inference can be made with regard to the cause of the population decline (Simpson and Norris, 2000). Although the population, community and ecosystem are important levels for the monitoring of toxic effects, the primary effect of xenobiotics is first revealed at the sub-cellular level by impaired biological functions (such as for biochemical and molecular variations, enzymatic activity modifications, DNA alterations). The rapid evaluation of early-warning signals can allow the activation of some procedures aimed at reducing the pollutant impact before the damage reaches higher hierarchical levels. To reach this goal, the application of biomarker techniques on an appropriate reference biological model, used in laboratory pollutant exposure, is strongly recommended as a sensitive approach to investigate the hazard of several classes of pollutants, including NPs (Matranga and Corsi, 2012). Biomarkers can offer complete and biologically relevant information on the potential impact of toxic pollutants on the health of organisms (Van der Oost et al., 1996).

Biomarkers were originally defined as any biochemical, histological, or physiological alterations or manifestations of environmental stress (NRC, 1987). More recently, this definition has been challenged by several authors (Adams, 1990; Engel and Vaughan, 1996) and the term biomarker is now more commonly used in a more restrictive sense, namely biochemical sub-lethal changes resulting from individual exposure to xenobiotics. Nowadays, any biological response to an environmental chemical at the sub-individual level, measured inside an organism or in its products (urine, faeces, hair, feathers), indicating a deviation from the normal status that cannot be detected in the whole organism, can be considered a biomarker (McCarthy et al., 1990).

The advantages of applying biomarkers are considerable (Handy et al., 2003):

- biomarker responses may indicate the presence of a biologically available contaminant, rather than a biological inert form of contamination;
- using a suite of biomarkers may reveal the presence of contaminants that were not suspected initially;
- biomarkers responses often persist long after a transient exposure to a contaminant that has then degraded and is no longer detectable. Thus, biomarkers may detect intermittent pollution events that routine chemical monitoring may miss;
- biomarker analyses are, in many cases, much easier to perform and are considerably less expensive than a wide range of chemical analyses.

Sometimes there is variability in biomarker responses that may be attributed to abiotic (temperature, salinity, dissolved oxygen, etc.) or biotic factors (genotype, phenotypic plasticity, tolerance, age, sex, body size, etc.). However, it is a widely held misconception that these sources of variability render the biomarker responses insensitive compared to traditional chemical monitoring techniques. Indeed, there are several options for minimizing variability, such as the use of a suite of biomarkers, since it is very important to measure several biomarkers at the same time in the same biological model. Moreover, if there is evidence that an abiotic/biotic factor can modulate the response during an exposure to a toxicant under controlled laboratory conditions, suitable exposures to differing conditions for that factor should be performed. In addition, combination of various approaches should be established to reduce the variability of specimens (Handy et al., 2003).

By screening multiple biomarker responses, important information will be obtained about organism toxicant exposure and stress. Biomarkers may also provide insight into the potential mechanisms of contaminant effects and the mode/mechanism of action (Crane et al., 2006).

1.6.1. The *in vivo* biomarker battery

The battery of biomarkers used in this thesis was selected based on i) the characteristics of nZnO, $nTiO_2$ and FC_{60} ii) the effects of these three NPs in other species, and iii) the potential mechanism of action of NPs. Various types of biomarkers were applied to obtain a general view about the effects of NPs in clam haemolymph, gills and digestive gland.

Haemolymph was used to measure different parameters and to perform assays useful to provide information about NP-induced modulations on haemocyte number, haemocyte diameter and volume, NP cytotoxicity and genotoxicity in haemocytes. Gills and digestive glands were used to assess potential modulation of anti-oxidant and detoxification enzyme activities, lipid peroxidation, protein carbonyl content and DNA damage due to NP action.

During the last decade, the multi-biomarker approach has been used in various ecotoxicology studies. In both field and laboratory exposures, biomarkers have been successfully applied in many aquatic organisms, including many species of

fish and aquatic invertebrates, mainly molluscs and crustaceans (Handy and Depledge, 1999; Galloway and Depledge, 2001; Forbes et al., 2006). Compared with other strategies in assessing exposure to contaminants, the advantages of these techniques include their fairly simple methodology, sensitivity for detecting low levels of changes and damage, flexibility, low costs, ease of application, the opportunity to conduct studies using relatively small amounts of a test substance, and the rapid production of data.

1.7. The "omics" technology: proteomics in ecotoxicology

Actually, the biological effects of emerging pollutants are often poorly understood. It is recognized that most pollutant effects depend on the determination of suites of responses. Moreover, there is increasing awareness that no single biomarker will serve to indicate the full effect of environmental pollutants, but it is absolutely necessary to use multiple biomarkers, to investigate the pollutant effects (Depledge and Galloway, 2005). At this regard, a new trend in ecotoxicology research is the application of so called "omics" technologies. These are methods that have the potential to monitor complete classes of cellular molecules such as messenger RNAs, proteins and intermediary metabolites in a single analysis (Ankley et al., 2006), compared to traditional analyses that rely on only one endpoint. By allowing simultaneous analysis of thousands of genes, proteins, or metabolites, these new global technologies have enabled a wider approach to biological questions, since toxicity generally involves not only changes in a single gene but rather a cascade of gene interactions (Aardema and MacGregor, 2002).

These methodologies have been already widely used in human medicine and in toxicology studies. In particular, proteomics is a well-established area of research in molecular medicine because the evaluation of changes in protein expression patterns can provide information on pathogenic signalling pathways and the identification of human disease markers (Petricoin and Liotta, 2003).

In the last decade, many authors have applied both traditional enzymatic and cellular biomarkers (Canesi et al., 2007; Sellami et al., 2015) and have demonstrated an effective methodology for characterising the modes of action and the mechanisms of toxicity for pollutants, with a high potential for identifying novel biomarkers (Dowling and Sheehan, 2006; Monsinjon and Knigge, 2007).

Snape et al. (2004) proposed the term "ecotoxicogenomics" to describe the integration of genomics (transcriptomics, proteomics, and metabolomics) into ecotoxicology, and defined it as "the study of gene and protein expression in non-target organisms that is important in responses to environmental toxicant exposures". Nonetheless, the use of "omics" approach is not widespread in ecotoxicology. Among "omics" technologies, environmental proteomics or ecotoxicoproteomics -the study of changes in the abundance of proteins and their post-translational modifications- has become a powerful tool for generating hypotheses regarding how the environment affects the biology of organisms. With

its rapidly expanding analytical tools, proteomics provides the means to study changes occurring at the level of the proteome -the entire protein pool- in response to both the external environment and ontogenetic events in animals, plants, and bacteria (Lemos et al., 2010). The approach has obvious applications to ecotoxicology since it has the potential both to identify previously unknown protein biomarkers and to gain insights into toxicity mechanisms.

Notwithstanding the improvement of proteomics methodologies, both for electrophoresis procedures and mass spectrometry analyses, the protein identification in the field of ecotoxicology is often challenging because of the limited information contained in the available databases, especially for non-model organisms, whose genomes have not been fully sequenced (Tomanek, 2011). Despite this drawback, proteomics has much to offer even in species poorly represented in sequence databases. More specific and new tools were developed to have greater results (e.g. *de novo* sequencing approaches, identification methods and tools), thus circumventing current limitations of sequence data (Habermann et al., 2004; Liska et al., 2004).

For example, in common procedure the protein identification is determined with subsequent matrix assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) with "LIFT" technique, and the peptide mass fingerprinting is used to screen a protein sequence database and, in some cases, this provides sufficient information for protein identification. Tandem mass spectrometry (MS/MS) fragment ion analysis of selected peptides can be used for improved identification. Although MALDI-TOF/TOF is a very suitable tool for analysis of peptides and proteins, thanks to its high sensitivity, fast data acquisition, ease of use, and robust instrumentation, identification of proteins in non-standard model organisms remains problematic. The database search fails if the sequence of the protein is not available or when unexpected modifications and amino acid substitutions are present in real samples. Recently, this limitation has been overcome to some extent using a *de novo* sequencing strategy, in which partial or complete amino-acid sequences are obtained using either manual or automated *de novo* peptide sequence analysis. This approach has been successfully applied in recent studies with incomplete or non-sequenced organisms in order to identify their proteins. Instead of using common tools to identify proteins, the linear ion trap combined with orbitrap mass spectrometer (LTQ-Orbitrap) yielding *de novo* protein sequences suitable for database searching could be used, in order to increase the possibility of identification (Waridel et al., 2007; Pedriali et al., 2013).

1.7.1. The redox proteomics

Proteomics provides a qualitative description of cellular changes and has shown in many studies that the systemic changes of proteome occurring during exposure are pollutant-specific. Moreover, proteins common to many pollutant-stress related responses include oxidative stress proteins, cytoskeletal proteins,

chaperones, proteases, and proteins involved in the detoxification of xenobiotics. Together, changes in these molecules suggest that the production of ROS leads to denaturation of proteins as well as wide-ranging modifications of cytoskeletal elements. In this scenario, post-translational modifications (PTMs) present a novel frontier to assess the biological effects of pollutants. Although the types of PTMs number in the hundreds (Walsh, 2006), only changing patterns of carbonylation, glutathionylation, thiol-modifications, and ubiquitination have been studied in response to pollutants (Sheehan, 2006). The former three are modifications caused by the increased production of ROS and can occur due to a change in the oxidative environment of the cell (Fig. 8).

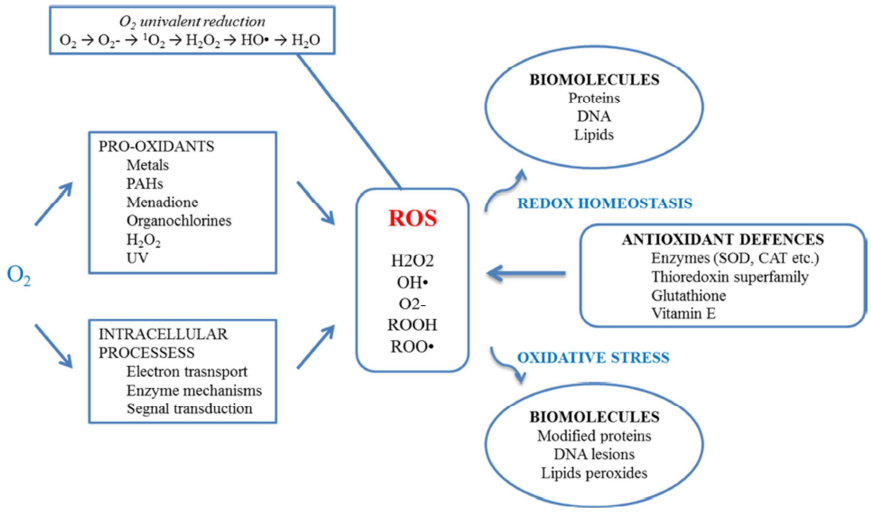

Fig. 8. Oxidative stress arises from ROS derived by univalent reduction of O_2, when antioxidant defences are overcome.

Radicals and non-radical oxidants can be generated by a wide variety of different processes in biological systems (e.g., by-products of mitochondrial electron transport and during inflammation), but they can also occur as a response to a wide range of exogenous agents (e.g., UV, pollutants). When this production overcomes the cellular defence mechanisms, oxidative stress occurs (Dalle-Donne et al., 2005). Most highly reactive oxidants, including many radicals, react with virtually all biological molecules, including DNA, RNA, cholesterol, lipids, carbohydrates, proteins and antioxidants. Since proteins are the most abundant non aqueous component of the cell, they are major targets of ROS (they adsorb ~ 68% of ROS) and numerous post-translational, reversible or irreversible modifications have been characterized, which may lead to a change in the structure and/or function of the oxidized protein (Davies, 2005).

Redox proteomics is an increasingly emerging branch of proteomics aimed at identifying and quantifying redox-based changes within the proteome both in redox signalling and under oxidative stress conditions (Butterfield et al., 2012; Sultana and Butterfield, 2011).

ROS can modify and inactivate proteins in a wide variety of ways. Sulphur-containing molecules are notoriously susceptible to oxidation. Cysteine thiols (-SH) can be irreversibly oxidised to sulphinic ($-SO_2H$) and cysteic ($-SO_3H$) acids or reversibly oxidised to sulphenic acid (-SOH), thiyl radicals (-S.) or nitrosothiols (-SNO). Methionines can be oxidised to sulphoxides and sulphones. Sulfur-containing residues (cysteine and methionine) are especially susceptible to oxidation which can have functional significance and lead to increased turnover of damaged proteins. Amino acid side-chains (e.g. lysine, arginine, proline) can be irreversibly converted to aldehyde/ketone groups collectively called protein carbonyls, causing inactivation, crosslinking or breakdown of protein (Ghezzi and Bonetto, 2003; Levine and Stadtman, 2001). These two modifications are readily detectable in one or two dimensional gel electrophoresis (1-DE and 2-DE) analysis by labelling modified proteins with specific reagents, such as 50-iodoacetamide fluorescein (IAF), a popular fluorescent dye for labelling protein thiols, and fluorescein-50-thiosemicarbazide (FTSC) for protein carbonyl groups (Fig. 9).

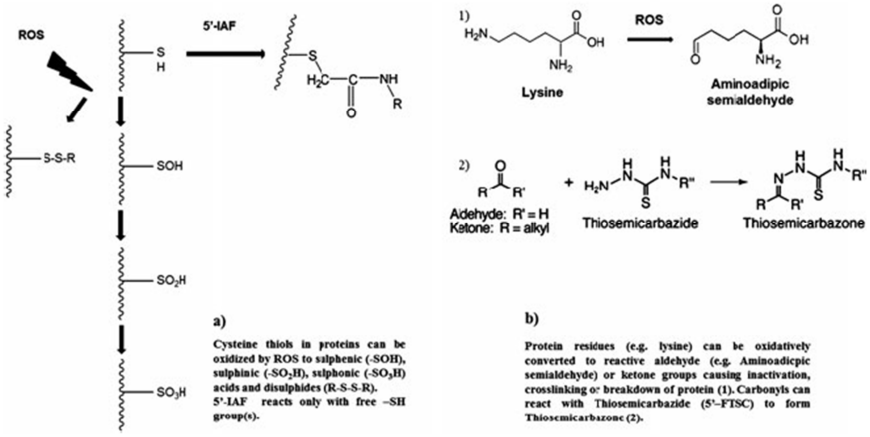

Fig. 9. Oxidative modifications of amino acid side chains: oxidation of cysteine –SH groups (a) and carbonylation (b) and their specific labeling.

Other reversible redox lesions of proteins include glutathionylation - which can protect cysteine residues from oxidation (Schafer and Buettner, 2001) - and formation of methionine sulphoxide, an indicator of cell ageing (O'Sullivan et al., 2005). Thus, given that a great number of pollutants are known to increase production of ROS, PTMs caused by ROS and subsequent changes in levels of protein degradation have the potential to be sensitive global markers of pollutant stress.

In the field of ecotoxicology, the redox proteomics approach is only recently recognized as a powerful tool to better understand the molecular mechanisms of toxicity (which can differ with differing identity of pro-oxidant). For example, *M. edulis* from polluted sites in Ireland showed greater levels of carbonylated proteins, but few changes in protein abundance in gill and digestive tissues were found in comparison to control sites (McDonagh et al., 2005). Carbonylation was

also used as a biomarker to demonstrate that p,p'-dichlorodiphenyldichloroethylene (p,p'-DDE) caused oxidative stress in the clam *Ruditapes decussatus* (Dowling et al., 2006). There is evidence that a number of abundant proteins in *Mytilus* are targets of ROS. Specifically, actin, protein disulfide isomerase, and other chaperones (heat shock protein gp96 and calreticulin) have been shown to form intra-molecular disulphide bonds in response to exposure to the pro-oxidant menadione, possibly sequestering ROS before they can cause uncontrollable damage to other proteins, lipids, or ribonucleotides (McDonagh and Sheehan, 2007).

Recently, polyaromatic hydrocarbon and anthracene exposure were shown to affect the gill oxidative stress status of the clams *Ruditapes decussatus* (Sellami et al., 2015). Moreover, changes in the redox status of gill proteins were revealed in *M. edulis* exposed to the pharmaceutical diclofenac (Jaafar et al., 2015). Chora et al. (2010) showed that nonylphenol exposure generated ROS in gill and digestive gland of *R. decussatus* resulting in a significant alteration of the proteome.

Redox proteomics was also used to study the effects of gold and copper oxide NPs on IAF/FTSC-labelled proteins in *M. edulis* (Tedesco et al., 2010; Hu et al., 2014).

Because of the range of irreversible and reversible modifications possibly occurring in proteins, redox proteomics offers a route to identify new protein targets for ROS toxicity with insights into likely mechanisms.

Aims

The aim of the PhD project was to evaluate the potential toxic effects of three widely used NPs: zinc oxide (nZnO), titanium dioxide ($nTiO_2$) and C_{60} fullerene (FC_{60}) to the clam *Ruditapes philippinarum*. Considering that wild organisms are generally exposed to mixtures of different pollutants, the combined effects of a mixture of all three NPs have also been investigated. In addition, combined effects of NPs and changing environmental parameter (salinity) were addressed to obtain information about possible variations in clam susceptibility to NPs under a global change scenario. Salinity is one of the dominant environmental factors controlling species distribution and influencing physiological processes in marine organisms. Among predicted changes in environmental parameters, there is an increasing concern about future alterations in seawater salinity values, mainly in estuarine and coastal areas, which will affect the performance of native and invasive species. Moreover, compared to freshwater, seawater has a more pronounced effect on the surface charge of NPs causing changes on their behaviour. In this context, to gain a better insight into the potential environmental impacts of NPs and also considering the relatively recent introduction of the studied species in the Northwestern Adriatic lagoons, the native species *Ruditapes decussatus* was used for a comparison.

To reach the above-mentioned purposes, many cellular and biochemical biomarkers, already validated in previous studies in our laboratory, have been measured in the perspective of a multi-biomarker approach. Further, new methods were adopted, such as proteomic analyses in the NP mixture exposure. In all the investigations, NP contents were measured in animal tissues (gills and digestive glands) to assess possible bioaccumulation.

The Manila clam has been chosen as model organism, previously used in a number of ecotoxicological studies, taking into account that bivalves are considered one of the most suitable target group to investigate NP toxicity (Canesi et al., 2012). However, information concerning the effects of NPs to clam species are lacking. Due to its filter-feeding and infaunal habits, *R. philippinarum* may be more susceptible to the effects of NPs, given that in seawater NPs tend to aggregate, adsorb to particulate matter, settle to the bottom and accumulate in sediments (Gagnè et al., 2015; Rocha et al., 2015).

Most of the published ecotoxicological studies were conducted with high NP concentrations that are unrealistic from an environmental point of view, if compared to PECs. Instead, in this project, we chose to test lower concentrations (1 and 10 μg/L) of NPs that were in the range of PEC values.

- ***In vivo* exposure to single NPs**

Clams were collected from a reference site located within a licensed area for clam culture in the Lagoon of Venice (Chioggia, Italy). Specimens were then acclimatized in laboratory conditions for 5 days before the exposure. They were maintained in large aquaria, which contained a sandy bottom and aerated natural seawater (salinity of 35 ± 1 psu, temperature of 17 ± 0.5 °C), and were fed daily with microalgae. Medium-term (7 days) exposures to three NPs (nZnO, $nTiO_2$, FC_{60}) were carried out (Tab. 1). A movement pump (Hydor, Koralia nano 900, USA) was positioned in every aquarium (both for control and treated clams) to facilitate the water circulation and to prevent NP sedimentation. Before exposure, the influence of the movement pump on clam behaviour was assessed, and no alterations were observed as clams quickly opened their valves, extended their siphons, and exhibited normal filtering activity.

$ZnCl_2$ (10 µg/L) was used to investigate possible contributions of Zn^{2+} release to nZnO toxicity, and bulk TiO_2 ($bTiO_2$, 10 µg/L) was used to understand the potential differing action of metal oxide compared with the respective NP. Throughout the exposure, clams were maintained in glass aquaria (without sediment) containing aerated seawater in the same thermo-haline conditions used during the acclimatisation period. The exposure was conducted in semi-static conditions, water, contaminants and food supply being renewed every day in the experimental tanks (35 L).

Tab. 1. Treatment conditions throughout the three NP experiments.

	Treatment conditions			
Experiment	**Control (µg/L)**	**related chemicals**	**NP low concentration**	**NP high concentration**
nZnO	0	$ZnCl_2$ 10 µg/L	nZnO 1 µg/L	nZnO 10 µg/L
$nTiO_2$	0	Bulk 10 µg/L	$nTiO_2$ 1 µg/L	$nTiO_2$ 10 µg/L
FC_{60}	0	-	FC_{60} 1 µg/L	FC_{60} 10 µg/L

For each experimental condition tested, two replicate tanks were set up. During exposure, tissues were collected at time intervals (after the first, T1, third, T3, and last ,T7, day of exposure), and five pools (5 animals per pool) from each experimental condition were prepared. Haemolymph, gills and digestive gland of clams were collected and frozen for subsequent analyses or immediately processed, depending on the various biological parameters measured. Immediately after collection, haemolymph was used to measure some cell parameters, such as total haemocyte count (THC), diameter and volume of haemocytes, haemocyte proliferation (XTT Cell Proliferation Assay), cytotoxicity (assessed by Lactate Dehydrogenase assay, LDH), Neutral Red uptake (NRU), and DNA damage

(Single Cell Gel Electrophoresis, SCGE, and Micronucleus assays). Frozen aliquots of haemolymph were subsequently used to measure lysozyme activity in both haemocyte lysate and cell-free haemolymph. Gills and digestive glands were frozen and then used to measure anti-oxidant enzyme activities (superoxide dismutase, SOD, and catalase, CAT), detoxification enzyme activities (glutathione S-transferase, GST), protein carbonyl content (PCC), lipid peroxidation (thiobarbituric acid reactive substances, TBARS) and DNA damage by DNA precipitation assay. Only in nZnO-exposed animals acetylcholinesterase (AChE) activity in gills and apoptosis (Cell Death Detection ELISA) in haemolymph were also measured.

For all the experiments, 4 pools of tissues (gills and digestive gland) were also collected in 7 days-treated clams to evaluate the potential bioaccumulation of NPs. To better understand the mechanism of action of the three NPs, analysis of zinc, titanium and FC_{60} concentrations were performed in both gills and digestive gland. Metals were quantified by the use of the inductively coupled plasma optical emission spectrometry (ICP-OES). FC_{60} content was measured through the use of High Performance Liquid Chromatography (HPLC).

- ***In vitro* exposure to $nTiO_2$ on haemocytes**

To get further insight into $nTiO_2$ effects at the cell level, an *in vitro* approach was used and phagocytic activity was assessed in clam haemocytes exposed to $nTiO_2$ (0, 1 and 10 µg/mL). This activity was evaluated in two series of experiments (with and without pre-treatment of haemocytes with NPs). In addition, the capability of $nTiO_2$ to interact with clam haemocytes (60 min) was evaluated with transmission electron microscope (TEM).

- ***In vivo* exposure to a mixture of NPs**

To assess mixture effects, clams were exposed for 7 days to i) 1 µg/L nZnO ii) 1 µg/L $nTiO_2$ iii) 1 µg/L FC_{60} fullerene and iv) all three NPs as a mixture (Fig. 10). Exposure duration and tissue collection were scheduled as in previous experiments. In haemolymph, DNA damage was evaluated using SCGE assays; only at T7, the production of intracellular superoxide anion was evaluated and the protein damage was investigated with one dimensional gel electrophoresis (1-DE) redox proteomics. In gills and digestive glands, SOD, CAT, and GST activities, PCC, lipid peroxidation, and DNA damage were measured; only at T7, protein damage and its modulation were investigated using 1-DE and two dimensional gel electrophoresis (2-DE) redox proteomics. To assess damaged proteins (1-DE and 2-DE) following oxidative stress, cytosolic proteins were labelled with two fluorescent molecules that detect different types of damage. IAF (50-iodoacetamido fluorescein) and FTSC (fluorescein-50-thiosemicarbazide) can detect thiol groups and carbonyl groups in proteins, respectively.

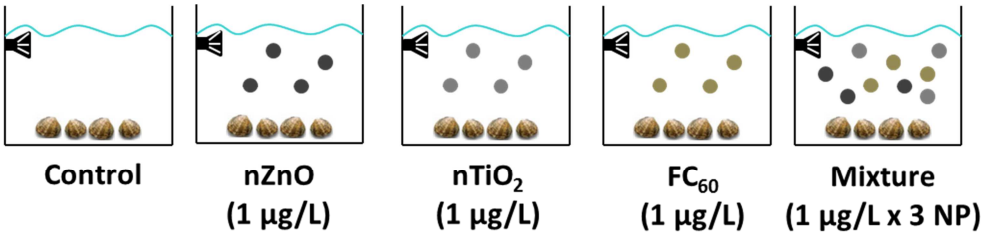

Fig. 10. Schematization of the different NP mixture experimental conditions.

The redox proteomics procedure was performed during the abroad period of the PhD, in collaboration with Prof. David Sheehan (University College Cork, Ireland). The identification of damaged proteins from the 2-DE analysis in gills and digestive gland was performed in collaboration with Dr. Maria Fedorova and Prof. Ralf Hoffman (Institute of Bioanalytical Chemistry, Universität Leipzig, Germany).

At T7, in controls and treated clams, the gills and digestive gland were also collected to measure the potential bioaccumulation of NPs. Analyses of NP content were performed as reported above for the exposure to single NPs.

- ***In vivo* exposure to a mixture of NPs under different salinity values**

To assess NP mixture effects under different salinity values, clams were exposed for 7 days to 18, 28 and 38 of salinity, both in the absence and in the presence of NP mixture (1 µg/L nZnO, 1 µg/L $nTiO_2$, and 1 µg/L FC_{60}) (Fig. 11).

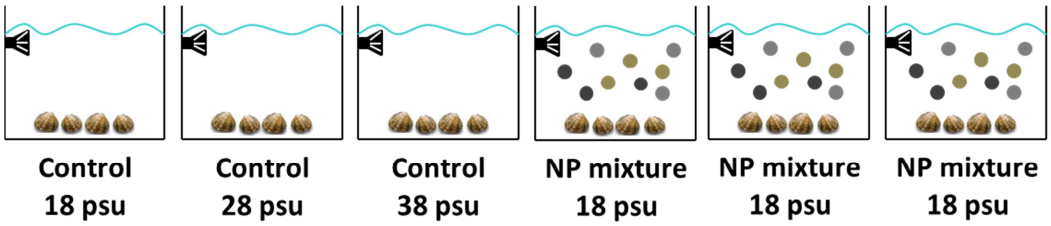

Fig. 11. Schematization of the experimental conditions tested.

Two phylogenetically similar species were used in this experiment, clams *R. philippinarum* and *R. decussatus* (Fig. 12), both collected from the Lagoon of Venice.

The values of salinity were chosen based on minimum, medium and maximum values measured in the Southern Venice lagoon from 2000 to 2009 (Zirino et al., 2014). For each species, three groups of animals were selected, placed in large aquaria and then acclimated to the three experimental salinity values, which were gradually achieved in 12 days. Exposure duration and tissue collection were scheduled as in previous experiments. Haemolymph was collected to measure DNA damage with Micronucleus assay, LDH activity, and NRU. Gills and digestive glands were collected to measure SOD, CAT, and GST activities, PCC and lipid peroxidation.

Fig. 12. Specimens of *R. philippinarum* (left) and *R. decussatus* (right).

At T7, gills and digestive glands of clams from all experimental conditions were collected to evaluate NP bioaccumulation. Metal oxide NP contents were quantified as previously reported. FC_{60} content was measured by using Ultra-Performance Liquid Chromatography coupled with a Q-Exactive mass spectrometry.

- **NP and bulk TiO_2 characterization**

In order to verify the NP properties as declared from the producer, thus obtaining information useful to understand better the toxicity of NPs, nZnO, $nTiO_2$, FC_{60} and $bTiO_2$ were characterized by a combination of analytical techniques. Mean average size and shape of primary particles were determined by TEM and X-ray diffraction (XRD). The particle size distribution was measured by laser diffraction, and the surface areas and the presence of micropores on NP surface were calculated by Brunauer-Emmett-Teller (BET) theory. To study the particle size distribution in water, the dynamic light scattering (DLS) was used.

- **nZnO exposure**

Considering the physico-chemical characteristics of nZnO, and the fact that nZnO toxicity is generally ascribed to two general mechanisms (e.g., the generation of ROS and the release of metal ions), the expectations were:

- modulation of antioxidant enzymes reflecting increased oxidative stress due to increased ROS production; as a consequence, damage to lipids, proteins and DNA was also expected;
- modulation of the immune parameters, since the immune system is considered as a potential and sensitive target for the effects of NPs (Canesi et al., 2012);
- effects of nZnO nanoparticles similar to those caused by zinc ions ($ZnCl_2$).

- **$nTiO_2$ exposure**

Based on the physico-chemical characteristics of $nTiO_2$, and the scarce information about $nTiO_2$ toxicity, the expectations were:

- modulation of antioxidant enzymes reflecting increased oxidative stress due to increased ROS production; as a consequence, damage to lipids, proteins and DNA was also expected;
- changes in the immune parameters (Canesi et al., 2012);
- digestive gland as the target tissue for the effects and accumulation of $nTiO_2$;
- different or similar effects of $nTiO_2$ and $bTiO_2$.

- **FC_{60} exposure**

Although FC_{60} is widely used and have a high potential to end up in the environment, it is one of the less investigated NPs in ecotoxicology studies. Considering the strong oxidising, hydrophobic and phototoxic properties of FC_{60}, and the general information about NP toxicity, the expected effects were:

- increased oxidative stress, and consequently damage to lipids, proteins and DNA;
- changes in the immune parameters (Canesi et al., 2012);
- internalization and bioaccumulation in tissues of FC_{60}.

- **NP mixture exposure**

Based on the results of our previous experiments on the three single NPs, the information in literature, the mechanism of action and the characteristics of NPs, the expectations were:

- a different mechanism of action of NPs alone or as a mixture (antagonist, synergic or additive);
- increased oxidative stress and damage to DNA, proteins, and lipids;

- a different bioaccumulation pattern in clam tissues exposed to NPs alone or as a mixture.

➢ **NP mixture under different salinity exposure**

Based on the results of the previous experiments, the information in literature, the mechanism of action and the characteristics of NPs, the expectations were:

- modulation of NP mixture toxicity under different salinity values;
- different levels of oxidative stress and damage to DNA, proteins, and lipids;
- different modulation of the haemocyte parameters;
- different responses between the two species considered;
- different bioaccumulation pattern in clam tissues exposed to NPs under different salinity values.

PAPER: I

Ilaria Marisa, Valerio Matozzo, Marco Munari, Andrea Binelli, Marco Parolini, Alessandro Martucci, Erica Franceschinis, Nicola Brianese, Maria Gabriella Marin

In vivo exposure of the marine clam _Ruditapes philippinarum_ to zinc oxide nanoparticles: responses in gills, digestive gland and haemolymph

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In vivo exposure of the marine clam *Ruditapes philippinarum* to zinc oxide nanoparticles: responses in gills, digestive gland and haemolymph

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Abstract

Potential nanoparticle (NP) toxicity poses a growing concern in marine coastal environments. Among NPs, zinc oxide nanoparticles (nZnO) are widely used in many common products that ultimately become deposited in coastal habitats from multiple non-point sources. In this study, we evaluated the in vivo effects of nZnO in the clam *Ruditapes philippinarum*.

Animals were exposed to nZnO (1 and 10 µg/L) and $ZnCl_2$ (10 µg/L) for 7 days. $ZnCl_2$ was used to compare the effects of the NPs to those of Zn^{2+} and to ascertain whether nZnO toxicity is attributable to the release of ions into the aquatic medium. At differing time intervals during the exposure, several biochemical and cellular responses were evaluated in the clam gills, digestive gland, and haemolymph. The results showed that nZnO, at concentrations close to the predicted environmental levels, significantly affected various parameters in clam tissues. Significant increases in catalase and superoxide dismutase activities and a decreasing trend of glutathione S-transferase activity indicated the involvement of oxidative stress in nZnO toxicity. In clams exposed to $ZnCl_2$ slight variations in antioxidant enzyme activities were detected respect to nZnO-treated clams. However, no damage to lipids, proteins or DNA was revealed in all exposure conditions, suggesting a protection of antioxidant enzymes in the tissues. Of the various haemolymph parameters measured, haemocyte proliferation increased significantly, in $ZnCl_2$-treated clams in particular. Under nZnO (10 µg/L) and $ZnCl_2$ exposure, DNA damage in haemocytes was also revealed, but it was lower in clams exposed to $ZnCl_2$. A decreasing trend in gill AChE activity of treated clams proposed a possible role of zinc ions in nZnO toxicity. However, the dissimilar modulation of the responses in the nZnO- and $ZnCl_2$-exposed clams suggested different mechanisms of action, with nZnO toxicity possibly depending not only on the release of zinc ions but also on NP specific features. Changes in the biological parameters measured in the clams were consistent with Zn accumulation in their gills and digestive glands.

Keywords: nanoparticles, zinc oxide nanoparticles, zinc, clams, biomarkers, bioaccumulation

1. Introduction

Currently, nanotechnology is one of the most promising fields of science and technology. Engineered nanomaterials (NPs) are used extensively in a variety of emerging technologies and commercial products (Maynard et al. 2006; Corsi et al. 2014; Rocha et al. 2015). Increased production and applications of NPs raise concerns due to their potential ecological impacts. Indeed, NPs are now recognised as novel environmental pollutants with toxic potential (Klaine et al. 2008). A large group of NPs is represented by metal oxides, among which zinc oxide is one of the most widely used. Due to their unique catalytic capacity, optoelectronic properties, antimicrobial activity and other characteristics, zinc oxide nanoparticles (nZnO) are currently used in various products including plastics, ceramics, glass, cement, rubber, lubricants, paints, pigments, foods (as nutritional supplement), batteries, and fire retardants (Ma et al. 2013). In addition, due to their excellent UV absorption and reflective properties, nZnO are common constituents of personal care products, including cosmetics and sunscreens (Baker et al. 2014). During the life cycle of NP-containing commercial products, the NPs are inevitably released into the environment and reach marine ecosystems from different sources and via various routes (Matranga and Corsi 2012). Consequently, coastal environments are considered the ultimate sink for NPs (Corsi et al. 2014), as well as for most contaminants. NPs can enter marine systems either directly (through aerial deposition, effluents, dumping and run-off) or indirectly, e.g., via river discharge (Baker et al. 2014). Due to lacking analytical tools to evaluate effective NP concentrations in the aquatic environments, only predicted environmental concentrations (PECs) are available in literature. Estimates of nZnO concentrations in the UK environments range from less than 100 μg/L (in water) to a few mg/Kg (in soil) (Boxall et al. 2007). Gottschalk et al. (2009) reported modelled nZnO concentrations of 10 ng/L in natural surface water and 430 ng/L in treated wastewater in Europe. In another work, nZnO PEC values of 76 μg/L in water and 3194 μg/Kg in soil were reported (Ferreira da Silva et al. 2011). Although nZnO PECs are low, it is important to stress that the environmental levels of these NPs are expected to increase continually given their widespread application (Corsi et al. 2014). Based on various surveys, in 2010, the global production of nZnO was estimated to be up to 1,000 t/year (Piccinno et al. 2012). Most studies of NP toxicity are related to freshwater environments, and relatively little is known regarding the potential biological risks of NPs on marine species (Matranga and Corsi 2012; Muller et al. 2014). Therefore, the need to understand the ecotoxicological impacts of NPs on marine ecosystems is becoming increasingly important (Corsi et al. 2014).

The toxicity of zinc oxide NPs has been reported for different taxa of bacteria, algae, plants, and aquatic and terrestrial invertebrates and vertebrates (Ma et al. 2013). Studies of the potential toxicity of nZnO to marine species are lacking. Nevertheless, the complexity of the seawater environment could further change the behaviour of NPs compared to freshwater ecosystems, greatly affecting aggregation, favouring deposition into sediments, and promoting dissolution processes responsible for the release of two potential toxic species: metal ions and NP aggregates (Keller et al. 2010). In addition, low solubility of zinc oxide in seawater suggests that it will partition in the sea bottom at the sediment/water interface (Gagnè et al. 2015), thus representing a major threat to benthic organisms.

The mechanisms of nZnO toxicity remains under debate as the prevailing role of either the NPs *per se* or the released Zn ions is not clearly demonstrated (Ma et al. 2013). Overall, the action and toxicity of nZnO remain unclear, especially in *in vivo* exposure and at environmentally relevant concentrations. In the sea urchin *Paracentrotus lividus*, nZnO has been shown to determine embryo- and spermiotoxicity (Manzo et al. 2013). In the mussel *Mytilus galloprovincialis*, immunomodulation effects of nZnO have been demonstrated after *in vitro* exposure of haemocytes to concentrations of 1, 5, 10 μg/mL (Ciacci et al. 2012). In another bivalve species, *Crassostrea gigas*, nZnO affected different responses in gills, leading to mitochondrial disruption and oxidative stress (Trevisan et al. 2014). These findings suggested that gills are the initial target of nZnO, even though NPs can accumulate in different tissues, as demonstrated after a long-term exposure (12 weeks) of *Mytilus galloprovincialis* to concentrations ranging from 0.1 to 2 mg/L (Hanna et al. 2013). Significant differences were detected in both the respiration and growth rates of treated animals compared to those of controls (Hanna et al. 2013) and in the energy budget (Muller et al. 2014).

To gain a better insight into the impact of NPs in marine species, in this study, we investigated the *in vivo* effects of nZnO in the clam *Ruditapes philippinarum*. Primarily due to their filter-feeding habit, bivalve molluscs may represent a unique target group for NP toxicity (Moore 2006; Canesi et al. 2012). Indeed, bivalves can filter large volumes of water, process microalgae, bacteria, sediments, particulates, and natural NPs and potentially accumulate different chemicals in their tissues. These organisms have been long recognised as valuable indicators of pollution, and extensive background information is now available on their biological responses to a wide range of both inorganic and organic chemicals (Dame 2011). Recently, in *R. philippinarum*, which is largely used in both coastal biomonitoring and ecotoxicological laboratory studies, the effects of NPs have been investigated in *in vitro* (Marisa et al. 2015) and *in vivo* exposures (Garcia-Negrete et al. 2013).

The aims of the present study were i) to evaluate the effects of a 7-day exposure to nZnO (1 and 10 μg/L) in three clam tissues (haemolymph, gills and digestive gland); ii) to assess the modulation of various biomarkers (antioxidant enzyme activities, levels of damage to molecules, neuromediator enzyme activity, and haemocyte parameters) possibly related to both physico-chemical characteristics of nZnO and two primary mechanisms of nZnO toxicity (e.g., the generation of oxygen species (ROS) and the release of metal ions); iii) to ascertain whether the toxicity of nZnO can be determined by the release of zinc ions in water by also exposing clams to $ZnCl_2$ (10 μg/L); and iv) to assess Zn bioaccumulation in the gills and digestive glands of both nZnO- and $ZnCl_2$-treated clams.

2. Materials and methods

2.1. Nanoparticle characterisation

Nanosized zinc oxide (declared size of <100 nm, percentage of zinc 79.1 - 81.5%, surface area 15 -25 m^2/g) was purchased from Sigma-Aldrich (Milano, Italy). nZnO particles were characterised via a combination of analytical techniques. The mean average diameter and shape of the primary particles were determined using a transmission electron microscope (TEM, FEI Tecnai G12) operated at 100 kV. Digital images were taken using a TVIPS F114 camera, and the size of the particles was measured using IMAQ Vision (National Instrument, USA). X-ray diffraction (XRD) characterisation was performed using a Bruker D8 Advance diffractometer. The analyses were performed in Bragg-Brentano configuration at 30 kV and 30 mA. The mean crystallite size was evaluated using the Sherrer equation. The surface areas and porosities of the nZnO were

characterised via nitrogen adsorption and desorption analyses at 77.35 K using an autosorb computer controlled surface analyser (AUTOSORB-1, Quantachrome). The surface areas were calculated according to the Brunauer-Emmett-Teller (BET) theory. The particle size distribution was measured via laser diffraction (Malvern Mastersizer Hydro 2000, Malvern Instruments, UK). The NPs were dispersed using a small amount of dispersant medium (distilled water) and sonicated for 10 min before analysis. The dispersion was poured into a Hydro 2000 dispersion unit (Malvern, UK) until the obscuration was in range. The analyses were performed in triplicate. The particle size distribution was then defined using the particle refractive index values of water and zinc oxide (1.330 and 1.554, respectively). The particle size distribution was evaluated as d(0.5) and SPAN. The latter is an index of particle size polydispersity and is expressed by the following equation: Span ¼ d(0.9) = d(0.1)/d(0.5), where d(0.9), d(0.1), and d(0.5) are the diameters at 90%, 10% and 50% cumulative volumes, respectively, of the particles.

2.2. Clams

Specimens of *R. philippinarum* were collected from a reference site located within a licensed clam culture area in the southern part of the Lagoon of Venice (Chioggia, Italy). These specimens were then acclimatised in the laboratory for 5 days before exposure to contaminants. Clams were maintained in large aquaria, which contained a sandy bottom and aerated natural seawater (salinity of 35 ± 1 psu, temperature of 16 ± 0.5 °C) and were fed daily with microalgae (*Phaeodactylum sp*.).

2.3. nZnO and $ZnCl_2$ exposure and tissues collection

nZnO stock solution (0.1 g/L) was prepared in Milli-Q water and sonicated at 4 °C using a Braun Labsonic U sonifier at 50% duty cycles for 30 min. $ZnCl_2$ was purchased from Sigma-Aldrich (Milano, Italy), and a stock solution (0.1 g/L) was prepared in Milli-Q water. Clams (35 per tank) were exposed for 7 days to 0 μg/L (control), 1 μg/L, 10 μg/L of nZnO and 10 μg/L of $ZnCl_2$. For each experimental condition tested, two replicate tanks were prepared. The nominal concentrations were chosen similar to the PEC values found in the literature. During exposure, the clams were maintained in glass aquaria (without sediment) containing aerated seawater (1 L per animal) in the same thermo-haline conditions used during the acclimatisation period. A movement pump (Hydor, Koralia nano 900, USA) was positioned in every aquarium (both for control and treated clams) to facilitate the water circulation and to prevent NP sedimentation. Before exposure, the influence of the movement pump on clam behaviour was assessed, and no alterations were observed as clams quickly opened their valves, extended their siphons, and exhibited normal filtering activity.

The seawater was renewed daily, and nZnO, $ZnCl_2$ and microalgae (at an initial concentration of approximately 150,000 cells/L) were supplied in the experimental tanks. Before adding NPs, the stock solution was sonicated, as reported above.

During exposure, the haemolymph, gills and digestive glands were collected after first (T1), third (T3), and last (T7) days of exposure. For each tissue, five pools (4 animals per pool, 2 from each replicate tank) from each experimental condition were prepared. Aliquots of each pooled tissue were frozen in liquid nitrogen and stored at -80 °C until analyses or immediately processed, depending on the various biological responses measured. All assays performed in this study had previously been validated (Matozzo et al. 2012a; Matozzo et al. 2012b; Matozzo et al. 2013; Parolini et al. 2010; Parolini et al. 2013). Unless reported, chemicals were purchased from Sigma-Aldrich, Milano (Italy).

2.4. Gill and digestive gland preparation and biochemical assays

Pooled gills and digestive glands were homogenised at 4 °C using an Ultra-Turrax homogeniser (model T8 basic, IKA) in four volumes of 50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M KCl, 0.5 M sucrose, and Protease Inhibitor Cocktail (P2714, Sigma–Aldrich) and then centrifuged at 12,000 × *g* for 40 min at 4 °C. Supernatants (SN) were collected for the analyses. SN protein concentrations were quantified according to Bradford (1976) using bovine serum albumin (BSA) as the standard.

2.4.1. Superoxide (SOD) dismutase assay

Total SOD activity was measured in the SN of both tissues using the xanthine oxidase/cytochrome c method proposed by Crapo et al. (1978). The cytochrome c reduction by superoxide anion generated by xanthine oxidase/hypoxanthine reaction was detected at 550 nm at room temperature using a Beckman Coulter (DU® Series 730) spectrophotometer. The reaction mixture contained

46.5 mM KH_2PO_4/K_2HPO_4 (pH 8.6), 0.1 mM EDTA, 195 mM hypoxanthine, 16 mM cytochrome c, and 2.5 mU xanthine oxidase. Enzyme activity is expressed as U SOD/mg protein, where one unit of SOD was defined as the amount of sample producing 50% inhibition in the assay conditions.

2.4.2. Catalase (CAT) assay

Gill and digestive gland CAT activity was measured according to the method of Aebi (1984). Decreases in the absorbance of a 50 mM H_2O_2 solution (ε = -0.0436 mM^{-1} cm^{-1}) in 50 mM phosphate buffer (pH 7.8) and 10 µL of tissue SN were continuously recorded at 240 nm for 30 seconds. The results are expressed in U CAT/mg protein, where one unit of CAT was defined as the amount of enzyme that catalysed the dismutation of 1 µmol of H_2O_2/min.

2.4.3. Glutathione S-transferase (GST) activity assay

GST activity was measured spectrophotometrically at 340 nm according to the method described in Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) as substrates. The reaction mixture contained SN, 20 mM CDNB, 0.1 M GSH, and 0.1 M phosphate buffer (pH 6.5). GST activity is expressed as nmol/min/mg protein.

2.4.4. Acetylcholinesterase (AChE) activity

Gill AChE activity was measured according to the method of Ellman et al. (1961), adapted to the microplate reader by Bocquené and Galgani (1998). Gill SN and buffer blanks were incubated for 5 min in microplates at room temperature with 0.75 mM dithiobisnitrobenzoate in 0.1 M Tris-HCl buffer, pH 7.5. The reaction was started by the addition of 3 mM acethylthiocholine. Samples were incubated for 10 min at room temperature. Changes in absorbance at 405 nm were then recorded for 5 min on a microplate reader (2100-C, Optic Ivymen System) at room temperature. The results are expressed as nmol/min/mg protein.

2.4.5. Lipid peroxidation (LPO)

LPO was quantified in both tissues SN using the malondialdehyde (MDA) assay, according to the method of Buege and Aust (1978). Absorbance was read spectrophotometrically at 532 nm, and the results are expressed as nmoles of thiobarbituric reactive substances (TBARS)/mg protein. TBARS, considered as ''MDA-like peroxide products'', were quantified by reference to MDA absorbance (ε = 156 x 10^3 M^{-1} cm^{-1}) (Damiens et al. 2007). The results were not expressed as MDA levels because TBA can react with a range of chemical compounds (Csallany et al. 1984).

2.4.6. Protein carbonyl measurement

Protein carbonyl content (PCC) was measured via the formation of labelled protein hydrazone derivatives, after 2,4-dinitrophenylhydrazide (DNPH) reaction, which were then quantified spectrophotometrically (Mecocci et al. 1999; Dalle-Donne et al. 2003). Briefly, SN was incubated in a solution of 10 mM DNPH in 2 N HCl. Separate blanks for each pool were prepared by adding to SN 2 N HCl without DNPH. The samples were left at room temperature for 1 h in the dark and vortexed every 15 min. After 30% trichloro-acetic acid (TCA) addition, the samples were kept on ice for 15 min and then centrifuged at 10,000 × *g* for 15 min at 4 °C. Pellets were washed three times with an ethanol–ethyl acetate mixture (1:1) to remove the free DNPH and lipid contaminants, resuspended in 6 M guanidine hydrochloride, and kept at 37 °C for 30 min in a water bath. Finally, the carbonyl content was calculated from the SN absorbance at 370 nm via the molar absorption coefficient of 22,000 mol/cm and expressed as nmol/mg protein.

2.4.7. DNA precipitation assay

DNA strand breaks were quantified using a fluorescence technique adapted from the alkaline precipitation assay (Olive 1988). Samples of both gills and digestive gland were weighed (Mettler Toledo, XS105 Dual Range analytical balance, 0.01 mg readability) before tissues preparation (see above), and the wet weight was recorded. Homogenate sample was mixed with 2% SDS containing 10 mM EDTA, 10 mM Tris-base, and 40 mM NaOH for 1 min. KCl (0.12 M) was added, and the solution heated at 60 °C for 10 min, mixed via inversion, and cooled at 4 °C for 30 min. This mixture was then centrifuged at 8,000 × *g* for 5 min at 4 °C. SN was added to a bisbenzimide 33258 (Hoechst) dye solution (1 µg/mL diluted with buffer containing 0.4 M NaCl, 4 mM sodium cholate, and 0.1 M Tris-acetate, pH 8.5–9; 1:6 v/v ratio) in a quartz cuvette and mixed for 5 min. Fluorescence was measured using a Varian Cary Eclipse fluorescence

spectrophotometer at 360 nm (excitation) and 450 nm (emission) against blanks containing identical constituents, with homogenisation buffer replacing the homogenate. Salmon sperm genomic DNA standards were added for DNA calibration, and the results are expressed as µg/g wet weight.

2.5. Zinc bioaccumulation in gills and digestive gland

At the end of the exposure (T7), 4 pools of gills and digestive glands per experimental condition (6 animals each) were collected to quantify zinc bioaccumulation. Tissue samples were freeze–dried, and approximately 150 mg were weighed and digested in TFM vessels with 4 mL of 69% nitric acid and 2 mL of 30% hydrogen peroxide. Digestion was performed in a Milestone MLS 1200 MEGA microwave oven. The heating programme consisted of five stages (2 min, 250 W - 2 min, 0 W - 6 min, 250 W - 5 min, 400 W and 5 min, 650 W). After cooling, samples were transferred into graduated flasks and diluted to 25 mL with Milli-Q water. The sample solutions were analysed via inductively coupled plasma optic emission spectroscopy (ICP-OES) using a Thermo Fischer Scientific iCAP 6300 DUO. Five calibration solutions (0, 0.5, 1, 3 and 6 ppm of Zn) were prepared by conventional dilution of Carlo Erba 1000 µg/mL mono-elemental standard solution of the analyte as nitrate. The same amount of reagents used for the digestion procedure was added to each calibration solution. Measurements were made at Zn 202.55 nm, and each sample was analysed in five replicas. The results are expressed as µg Zn/g dry weight. The detection limit of Zn was 0.9 µg/L.

2.6. Haemolymph parameters

2.6.1. Total haemocyte count (THC) and haemocyte diameter and volume

THC and haemocyte diameter and volume were determined using a Model Z2 Coulter Counter electronic particle counter/size analyser (Coulter Corporation, FL, USA). THC is expressed as the number of haemocytes ($x10^6$)/mL of haemolymph. Haemocyte diameter and volume are expressed in µm and in femtolitres (fL), respectively.

2.6.2. Haemocyte proliferation

Haemocyte proliferation was evaluated using a colorimetric method and measured using a commercial kit (Cell proliferation Kit II, Roche). The assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye in metabolically active (viable) cells. This assay has been validated in our previous studies (Matozzo et al. 2012a,b) according to the evidence of cell division in circulating haemocytes of Manila clams (Matozzo et al. 2008). The data were normalised to the THC values recorded for the clams from each experimental condition and expressed as the optical density (OD) at 450 nm.

2.6.3. Cytotoxicity assay

Cytotoxicity was evaluated using a colorimetric assay based on the measurement of lactate dehydrogenase (LDH) activity in cell-free haemolymph. LDH is a stable cytoplasmic enzyme that is released by damaged cells (at cell membrane level) into the haemolymph. A commercial kit (Cytotoxicity Detection Kit, Roche) was used to assess cell damage. The results, normalised to THC values, are expressed as the optical density (OD) at 490 nm.

2.6.4. DNA fragmentation

A photometric enzymatic immunoassay was used for the quantitative determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after induced cell death (apoptosis). One of the documented events of apoptosis is DNA fragmentation due to the activation of endogenous endonucleases. Endonucleases cleave double-stranded DNA at the most accessible internucleosomal linker region, generating mono- and oligonucleosomes. The DNA fragments of apoptotic haemocytes were determined using a commercial kit (Cell Death Detection ELISA, Roche) and according to the manufacturer's instructions. The results, normalised to THC values, were expressed as the optical density (OD) at 405 nm.

2.6.5. Single cell gel electrophoresis (SCGE) assay

The SCGE assay was performed using the alkaline (pH>13) version of the assay developed by Singh et al. (1988). Thirty microlitres of each haemolymph pool were mixed with an aliquot of low melting agarose (LMA - 0.7%) and added to a coated slide (previously dipped in 1% normal melting agarose). The slides were covered with cover-glass and placed at 4 °C for 40 min until the

agarose layer solidified. A third agarose (LMA) layer was added to the slides in the same way. After agarose solidification, the slides were placed in a lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 8 mM Tris–HCl, 1% Triton X-100 and 10% DMSO, pH 10) in a Coplin jar at 4 °C in the dark for at least one hour. Alkaline DNA unwinding was performed for 30 min in a gel electrophoresis chamber containing freshly prepared buffer (1 mM Na_2EDTA, 300 mM NaOH, pH 13) and then in an ice-water bath (4 °C). Electrophoresis was then performed at 0.78 V/cm and 300 mA for 25 min. The slides were washed after electrophoresis in a neutralisation buffer (0.4 M Tris - HCl, pH 7.5) and fixed in absolute ethanol. After staining with DAPI (4,6-diamidino-2-phenylindole) DNA dye, a coverslip was placed over the slides. Imaging was performed using a fluorescence microscope (Leica 5000B, Germany) equipped with an FITC filter (I3, excitation BP 450-490, emission LP 515) at 10× magnification. All steps were performed in the dark to minimise additional UV-induced DNA damage. Positive controls were performed with H_2O_2 to check the effectiveness of the electrophoresis conditions. One hundred cells per slide for a total of 500 cells per condition were analysed using an image analysis system (Comet Score®). The ratio between the migration length and the diameter of the comet head (LDR) was chosen to represent DNA damage. According to Binelli et al. (2009), the LDR value was coupled to the percentage of tail DNA obtained by the Comet Score®.

2.6.6. Micronucleus (MN) test

The MN test was performed according to the method of Pavlica et al. (2000). One hundred fifty microlitres of each haemolymph pool were placed on a slide and left for 15 min in a humidified chamber at room temperature to allow the haemocytes to adhere. Haemocytes on the slides were subsequently fixed in a glutaraldehyde (25% in 0.45 µm filtered seawater) solution for 5 min. After rinsing with PBS, the slides were stained with Hoechst dye solution (1 µg/mL) for 5 min and then washed and mounted in glycerol–McIlvaine buffer (1:1). The slides were kept in the dark at 4 °C prior to examination under the microscope. All samples were coded and blindly evaluated always by only one observer. Using a pre-arranged pathway to reduce the observer's subjectivity, slides were scored under the fluorescent microscope Leica 5000B equipped with a submerged lens at 100× magnification. Four hundred cells were counted for each slide for a total of 2000 cells/treatment. Only intact and non-overlapping haemocytes were considered. Micronuclei were identified according to the criteria proposed by Kirsch-Volders et al. (2000), and the MN frequency (MN‰) was calculated.

2.7. Statistical analysis

The normal distribution (Shapiro-Wilk test) and the homogeneity of the variance (Bartlett test) of the data were assessed. The data were statistically compared using a two-way ANOVA test, with exposure time and contaminant concentrations as variables and biomarkers as cases. The ANOVA was followed by a Fischer LSD post-hoc test to evaluate significant differences (*$p<0.05$; **$p<0.01$, ***$p<0.001$) between treated samples and related controls (time to time) and among exposures. The data regarding the bioaccumulation of zinc in gills and digestive gland were statistically compared using a one-way ANOVA test followed by Tukey's HSD test.

The results are expressed as the mean ± standard deviation. The STATISTICA 10 software package (StatSoft, Tulsa, OK) was used for statistical analyses.

3. Results

Throughout the experiment, animal behaviour was considered normal by checking visible siphons pumping in and out and assessing their response to mechanical stimuli (rapid siphon retraction and shell valve closure). No clam mortality was observed at all the concentrations tested.

3.1. Nanoparticle characterisation

A TEM image and a size histogram of NPs are shown in Fig. 1A and 1B, respectively. The nZnO mean diameter, obtained from 2000 NP measurements, was approximately 52 nm ± 22 (s.d.), in agreement with the declared range size. The XRD patterns (Fig. 1C) revealed the zincite (hexagonal) crystal structure. The average crystallite diameter of nZnO, evaluated according to the Sherrer equation,

was estimated as 40 nm. The NP specific surface area estimated via BET was 9 m^2/g with no surface porosity. nZnO dispersed in water showed a d(0.5) of 9.791 µm and 3.423 of SPAN (Fig. 1D), as determined via laser diffraction.

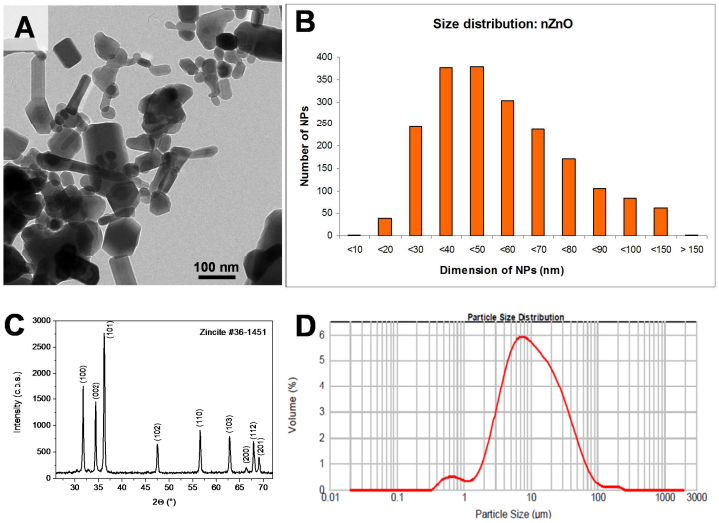

Fig. 1. A: TEM image of nZnO. B: size (diameter) distribution histogram of nZnO estimated via TEM. C: XRD pattern of the ZnO powder. The crystalline phase is Zincite (JCPDS #36-1451). D: size distribution of nZnO estimated via laser diffraction.

3.2. Gill and digestive gland assays

The two-way ANOVA results for the various parameters measured in clam gills and digestive glands are shown in Table 1.

Table 1. Two-way ANOVA results for the biochemical responses measured in the gills and the digestive gland of *R. philippinarum* throughout the exposure to nZnO (1, 10 µg/L) and $ZnCl_2$ (10 µg/L). Statistically significant effects of the variables "contaminants", "time", and "contaminants/time" interaction are indicated in bold.

		Gills					Digestive gland				
	Source of variation	SS	*df*	MS	F	p	SS	*df*	MS	F	p
SOD activity	Contaminants	**6202.5**	**3**	**2067.5**	**8.73**	**0.000**	**932.9**	**3**	**311.0**	**3.66**	**0.050**
	Time exposure	**4912.3**	**2**	**2456.2**	**10.37**	**0.000**	526.6	2	263.3	2.25	0.115
	Contaminants*time	1969.2	6	328.2	1.38	0.239	424.1	6	70.7	0.60	0.723
CAT activity	Contaminants	**739.92**	**3**	**246.64**	**3.16**	**0.032**	**614.34**	**3**	**204.7**	**4.49**	**0.007**
	Time	425.35	2	212.67	2.73	0.075	**4553.07**	**2**	**2276.5**	**49.91**	**0.000**
	Contaminants*time	621.11	6	103.52	1.33	0.262	**978.64**	**6**	**163.1**	**3.57**	**0.005**
GST activity	Contaminants	813366	3	27112	1.04	0.381	371291	3	12376	0.53	0.662
	Time	1429764	2	71488	2.75	0.073	**525358**	**2**	**26267**	**11.28**	**0.000**
	Contaminants*time	1232239	6	20537	0.79	0.581	107982	6	17997	0.77	0.594
AChE activity	Contaminants	0.43918	3	0.1463	1.37	0.262	/	/	/		/
	Time	**0.91289**	**2**	**0.4564**	**4.27**	**0.019**	/	/	/		/
	Contaminants*time	**1.57196**	**6**	**0.2619**	**2.45**	**0.037**	/	/	/		/
LPO	Contaminants	**0.01012**	**3**	**0.0033**	**4.304**	**0.009**	**0.13012**	**3**	**0.04**	**4.56**	**0.006**
	Time	0.00088	2	0.0004	0.56	0.574	0.04116	2	0.02	2.16	0.125
	Contaminants*time	**0.01237**	**6**	**0.0020**	**2.63**	**0.027**	0.02685	6	0.00	0.47	0.826
PCC	Contaminants	**148.97**	**3**	**49.66**	**4.28**	**0.009**	58.88	3	19.6	0.87	0.458
	Time	**1343.81**	**2**	**671.91**	**57.9**	**0.000**	**1118.23**	**2**	**559.1**	**25.02**	**0.000**
	Contaminants*time	14.52	6	2.42	0.20	0.972	171.21	6	28.5	1.27	0.285
DNA damage	Contaminants	0.1568	3	0.0523	0.09	0.963	1.2427	3	0.41	0.42	0.732
	Time	0.0001	2	0.0007	0.12	0.542	1.0579	4	0.39	0.41	0.800
	Contaminants*time	1.6769	6	0.2795	0.50	0.804	1.1399	6	0.19	0.19	0.976

A significant concentration-dependent ($p<0.001$) and time-dependent ($p<0.001$) increase in the activity of SOD was found in the gills: $ZnCl_2$ treated clams exhibited significantly higher values of SOD activity with respect to controls from T3, whereas nZnO- (10 µg/L) treated clams exhibited significantly higher values of SOD activity only at the end of the exposure (Fig. 2A).

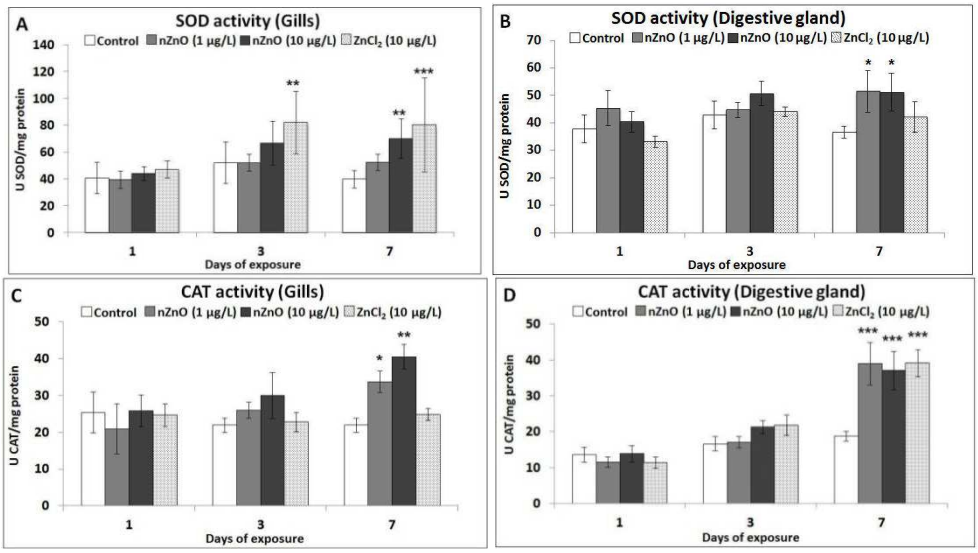

Fig. 2. SOD activity (A - B) expressed as U SOD/mg protein and CAT activity (C - D) expressed as U CAT/mg protein in *R. philippinarum* after 1, 3 and 7 days of exposure to nZnO (1, 10 µg/L) and $ZnCl_2$ (10 µg/L). The values are reported as the means ± SD (standard deviation); n= 5 pools. Asterisks denote significant differences compared to controls at the same time of exposure: *p<0.05, **p<0.01, *** p<0.001.

SOD activity was significantly affected by the contaminants in the digestive gland (p=0.050); a significant increase was found at T7 in clams exposed to both concentration of NPs compared to control (Fig. 2B).

CAT activity was affected significantly in both tissues due to exposure to contaminants (p=0.032 in gills, p=0.007 in digestive gland). In the gills, the CAT activity did not change under $ZnCl_2$ exposure, whereas an increase was observed in clams exposed to 1 and 10 µg/L of nZnO at T7, when a significantly higher enzyme activity (p=0.006) was also detected at the highest nZnO concentration with respect to the Zn^{2+} treated clams (Fig. 2C). In the digestive gland, a significant increase in CAT activity was found under both nZnO and $ZnCl_2$ exposure (time and concentration/time interaction: p<0.001 and p=0.005, respectively). At T7, the enzyme activity was significantly higher in all treatments with respect to control (Fig. 2D). The GST activity exhibited a not significant decreasing trend in the digestive gland with increasing exposure time (two-way ANOVA, p=0.059, Fig. 3A), whereas no significant variation was found in the gills.

In the gills, AChE activity was significantly affected by exposure time and concentration/time interaction (p=0.019, p=0.037, respectively). Pair-wise comparisons highlighted significant differences only at T1 between controls and clams exposed to the lower nZnO concentration. Although the AChE activity values were not significantly different in control and treated clams after 7 days of exposure, a similar decreasing trend was observed in the nZnO- and $ZnCl_2$-treated clams (Fig. 3B).

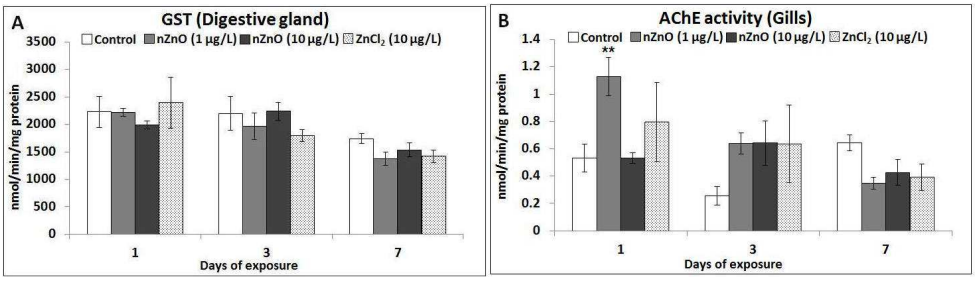

Fig. 3. GST activity in the digestive gland (A) and AchE activity in the gills (B) expressed as nmol/min/mg protein of *R. philippinarum* after 1, 3 and 7 days of exposure to nZnO (1, 10 µg/L) and $ZnCl_2$ (10 µg/L). The values are reported as the means ± SD; n= 5 pools. Asterisks denote significant differences compared to controls at the same time of exposure: **p<0.01.

Lipid peroxidation was significantly influenced by the contaminants in both tissues (p=0.009 in gills, p=0.006 in digestive glands) and by concentration/time interaction in gills only (p=0.027). In the gills, the TBARS levels increased at lower nZnO concentration only during the first phase of the exposure (T1, T3), but similar values were found in all conditions tested at T7 (Fig. 4A). In the digestive gland, the TBARS values measured in the treated clams were always lower than in the controls, with a significant reduction with respect to controls in the $ZnCl_2$-exposed clams at T3 (Fig. 4B). PCC values were significantly affected by the exposure time in both tissues (p<0.001) and by the contaminants in the gills. The only significant differences in the pair-wise comparisons were between nZnO- (10 µg/L) and $ZnCl_2$-treated clams at T3 in the gills (Fig. 4C) and between controls and the $ZnCl_2$-treated clams at T1 in the digestive gland (Fig. 4D).

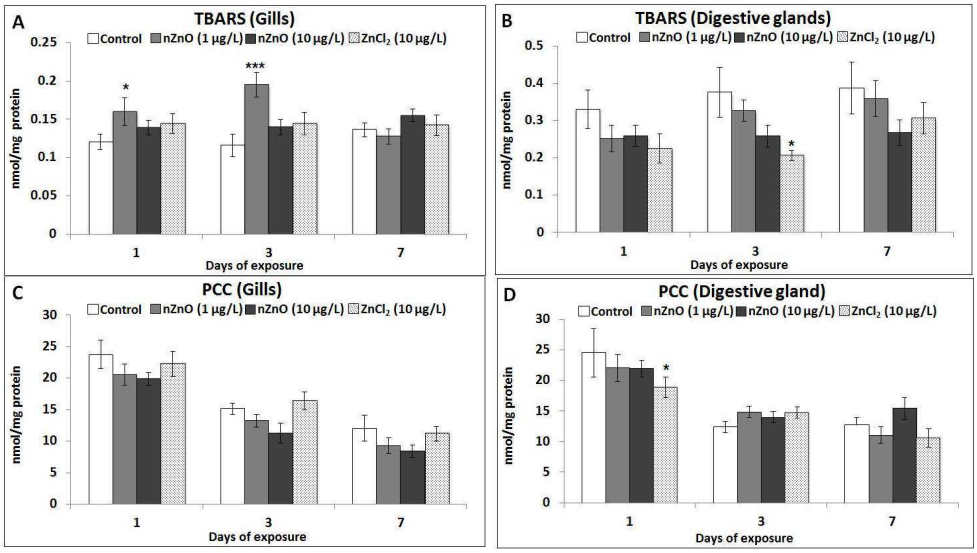

Fig. 4. TBARS (A - B) and PCC (C - D) levels expressed as nmol/mg protein in *R. philippinarum* after 1, 3 and 7 days of exposure to nZnO (1, 10 µg/L) and $ZnCl_2$ (10 µg/L). The values are expressed as the means ± SD; n= 5 pools. Asterisks denote significant differences compared to controls at the same time of exposure: *p<0.05, *** p<0.001.

DNA damage detected via the DNA precipitation assay was not significantly different in the gills and digestive glands of the control and treated clams.

3.2.1. Zinc bioaccumulation in gills and digestive gland

The total zinc content in the gills and digestive gland of the clams exposed for 7 days to nZnO (1-10 µg/L) and $ZnCl_2$ is reported in Fig. 5A, B. The results demonstrated significant accumulation of Zn in both the gills (p=0.020) and the digestive gland (p=0.002) in all treatments compared to controls. In both tissues, no difference was found among the treated clams.

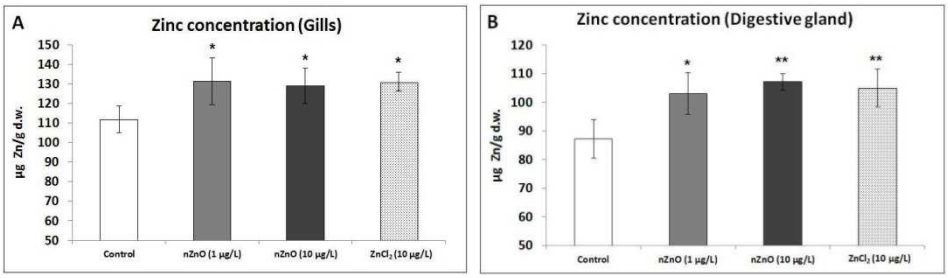

Fig. 5. Levels of zinc expressed as µg Zn/g dry weight in the gills (A) and the digestive gland (B) of *R. philippinarum* after 7 days of exposure to nZnO (1, 10 µg/L) and $ZnCl_2$ (10 µg/L). The values are expressed as the means ± SD; n= 4 pools. Asterisks denote significant differences compared to controls: *p<0.05, **p<0.01.

3.3. Haemolymph parameters

The two-way ANOVA results for the various parameters measured in the haemolymph are shown in Table 2. Only haemocyte proliferation and the SCGE assay showed significant variations among treatments, whereas no significant effect of treatment, time of exposure and their interaction was detected in THC, haemocyte diameter and volume, LDH activity, DNA fragmentation and MN frequency values.

Table 2. Two-way ANOVA results for the biochemical and cellular responses measured in the haemolymph of *R. philippinarum* throughout the exposure to nZnO (1, 10 µg/L) and $ZnCl_2$ (10 µg/L). Statistically significant effects of the variables "contaminants", "time" and "contaminants/time" interaction are indicated in bold.

		Haemolymph				
	Source of variation	**SS**	***df***	**MS**	**F**	**p**
THC	Contaminants	6,767138E+13	3	2,255713E+13	2.41	0.077
	Time	4,690825E+13	2	2,345412E+13	2.51	0.091
	Contaminants*time	4,237941E+13	6	7,063235E+12	0.75	0.606
Haemocyte Diameter	Contaminants	0.2458524	3	0.081950817	0.58	0.633
	Time	0.2634223	2	0.13171115	0.93	0.403
	Contaminants*time	0.4746785	6	0.079113083	0.56	0.762
Haemocyte volume	Contaminants	1110.119	3	370.0396	0.57	0.636
	Time	2530.507	2	1265.254	1.95	0.152
	Contaminants*time	2846.538	6	474.4231	0.73	0.625
Haemocyte proliferation	Contaminants	**2.508659**	**3**	**0.83622**	**11.59**	**0.000**
	Time	0.398485	2	0.199242	2.76	0.073
	Contaminants*time	0.288901	6	0.04815	0.66	0.676
Cytotoxicity	Contaminants	0.000524	3	0.000175	0.31	0.814
	Time	0.000696	2	0.000348	0.62	0.375
	Contaminants*time	0.004695	6	0.000782	1.41	0.229
DNA fragmentation	Contaminants	0.07618	3	0.025393	2.35	0.083
	Time	0.033458	2	0.016729	2.15	0.076
	Contaminants*time	0.066264	6	0.011044	1.02	0.421
LDR value (SCGE assay)	Contaminants	**1.0171**	**3**	**0.3390**	**16.29**	**0.0000**
	Time	**0.1885**	**2**	**0.0942**	**4.52**	**0.0147**
	Contaminants*time	**0.5368**	**6**	**0.0895**	**4.29**	**0.0011**
% of tail DNA (SCGE assay)	Contaminants	**392.1812**	**3**	**130.7271**	**13.22**	**0.000**
	Time	**82.15225**	**2**	**41.07613**	**4.15**	**0.020**
	Contaminants/*ime	**167.1554**	**6**	**27.85923**	**2.81**	**0.017**
MN frequency	Contaminants	27.083	3	9.028	0.85	0.250
	Time	25.215	2	13.810	0.56	0.658
	Contaminants*time	22.396	6	3.733	0.60	0.658

Haemocyte proliferation was significantly affected by the exposure to the contaminants ($p<0.001$). At each exposure time, haemocyte proliferation increased with increasing nZnO concentration and peaked at the highest value in the $ZnCl_2$-treated clams. Differences with respect to controls were significant from T1 in the clams exposed to $ZnCl_2$ but significant at T7 only in the clams exposed to nZnO (10 µg/L).
Moreover, at T3, haemocyte proliferation was significantly higher ($p=0.028$) in the clams exposed to $ZnCl_2$ than in the nZnO- (10 µg/L) treated clams (Fig. 6A).
Both SCGE assay endpoints (LDR value and the percentage of DNA in the comet tail) highlighted significant primary genetic damage due to contaminants in the clam haemocytes ($p<0.001$), a significant effect of exposure time ($p=0.014$, $p=0.020$, respectively), and a significant contaminants/exposure time interaction ($p=0.001$, $p=0.017$, respectively). In particular, the LDR values significantly increased in the presence of nZnO (10 µg/L) and $ZnCl_2$ from T3, reaching maximum values at T7 (Fig. 6B). A statistically significant increase in the percentage of tail DNA was observed just after the first day of exposure at the lowest nZnO concentration, whereas at T3 and T7 genetic damage significantly increased in clams exposed to the highest nZnO concentration and to $ZnCl_2$ (Fig. 6C). Moreover, at T3, the percentage of tail DNA was significantly higher in the nZnO- (10 µg/L) treated clams with respect to the $ZnCl_2$-treated clams ($p=0.034$).

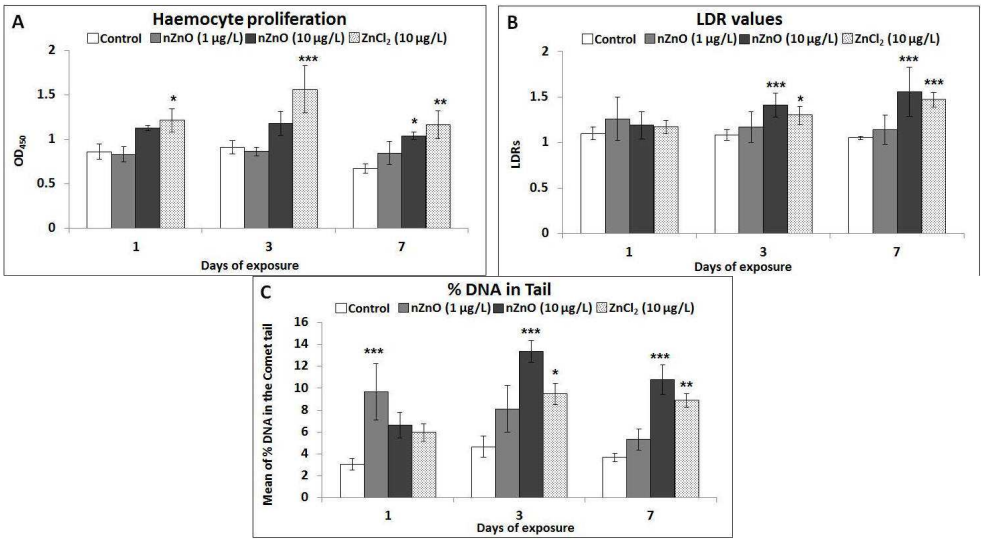

Fig. 6. Haemocyte proliferation (A) expressed as OD_{450} and SCGE results expressed as length/diameter ratio (B) and the mean percentage of tail DNA (C) in *R. philippinarum* after 1, 3 and 7 days of exposure to nZnO (1, 10 µg/L) and $ZnCl_2$ (10 µg/L). The values are expressed as the means ± SD; n=5 pools. Asterisks denote significant differences compared to controls at the same time of exposure: *$p<0.05$, **$p<0.01$, *** $p<0.001$.

4. Discussion

The Manila clams are often used to monitor marine coastal pollution and to understand the potential effects determined by various groups of pollutants in laboratory experiments (Blasco and Puppo 1999; Ji et al. 2006; Matozzo et al. 2010; Milan et al. 2013; Marisa et al. 2015). Due to its prevalence in lagoon and coastal areas which are characterised by many anthropogenic impacts, *R. philippinarum* is considered a model species to assess environmental

contamination. The use in ecotoxicological studies is also supported by the relevant economic value of the species that is both fished and farmed (Melaku Canu et al. 2011; Boscolo Brusà et al. 2013). As an infaunal filter-feeder, the Manila clam is particularly exposed to the impact of contaminants having in sediments their ultimate sink, such as NPs (Corsi et al., 2014). Considering the overall NP behaviour and characteristics, nZnO could more deposit in the sea bottom at the sediment-water interface; bioturbation and resuspension of sediments could promote particle exchange between the sediment and the water column (Keller et al. 2010; Majedi et al. 2013; Rocha et al. 2015).

Oberdörster et al. (2005) reported that the primary elements of NP toxicity-screening strategies are physicochemical characterisation and the elucidation of biological effects in *in vivo* studies.

The bioavailability, uptake, accumulation and toxicity of NPs in aquatic organisms depend on several physico-chemical properties, such as particle size/shape, surface charge and structure, particle chemistry, solubility, and aggregation state (Ma et al. 2013; Baker et al. 2014). In this study, as shown via the TEM analysis, the nZnO had a mean diameter of 52 nm, in agreement with the mean crystallite size evaluated via XRD. The NPs were monocrystalline with a hexagonal structure, and a range of various morphologies was present, including spherical, elongated and faceted particles. The specific surface area evaluated via BET was also in agreement with the specifications for nZnO. nZnO were highly hydrophobic, thus aggregating strongly in aqueous solutions, as confirmed by our observations.

Currently, no data are available regarding NP pollution in the marine environment, as a consequence of the difficulty to detect and quantify NPs in complex matrices. Only the PECs can be used to understand the potential concentration of NPs in seawater (Ma et al. 2013). In addition, information on the ecotoxicological effects of nZnO has been very limited across all taxa, especially compared to $nTiO_2$, which possess many similar properties to nZnO and have been the most extensively studied among metal oxide NPs (Ma et al. 2013; Rocha et al. 2015). Most of the published ecotoxicological studies were conducted with high NP concentrations that are unrealistic from an environmental point of view compared to PECs (Mouneyrac et al. 2014). Instead, in this study, we chose lower concentrations (1 and 10 μg/L) of nZnO that were in the range of PEC values. To assess the effects of nZnO on the clam *R. philippinarum*, a multi-biomarker approach was used. Biomarkers have been shown to be sensitive and early warning indicators of exposure to pollutants and to provide information regarding alterations in the organism physiology and biochemical mechanisms of a contaminant's action (Mouneyrac et al. 2014).

The battery of biomarkers was selected based on i) the characteristics of nZnO, ii) the effects of nZnO in other species, and iii) the potential mechanism of action of NPs. To gain a better insight on the effects of nZnO in *R. philippinarum*, we investigated various biological responses in three tissues (gills, digestive glands and haemolymph). Indeed, in bivalves, NPs are known to be filtered by the gills, accumulate in the digestive gland, and transferred to the haemolymph through the epithelium of the digestive gland tubules (Moore et al. 2009; Rocha et al. 2015).

In literature, the primary general mode of action of NPs is the direct and indirect induction of oxidative stress, when antioxidant defences are overwhelmed by reactive oxygen species (ROS) produced in organism tissues (Al-Subiai et al.

2012; Rocha et al. 2015). In nZnO-exposed *Escherichia coli,* ROS production results in membrane damage, thus highlighting antibacterial properties of the NPs (Zhang et al. 2007). Normally, antioxidants, such as SOD and CAT enzymes, allow cells to protect themselves from damage due to ROS (Farber 1994; White 2000). In this study, SOD activity exhibited a different behaviour in the two tissues. Interestingly, in both tissues, SOD activity increased with increasing time of exposure, but it was more responsive to zinc ions in the gills and to nZnO in the digestive gland. An increase in this enzyme activity was also detected in the digestive gland of the freshwater mussel *Unio tumidus* after 14 days of nZnO (3.1 µM) exposure (Falfushynska et al. 2015). In another study, SOD activity of the earthworm *Eisenia fetida* exhibited a decreasing trend, with a response significantly lower than that of controls at the highest nZnO concentration (0.5 g/Kg) (Hu et al. 2010). Conversely, in the freshwater snail *Biomphalaria alexandrina,* SOD activity varied depending on the concentrations of nZnO (7 and 35 µg/mL). At lower concentration, the activity decreased in the soft tissues and haemolymph, whereas at higher concentration, the activity increased (Fahmy et al. 2014).

Many studies have found varying responses of CAT to increased metal concentrations, with some organisms exhibiting increased activity, others exhibiting depressed activity, and still others showing no catalase response (Regoli et al. 1998). In the present study, we observed a significant increase in CAT activity in both the gills and the digestive gland of nZnO-treated clams, but a similar increase was found only in the digestive gland of clams exposed to zinc ions. The obtained results are in accordance with the findings of Ali et al. (2012) that showed an increase in CAT activity in the freshwater snail *Lymnaea luteola* exposed to nZnO (10, 21, 32 µg/mL) for 96 hours, even though the concentrations of nZnO tested in that study were thousands of times higher than the concentration considered here. A modulation of CAT activity was also shown in *E. fetida*: the enzyme activity increased at low concentrations (0.1 – 0.5 g/Kg) or decreased at high concentration (5 g/Kg) (Hu et al. 2010). Similar results were found in *B. alexandrina,* after 3 weeks of exposure to nZnO with a modulation of CAT activity in both the haemolymph and soft tissues, despite the different patterns of variation, depending on concentration and tissue (Fahamy et al. 2014). Although CAT response was differently modulated, in those studies and in the present study the enzyme activity was always significantly affected in nZnO exposed animals with respect to controls.

GST is an enzyme that participates in the detoxification process due to conjugation reaction between GSH and xenobiotics (Cummins et al. 2011). Thiol compounds, such as reduced and oxidised GSH, represent the initial protective substances against heavy-metal ions and other pollutants. Inhibition of GST activity occurs either through direct action of metal on the enzyme or indirectly via the production of ROS that interact with the enzyme, depletion of its substrate (GSH) and/or down regulation of GST genes through different mechanisms (Roling and Baldwin 2006). In this study, primarily in the digestive gland, GST activity exhibited a decreasing trend under nZnO and $ZnCl_2$ exposure. This effect may be related to the depletion of glutathione following increased antioxidant response. A similar variation pattern was observed by Ali et al. (2012) and Fahmy et al. (2014) that reported significantly reduced GST activity in two freshwater

snails species, *L. luteola* and *B. alexandrina*, respectively, following zinc oxide NP exposure.

Overall, the modulation of CAT, SOD, and GST activities highlighted oxidative stress conditions progressively increasing throughout the experiment in clams exposed to nZnO. The consequences of ROS production depend on the intensity of the stress and on physicochemical conditions in the cell. It has been generally accepted that active oxygen produced under elevated stress could cause different damage to molecules (Valko et al. 2004; Fahmy et al. 2014). The measurement of the end-products of lipid peroxidation (TBARS content), the content of carbonyl groups of proteins and strand breaks of DNA could provide an idea of the potential effect of oxidative injury caused to nZnO and Zn^{2+}. Despite evidence of oxidative stress in contaminant-exposed clams, at the end of the exposure damage to lipids, proteins and DNA were not detected compared to controls, either in the gills or in the digestive gland. In the gills, the TBARS level increased at T1 and T3 but dropped to control values at T7, likely a result of steady increases in CAT activity. The results obtained are in good agreement with those from previous studies in which increased levels of protection from oxidative stress led to decreased levels of damage (Rodriguez-Ariza et al. 1993; Falfushinska et al. 2015). As in our study, the results obtained in *U. tumidus* exposed to nZnO demonstrated no lipid or DNA damage and even a decrease in protein damage, expressed as protein carbonyl concentration (Falfushinska et al. 2015). In other studies, nZnO acted in different ways depending on the species, the time of exposure and the concentrations of NPs. Ali et al. (2012) and Fahmy et al. (2014) found LPO increases in freshwater snail species exposed to nZnO. Similarly, in the digestive gland of the freshwater mussel *Elliptio complanata*, an increase in lipid damage was shown after 21 days of exposure to 2 µg/L of nZnO (Gagnè et al. 2013). Interestingly, significant increases in the MDA levels and protein damage were observed in the gills of nZnO-treated oysters, *Crassostrea virginica*, after 48 hours of exposure (4 mg/L), whereas no effects were detected for other classical antioxidant-related parameters (Trevisan et al. 2014). Notably, most exposures resulting in increased oxidative damage were performed using NP concentrations higher than that considered in our study (Ali et al. 2012; Fahmy et al. 2014; Trevisan et al. 2014) or using longer exposure times (Gagnè et al. 2013; Fahmy et al. 2014).

AChE is an enzyme essential for the degradation of the neurotransmitter acetylcholine in cholinergic synapses and thus responsible for the correct transmission of nerve impulses in both vertebrates and invertebrates. Due to the role of AChE, its activity is widely used as a biomarker of neurotoxicity. Inhibition of AChE activity occurs after exposure to some contaminants, such as organophosphorus and carbamate insecticides, metals, detergents and other organic pollutants (Pope 1999; Frasco et al. 2005). This biomarker was also used to investigate potential NP neurotoxicity in bivalves, taking into consideration that the metal oxide NPs may release metal ions into water. Zinc is known to inhibit the activity of AChE (Frasco et al. 2005), and zinc ions can be released into aquatic compartments through nZnO dissolution (David et al. 2012). However, the measurement of AChE activity in NP ecotoxicological studies remains controversial. Indeed, exposure to CuO, ZnO and Ag NPs did not change AChE activity in the clam *Scrobicularia plana* (Buffet et al. 2011, 2012, 2013). Conversely, Au NPs increased AChE activity in the same species, and this finding

was associated with a phenomenon of overcompensation related to increased levels of acetylcholine (Pan et al. 2012). In addition, no obvious neurotoxic effects were observed in *Mytilus* sp. after *in vitro* exposure to nano-Fe (Kadar et al. 2010). Only Gomes et al. (2011) reported a significant inhibition of this enzyme in *M. galloprovincialis* exposed to CuO NPs. In our study, only at the end of our exposure, a slight not significant AChE inhibition was observed in the nZnO- and $ZnCl_2$-treated clams, suggesting the possible role of zinc ion release in the mode of action of nZnO.

Mainly due to their feeding habits, bivalve molluscs accumulate NPs and are a target of their toxicity. As filter-feeders, they can remove NPs from the water column, both as single particles and aggregates (Rocha et al. 2015). The accumulation of NPs in bivalves is a cause for environmental concern, considering the potential toxic impacts, trophic transfer, and even exposure to humans (Hanna et al. 2013). It is ascertained in various aquatic species that nZnO are accumulated in tissues. Marine mussels, *M. galloprovincialis,* exposed to nZnO (0.1–2 mg/L) for 12 weeks, exhibited strong Zn accumulation in both somatic tissues and gonads, the extent to which was dependent on mussel size and nZnO concentration (Hanna et al. 2013). In *E. complanata,* Zn was accumulated after 21 days of exposure to an environmentally realistic concentration of nZnO (2 µg/L) (Gagné et al. 2013). Conversely, environmentally relevant concentrations of nZnO and the equivalent levels of free Zn^{2+} did not lead to Zn accumulation in the digestive gland of *U. tumidus* (Falfushynska et al. 2015). In *C. virginica,* a chemical analysis of gills and digestive gland indicated that nZnO were initially incorporated by the gills, although they were preferably accumulated in the digestive gland after 96 h (Trevisan et al. 2014). The potential for trophic transfer and biomagnification of the accumulated Zn is highlighted by the findings of Blackmore and Wang (2004) for the rock oyster, *Saccostrea cucullata*. Soluble and insoluble forms of Zn accumulated in the bivalves and were then transferred to the predatory whelk, *Thais clavigera*, with a trophic transfer factor >1. However, for all studies evaluating nZnO accumulation, it remains unknown whether the Zn that accumulated in bivalve tissues was present as an ion released from NPs or as NPs only.

In our study, control clams showed similar Zn contents in gills and digestive gland, with values in the range of those previously reported by Irato et al. (2003) in *R. philippinarum* from the same lagoon area. Compared to controls, clams exposed to nZnO and $ZnCl_2$ revealed a significant accumulation of zinc in both tissues. The amount of accumulated Zn was similar for the various treatments and for the two tissues. The absence of a clear relationship between increasing exposure concentrations and Zn content in the 1 and 10 µg/L nZnO-treated clams could be due to the reduced exposure time and the low concentrations tested. In this regard, it must be considered that Hanna et al. (2013) did not find differences in Zn accumulation of large-size mussels exposed for a longer period (12 weeks) to higher nZnO concentrations (0.1 and 0.5 mg/L). In this study, no difference in Zn accumulation was detected in the clams exposed at the highest concentration of nZnO and at the same concentration of Zn^{2+} ions. Similar results were obtained in two marine invertebrates, the clam *S. plana* and the polychaete *Hediste diversicolor* (Mounyarc et al. 2014). Most studies have suggested that the accumulation of NPs by bivalves can occur preferentially in digestive organs (Moore 2006; Al-Subiai et al. 2012; García-Negrete et al. 2013). However, it has

been demonstrated that gills can also be a primary target for NPs (Koehler et al. 2008; Trevisan et al. 2014). Overall, NP accumulation is not only species-specific but also occurs through different routes depending on the tissue. Furthermore, the high ionic strength in the marine environment induces aggregation/agglomeration of NPs, which can interfere with their uptake in tissues of filter-feeding organisms, reducing NP capability to enter cells via endocytosis (Canesi et al. 2010a, 2010b; Gomes et al. 2011, 2012). Some studies have proposed the preferential accumulation of aggregates rather than single metal NPs (García-Negrete et al. 2013), but recently it was demonstrated that soluble metals and NPs accumulate more than micrometre-sized particles (Dai et al. 2013; Rocha et al. 2015).

Several studies have demonstrated the adverse effects of contaminants on haemocyte functionality in bivalves (Renault 2015). The haemocytes of the clam *R. philippinarum* play a primarily role in internal defence but are also responsible for other physiological processes, including wound and shell repair, shell production, digestion and transport of nutrients, and excretion (Cima et al. 2000; Donaghy et al. 2009). NPs are known to alter many morphological and functional characteristics of bivalve haemocytes (Gagné et al. 2008; Ciacci et al. 2012; Couleau et al. 2012; Barmo et al. 2013; Katsumiti et al. 2014). The immunocytotoxicity, immunoactivity and immunoefficiency are dependent on the size, composition and concentration of NPs and on the bivalve species (Rocha et al. 2015). In our experiment, nZnO induced moderate effects on haemocyte parameters measured in the clams. Indeed, responses from only two assays (haemocyte proliferation and SCGE assay) were significantly affected by the exposure. Haemocyte proliferation significantly increased in the treated animals, although a different pattern of variation was shown for nZnO and $ZnCl_2$. Indeed, this cell parameter can vary markedly in molluscs, depending on stress conditions (Matozzo et al. 2012c).

Genotoxicity is considered one of the most important toxic endpoints in chemical toxicity testing and risk assessment. However, little is known regarding the genotoxicity of nZnO, especially towards marine organisms. The SCGE assay is a well-established technique that has been applied to the study of DNA single strand breaks induced by a variety of toxic agents, such as chemical compounds, ionising radiation and NPs (Ali et al, 2012). In the present study, the results of the SCGE assay suggested that nZnO determined DNA damage in the clam haemocytes. The LDR values and the percentage of tail DNA significantly increased during the experiment but only in clams treated at the higher concentration of nZnO and under $ZnCl_2$ exposure. The damage detected could be classified as low level, which was also confirmed by the unchanged results of the micronucleus assay in the treated clams compared to controls. Cellular internalisation of NPs may promote direct interaction with DNA inside the nucleus or during mitosis. NPs could also determine DNA alteration indirectly through ROS generation in the cells (Moore 2006; Handy et al. 2008; Karlsson 2010). Of the three tissues analysed, haemolymph was less responsive to nZnO exposure. This finding is consistent with the role of haemocytes as the ultimate site of NP uptake. As above reported, a 7-day exposure at environmentally relevant concentrations of nZnO was not enough to highlight the major effects on haemocytes. Compared to other studies that have investigated nZnO effects on mollusc haemocytes, limited changes were found in the haemocyte parameters of *R. philippinarum*. However,

it must be considered that differing exposure conditions and remarkably higher nZnO concentrations were used in those studies. In an *in vitro* exposure to nZnO (1, 5, 10 µg/mL), Ciacci et al. (2012) reported a modulation of mussel haemocyte parameters, with a decrease in lysosomal membrane stability and phagocytic activity. Three weeks of *in vivo* exposure to nZnO (7 and 35 µg/mL) affected many biochemical responses in the haemolymph of the snail *B. alexandrina*, significantly inducing malondialdehyde and nitric oxide and decreasing glutathione and glutathione S-transferase levels (Fahmy et al. 2014).
In some studies, the toxicity of nZnO on marine organisms was shown to be significantly influenced by the release of Zn^{2+} ions (Miller et al. 2010; Wong et al. 2010). The present study clearly demonstrated that the effects of nZnO in the various clam tissues could not be solely explained by the dissolved zinc. Although there is some overlap in the responses to nZnO and Zn^{2+}, marked differences were also found, suggesting different intracellular mechanisms of action of these two forms of Zn. Therefore, the toxicity of nZnO depends not only on the release of zinc ions but also on the particular characteristics of the NPs (e.g., size, surface, and shape). Although Ciacci et al. (2012) stated that in mussels, the haemocyte responses to higher concentrations of nZnO and Zn^{2+} were comparable, our results are in agreement with those reported by Falfushynska et al. (2015) and Gagnè et al. (2015) who both supported the notion that the mechanisms of the cellular effects are distinct for metal NPs and the respective metals.

5. Conclusions

Based on the results obtained in this study, nZnO determined a modulation of the biological parameters measured, mostly at the biochemical level, in *R. philippinarum* exposed to low concentrations similar to PEC values. The observed increase in the antioxidant enzyme activities throughout the exposure to nZnO and zinc ions suggest enhanced ROS production. As evidence of damage to cell components was not found, we hypothesise that antioxidant defence was sufficient to cope with increased oxidative stress and protect the cells. Oxidative stress is reported as the primary effect of NP exposure in previous studies on both aquatic and terrestrial species, which was confirmed here also in the clam *R. philippinarum* exposed to nZnO. Among the tissues analysed, gills and digestive gland were the most affected by nZnO exposure. Changes in the parameters measured in gills and digestive gland are consistent with their increased Zn content after 7 days of exposure. Overall results suggest that the mechanisms of action of nZnO and Zn ions are not fully comparable.
To our knowledge, this is the first study that investigated the effects of nZnO in Manila clams, thus providing a new topic of discussion for NP ecotoxicological studies. Indeed, considering the biological and ecological features of *R. philippinarum*, this species could be subject to the risk of NP exposure. In this regard, the interaction between nZnO exposure and changes in environmental parameters should be addressed in future studies, possibly matching long-term experiments and NP concentration similar to PEC values.

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PAPER: II

Ilaria Marisa, Maria Gabriella Marin, Federico Caicci, Erica Franceschinis, Alessandro Martucci, Valerio Matozzo

In vitro exposure of haemocytes of the clam _Ruditapes philippinarum_ to titanium dioxide (TiO_2) nanoparticles: Nanoparticle characterisation, effects on phagocytic activity and internalisation of nanoparticles into haemocytes

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In vitro exposure of haemocytes of the clam *Ruditapes philippinarum* to titanium dioxide (TiO_2) nanoparticles: Nanoparticle characterisation, effects on phagocytic activity and internalisation of nanoparticles into haemocytes

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ABSTRACT

The continuous growth of nanotechnology and nano-industries, the considerable increase of products containing nanoparticles (NPs) and the potential release of NPs in aquatic environments suggest a need to study NP effects on aquatic organisms. In this context, *in vitro* assays are commonly used for evaluating or predicting the negative effects of chemicals and for understanding their mechanisms of action. In this study, a physico-chemical characterisation of titanium dioxide NPs (*n*-TiO_2) was performed, and an *in vitro* approach was used to investigate the effects of *n*-TiO_2 on haemocytes of the clam *Ruditapes philippinarum*. In particular, the effects on haemocyte phagocytic activity were evaluated in two different experiments (with and without pre-treatment of haemocytes) by exposing cells to P25 *n*-TiO_2 (0, 1 and 10 μg/mL). In addition, the capability of *n*-TiO_2 to interact with clam haemocytes was evaluated with a transmission electron microscope (TEM). In this study, *n*-TiO_2 particles showed a mean diameter of approximately 21 nm, and both anatase (70%) and rutile (30%) phases were revealed. In both experiments, *n*-TiO_2 significantly decreased the phagocytic index compared with the control, suggesting that NPs are able to interfere with cell functions. The results of the TEM analysis support this hypothesis. Indeed, we observed that TiO_2 NPs interact with cell membranes and enter haemocyte cytoplasm and vacuoles after 60 min of exposure. To the best of our knowledge, this is the first study demonstrating the internalisation of TiO_2 NPs into *R. philippinarum* haemocytes. The present study can contribute to the understanding of the mechanisms of action of TiO_2 NPs in bivalve molluscs, at least at the haemocyte level.

1. Introduction

Nanotechnology is one of the fastest growing and most promising technologies but may present a variety of hazards for environmental and human health (Moore, 2006). The nanotechnology market, as a unified market, was first quantified in 2001 by the National Science Foundation, which predicted its value to be 1 trillion dollars by 2015, but the value of the market has increased steadily over time (Nel et al., 2006). In particular, metal nanoparticles (NPs) represent the highest-volume component of total metal oxide production (Kumar, 2006). However, due to the relative novelty of this technology, information about the potential risks that NPs can pose to non-target organisms are scarce. NPs are widely used in many consumer products and in a variety of disciplines, including medicine, cosmetics, renewable energy, electronic devices and environmental remediation. They show unique physico-chemical properties, such as large surface area, charge and shape, that differ from those of their respective bulk materials (Handy et al., 2008). These features may result in i) direct generation of reactive oxygen species (ROS), ii) a high affinity for organic and metallic pollutants, and iii) an ability to penetrate cells (Al-Subiai et al., 2012). The development of nanotechnologies has

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introduced remarkable amounts of manufactured NPs into the environment, including aquatic ecosystems. NPs can reach marine coastal areas from various sources and by various routes, which can affect their chemical nature, fate, behaviour and toxicity (Matranga and Corsi, 2012; Rana and Kalaichelvan, 2013).

Among NPs, titanium dioxide (TiO_2) is widely used because of its distinctive physico-chemical properties (photocatalysis, inertness, opacity, resistance to fading). *n*-TiO_2 commonly occurs in many consumer products (sunscreens, cosmetic, medicine, paints, textiles, plastics, paper and wood preservatives), industrial products, and photocatalytic processes (Couleau et al., 2012; Jacobash et al., 2014; Frenzilli et al., 2014). A review published by the United States Environmental Protection Agency (USEPA) estimated the annual production of *n*-TiO_2 to be 2000 metric tons in 2005, with 65% of this production used in products such as cosmetics and sunscreen lotions (USEPA, 2009). Jovanović (2014) reported that from 1916 to 2011, an estimated total of 165,050,000 metric tonnes of TiO_2 pigment were produced worldwide. The predicted levels of *n*-TiO_2 in the aquatic environment are on the order of a few μg/L (Menard et al., 2011).

From an ecotoxicological perspective, it has been demonstrated that the immune system of aquatic organisms is a sensitive target for NPs, which can induce changes in the immune responses of exposed cells/animals (Ciacci et al., 2012; Couleau et al., 2012; Jovanović and Palić, 2012; Barmo et al., 2013). Like other invertebrates, bivalves rely on an innate, non-lymphoid immune system involving both cellular and humoural components (Baracco et al., 1999; Wootton et al., 2003). Haemocytes are primarily involved in defence against pathogens but are also responsible for other physiological process, including wound and shell repair, shell production, digestion and transport of nutrients, and excretion (Matozzo et al., 2007; Donaghy et al., 2009). In molluscs, phagocytosis is one of the most important mechanisms for eliminating nonself materials (Takahashi and Muroga, 2008).

It is well known that both biotic and abiotic factors can strongly affect haemocyte parameters in bivalves. However, most previous studies (both *in vitro* and *in vivo*) have been focused on the evaluation of contaminant effects on bivalve haemocyte functionality (Pipe and Coles, 1995; Galloway and Depledge, 2001; Matozzo, 2014). Regarding NPs, recent studies have demonstrated that this distinctive class of contaminants can affect various biological responses at the cellular, subcellular and molecular levels (Canesi et al., 2012; Matranga and Corsi, 2012; Baker et al., 2014). In particular, *n*-TiO_2 (photoinducible, redox active and a potential generator of ROS) has been shown to induce immunostimulation or immune suppression in molluscs (Ciacci et al., 2012; Couleau et al., 2012; Barmo et al., 2013; Grimaldi et al., 2013; Wang et al., 2014).

Despite these findings, to the best of our knowledge only one study has investigated the negative effects of NPs in the clam *Ruditapes philippinarum* (Garćia-Negrete et al., 2013). To provide further information concerning NP toxicity in this clam species and in bivalves in general, an *in vitro* approach was used to evaluate the effects of *n*-TiO_2 on *R. philippinarum* haemocytes. In *R. philippinarum*, four cell types have previously been identified, namely, haemoblasts, serous cells, and two types of immunocytes (granulocytes and hyalinocytes) (Cima et al., 2000). In this study, *n*-TiO_2 features (size, shape and particle size distribution) were determined and NPs effects on phagocytic activity were evaluated in two experiments. In the former, haemocytes were first exposed to *n*-TiO_2 and then incubated with yeast cells; in the latter, haemocytes were incubated with a yeast suspension containing *n*-TiO_2. The aim of this experimental design was to investigate whether pre-treatment of haemocytes was able to induce more marked effects on clam haemocytes. In addition, to evaluate possible interactions between haemocytes and NPs, an electron microscope analysis of haemocytes was performed.

2. Materials and methods

2.1. Nanoparticle characterisation

Nanosised titanium dioxide P25 (declared size of 21 nm and ≥99.5% purity) was purchased from Sigma–Aldrich (Milano, Italy). *n*-TiO_2 particles were characterised by a combination of analytical techniques. The mean average diameter and shape of the primary particles were determined with a TEM (FEI Tecnai G12) operated at 100 kV. Digital images were taken with a TVIPS F114 camera, and the size of the particles was measured by IMAQ Vision (National Instrument, USA).

X-ray diffraction (XRD) characterisation was performed with a Bruker D8 Advance diffractometer. The analyses were performed in Bragg-Brentano configuration at 30 kV and 30 mA. The mean crystallite size was evaluated using the Sherrer equation.

The surface areas and porosities of TiO_2 NPs were characterised by nitrogen adsorption and desorption analysis at 77.35 K with an autosorb computer controlled surface analyser (AUTOSORB-1, Quantachrome). The surface areas were calculated with Brunauer-Emmett-Teller (BET) theory.

The particle size distribution was measured by laser diffraction (Malvern Mastersizer Hydro 2000, Malvern Instruments, UK). The NPs were dispersed using a small amount of dispersant medium (distilled water) and sonicated for 10 min before analysis. The dispersion was poured into the Hydro 2000 dispersion unit (Malvern, UK) until the obscuration was in range. The analysis was performed in triplicate. Particle size distribution was then defined using the particle refractive index values of water and titanium dioxide (1.330 and 2.741, respectively). The particle size distribution was evaluated as $d(0.5)$ and SPAN. The latter is an index of particle size polydispersity and is expressed by the following equation: $\text{Span} = d(0.9) - d(0.1)/d(0.5)$, where $d(0.9)$, $d(0.1)$, and $d(0.5)$ are the diameters at 90%, 10% and 50% cumulative volume, respectively, of the particles.

2.2. Clams

Specimens of *R. philippinarum* were collected from a reference site that was located inside a licensed area for clam culture in the southern basin of the Lagoon of Venice (Italy) and were acclimatised in the laboratory for 7 days before the beginning of the experiments. Clams were maintained in large aquaria containing a sandy bottom and aerated seawater (salinity of 35 ± 1 psu, temperature of 17 ± 0.5 °C) and were fed with microalgae (*Isochrysis galbana*) daily.

2.3. Haemolymph collection and haemocyte cultures

For eah experiment (see below), pools of haemolymph (from 5 clams each) were used. Haemolymph was collected from the adductor muscles with a plastic syringe, stored on ice, and added to an equal volume of 0.38% sodium citrate in 0.45 μm filtered sea water (FSW), pH 7.5, to prevent clotting. Haemolymph was centrifuged at 800 × *g* for 10 min. Haemocytes were resuspended in FSW to prepare short-term cell cultures.

Short-term haemocyte cultures were prepared according to Ballarin et al. (1994). Sixty microlitres of haemocyte suspension were placed in the centre of culture chambers made by a Teflon ring (15 mm internal diameter and 1 mm thick) smeared with petroleum jelly, glued to a siliconised glass slide, and covered with a coverslip. Chambers were kept upside down for 30 min at room

temperature to allow haemocytes to settle and adhere to coverslips.

2.4. n-TiO_2 solutions, in vitro exposure of haemocytes and phagocytosis assay

n-TiO_2 stock solution (1 g/L) was prepared in artificial seawater (ASW) (Red Sea Salt, Red Sea Fish Pharm, Israel) and sonicated at 4 °C with a Braun Labsonic U sonifier at 50% duty cycles for 30 min. Two different experiments were performed to investigate the effects of *n*-TiO_2 on phagocytic capability of haemocytes. Each experiment was repeated three times, and three different pools of haemolymph were used in each experiment. Two slides were prepared for each experimental condition.

Experiment A (pre-treatment of haemocytes with *n*-TiO_2). After the adhesion of the haemocytes to the coverslips, FSW was removed from the culture chambers and replaced with an equal volume of FSW (control) or *n*-TiO_2 solution (1 μg/mL and 10 μg/mL in FSW). Cells were then incubated for 60 min at room temperature. Thereafter, the *n*-TiO_2 solutions were removed from the culture chambers, and the haemocytes were washed three times in FSW prior to a phagocytosis assay. FSW was then removed from the culture chambers and replaced with an equal volume of a yeast suspension (*Saccharomyces cerevisiae*) in FSW (yeast: haemocyte ratio = 10:1) (Cima et al., 2000). The haemocytes were incubated at room temperature for 60 min.

Experiment B (concomitant incubation of haemocytes with yeast plus *n*-TiO_2). After adhesion of the haemocytes to the coverslips, FSW was removed from the culture chambers and replaced with an equal volume of a yeast suspension containing *n*-TiO_2 (1 μg/mL and 10 μg/mL). The yeast suspension was prepared in FSW (yeast: haemocyte ratio = 10:1), divided into three aliquots (one for each experimental conditions), centrifuged at 12,000 × g for 3 min, and resuspended in an equal volume of FSW (control) or *n*-TiO_2 solution (1 μg/mL and 10 μg/mL). The cells were incubated for 60 min at room temperature.

In both the experiments, the haemocyte monolayers were washed several times in FSW after incubation to eliminate uningested yeast cells, fixed in a solution of 1% glutaraldehyde (Fluka) and 1% sucrose in FSW at 4 °C for 30 min, washed in 0.1 M phosphate buffer saline (PBS), for 10 min, stained with 10% Giemsa (Fluka) for 5 min, mounted on glass slides with an aqueous medium (Acquovitrex, Carlo Erba, Milano, Italy), and observed with a Leica DM-LB light microscope. Two hundred cells per slide were counted, and the phagocytic index was expressed as the percentage of cells containing ingested yeast particles.

2.5. Electron microscopy

A haemocyte suspension was prepared as described above and used for analysis by electron microscopy. Three hundred microlitres of the cell suspension were placed in a 12-well microplate. Haemocytes were incubated for 30 min at room temperature to allow them to settle and adhere to the well bottom. After cell adhesion, FSW was discharged by pipetting, and an equal volume of *n*-TiO_2 solution (10 μg/mL in ASW) was added. The controls received ASW only. The haemocytes were treated for 60 min at room temperature, washed with 0.1 M cacodylate buffer in ASW, and then fixed in 0.1 M cacodylate buffer in ASW containing 2.5% glutaraldehyde for 30 min.

The cells were postfixed in osmium tetroxide for 60 min and 0.25% uranyl acetate overnight. Subsequently, the samples were dehydrated through a graded ethanol series and embedded in EPON 812 resin overnight at 37 °C, 1 day at 45 °C and 1 day at 60 °C. Ultrathin sections (80 nm) were cut parallel to the substrate and observed with a transmission electron microscope (TEM, FEI Tecnai G12) operated at 100 kV. Digital images were taken with a TVIPS F114 camera.

2.6. Statistical analysis

The normal distribution (Shapiro–Wilk test) and homogeneity of the variance (Bartlett test) of the data were assessed. The data were statistically compared using a one-way ANOVA test, followed by a post-hoc test (Bonferroni). The results are expressed as the mean ± standard deviation.

3. Results

3.1. Nanoparticle characterisation

A TEM image of NPs and a size histogram of particles are provided in Fig. 1A and B, showing a mean diameter of approximately 21 nm, in agreement with the declared size. XRD patterns (Fig. 1C) reveal the presence of both anatase (70%) and rutile (30%) phases. The average crystallite diameter of *n*-TiO_2, evaluated from the line broadening of the main diffraction peaks, has been estimated to be 21–24 nm. The NPs specific surface area estimated by BET was 31 m^2/g with no porosity.

TiO_2 NPs dispersed in water showed a d(0.5) of 4.177 μm and 2.462 of SPAN (Fig. 1D), as determined by laser diffraction.

3.2. Phagocytosis assay

This study demonstrated that *n*-TiO_2 significantly affected the phagocytic capability of clam haemocytes. In particular, in experiment A, pre-treatment of haemocytes with *n*-TiO_2 caused a significant ($p < 0.01$ at 1 μg/mL, $p < 0.001$ at 10 μg/L) decrease in the percentage of haemocytes containing ingested yeast particles, compared with the controls (Fig. 2). In experiment B, the percentage of phagocytising haemocytes was significantly ($p < 0.001$) lower after 60 min of incubation with the solution containing yeast plus *n*-TiO_2 (10 μg/mL), compared with the controls (Fig. 3).

3.3. Electron microscopy

The results of TEM analysis demonstrated that TiO_2 NPs interact with clam haemocytes. Note that after 60 min of exposure to the highest concentration (10 μg/mL), TiO_2 NPs were not only in very close contact with cell surface membrane but also internalised by haemocytes. The nature of the NPs observed inside and outside the haemocytes was confirmed by analyses of the size, morphology and electron density of *n*-TiO_2 (see Section 3.1.). Representative images of control haemocytes are provided in Fig. 4, whereas those of haemocytes that were incubated with *n*-TiO_2 are shown in Fig. 5. The NPs were in contact with the cell membrane (Fig. 5B–E), entered the cytoplasm (Fig. 5D) and vacuoles (Fig. 5A) and interacted with the membranes of other cell organelles. Exposure to *n*-TiO_2 did not apparently affect the haemocyte morphology, but stressed cells (e.g., apoptotic nuclei) were observed (Fig. 5B).

4. Discussion

P25 (TiO_2) NPs are among those most frequently used in commercial products, such as sunscreens (Wokovich et al., 2009). The bioavailability, uptake, accumulation and toxicity of NPs in aquatic organisms depend on several physico-chemical properties, such as particle size/shape, surface charge and structure, particle chemistry and solubility and aggregation state (Scown et al., 2010; Bhatt and Tripathi, 2011). In this study, a characterisation of the primary

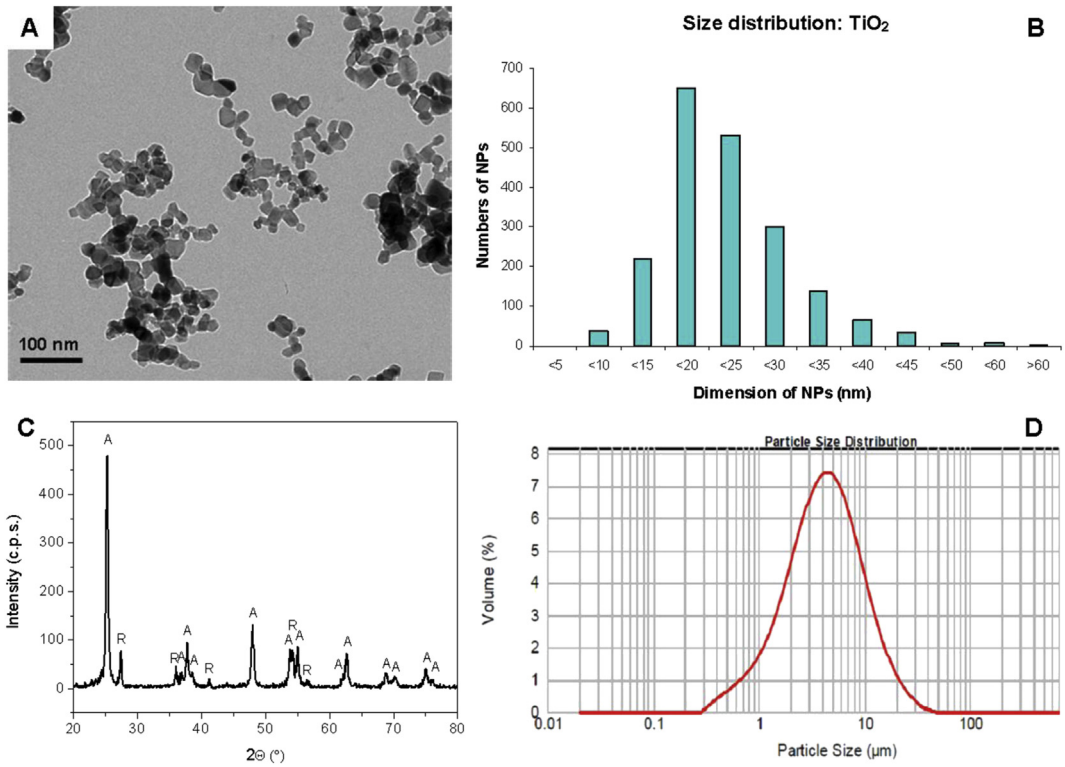

Fig. 1. A: TEM image of *n*-TiO_2. B: size (diameter) distribution histogram of *n*-TiO_2 estimated by TEM. C: XRD pattern of *n*-TiO_2 powder; the crystalline phases are anatase (A) (JCPDS #84-1285) and rutile (R) (JCPDS #87-0920). D: size distribution of *n*-TiO_2 estimated by laser diffraction.

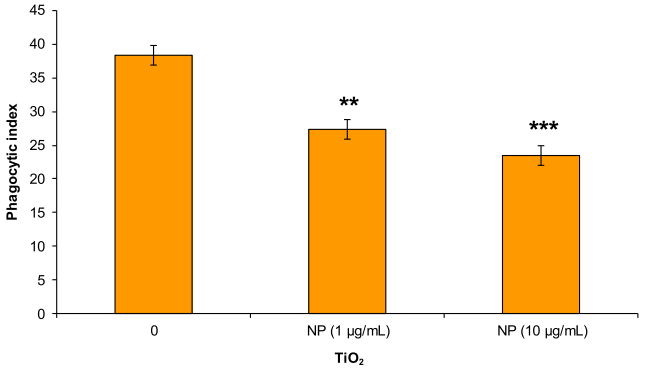

Fig. 2. Phagocytic index, expressed as the percentage of haemocytes containing ingested yeast particles, after exposure of haemocytes to *n*-TiO_2. Significant results, in comparison with controls, are indicated by asterisks. Values are means ± standard deviation ($n = 3$); $^{**}p < 0.01$, $^{***}p < 0.001$.

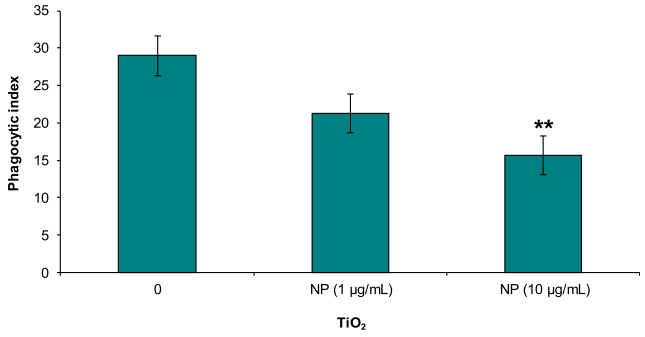

Fig. 3. Phagocytic index, expressed as the percentage of haemocytes containing ingested yeast particles, after incubation of haemocytes with a solution containing yeast + *n*-TiO_2. Significant results, in comparison with controls, are indicated by asterisks. Values are means ± standard deviation ($n = 3$); $^{**}p < 0.01$.

particles was performed to verify the declared properties. A TEM analysis showed that the TiO_2 NPs have a mean diameter of 21 nm, in agreement with the mean crystallite size evaluated from XRD. Hence, we demonstrated that the NPs used in this study were monocrystalline. An XRD analysis also showed that although anatase was the predominant phase, the rutile phase was also present. The specific surface area evaluated by BET is also in agreement with the specifications for P25. TiO_2 NPs are highly hydrophobic; therefore, they aggregate strongly in aqueous solutions (Ates et al., 2013), as confirmed by our observations.

In vitro assays are commonly used for assessing or predicting the toxic effects of chemicals and for elucidating their mechanisms of action. Furthermore, *in vitro* assays have low costs, rapid performance, high reproducibility and reduce the use of experimental animals (Olabarrieta et al., 2001). *n*-TiO_2 is the most widely produced nanomaterial (Robichaud et al., 2009), and its levels in aquatic ecosystems could negatively influence aquatic organisms. In this study, an *in vitro* approach was used to evaluate the effects of *n*-TiO_2 on haemocytes of the clam *R. philippinarum*. Haemocytes of bivalves play a key role in internal defence, and the predominant cellular mechanism in the haemocyte-mediated immune response is phagocytosis (Cima et al., 2000; Donaghy et al., 2009). Several studies have demonstrated the adverse effects of contaminants on haemocyte functionality in molluscs. Regarding NPs, it has been observed that they can alter haemocyte parameters in bivalves, but the precise mechanism of action is still poorly understood. In bivalves, NPs are filtered by the gills, directed to the digestive gland, and subsequently translocated in the haemolymph and the haemocytes (Browne et al., 2008; Canesi et al., 2012). Both *in vitro* and *in vivo* studies have demonstrated that *n*-TiO_2 - in the low mg/l

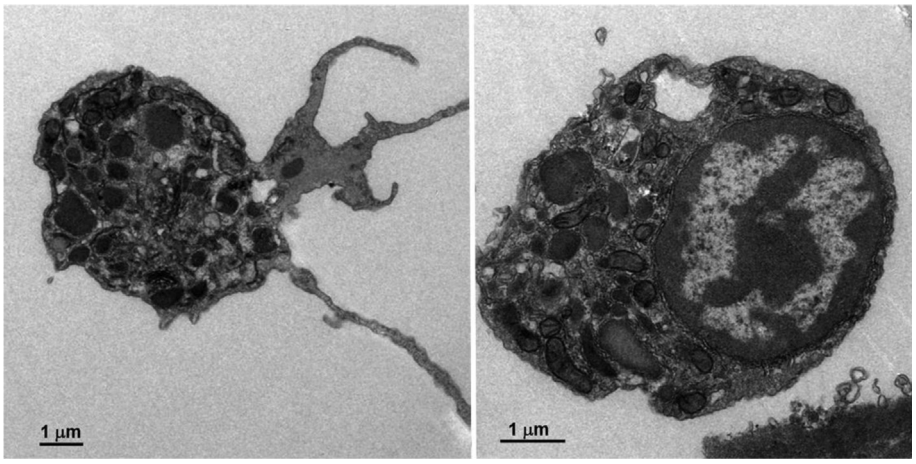

Fig. 4. TEM images of *R. philippinarum* haemocytes. Images obtained from control (ASW) cells.

range - caused adverse effects on various aquatic organisms (Canesi et al., 2010; Menard et al., 2011; Couleau et al., 2012; Ates et al., 2013). *n*-TiO_2 can also affect different parameters of bivalve immunocytes, both *in vitro* and *in vivo* (Canesi et al., 2010; Colau et al., 2012; Barmo et al., 2013).

Given that information concerning the toxic effects of NPs on the clam *R. philippinarum* is scarce, the present study represents a first attempt to evaluate *in vitro* effects of *n*-TiO_2 in haemocytes of this bivalve species. The results obtained in both experiments (A and B) confirm the hypothesis that *n*-TiO_2 is able to significantly reduce the phagocytic capability of clam haemocytes. Moreover, these results suggest that the negative effects can be mediated by the interaction between cells and NPs, as indicated by the detectable uptake of particles by haemocytes (see results of TEM

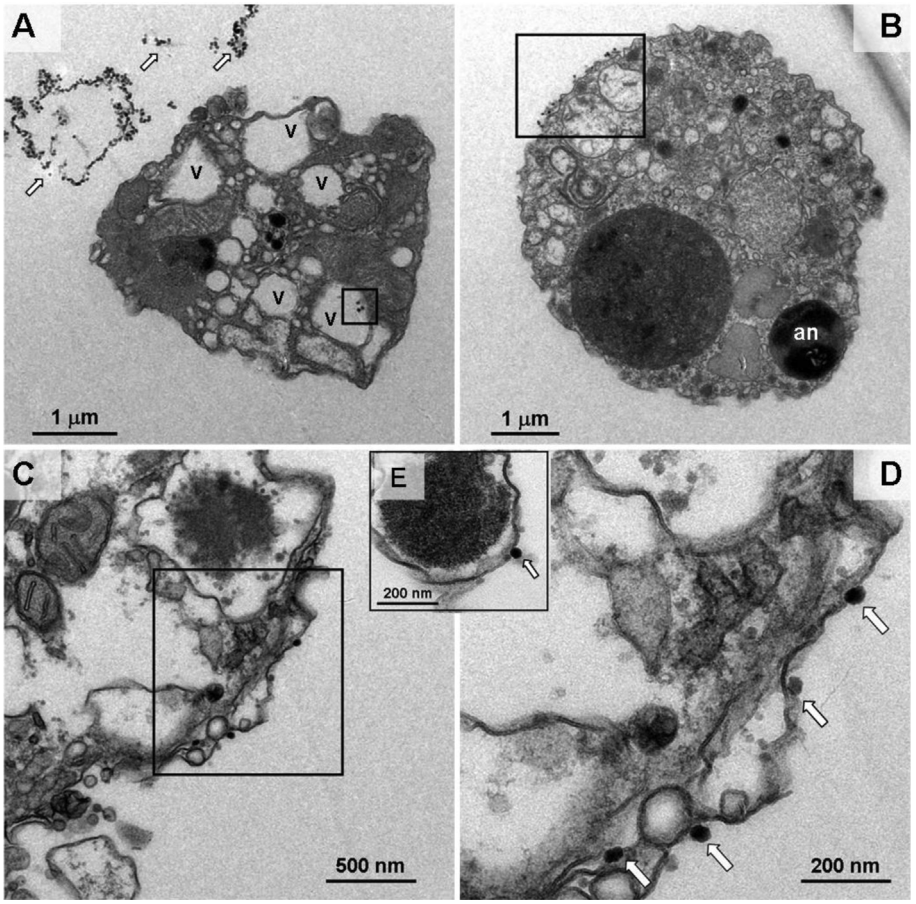

Fig. 5. TEM images of *R. philippinarum* haemocytes. Images obtained from *n*-TiO_2-exposed cells (10 μg/L, 60 min). A: NPs are visible in cells and in extracellular space (arrows); the square highlights the presence of *n*-TiO_2 in a vacuole (V). B: a stressed cell showing an apoptotic nucleus (an); the square highlights *n*-TiO_2 on the surface of haemocyte membrane. C and D: enlargements highlighting NP agglomerates on haemocyte membrane (arrows) and within cytoplasm. E: a detailed image of NPs entering a cell (arrow).

analysis). Considering that no information is available in the literature concerning *n*-TiO_2 levels in seawater, the *n*-TiO_2 concentrations that were tested in this study were chosen based on the reported data on *in vitro* NP toxicity in both aquatic organisms (e.g., *Mytilus*) and vertebrate cells (Reeves et al., 2008; Canesi et al., 2010; Ciacci et al., 2012).

The two experiments were conducted to better understand the effects of *n*-TiO_2 on clam haemocytes. In the first experiment, pre-treatment of haemocytes with *n*-TiO_2 before incubation with yeast caused a significant decrease in the phagocytic index at the two concentrations (1 and 10 μg/mL) tested. In a previous *in vitro* study, cells from *Mytilus galloprovincialis* exposed to *n*-TiO_2 (1, 5, 10 μg/mL) showed a biphasic response: phagocytosis of Neutral Red-conjugated zymosan particles increased at the lowest concentration, whereas it decreased at the highest concentrations (Ciacci et al., 2012). In that study, a slight increase in phagocytic activity was also observed in haemocytes treated with 5 μg/mL *n*-SiO_2, whereas *n*-CeO_2 inhibited phagocytosis at all the concentrations tested (Ciacci et al., 2012). Canesi et al. (2014) demonstrated that *in vitro* exposure of mussel haemocytes to *n*-TiO_2 (10 μg/mL) significantly decreased phagocytic activity. In a series of *in vivo* studies, *n*-TiO_2 (like other NPs) was shown to affect the phagocytic capability of haemocytes of three bivalve species, namely, *Crassostrea virginica*, *M. galloprovincialis* and *Dreissena polymorpha* (Chalew et al., 2012; Couleau et al., 2012; Barmo et al., 2013). In our study, the concomitant incubation of haemocytes with *n*-TiO_2 and yeast (experiment B) caused a decrease in the phagocytic index only at the highest concentration tested. According to Ciacci et al. (2012), the possibility that, at higher concentrations, *n*-TiO_2 agglomerates may compete with yeast particles (the latter becoming less available for haemocytes) cannot be excluded. In this study, the duration of exposure of haemocytes to *n*-TiO_2 was chosen on the basis of previous surveys concerning the evaluation of *in vitro* effects of NPs in bivalves (Ciacci et al., 2012), while the duration of incubation of haemocytes with yeast was chosen on the basis of our previous study on the evaluation of the phagocytic capability of *R. philippinarum* haemocytes (Cima et al., 2000). Overall, the results of our study indicate that clam haemocyte functionality can be affected by TiO_2 NPs. We are aware that phagocytosis is only one of the possible defence mechanisms against foreign materials. However, in many bivalve species phagocytosis is the main defence mechanism. In *R. philippinarum* in particular, both granulocytes and hyalinocytes are competent phagocytes (Cima et al., 2000).

In this study, the observed effects on phagocytosis can be explained (at least in part) by the interaction between *n*-TiO_2 and haemocytes, as revealed by TEM analysis. Both granulocytes and hyalinocytes of *R. philippinarum* were able to internalise *n*-TiO_2. TiO_2 NPs interacted with the cell membrane and entered the haemocytes. As a result, they were primarily localised in the cytoplasm and vacuoles. To the best of our knowledge, this is the first study describing the internalisation processes of an NP in haemocytes of the clam *R. philippinarum*. Indeed, the internalisation of gold NPs has previously been demonstrated in digestive gland cells of *R. philippinarum* (Garćia-Negrete et al., 2013). Couleau et al. (2012) have recently demonstrated by TEM that TiO_2 NPs can be internalised into haemocytes of *D. polymorpha*. In that study, the authors suggested a putative link between phagocytosis inhibition and intracellular uptake of NPs. At the haemocyte level, TiO_2 NP internalisation may occur through endocytic pathways leading to the endosomal and lysosomal compartments or else via cell surface lipid raft-associated domains termed caveolae (Moore, 2006). In certain cases, TiO_2 NPs were in contact with the cell surface membrane of *R. philippinarum* haemocytes and did not enter the cells. Regarding this NP behaviour, note that the interaction between the cell membrane and NPs can activate various signalling pathways. Indeed, it has been demonstrated that certain NPs, including TiO_2, may alter the phosphorylation levels of the mitogen activated protein kinases (MAPKs), which play a key role in the activation of the immune system (Canesi et al., 2010; Couleau et al., 2012). In *M. galloprovincialis* haemocytes, *in vitro* exposure to *n*-TiO_2 (for the same duration and at the same concentrations tested in our study) resulted in the presence of agglomerates of NPs within the endosome and in the nucleus (Ciacci et al., 2012). After *in vivo* exposure of *D. polymorpha*, TiO_2 was identified in the cytoplasm of haemocytes by scanning electron microscopy (Couleau et al., 2012). Moreover, in this study, some apoptotic cells could be observed under TEM analysis. Similar results have been found in *M. galloprovincialis* exposed *in vivo* to *n*-TiO_2 (100 μg/L, 96 h), where the current findings were confirmed by a decrease in the number of circulating haemocytes (Barmo et al., 2013).

In summary, this preliminary study demonstrates that TiO_2 NPs affect haemocyte phagocytosis in *R. philippinarum*. Based on the TEM results, we can state that the effects on haemocyte functionality are mediated (at least in part) by internalisation of NPs within haemocytes. In any case, further studies are needed to better understand the mechanisms of action of *n*-TiO_2, as well as of other NPs, at the immune level in *R. philippinarum*. In this context, we highlight that a series of *in vivo* studies are underway in our laboratory and that a battery of immunomarkers (plus other biomarkers) are currently measured. Overall, we suggest that the results of our study can provide a new topic for discussion in ecotoxicological studies.

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PAPER: III

Ilaria Marisa, Valerio Matozzo, Alessandro Martucci, Erica Franceschinis, Nicola Brianese, Maria Gabriella Marin

Sub-lethal effects and bioaccumulation of titanium dioxide nanoparticles and bulk in marine bivalve *Ruditapes philippinarum*

Sub-lethal effects and bioaccumulation of titanium dioxide nanoparticles and bulk in marine bivalve *Ruditapes philippinarum*

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Abstract

Titanium dioxide nanoparticle ($nTiO_2$) is one of the most common nanoparticles (NPs) and is part of many commercial products. During recent years several studies have been performed demonstrating that this NP can affect various responses at different levels of biological organization.

In this study, *Ruditapes philippinarum* was selected to assess the potential adverse effects of $nTiO_2$ under *in vivo* exposure. Clams were exposed for 7 days to environmentally realistic concentrations of $nTiO_2$ (0, 1, 10 μg/L) and bulk TiO_2 ($bTiO_2$, 10 μg/L). $bTiO_2$ was used to assess the potential differing action of metal oxide compared with the respective NP. At various time intervals during the exposure, cellular and biochemical responses were evaluated in clam gills, digestive gland and haemolymph. The titanium content in gills and digestive gland was determined after 7 days of exposure.

Both forms of titanium dioxide caused alterations in several parameters measured. Significant increases in antioxidant enzyme activities, and changes in glutathione S-transferase activity, were shown, highlighting an increase of oxidative stress under $nTiO_2$ exposure. In clams exposed to $bTiO_2$ slighter variations in antioxidant enzyme activities were detected. Only in digestive gland of clams exposed to the higher concentration of $nTiO_2$ an increase in lipid peroxidation was also found. Furthermore, only $bTiO_2$ treated clams exhibited significant increase of gill protein carbonyl content with respect to control. Both in gills and digestive gland an increase of Ti content was measured, although a significant difference respect to control was observed in $nTiO_2$- (10 μg/L) treated clams only. Results highlighted that both tissues are a target for $nTiO_2$ bioaccumulation. The findings obtained in haemolymph analyses confirmed haemocytes as sensitive target of $nTiO_2$ toxicity. In particular, significant increases were found in total haemocyte count (THC), matching increases in haemocyte proliferation. Increases in diameter and volume of haemocytes were also shown. Regarding these parameters, no variations were detected in THC and haemocytes diameter in clams exposed to $bTiO_2$. Under $nTiO_2$ (1-10 μg/L) and $bTiO_2$ exposure, DNA damage in haemocytes was also revealed by both Single Cell Gel Electrophoresis and Micronucleus tests, but it was lower in clams exposed to $bTiO_2$. Different responses in $nTiO_2$- and $bTiO_2$-exposed clams suggested different mechanism of

action for NP and bulk form, the toxicity of $nTiO_2$ depending not only on TiO_2 characteristics, but also on NP specific features. Moreover, both $nTiO_2$ concentrations tested affected the clam responses.

Keywords: titanium dioxide nanoparticles, bulk titanium dioxide, clams, biomarkers, bioaccumulation

1.Introduction

Titanium dioxide nanoparticle ($nTiO_2$) is one of the most produced industrial metal oxide nanoparticles (NPs) worldwide with up to 10,000 t of manufacture (Piccinno et al., 2012). Thanks to their unique physical and chemical properties, $nTiO_2$ are used in a variety of applications and products, including medicines, personal care products (e.g. sunscreens, toothpastes, cosmetics, soaps), paints, paper, sporting goods, self-cleaning surface coatings, solar cells, disinfectant sprays, as well as in the environmental decontamination of air, soil, and water (EPA, 2009; Menard et al., 2011; Pulicharla et al., 2014). For its common uses, $nTiO_2$ has different sources of pollution in aquatic environments (Gondykas et al., 2014; Menard et al., 2011), and potentially affect the estuarine and coastal habitats, which represent the ultimate sinks of various pollutants, including NPs (Corsi et al., 2014). Since there is increasing need to address any potential risk for marine organisms and ecosystems, and to safeguard the marine environment, major scientific gaps related to $nTiO_2$ toxicity need to be filled.

Currently very few data exist regarding measured environmental concentrations of $nTiO_2$, and in particular no data are available for the marine environment (Griffitt et al., 2008). To overcome this deficiency the only estimates are given by the predicted environmental concentrations (PECs) whose values are in the range of low µg/L concentrations (Gottschalk et al., 2013).

$nTiO_2$ released into the aquatic systems interact with aquatic organisms and induce toxic effects at different levels of biological organization. The $nTiO_2$ potential toxicity to aquatic organisms has recently been reviewed (Clemente et al., 2011; Menard et al., 2011; Minetto et al., 2014), but $nTiO_2$ mode of action and the related biological risk remain unclear. The effects of $nTiO_2$ on marine organisms have been investigated in terms of physiological and reproductive alterations, immunotoxicity, genotoxicity, cytotoxicity and induction of oxidative stress (Menard et al., 2011; Minetto et al., 2014). The results of these investigations are often very controversial due to the lack of homogeneity in the studies' methodology, and the scarcity of the related effect data make the results really hard to approach (Borgelaut et al., 2015).

From the perspective of NP ecotoxicology, $nTiO_2$ are by far the most extensively studied metal oxide NPs in bivalve species. Despite this, most studies assessing $nTiO_2$ toxicity have been performed using only few species and not environmentally relevant NP concentrations (Rocha et al., 2015).

To gain a better insight into the impact of $nTiO_2$ in marine bivalve species, in this study, we investigated the *in vivo* effects of these NPs in the clam *Ruditapes philippinarum*. Due to its ecological characteristics, the Manila clam could be more affected by NP toxicity. Indeed, it is estimated that NPs released into seawater, will most probably aggregate and partition to sediment and suspended particulate matter. Aggregated particles are generally less mobile and can interact with filter feeders and sediment-dwelling organisms (Boxall et al., 2007; Farré et

al., 2009). Recently, in *R. philippinarum*, the effects of $nTiO_2$ have been investigated in an *in vitro* study (Marisa et al., 2015), where $nTiO_2$ significantly decreased the phagocytic activity of haemocytes, interacted with cell membrane and enter cytoplasm and vacuoles after 60 min of exposure.
Considering the physico-chemical characteristics, the primary mechanism of action identified in NPs (e.g., oxidative stress), and the information in literature regarding $nTiO_2$ uptake, bioaccumulation and effects in other species, the aims of the present study were i) to evaluate the effects throughout a 7-day exposure to $nTiO_2$ (1 and 10 µg/L) in three clam tissues (haemolymph, gills and digestive gland) ii) to assess the modulation of various biomarkers (antioxidant enzyme activities, levels of damage to molecules, and haemocyte parameters); iii) to ascertain whether or not the same toxicity can be ascribed to $nTiO_2$ and bulk TiO_2 exposure ($bTiO_2$, 10 µg/L); and iv) to assess Ti bioaccumulation in the gills and digestive gland of both $nTiO_2$- and $bTiO_2$-treated clams.

2. Materials and methods

2.1. Titanium dioxide characterisation

Nanosised titanium dioxide (P25, declared size of 21 nm, percentage of titanium >99.5%, surface area 35 - 65 m^2/g) and bulk titanium dioxide (percentage of titanium 100%) were purchased from Sigma-Aldrich (Milano, Italy).
$nTiO_2$ was already characterised in Marisa et al. (2015) and the same analyses were used also in this study to characterised the $bTiO_2$.

2.2. Clams

Specimens of *R. philippinarum* were collected from a reference site located within a licensed clam culture area in the southern part of the Lagoon of Venice (Italy). These specimens were then acclimatised in the laboratory for 5 days before exposure to contaminants. Clams were maintained in large aquaria, which contained a sandy bottom and aerated natural seawater (salinity of 35 ± 1 psu, temperature of 16 ± 0.5 °C) and were fed daily with microalgae (*Isochrysis galbana*).

2.3. $nTiO_2$ and $bTiO_2$ exposure and tissues collection

Stock solutions of $nTiO_2$ and $bTiO_2$ (0.1 g/L) were prepared in Milli-Q water and sonicated at 4 °C using a Braun Labsonic U sonifier at 50% duty cycles for 30 min prior to each administration into the experimental tanks. Clams (35 per tank) were exposed for 7 days to 0 µg/L (control), 1 µg/L, 10 µg/L of $nTiO_2$ and 10 µg/L of $bTiO_2$. For each experimental condition tested, two replicate tanks were prepared. During exposure, the clams were maintained in glass aquaria (without sediment) containing aerated seawater (1 L per animal) in the same thermo-haline conditions used during the acclimatisation period. A movement pump (Hydor, Koralia nano 900, USA) was positioned in every aquarium (both for control and treated clams) to facilitate the water circulation and to prevent NP sedimentation (Marisa et al., paper I).
The seawater was renewed daily, and $nTiO_2$, $bTiO_2$ and microalgae (at an initial concentration of approximately 150,000 cells/L) were supplied in the experimental tanks. Before adding contaminants, the stock solution was sonicated, as reported above.
During exposure, the haemolymph, gills and digestive glands were collected after first (T1), third (T3), and last (T7) days of exposure. For each tissue, five pools (5 animals per pool, 2 or 3 from each replicate tank) from each experimental condition were prepared. Aliquots of each pooled tissue were frozen in liquid nitrogen and stored at -80 °C until analyses or immediately processed, depending on the various biological responses measured. All assays performed in this study had previously been validated (Marisa et al., paper I; Matozzo et al., 2012a; Matozzo et al., 2012b; Matozzo et al., 2013; Parolini et al., 2010; Parolini et al., 2013).

2.4. Gill and digestive gland preparation and biochemical assays

Pooled gills and digestive glands were homogenised at 4 °C using an Ultra-Turrax homogeniser (model T8 basic, IKA) in four volumes of 50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M KCl, 0.5 M sucrose, and Protease Inhibitor Cocktail (P2714, Sigma–Aldrich) and then centrifuged at 12,000 × *g* for 40 min at 4 °C. Supernatants (SN) were collected for the analyses. SN protein

concentrations were quantified according to Bradford (1976) using bovine serum albumin (BSA) as the standard.
Total superoxide dismutase (SOD) activity was measured in the SN of both tissues using the xanthine oxidase/cytochrome c method proposed by Crapo et al. (1978). Enzyme activity is expressed as U SOD/mg protein, where one unit of SOD was defined as the amount of sample producing 50% inhibition in the assay conditions. Gill and digestive gland catalase (CAT) activity was measured according to the method of Aebi (1984). The results are expressed in U CAT/mg protein, where one unit of CAT was defined as the amount of enzyme that catalysed the dismutation of 1 µmol of H_2O_2/min. Glutathione S-transferase (GST) activity was measured spectrophotometrically according to the method described in Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) as substrates. GST activity is expressed as nmol/min/mg protein. Lipid peroxidation (LPO) was quantified in both tissues' SNs using the malondialdehyde (MDA) assay, according to the method of Buege and Aust (1978). Absorbance was read spectrophotometrically at 532 nm, and the results are expressed as nmoles of thiobarbituric reactive substances (TBARS)/mg protein. TBARS, considered as ''MDA-like peroxide products'', were quantified by reference to MDA absorbance (Damiens et al., 2007). The results were not expressed as MDA levels because TBA can react with a range of chemical compounds (Csallany et al., 1984). Protein carbonyl content (PCC) was measured via the formation of labelled protein hydrazone derivatives, after 2,4-dinitrophenylhydrazide (DNPH) reaction, which were then quantified spectrophotometrically (Dalle-Donne et al., 2003; Mecocci et al., 1999). The carbonyl content was calculated from the SN absorbance via the molar absorption coefficient of 22,000 mol/cm and expressed as nmol/mg protein. DNA strand breaks were quantified using a fluorescence technique adapted from the alkaline precipitation assay (Olive, 1988). Samples of both gills and digestive gland were weighed (Mettler Toledo, XS105 Dual Range analytical balance, 0.01 mg readability) before tissue preparation (see above), and the wet weight was recorded. Salmon sperm genomic DNA standards were added for DNA calibration, and the results are expressed as µg/g wet weight.

2.5. Titanium bioaccumulation in gills and digestive gland

At the end of the exposure (T7), 4 pools of gills and digestive glands per experimental condition (6 animals each) were collected to quantify titanium bioaccumulation. Tissue samples were freeze–dried, and approximately 150 mg were weighed and digested in TFM vessels with 4 mL of 69% nitric acid and 1.5 mL of 30% hydrogen peroxide and 0.4 mL of 47% hydrofluoric acid. Digestion was performed in a Milestone MLS 1200 MEGA microwave oven. The heating programme consisted of five stages (2 min, 250 W - 2 min, 0 W - 6 min, 250 W - 5 min, 400 W and 5 min, 650 W). After cooling, 5 mL of saturated boric acid solution were added, and the heating program performed again (20 min, 400 W). Samples were then transferred into graduated flasks and diluted to 25 mL with Millipore Milli-Q water. The sample solutions were analysed by inductively coupled plasma optic emission spectroscopy (ICP-OES) using a Thermo Fischer Scientific iCAP 6300 DUO. Five calibration solutions (0, 0.5, 1, 3 and 6 ppm of Ti) were prepared by conventional dilution of a Carlo Erba 1000 µg/mL mono-elemental standard solution of the analyte as nitrate. The same amount of reagents used for the digestion procedure was added to each calibration solution. Measurements were made at Ti 323,45 nm and each sample was analysed in five replicas. The results are expressed as µg Ti/g dry weight. The detection limits of Ti was 0.3 µg/L.

2.6. Haemolymph parameters

Total haemocyte count (THC) and haemocyte diameter and volume were determined using a Model Z2 Coulter Counter electronic particle counter/size analyser (Coulter Corporation, FL, USA). THC is expressed as the number of haemocytes ($x10^6$)/mL of haemolymph. Haemocyte diameter and volume are expressed in µm and in femtolitres (fL), respectively. Haemocyte proliferation was evaluated using a colorimetric method and measured using a commercial kit (Cell proliferation Kit II, Roche). This assay has been validated in our previous studies (Matozzo et al., 2012a,b) according to the evidence of cell division in circulating haemocytes of Manila clams (Matozzo et al., 2008). The data were normalised to the THC values recorded for the clams from each experimental condition and expressed as the optical density (OD) at 450 nm. Cytotoxicity was evaluated using a colorimetric assay based on the measurement of lactate dehydrogenase (LDH) activity in cell-free haemolymph. A commercial kit (Cytotoxicity Detection Kit, Roche) was used to assess cell damage. The results, normalised to THC values, are expressed as the optical density (OD) at 490 nm. The Neutral Red uptake assay (NRU) provides a

quantitative estimation of viable cells, and it was performed according to the modified method of Cajaraville et al. (1996). This test is based on the ability of cells to incorporate and bind the vital dye neutral red, and also, it was used to evaluate the capability of haemocytes to perform pinocytosis. The results, normalised to THC values, are expressed as the optical density (OD) at 550 nm. Lysozyme activity was quantified in cell-free haemolymph (CFH) and in haemocyte lysate (HL) (Matozzo et al., 2012b). The results, normalised to THC values, were expressed as µg lysozyme/mg protein. Protein concentrations in CFH and HL were quantified according to Bradford (1976). The SCGE (Single Cell Gel Electrophoresis) assay was performed using the alkaline (pH>13) version of the assay developed by Singh et al. (1988) with some modifications (for more details see Marisa et al., paper I). Imaging was performed using a fluorescence microscope (Leica 5000B, Germany) equipped with an FITC filter (I3, excitation BP 450-490, emission LP 515) at 10× magnification. One hundred cells per slide for a total of 500 cells per condition were analysed using an image analysis system (Comet Score®). The ratio between the migration length and the diameter of the comet head (LDR) and the percentage of tail DNA were chosen to represent DNA damage. The Micronucleus (MN) test was performed according to the method of Pavlica et al. (2000). The slides were kept in the dark at 4 °C prior to examination under the microscope. Using a pre-arranged pathway, slides were scored under the fluorescent microscope Leica 5000B equipped with a submerged lens at 100× magnification. Four hundred nucleus were counted for each slide, for a total of 2000 nucleus/treatment. Only intact and non-overlapping haemocytes were considered. Micronuclei were identified according to the criteria proposed by Kirsch-Volders et al. (2000), and the MN frequency (MN‰) was calculated.

2.7. Statistical analysis

All biomarker data from gills, digestive gland and haemolymph were regrouped and analysed all together using PERMANOVA analysis (PRIMER-6 PERMANOVA plus software package). The variables considered were i) concentration, ii) time of exposure, and iii) replicate. Furthermore, a multidimensional scaling (MDS, PRIMER software package) was used for all parameters considered, to highlight specific patterns due to tissues, concentration of contaminant and time of exposure. Statistical differences due to experimental conditions were evaluated both between and within tissues (gills and digestive gland).

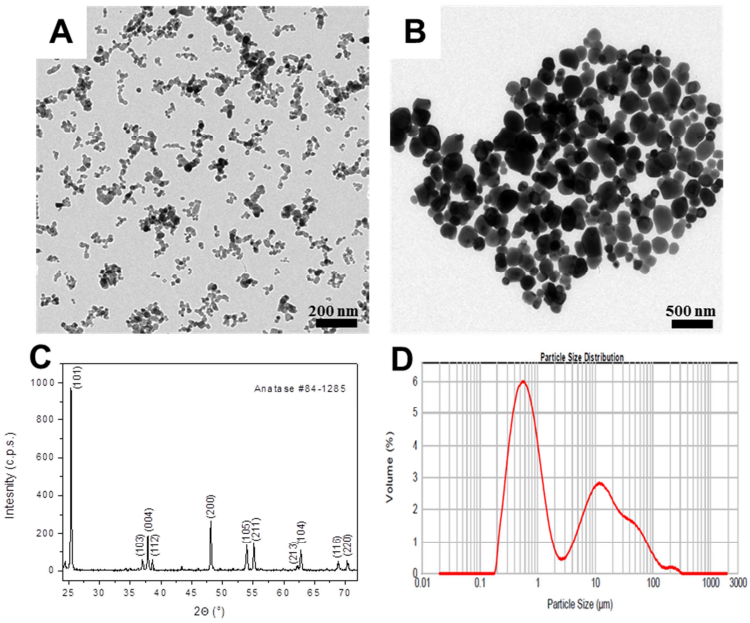
Instead, for every single biomarker the normal distribution (Shapiro-Wilk test) and the homogeneity of the variance (Bartlett test) were assessed (STATISTICA 10 software package). The data were statistically compared using a two-way ANOVA test, with exposure time and concentration of contaminant as variables and biomarkers as cases. The ANOVA was followed by a Fischer LSD post-hoc test to evaluate significant differences (*$p<0.05$; **$p<0.01$, ***$p<0.001$) between treated samples and related controls (time to time) and among exposure times.

The data regarding the bioaccumulation of titanium in gills and digestive gland were statistically analysed using a one-way ANOVA test followed by Tukey's HSD test (STATISTICA 10 software package).

3. Results

3.1 Nanoparticle characterisation

TEM images of $nTiO_2$ and $bTiO_2$ are provided in Fig. 1A and 1B, respectively. The complete results of $nTiO_2$ characterisation were reported in Marisa et al. (2015). Bulk TiO_2 mean diameter, obtained from 2000 particle measurements, was 180.9 nm ± 141 (s.d.). XRD patterns (Fig. 1C) reveal the presence of anatase phase. The average crystallite diameter of $bTiO_2$, evaluated form the Sherrer equation, has been estimated to be 50 nm. The specific surface area estimated by BET was 1 m^2/g, and no porosities were detected. $bTiO_2$ dispersed in water showed a d(0.5) of 1.158 µm and 31.229 of SPAN, as determined by laser diffraction (Fig. 1D). In Fig. 1E the summary of $nTiO_2$ and $bTiO_2$ characterisation results were reported.



E

TiO_2	Cristale structure	Average size (nm)	Average size distribution d(0.5) µm	Average size distribution (SPAN)	Average crystallite diameter (nm)	Surface area (m^2/g)
NP	Anatase (70%) / Rutile (30%)	21	4.177	2.462	21-24	31
Bulk	Anatase	180	1.158	31.229	50	1

Fig. 1. A, B: TEM image of $nTiO_2$ and $bTiO_2$. C: XRD pattern of the $bTiO_2$ powder. The crystalline phase is Anatase (#84-1285). D: size distribution of $bTiO_2$ estimated via laser diffraction. E: Scheme reports the $nTiO_2$ and $bTiO_2$ characteristics.

3.2 PERMANOVA and MDS results

PERMANOVA highlighted significant differences in the response of biomarkers due to tissue (F=45.212 and p=0.009), concentration (F= 6.4808 and p=0.001), time of exposure (F=4.358 and p=0.003), tissue/time interaction (F=3.5721, p=0.004), concentration/time interaction (F=2.0297 and p=0.011), and tissue/concentration/time interaction (F=1.8265 and p=0.026).

In gills, biomarker responses were significantly influenced by concentration (F=2.8717 and p=0.001), time of exposure (F=2.9881 and p=0.024) and concentration/time interaction (F=2.0722 and p=0.008).

In digestive gland, the responses of biomarkers were affected by concentration (F=8.5789 and p=0.001), time of exposure (F=5.9962 and p=0.001) and concentration/time interaction (F=3.0411 and p=0.001).

In haemolymph, all parameters tested were significantly affected by concentrations (F=3.0411 and p=0.001) and time of exposure (F=4.0877 and p=0.003).

The MDS results showed a clear separate distribution of the parameters analysed between gills and digestive gland (2-D stress=0.18). The MDS representation made possible to have a rapid visual discrimination only among days of exposure (T1, T3 and T7) in digestive gland, where a clear separate distribution of parameters analysed was shown (2-D stress=0.19).

3.3 Gill and digestive gland assays

Only in digestive gland of exposed clams SOD activity was significantly affected by exposure to $nTiO_2$ and $bTiO_2$ (p=0.004), time of exposure and

concentration/time interaction ($p<0.001$ and $p=0.007$, respectively). A significant increase was found at T7 in clams exposed to all the experimental conditions respect to control (Fig. 2A).

CAT activity was affected significantly in gills and digestive gland by exposure to concentration ($p=0.001$, $p<0.001$, respectively), and time of exposure ($p=0.033$, $p<0.001$, respectively). In gills, CAT activity did not change under $bTiO_2$ exposure, whereas significant increase respect to control was observed in clams exposed to 1µg/L of $nTiO_2$ at T3, and to both $nTiO_2$ concentrations at T7. Moreover, at T7 the CAT activity was significantly higher in both $nTiO_2$ (1-10 µg/L)- respect to $bTiO_2$- treated clams ($p=0.011$ and $p=0.009$, respectively) (Fig. 2B). In digestive gland, a significant increase in CAT activity was found under the lower $nTiO_2$ concentration at T1, and under the higher concentration of $nTiO_2$ at T3 respect to controls. An increase in enzyme activity was also observed at T7 in all treated clams (both $nTiO_2$ and $bTiO_2$) compared to control. At T3 the CAT activity was significantly higher in $nTiO_2$ (10 µg/L)- respect to $nTiO_2$ (1 µg/L)- and $bTiO_2$ - treated clams ($p=0.011$ and $p=0.003$, respectively) (Fig. 2C).

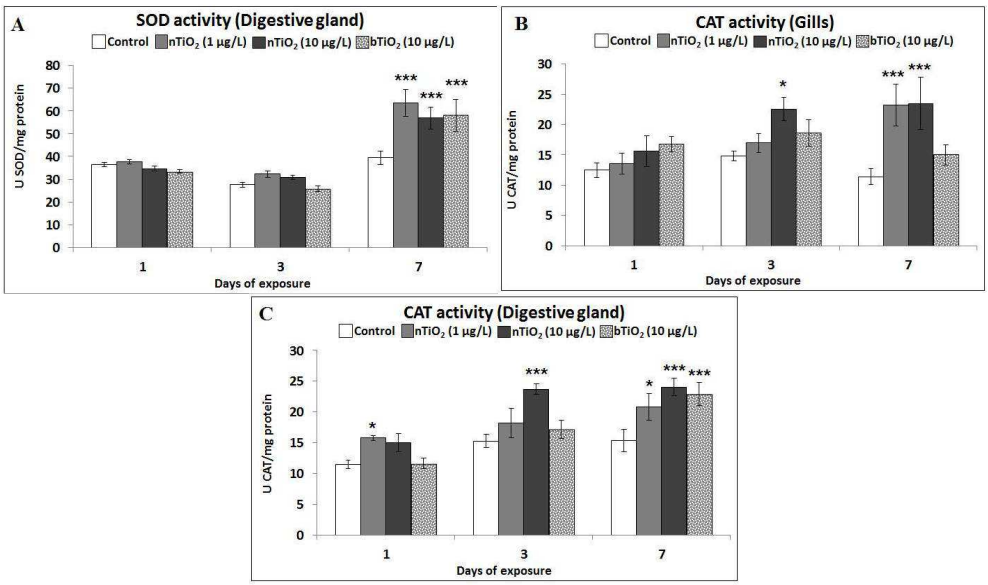


Fig. 2. SOD activity (A) expressed as U SOD/mg protein and CAT activity (B, C) expressed as U CAT/mg protein in *R. philippinarum* after 1, 3 and 7 days of exposure to $nTiO_2$ (1, 10 µg/L) and $bTiO_2$ (10 µg/L). The values are reported as the means ± SD (standard deviation); n= 5 pools. Asterisks denote significant differences compared to controls at the same time of exposure: *$p<0.05$, *** $p<0.001$.

The activity of GST was significantly influenced by the concentration and concentration/time interaction in gills ($p= 0.005$, $p= 0.002$, respectively) and only by time in digestive gland ($p= 0.003$). In gills, GST activity significantly decreased at T3 in all treated clams compared to control, and this change condition was maintained at T7 in animals exposed to $nTiO_2$ (10 µg/L) and $bTiO_2$ (Fig. 3A). Instead, in digestive gland the GST activity increased, and the only significant increase in pair-wise comparisons was between control and $nTiO_2$ (1 µg/L) at T7. Furthermore, GST activity was significant higher in $nTiO_2$ (1 µg/L)- respect $bTiO_2$-treated clams at T7 ($p= 0.013$) (Fig. 3B).

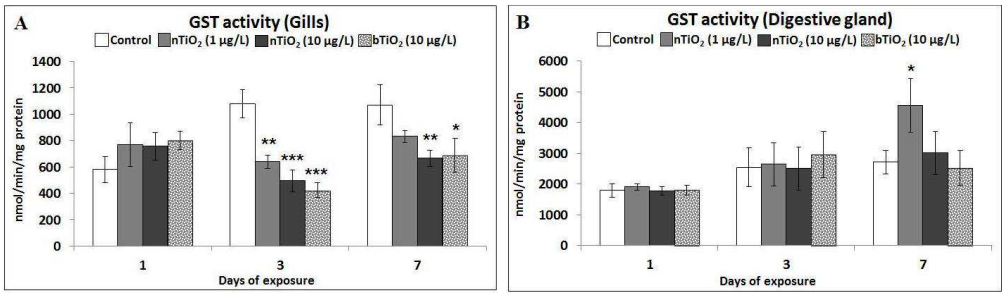


Fig. 3. GST activity (A, B) expressed as nmol/min/mg protein in *R. philippinarum* after 1, 3 and 7 days of exposure to $nTiO_2$ (1, 10 µg/L) and $bTiO_2$ (10 µg/L). The values are reported as the means ± SD (standard deviation); n= 5 pools. Asterisks denote significant differences compared to controls at the same time of exposure: *p<0.05, **p<0.01, *** p<0.001.

In digestive gland, lipid peroxidation was significantly affected by concentration, time of exposure and concentration/time interaction (p=0.047, p<0.001, p=0.003, respectively), whereas in gills no damage to lipids was detected. At T1 in digestive gland, all treated clams showed significant lower levels of lipid damage compared to control. However, the trend changed at T7, when an increase of damage in $nTiO_2$ (10 µg/L) exposed clams respect to control was detected, and significantly higher damage was found in $nTiO_2$ (10 µg/L)- treated clams respect to $bTiO_2$-treated animals (p<0.001) (Fig. 4A).

PCC values was significantly influenced by time of exposure and concentration/time interaction in gills (p<0.001 and p=0.017, respectively). A significant concentration-dependent (p=0.026) and time-dependent (p<0.001) modulation was found in the digestive gland. Respect to control, only $bTiO_2$ treated clams exhibited higher PCC values at T7 in gills (Fig. 4B), and only at T1 in digestive glands (Fig. 4C).

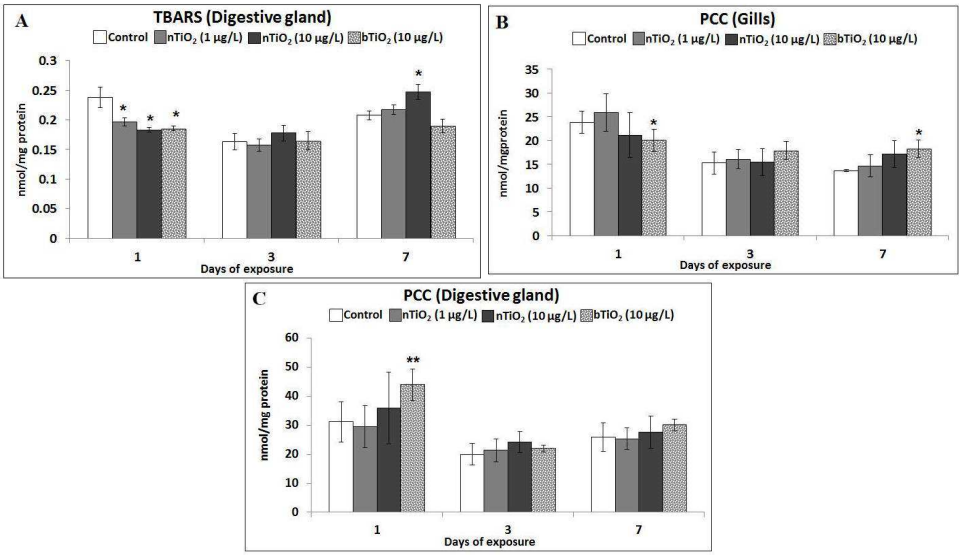


Fig. 4. TBARS (A) and PCC (B, C) levels expressed as nmol/mg protein in *R. philippinarum* after 1, 3 and 7 days of exposure to $nTiO_2$ (1, 10 µg/L) and $bTiO_2$ (10 µg/L). The values are expressed as the means ± SD; n= 5 pools. Asterisks denote significant differences compared to controls at the same time of exposure: *p<0.05.

In both gills and digestive gland, no significant damage to DNA was detected in treated clams compared to controls.

3.3.1. Titanium bioaccumulation in gills and digestive gland

The titanium content in the gills and digestive gland of the clams exposed for 7 days to $nTiO_2$ (1-10 µg/L) and $bTiO_2$ is reported in Fig. 5A and B. The results demonstrated significant accumulation of Ti in both the gills ($p=0.049$) and the digestive gland ($p=0.001$). In both tissues, only the clams exposed to 10 µg/L $nTiO_2$ showed a statistically significant increase of Ti content compared to controls. In gills, no difference was found among the treated clams, whereas in digestive gland, the Ti content was significantly higher in clam exposed to $nTiO_2$ (10 µg/L) respect to the $nTiO_2$ (1 µg/L)-treated clams ($p=0.001$).

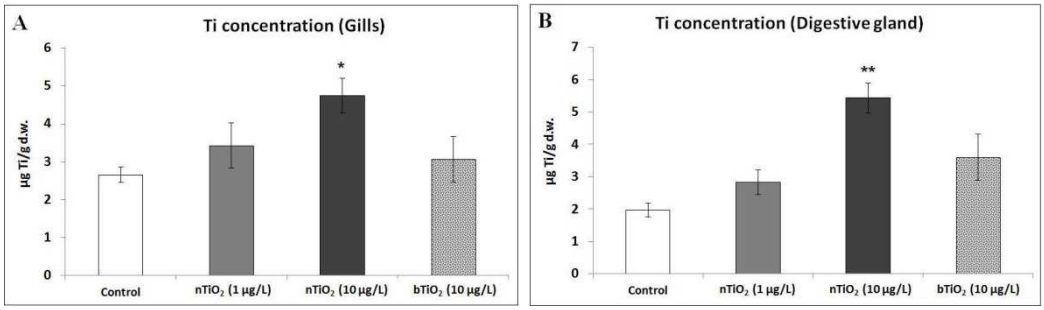


Fig. 5. Levels of titanium expressed as µg Ti/g dry weight in the gills (A) and the digestive gland (B) of *R. philippinarum* after 7 days of exposure to $nTiO_2$ (1, 10 µg/L) and $bTiO_2$ (10 µg/L). The values are expressed as the means ± SD; n= 4 pools. Asterisks denote significant differences compared to controls: *p<0.05, **p<0.01.

3.4. Haemolymph assays

THC was significantly affected by the exposure to the concentration ($p<0.001$). Differences with respect to controls were significant in the clams exposed to $nTiO_2$ (10 µg/L) at T3 and T7, and only in the clams exposed to $nTiO_2$ (1 µg/L) at T7 (Fig. 6A). Haemocyte diameter showed significant modulation due to concentration and time of exposure ($p<0.001$ and $p=0.002$, respectively). A statistically significant increase in the haemocyte diameter was observed just after the first day of exposure at the higher $nTiO_2$ concentration (10 µg/L), whereas an increase in all experimental conditions was detected at T3. However, significantly higher values with respect to controls were maintained only in both $nTiO_2$-exposed clams at T7 (Fig. 6B).

Haemocyte volume was statistically changed due to concentration ($p<0.001$) and time of exposure ($p=0.023$). From the beginning until the end of exposure, $nTiO_2$-(10 µg/L) and $bTiO_2$-treated clams exhibited significantly increased haemocyte volume respect to control clams, and the same significant increase was also observed from T3 to the end of exposure in clams exposed to the lower concentrations of $nTiO_2$ (Fig. 6C).

Haemocyte proliferation was significantly affected by the exposure to concentration ($p<0.001$) and time of exposure ($p=0.007$). In the pair-wise comparisons, significant increases respect to controls were found in the $nTiO_2$-(10 µg/L) and $bTiO_2$- treated clams from T1, and in clams exposed to $nTiO_2$ (1 µg/L) from T3 (Fig. 6D).

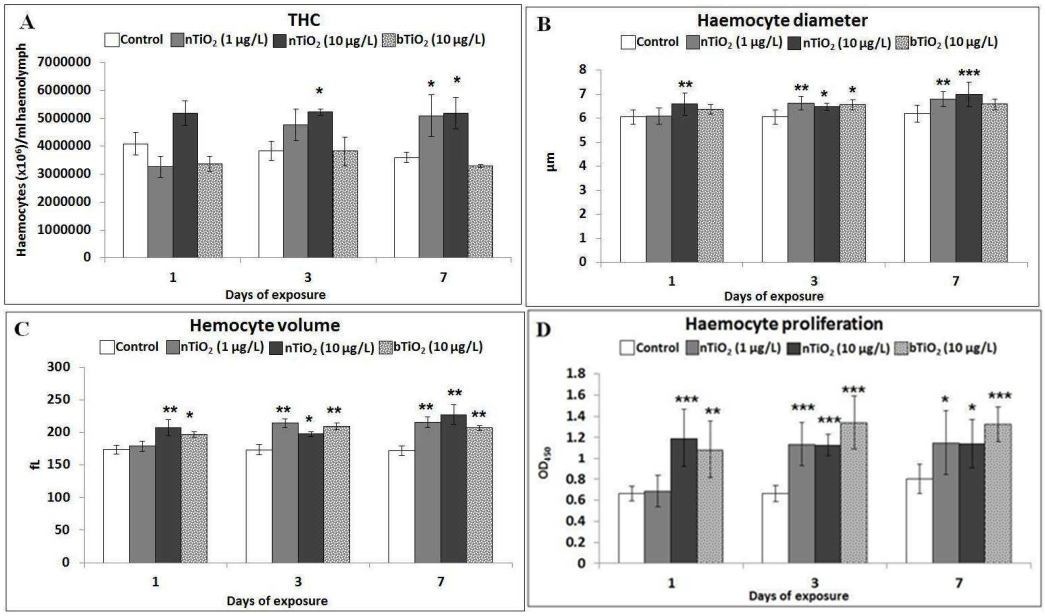


Fig. 6. THC (A) expressed as the number of haemocytes ($x10^6$)/mL of haemolymph, diameter of haemocytes (B) expressed in µm, volume of haemocytes (C) expressed in femtolitres (fL) and haemocyte proliferation (D) expressed as OD_{450} in *R. philippinarum* after 1, 3 and 7 days of exposure to nTiO2 (1, 10 µg/L) and bTiO2 (10 µg/L). The values are reported as the means ± SD (standard deviation); n= 5 pools. Asterisks denote significant differences compared to controls at the same time of exposure: *p<0.05, **p<0.01, *** p<0.001.

LDH activity was significantly affected by concentration (p=0.001). Pair-wise comparisons highlighted significant increased only at T1 between control and clams exposed to the higher $nTiO_2$ concentration (Fig. 7A). NRU showed a significant modulation due to time of exposure (p=0.001). Post-hoc analysis showed a significantly increased of NRU in $nTiO_2$-(10 µg/L) treated clams respect to control only at T3 (Fig. 7B). Only the lysozyme activity in HL was affected by the concentration (p=0.047), but no significant differences between conditions were detected during exposure by pair-wise comparisons (time to time).

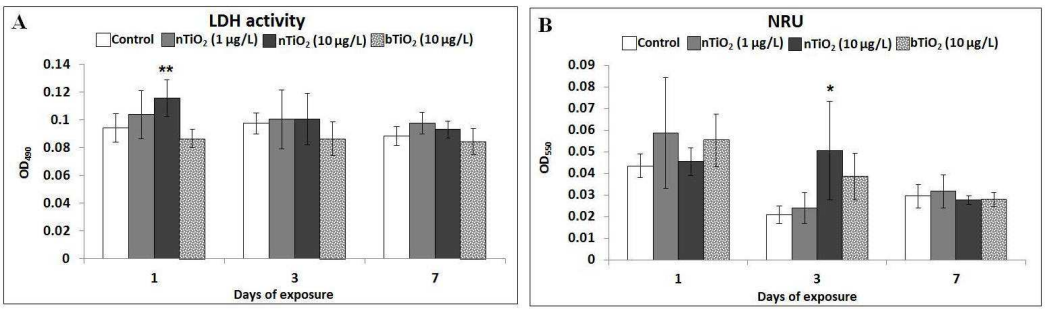


Fig. 7. LDH activity in haemolymph (A) expressed as OD_{490} and NRU levels (B) expressed as OD_{450} of *R. philippinarum* after 1, 3 and 7 days of exposure to nTiO2 (1, 10 µg/L) and bTiO2 (10 µg/L). The values are reported as the means ± SD (standard deviation); n= 5 pools. Asterisks denote significant differences compared to controls at the same time of exposure: *p<0.05, **p<0.01.

Both SCGE assay endpoints (LDR value and the percentage of DNA in the comet tail) highlighted significant primary genetic damage due to concentration in the

clam haemocytes ($p<0.001$). In particular, the LDR values significantly increased in the presence of both $nTiO_2$ concentrations from the beginning of exposure. Moreover, at T7, LDR value was significantly higher in the $nTiO_2$- (1 µg/L) treated clams with respect to the $nTiO_2$- (10 µg/L) treated clams ($p=0.024$). Clams exposed to $bTiO_2$ increased significantly the LDR values only at T1 and T7 (Fig. 8A). Similar results were observed in the percentage of tail DNA, whose values significantly increased in the presence of both $nTiO_2$ concentrations from the beginning of exposure, whereas an increase only at T1 and T7 was found in $bTiO_2$- treated clams (Fig. 8B).

The frequency of micronucleus was affected by concentration ($p<0.001$), time of exposure ($p<0.001$) and concentration/time interaction ($p<0.001$). Differences with respect to controls were significant from T1 in all treated clams, with an increase throughout the exposure. At T7, a significantly higher DNA damage ($p<0.001$) was also detected at the higher $nTiO_2$ concentration with respect to the lower $nTiO_2$ treatment (Fig. 8C).

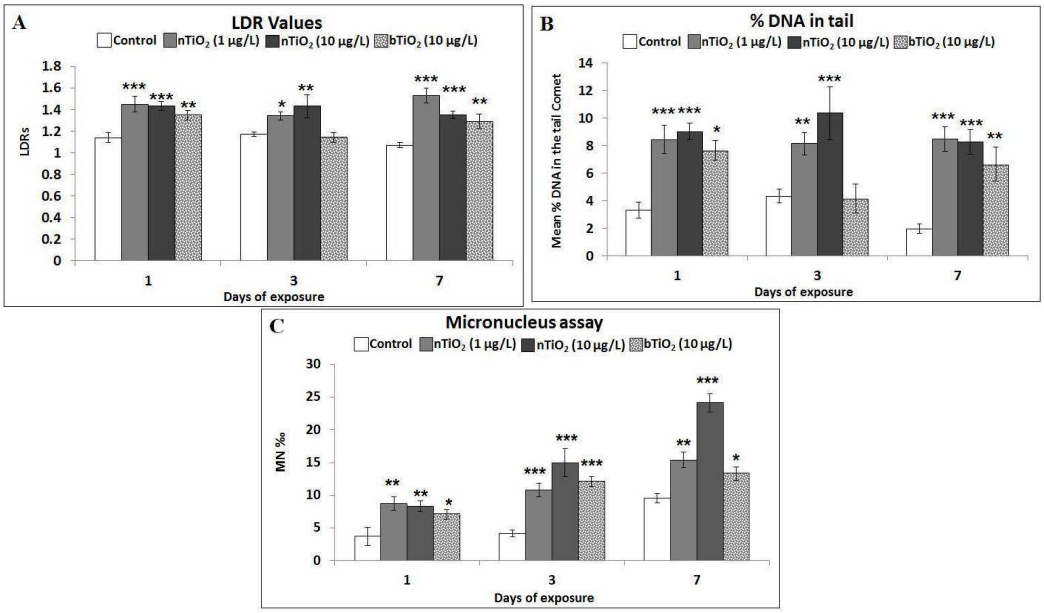


Fig. 8. SCGE results, expressed as length/diameter ratio (A) and the mean percentage of tail DNA (B), and MN results (C) expressed as frequency (MN‰) in *R. philippinarum* after 1, 3 and 7 days of exposure to $nTiO_2$ (1, 10 µg/L) and $bTiO_2$ (10 µg/L). The values are reported as the means ± SD (standard deviation); n= 5 pools. Asterisks denote significant differences compared to controls at the same time of exposure: *$p<0.05$, **$p<0.01$, *** $p<0.001$.

4. Discussion

It is generally assumed that the toxicity of NPs depends on their characteristics (e.g. size, shape, material), and is further influenced by the surrounding medium, though the key elements and parameters controlling NP toxicity are not yet clearly identified (Minetto et al., 2015; Rocha et al., 2015). NPs differ from bulk particles in terms of their heterogeneous size distribution, surface charge and area, composition, and degree of dispersion. Therefore, in a ecotoxicology study, it is important to determine not only the exposure effects on organisms but also the bioaccumulation, the characteristics of NP tested and the comparison with bulk counterpart to have more information useful to understand the NP toxicity (Hasselov et al., 2008; Rocha et al., 2015). Titanium dioxide has different phases, i.e., rutile and anatase, which are both used in commercial goods. These phases

are known to be intimately linked to TiO_2 toxicity (Kakinoki et al., 2004; Gurr et al., 2005). Anatase is 100 times more toxic than rutile, and a mixture of anatase and rutile is considered more toxic than the individual phases; also the nanometer size of the material may greatly increase the toxicity of TiO_2 (Gurr et al., 2005; Gerloff et al., 2012; Zhang et al., 2012).
The $nTiO_2$ used in this study are classified as P25, a type of $nTiO_2$ widely used in common products and one of the three TiO_2 raw materials utilized in sunscreens (Wokovich et al., 2009). In this study, $nTiO_2$ and $bTiO_2$ characterization confirmed the few information given from the producer. The two particles had only the TiO_2 constituent in common, in fact they were different in size. The nano-meter size being confirmed for the NPs. The two TiO_2 particles differed also in the XRD pattern, that identified a mixture of two phases (anatase and rutile) in $nTiO_2$ and only the anatase in $bTiO_2$. Both TiO_2 particles are highly hydrophobic, therefore they aggregate substantially in aqueous solutions (Brunelli et al., 2013). The size distribution results obtained for $nTiO_2$ and $bTiO_2$ confirmed this behaviour, showing the highest hydrophobic ability in bulk counterpart.
As an infaunal filter-feeder, the Manila clam is particularly exposed to the impact of $nTiO_2$ having in sediments their ultimate sink. Indeed, seawater has a more pronounced effect on the surface of $nTiO_2$ causing more particle collisions and consequently more aggregation/agglomeration and thus sedimentation respect to freshwater (Keller et al., 2010; Brunelli et al., 2013; Romanello et al., 2013). Furthermore, bioturbation and resuspension of sediments can lead to an increase in NP concentration at the sediment-water interface, promoting particle exchange between sediment and water column (Rocha et al., 2015).
Most published studies aimed at assessing NP toxicity were conducted using high concentrations that are unrealistic from an environmental point of view compared to PECs (Mouneyrac et al., 2014). Instead, in this study, we chose lower concentrations (1 and 10 µg/L) of $nTiO_2$ that were in the range of PEC values.
To assess the potential toxic effects of $nTiO_2$ on the clam *R. philippinarum*, a multi-biomarker approach was used, and the battery of biomarkers was selected based on the information available on NP toxicity and on the effects determined by $nTiO_2$ in other species. In bivalves, NPs are known to be filtered by the gills, accumulate in the digestive gland, and transferred to the haemolymph through the epithelium of the digestive gland tubules (Moore et al., 2009; Rocha et al., 2015). Indeed, we investigated various biological responses in these three tissues (gills, digestive gland and haemolymph). Evidence of adverse effects of a given contaminant at sub-lethal concentrations is extremely important in environmental risk assessment, since it may generate a cascade effect with consequences at different biological levels. Thus, the use of biomarkers offers the advantage of allowing for the detection of potentially toxic exposure well before real adverse effects occur (Clemente et al., 2011). Biomarkers have been shown to be sensitive and early warning indicators of exposure to pollutants and to provide information regarding alterations in the organism physiology and biochemical mechanisms of a contaminant's action (Mouneyrac et al., 2014).
Once in contact with cells or inside cells (stored within lysosomes, the endoplasmatic reticulum, or the Golgi apparatus), NPs can directly or indirectly induce oxidative stress and consequently damage to macromolecule (Moore, 2006). Since oxidative stress is probably the main cause of the toxicity of $nTiO_2$, the investigation on biomarkers associated with this effect, such as antioxidant

enzymes, should be used in the case of organisms exposed to the particles. While some studies have reported no adverse effects, others have described changes in the activities of the antioxidant enzymes SOD, CAT, GST and glutathione peroxidase (GPx) in aquatic organisms exposed to $nTiO_2$ (Clemente et al., 2011; Clemente et al., 2014). For example, *Mytilus galloprovincialis* exposed to $nTiO_2$ (1-10-100 µg/L) for 96 hours showed a modulation of glutathione-related enzymes, and an increase of CAT in digestive gland (Barmo et al., 2013). Also in Farkas et al. (2015), *Mytilus edulis* was exposed to $nTiO_2$ (0.2 mg/L and 2 mg/L) for 96 hours and an increase in antioxidant enzyme (SOD, CAT and GPx) was determined in digestive gland.

The findings in this study highlighted oxidative stress conditions progressively increasing throughout the experiment in both gills and digestive gland of clams exposed to $nTiO_2$. In particular, SOD activity exhibited a different results in the two tissues analysed, and only in digestive gland there was a significant increase in all TiO_2 treated clams compared to control. Conversely, CAT activity increased in gills, but only in clams exposed to both concentrations of $nTiO_2$, and did not increase in $bTiO_2$ treated clams. On the other hand, in digestive gland CAT activity, alike SOD activity, increased in all treated clams compared to control. GST activity changed the trend during the exposure when considering the two tissues. The decrease in gill GST activity confirmed in all treated clams an underway oxidative stress in cells.

GST is an enzyme that participates in the detoxification process due to conjugation reaction between GSH and xenobiotics (Cummins et al., 2011). Thiol compounds, such as reduced and oxidised GSH, represent the initial protective substances against heavy-metal ions and other pollutants. Inhibition of GST activity occurs either through direct action of metal on the enzyme or indirectly via the production of ROS that interact with the enzyme, depletion of its substrate (GSH) and/or down regulation of GST genes through different mechanisms (Roling and Baldwin, 2006). Instead, in digestive gland the GST activity increased, but only at the low concentration of $nTiO_2$, indicating the reaction of cells to metal particles. The present result is in accordance with the literature that has described increases in the activity of GST in mollusks and fish exposed to $nTiO_2$ (Canesi et al., 2010; Clemente et al., 2014).

These data suggest that the increase in antioxidant defences would be due to enhanced ROS production in TiO_2-exposed clams, and thus to the urgent need to protect the cells from damage (Torres et al., 2002). Indeed, in this study only a low damage to proteins and lipids were detected at the end of exposure. In gills, only $bTiO_2$-treated clams showed an increase in protein damage compared to controls. In digestive gland, only the $nTiO_2$ (10 µg/L)-exposed clams showed an increase in lipid peroxidation. The lack of damage in digestive gland of $bTiO_2$-treated clams could be related to size and characteristics of the contaminant, since micrometer-size materials are known to infiltrate biological systems lesser than nano-size materials (Moore, 2006). Low damage to molecules was consistent with low concentrations of contaminants and short *in vivo* exposure. The increase in LPO levels observed in the digestive gland could be explained by the ability of $nTiO_2$ to interact with the cell membrane (Moore, 2006; Marisa et al., 2015), and it is in agreement with the view that the digestive gland represents the target tissue for $nTiO_2$ toxicity, showing more effects than other tissues (Rocha et al., 2015). In two fish species, *Carassius auratus* and *Oncorhynchus mykiss*, $nTiO_2$ determined

high lipid damage, however animals were subject to NP concentrations higher (mg/L) than those in our study (Federici et al., 2007; Ates et al., 2013). In the oyster *Crassostrea virginica* exposed to $nTiO_2$ (5, 50, 500, and 5000 µg/L) for 48 h, Johnson et al. (2015) did not find lipid peroxidation.

In our study, DNA damage in gills and digestive gland did not increase in exposed clams, as observed also in gills of *M. galloprovincialis* by Della Torre et al. (2015), even though at $nTiO_2$ concentration (0.1 mg/L) higher than those we tested.

It is assumed that $nTiO_2$ can be accumulated in different tissues of various species, as shown, for example, in Hu et al. (2010), Ates et al. (2012) and Canesi et al. (2014). In marine mussels $nTiO_2$-bioaccumulation occurred more in the digestive gland than in the gills (Canesi et al., 2014; D'Agata et al., 2013)

In our study, a significant accumulation of Ti was revealed in both gills and digestive gland only in clams exposed to $nTiO_2$ (10 µg/L) compared to controls, and no different pattern of accumulation was found in the two tissues. This suggested that also the gills act as a target tissue for bioaccumulation of $nTiO_2$. Conversely, no significant evidence of $bTiO_2$ accumulation was found in both gills and digestive gland. It is possible to envisage a scenario where larger (bulk) particles are rejected and excreted as pseudofaeces at the gills, and did not reach the digestive gland. Differently, NPs can interact with gills and thereafter they can be transported to the digestive gland (D'Agata et al., 2013).

In our results, control clams showed similar Ti contents in gills and digestive gland, this amount being originated from a natural background of Ti in the environment (Borgelaut et al., 2015). In nature, Ti occurs only in the form of oxide or oxides mixed with other elements. Mineral deposits are usually of volcanic origin, and they are also found in beach sand (Winkler, 2003). In organism tissues, Ti content is expected to be in the range 1−50 µg/g dry weight, mostly arising from the natural abundance of TiO_2 particles (Federici et al., 2007; Bigorgne et al., 2011). TiO_2 occurs at 80 µg/L in the Seine river (Geertsen et al., 2014), while the PECs of manufactured $nTiO_2$ is about 1 µg/L (Sun et al., 2014). The main reserves of TiO_2 are located in Canada, USA, Scandinavia, South Africa, Mediterranean Sea, and Australia (Titaniumart, 2010).

Bivalve $nTiO_2$ accumulation was also reported in other studies. In Barmo et al. (2013), mussel exposed to $nTiO_2$ (1, 10 and 100 µg/L) for 96 h showed an increase of Ti content in the digestive gland and in the haemocytes, although without a clear dose-dependent pattern, in contrast to what observed in a previous study conducted at higher NP concentrations and with shorter times of exposure (Canesi et al., 2010). This could be partly due to different behaviour of $nTiO_2$ at lower concentration range in seawater respect to higher concentrations, possibly resulting in different uptake by the organisms. It was demonstrated that $nTiO_2$ create less aggregates, with smaller size and thus higher sedimentation time when present at lower concentrations respect to higher concentrations (Brunelli et al., 2013).

The immune system is considered a sensitive target for the effects of various environmental contaminants, as well as for those of NPs, in different species (Jovanovíc and Palíc, 2012). Variations in haemocyte parameters can elicit severe problems for the organisms, considering the haemocytes' role in internal defence and various physiological processes (Cima et al., 2000; Donaghy et al., 2009). $nTiO_2$ are known to alter many morphological and functional characteristics of

mollusc haemocytes (Couleau et al., 2012; Barmo et al., 2013; Ali et al., 2015). The above-quoted studies corroborated the hypothesis that immune cells could be one of the target tissue useful to understand the action of $nTiO_2$ toxicity (Canesi et al., 2012).

Among immunomarkers, THC is one of the most commonly used parameter to evaluate negative effects of stressors (including pollutants) in bivalves (Oliver and Fisher, 1999). Decreases in THC can be caused by haemocyte lysis or by increased cell movement from haemolymph to peripheral tissues. In the other hand, an increases in THC can be due to the proliferation or movement of cells from tissues into haemolymph. In this study, THC increased in haemolymph of treated clams, with a simultaneous increase in haemocyte proliferation. Since cytotoxicity did not show any variation, haemocyte proliferation suggested a general stress condition for cells after the exposure to contaminants.

In Barmo et al. (2013), the responses in *M. galloprovincialis* were opposite to our findings, indeed the THC did not change in mussels exposed to the lowest concentration of $nTiO_2$ and decreased in the other concentrations tested compared to controls. Instead, similarly to our results, in the green-lipped mussel *Perna viridis* exposed to $nTiO_2$ (0, 2.5 mg/L and 10 mg/L) for 216 h, under various combinations of oxygen levels, the THC increased during all the exposure, and no evidence of $nTiO_2$ cytotoxic effects was detected in an *in vitro* related haemocyte experiment (Wang et al., 2014). In the cephalopod mollusc *Octopus vulgaris*, intramuscular injections of $nTiO_2$ (1 and 10 mg/kg) did not lead to a specific response in THC values (Grimaldi et al., 2013).

During our experiment, an increase of diameter and volume of haemocytes in treated clams suggested an uptake of liquid from haemolymph, but this hypothesis was not confirmed by an increase in NRU values. On the other hand, in all clams exposed to TiO_2, the results of the cytotoxicity test indicated an overall maintenance of cell membrane integrity (except for $nTiO_2$ at T1). Lysozyme activity did not change throughout the present study, both in CFH and HL. Conversely, a significant increase was observed in other study on *O. vulgaris* (Grimaldi et al., 2013). Nevertheless, it is important to note that the concentrations of $nTiO_2$ tested in that study were thousands of times higher than the concentration considered here and also the experimental approach was completely different (Grimaldi et al., 2013).

It is known that NPs can determine DNA damage, directly and/or indirectly through different mechanisms (Moore, 2006; Handy et al., 2008; Karlsson, 2010). Indeed, the haemocytes of the freshwater snail *Limnea luteola* exposed to $nTiO_2$ (28-56-84 µg/mL) for 96 h showed high DNA damage correlated also with an high oxidative stress, lipid peroxidation, decreased GSH level and GST activity. In *L. luteola* $nTiO_2$ determined also high cytotoxicity (Ali et al., 2015). Production of ROS could cause DNA oxidation and strand breaks leading to a great amount of cell death. Moreover, the $nTiO_2$-mediated induction of ROS production is one of the main common biological responses in immune cells (Jovanovíc and Palíc, 2012). In the blue mussel *M. edulis* exposed to $nTiO_2$ (0.2 and 2.0 mg/L) for 96 h the MN frequency increased with increasing NP dose (Farkas et al., 2015). Also in the marine fish *Trachinotus carolinus* exposed to $nTiO_2$ (1.5 µg/g and 3.0 µg/g) by intraperitoneal injection, the NP determined high DNA damage in the erythrocytes.

In this study, the results of both SCGE and MN assay indicated an high level of DNA damage with a major amount in haemocytes of $nTiO_2$-exposed clams respect to the bulk-exposed ones. An high DNA damage could be the beginning of other dysfunctions in cells at both biochemical and physiological level (Depledge, 1998). All these findings indicated that $nTiO_2$ at haemocyte level determined high genotoxicity, also at low concentrations, such as those we used.
The toxicity of $nTiO_2$ showed to be mainly related to their "nano" properties, although the $bTiO_2$ also affected exposed clams, confirming the toxicity of Ti. $nTiO_2$ exhibited specific physico-chemical characteristics that differed from the bulk counterpart. Moreover, due to their small sizes, $nTiO_2$ are more likely to infiltrate biological systems compared to the larger molecules that cannot infiltrate (Moore, 2006) and diffuse through cell membranes (Lin et al., 2010). Also Libralato et al. (2013) affirmed the different action and effects of $nTiO_2$ and $bTiO_2$, when comparing the relative embryotoxicity in mussels .

Conclusion

To our knowledge, this is the first study that investigated the effects of $nTiO_2$ in Manila clams, thus providing a new topic for discussion in NP ecotoxicological studies. Due to its biological and ecological features, *R. philippinarum* could be particularly subject to the risk of NP exposure. Indeed, the experimental results of this work indicated the sensitivity of *R. philippinarum* to $nTiO_2$. The NPs determined a modulation of the most biological parameters measured, both at the biochemical and cellular level, in clams exposed to low concentrations similar to PEC values.
Our results showed that gills can be considered a target of effect and bioaccumulation, providing a general understanding of the $nTiO_2$ mode of action. Nonetheless, the digestive gland was confirmed as the primary target tissue also for this NP. More severe effects of $nTiO_2$ compared to bulk form were highlighted, arising by their specific NP characteristics.
The oxidative stress appeared to be an important cause of $nTiO_2$ toxicity, since primarily oxidative stress-related responses were observed in clams exposed to $nTiO_2$. Although the *in vivo* exposure was relatively short and with low NP concentrations, treated clams showed a low lipid damage.
Haemocytes were seriously affected by $nTiO_2$ exposure, with high modulation of various cell parameters and also with an high DNA damage.
In future studies the toxicity of $nTiO_2$ needs to be investigated further for chronic effects, using longer exposure at concentrations similar to PEC values.

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PAPER: IV

Ilaria Marisa, Valerio Matozzo, Alessandro Martucci, Maria Gabriella Marin

Toxicological effects and bioaccumulation induced by C_{60} fullerene (FC_{60}) exposure in marine bivalve *Ruditapes philippinarum*

Toxicological effects and bioaccumulation induced by C_{60} fullerene (FC_{60}) exposure in marine bivalve *Ruditapes philippinarum*

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Abstract

C_{60} fullerene (FC_{60}), with its unique physical properties, has been produced for many applications in recent decades. The increased likelihood of direct release into the environment has raised interest in understanding the corresponding biological effects of FC_{60} to aquatic organisms. Nowadays, only few studies have analysed FC_{60} effects and bioaccumulation in marine organisms under a *in vivo* exposure with low concentrations. For these reasons the information concerning FC_{60} ecotoxicity is still lacking.

To provide new data about FC_{60} toxicity, in this study, *Ruditapes philippinarum* was selected to assess potential adverse effects of the contaminant. Clams were exposed for 7 days to low concentrations of FC_{60} (0, 1, 10 µg/L). At various time intervals during the exposure, cellular and biochemical responses were evaluated in clam gills, digestive gland and haemolymph. The FC_{60} content in gills and digestive gland was determined in all experimental conditions after 7 days of exposure through High Performance Liquid Chromatography analysis.

In gills, a significant modulation in antioxidant enzyme activities until the end of the exposure, and changes in glutathione S-transferase activity during the first phases of exposure only, were shown, highlighting an increase in oxidative stress. Moreover, damage to lipids and proteins was detected in FC_{60} (10 µg/L)- treated clams after 7 days of exposure. In digestive gland, slighter variations in antioxidant enzyme activities and damage to molecules were detected. In the first phases of exposure, superoxide and glutathione S-transferase activity showed a significant modulation. Only changes in catalase activity and lipid peroxidation were detected until the end of exposure.

FC_{60} accumulation was found in both gill and digestive gland tissues. In particular, significantly higher values compared to control were measured in gills of FC_{60}- (1-10 µg/L) treated clams, and in digestive gland of FC_{60}- (1 µg/L) treated clams.

Haemolymph presented lower responses until the end of exposure. Indeed, only Single Cell Gel Electrophoresis and Neutral Red uptake assays showed increased values in FC_{60} exposed clams. Moreover, volume and diameter of haemocytes, haemocyte proliferation, and Micronucleus assay highlighted significant variations in treated clams, but only in the first phases of exposure, and no changes were detected after 7 days.

Gill clams could be considered the target tissue for FC_{60} toxicity under the exposure conditions used at least, and in particular the high damage detected to lipids and proteins could contribute to long-term problems for organism.

Keywords: C_{60} fullerene, nanoparticles, clams, biomarkers, bioaccumulation

1. Introduction

Among carbon nanoparticles (NPs), C_{60} fullerene (FC_{60}) is a molecule possessing 60 carbon atoms, and is a hollow sphere like a football/soccer ball. Due to its three-dimensional shape, its unsaturated bonds and its electronic structure, fullerene has unique chemical and physical characteristics (Costa et al., 2012). Since its discovery (Kroto et al., 1985), FC_{60} has received considerable attention in various fields (Kim et al., 2010), and nowadays it has many important commercial and industrial applications, including drug delivery, electronics, superconductors, and cosmetics (Langa and Nierengarten, 2011; Pulicharla et al., 2015). It is one of the most ubiquitous and produced carbon NPs (Sanchìs et al., 2015), and it is also present in polluted air as a result of fuel combustion (Nielsen et al., 2008). Moreover, in view of its large production and applications, FC_{60} is inevitably released into the aqueous environment (Gottshalk et al., 2009). Once released into aquatic systems, FC_{60} tends to take the form of colloid via several mechanisms (Andrievsky et al., 1995) and be associated with biomolecules or natural organic matter (Hyung et al., 2007). FC_{60} possesses strong hydrophobic characteristic, and consequently, in molecular form has the tendency to associate with hydrophobic substances in the environment (e.g., natural organic matter, lipids, and living organisms).

Despite this information, a very few data on the presence and the behaviour of FC_{60} in the aquatic environment have been reported, mainly due to the lack of analytical methods able to detect and to quantify NPs. Only predicted environmental concentrations (PECs) are available in literature. PECs of FC_{60} in waste water treatment plants were estimated to be in the range of few ng/L to about 100 µg/L (Gottschalk et al., 2009). Instead, Ferreira da Silva et al. (2011) reported FC_{60} PECs in water in the range of 0.31-5 µg/L.

Coastal marine environments tend to be major depositional areas for many pollutants, as well as for NPs. NPs can enter marine systems via different sources, either directly or indirectly (Corsi et al., 2014). These environments are commonly inhabited by various species of organisms, among which bivalve molluscs are considered target species for NP exposure and thus for investigating their toxicity (Moore, 2006: Canesi et al., 2012). As filter-feeding organisms, bivalves spend their lives filtering large volumes of water, processing microalgae, bacteria, sediments, particulates, and also pollutants. In addition, the NP characteristics, together with the results from studies comparing their presence in different types of water, suggest that NPs will partition in the sea bottom at the sediment/water interface, thus representing a major threat to benthic organisms (Rocha et al., in press). In this study, the use of an infaunal filter-feeder, the marine clam *Ruditapes philippinarum*, could give a better insight on the real risk of NP exposure in coastal habitats. Recently, in Manila clam, largely used in laboratory studies to assess the toxicity of contaminants, the effects of NPs have been investigated in *in vitro* (Marisa et al. 2015) and *in vivo* exposures (Garcia-Negrete et al., 2013; Marisa et al., paper I; Marisa et al., paper III).

Ecotoxicological studies on FC_{60} were carry out in various organisms, including bacteria (Letts et al., 2011), algae (Baun et al., 2008), crustaceans (Tao et al., 2011), fishes (Oberdöster, 2004; Kuznetsova et al., 2014), insects (Waissi-Leinonen et al., 2015), worms (Wang et al., 2014), and bivalve molluscs

(Ringwood et al., 2009; Al-Subiai, et al., 2012). FC_{60} has been shown to determine DNA damage (Al-Subiai, et al., 2012), bactericidal action (Trpkovic et al., 2012), induction of oxidative stress (Zhu et al., 2008), and the inhibition of growth, development and reproduction (Tao et al., 2009; Waissi-Leinonen et al., 2015). Moreover, FC_{60} can enter living organisms via several routes and accumulate in tissues (Oberdöster et al., 2005; Oberdöster et al., 2006).
The primary mechanism of FC_{60} toxicity has generally been attributed to its ability to generate reactive oxygen species (ROS), although other studies have reported contradictory results, indicating the need for further investigations to understand the potential oxidized action of this NP (Shinohara et al., 2009; Al-Subiai et al., 2012; Costa et al., 2012).
The information on FC_{60} toxicity in marine species is still lacking, although the increasing use and inevitable release of this NP into aquatic environments highlight the need to assess better its potential impact to coastal environments.
To fill this gap, the present study was aimed at evaluating potential sub-lethal effects of FC_{60} on *R. philippinarum*, by analysing various biomarkers (antioxidant enzyme activities, levels of damage to molecules, and haemocyte parameters) in three tissues (haemolymph, gills and digestive gland) throughout a 7-day exposure to 1 and 10 μg/L of FC_{60}. The biochemical and cellular parameters measured were selected based on both physico-chemical characteristics of FC_{60} and information on FC_{60} toxicity in other species. To confirm the FC_{60} ability to penetrate and accumulate in different tissues, the FC_{60} content in gills and digestive glands was measured. Physico-chemical characterisation of FC_{60} was also performed by various techniques to validate the manufacturer information on particle features.

2. Materials and methods

2.1. FC_{60} characterisation

Nanosised pownder of FC_{60} (purity>97.5 %) was purchased from Sigma-Aldrich (Milano, Italy). The images of FC_{60} were determined using a transmission electron microscope (TEM, FEI Tecnai G12) operated at 100 kV, with a TVIPS F114 camera. X-ray diffraction (XRD) characterisation was performed using a Bruker D8 Advance diffractometer. The analyses were performed in Bragg-Brentano configuration at 30 kV and 30 mA. The mean crystallite size was evaluated using the Sherrer equation.

2.2. Clams, FC_{60} exposure and tissue collection

Specimens of *R. philippinarum* were collected from a reference site located within a licensed clam culture area in the southern part of the Lagoon of Venice (Italy). These specimens were then acclimatised in the laboratory for 5 days before exposure to the contaminant. Clams were maintained in large aquaria, which contained a sandy bottom and aerated natural seawater (salinity of 35 ± 1 psu, temperature of 16 ± 0.5 °C) and were fed daily with microalgae (*Isochrysis galbana*).
Stock solution of FC_{60} (0.1 g/L) was prepared in Milli-Q water and sonicated at 4 °C using a Braun Labsonic U sonifier at 50% duty cycles for 30 min. Clams (35 per tank) were exposed for 7 days to nominal concentrations of 0 (control), 1, and 10 μg FC_{60}/L. For each experimental condition tested, two replicate tanks were prepared. During exposure, the clams were maintained in glass aquaria (without sediment) containing aerated seawater (1 L per animal) in the same thermo-haline conditions used during the acclimatisation period. A movement pump (Hydor, Koralia nano 900, USA) was positioned in every aquarium (both for control and treated clams) to facilitate the water circulation and to prevent FC_{60} particle sedimentation (Marisa et al., paper I).
The seawater was renewed daily, and FC_{60} and microalgae (at an initial concentration of approximately 150,000 cells/L) were supplied in the experimental tanks. Before adding the contaminant, the stock solution was sonicated, as reported above.
Throughout the exposure, clam haemolymph, gills and digestive gland were collected after first (T1), third (T3), and last (T7) days of exposure. For each tissue, six pools (5 animals per pool, 2 or

3 from each replicate tank) from each experimental condition were prepared. Aliquots of each pooled tissue were frozen in liquid nitrogen and stored at -80 °C until analyses or immediately processed, depending on the various biological responses measured. All assays performed in this study had previously been validated (Marisa et al., paper I; Marisa et al., paper III; Matozzo et al., 2012a; Matozzo et al., 2012b; Matozzo et al., 2013; Parolini et al., 2010; Parolini et al., 2013).

2.3. Gill and digestive gland preparation and biochemical assays

Pooled gills and digestive glands were homogenised at 4 °C using an Ultra-Turrax homogeniser (model T8 basic, IKA) in four volumes of 50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M KCl, 0.5 M sucrose, and Protease Inhibitor Cocktail (P2714, Sigma–Aldrich) and then centrifuged at 12,000 ×*g* for 40 min at 4 °C. Supernatants (SN) were collected for the analyses. SN protein concentrations were quantified according to Bradford (1976) using bovine serum albumin (BSA) as the standard.

Total superoxide dismutase (SOD) activity was measured in the SN of both tissues using the xanthine oxidase/cytochrome c method proposed by Crapo et al. (1978). Enzyme activity is expressed as U SOD/mg protein, where one unit of SOD was defined as the amount of sample producing 50% inhibition in the assay conditions. Gill and digestive gland catalase (CAT) activity was measured according to the method of Aebi (1984). The results are expressed in U CAT/mg protein, where one unit of CAT was defined as the amount of enzyme that catalysed the dismutation of 1 μmol of H_2O_2/min. Glutathione S-transferase (GST) activity was measured according to the method described in Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) as substrates. GST activity is expressed as nmol/min/mg protein. Lipid peroxidation (LPO) was quantified in both tissues' SN using the malondialdehyde (MDA) assay, according to the method of Buege and Aust (1978). The results are expressed as nmoles of thiobarbituric reactive substances (TBARS)/mg protein. TBARS, considered as ''MDA-like peroxide products'', were quantified by reference to MDA absorbance (Damiens et al., 2007). The results were not expressed as MDA levels because TBA can react with a range of chemical compounds (Csallany et al., 1984). Protein carbonyl content (PCC) was measured via the formation of labelled protein hydrazone derivatives, after 2,4-dinitrophenylhydrazide (DNPH) reaction, which were then quantified spectrophotometrically (Dalle Donne et al., 2003; Mecocci et al., 1999). The carbonyl content was calculated from the SN absorbance via the molar absorption coefficient of 22,000 mol/cm and expressed as nmol/mg protein. DNA strand breaks were quantified using a fluorescence technique adapted from the alkaline precipitation assay (Olive, 1988). Samples of both gills and digestive gland were weighed (Mettler Toledo, XS105 Dual Range analytical balance, 0.01 mg readability) before tissue preparation (see above), and the wet weight was recorded. Salmon sperm genomic DNA standards were added for DNA calibration, and the results are expressed as μg/g wet weight.

2.4. FC_{60} bioaccumulation in gills and digestive gland

At the end of the exposure (T7), 4 pools of gills and digestive glands per experimental condition (6 animals each) were collected to quantify FC_{60} bioaccumulation. Tissue samples were carefully washed with pure toluene to remove surface adsorbed FC_{60} particles. Then, tissues were extracted into toluene (1:6, w:v ratio) using ultra-sonication for 30 min (35 kHz). Extracts were centrifuged (9000 ×*g*) and the SN was used in HPLC (High Performance Liquid Chromatography) analysis. FC_{60} was separated using Eclipse XDB-C18 4.6 mm ID x 250 mm 5μm 80 Å column.

The mobile phase for columns was toluene (Sigma-Aldrich HPLC grade), at a flow-rate of 1.0 mL/min. Sample injections were performed manually with volumes of 100 μL. The eluent was monitored at 335 nm using a HPLC Agilent 1100, Chemstation rev. A10.02. For external calibration, a standard curve was generated for FC_{60} concentrations ranging from 0.001 to 0.8 mg/mL. The FC_{60} concentration of each sample was calculated by comparison to the standard curve, and the results were expressed as μg FC_{60}/g wet weight.

2.5. Haemolymph parameters

Total haemocyte count (THC) and haemocyte diameter and volume were determined using a Model Z2 Coulter Counter electronic particle counter/size analyser (Coulter Corporation, FL, USA). THC is expressed as the number of haemocytes ($x10^6$)/mL of haemolymph. Haemocyte diameter and volume are expressed in μm and in femtolitres (fL), respectively. Haemocyte proliferation was evaluated using a colorimetric method and measured using a commercial kit (Cell proliferation Kit II, Roche). This assay has been validated in our previous studies (Matozzo

et al., 2012a,b) according to the evidence of cell division in circulating haemocytes of Manila clams (Matozzo et al., 2008). The data were normalised to the THC values recorded for the clams from each experimental condition and expressed as the optical density (OD) at 450 nm. Cytotoxicity was evaluated using a colorimetric assay based on the measurement of lactate dehydrogenase (LDH) activity in cell-free haemolymph. A commercial kit (Cytotoxicity Detection Kit, Roche) was used to assess cell damage. The results, normalised to THC values, are expressed as the optical density (OD) at 490 nm. The Neutral Red uptake (NRU) assay provides a quantitative estimation of viable cells, and it was performed according to the modified method of Cajaraville et al. (1996). This test is based on the ability of cells to incorporate and bind the vital dye neutral red, thus it was used to evaluate the capability of haemocytes to perform pinocytosis. The results, normalised to THC values, are expressed as the optical density (OD) at 550 nm. Lysozyme activity was quantified in cell-free haemolymph (CFH) and in haemocyte lysate (LH) (Matozzo et al., 2012b). The results, normalised to THC values, were expressed as µg lysozyme/mg protein. Protein concentrations in CFH and LH were quantified according to Bradford (1976). The SCGE assay (Single Cell Gel Electrophoresis) was performed using the alkaline (pH>13) version of the assay developed by Singh et al. (1988) with some modifications (for more details, see Marisa et al., paper I). After haemocyte electrophoretic treatment imaging was performed using a fluorescence microscope (Leica 5000B, Germany) equipped with an FITC filter (I3, excitation BP 450-490, emission LP 515) at 10× magnification. One hundred cells per slide for a total of 600 cells per condition were analysed using an image analysis system (Comet Score®). The ratio between the migration length and the diameter of the comet head (LDR) and the percentage of tail DNA were chosen to represent DNA damage. The Micronucleus (MN) test was performed according to the method of Pavlica et al. (2000). After haemocyte preparation on slides, the slides were kept in the dark at 4 °C prior to examination under the microscope. Four hundred cells were counted for each slide for a total of 2400 cells/treatment, slides were scored under the fluorescent microscope Leica 5000B equipped with a submerged lens at 100× magnification. Only intact and non-overlapping haemocytes were considered. Micronuclei were identified according to the criteria proposed by Kirsch-Volders et al. (2000), and the MN frequency (MN‰) was reported.

2.6. Statistical analysis

The normal distribution (Shapiro-Wilk test) and the homogeneity of the variance (Bartlett test) of the data were assessed. The data were statistically compared using a two-way ANOVA test, with concentration of contaminant and time of exposure as variables and biomarkers as cases. The ANOVA was followed by a Fischer LSD post-hoc test to evaluate significant differences between treated samples and related controls (time to time) and between differing exposure times at the same experimental condition. The data regarding the bioaccumulation of FC_{60} in gills and digestive gland were statistically compared using a one-way ANOVA test followed by Tukey's HSD test.

The STATISTICA 10 software package (StatSoft, Tulsa, OK) was used for statistical analyses.

3. Results

3.1. Nanoparticle characterisation

A TEM image of FC_{60} was revealed in Fig. 1A. The FC_{60} mean crystallite diameter is 49 nm and the crystalline phase is cubic, in Fig. 1B was shown the XRD patter of FC_{60} powder.

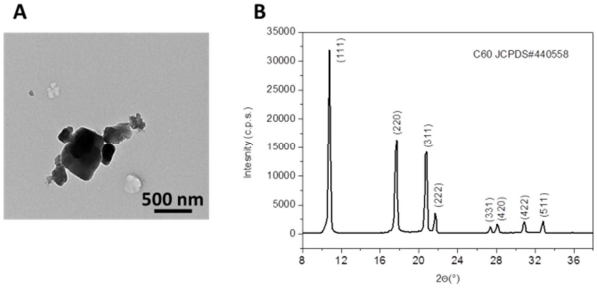

Fig. 1. A: TEM image of FC_{60}. B: XRD pattern of the FC_{60} powder. The crystalline phase is C_{60} (JCPDS#440558).

3.2. Gill and digestive gland assays

SOD activity was significantly affected by concentration ($p<0.001$) and time of exposure ($p<0.001$) in the gills; FC_{60} (10 µg/L) treated clams exhibited significantly higher values of SOD activity with respect to control only at T7 (Fig. 2A). A significant time-dependent and concentration/time interaction-dependent variation in the activity of SOD was found in the digestive gland ($p<0.001$; $p=0.009$, respectively); a significant increase was found at T3 in clams exposed to both concentrations of FC_{60} compared to control, and no significant difference was detected between the two treatments (Fig. 2B).

CAT activity was affected significantly in both tissues due to exposure to concentration ($p=0.002$ in gills, $p<0.001$ in digestive gland) and to time of exposure ($p<0.001$ in gills, $p<0.001$ in digestive gland). In the gills, the CAT activity decreased in clams exposed to both concentrations of FC_{60} at T3 and T7 compared to controls (Fig. 2C). In the digestive gland, a significant increase in CAT activity was found under FC_{60} (1 µg/L) exposure from the beginning to the end of the exposure compared to controls (Fig. 2D).

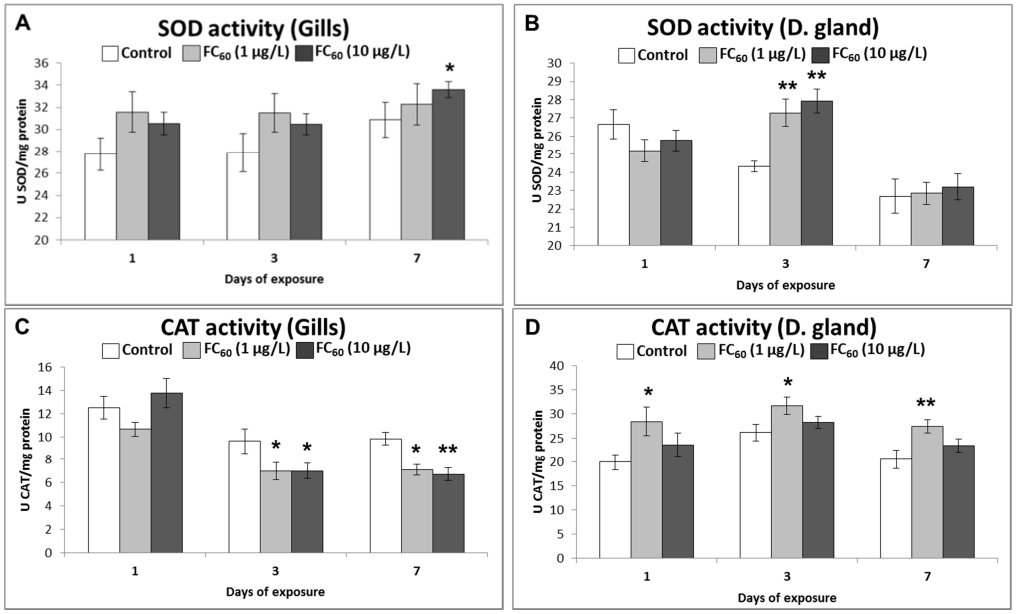

Fig. 2. SOD activity (A, B) expressed as U SOD/mg protein, and CAT activity (C, D) expressed as U CAT/mg protein in *R. philippinarum* after 1, 3 and 7 days of exposure to FC_{60} (1, 10 µg/L). The values are reported as the means ± SD (standard deviation); n= 6 pools. Asterisks denote significant differences compared to controls at the same time of exposure: *p<0.05, **p<0.01.

The GST activity exhibited in gills a significant modulation determined by FC_{60} concentration ($p<0.001$), time of exposure ($p<0.001$) and concentration/time interaction ($p<0.001$). In particular, only in clams exposed to the lower FC_{60} concentration GST activity significantly increased respect to controls at T1 and T3; at T3 the activity was statistically ($p<0.001$) higher in clams exposed to 1 µg/L respect to those at 10 µg/L of FC_{60}. At T7 no variations were detected between all experimental conditions (Fig. 3A). In digestive gland, the GST activity was affected by time of exposure ($p=0.002$) and concentration/time interaction ($p<0.001$). Pair-wise comparisons highlighted significant difference only at T3 between controls and clams exposed to the higher FC_{60} concentration,

and the activity was statistically higher ($p<0.001$) in clams exposed to 10 µg/L respect to 1 µg/L of FC_{60} (Fig. 3B).

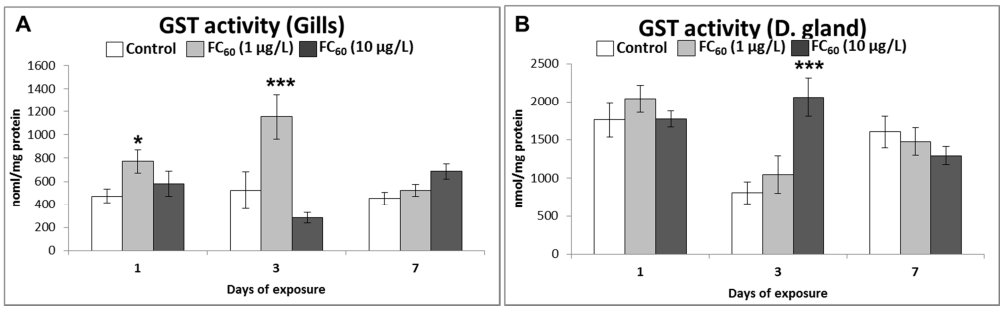

Fig. 3. GST activity (A, B) expressed as nmol/min/mg protein in *R. philippinarum* after 1, 3 and 7 days of exposure to FC_{60} (1, 10 µg/L). The values are reported as the means ± SD (standard deviation); n= 6 pools. Asterisks denote significant differences compared to controls at the same time of exposure: *p<0.05, *** p<0.001.

LPO was significantly influenced in gills by concentration ($p<0.001$), time of exposure ($p=0.002$), and concentration/time interaction ($p=0.045$). The TBARS levels increased significantly at the higher FC_{60} concentration at T1, and they were maintained for all the exposure. In clams exposed to the lower concentration of FC_{60}, an increase of lipid peroxidation respect to control was shown at T3 only (Fig. 4A). In the digestive gland, the TBARS values measured in the treated clams were affected only by time of exposure ($p<0.001$). A significant increase in lipid damage was detected in both FC_{60}-treated clams compared to control only at the end of exposure (Fig. 4B).

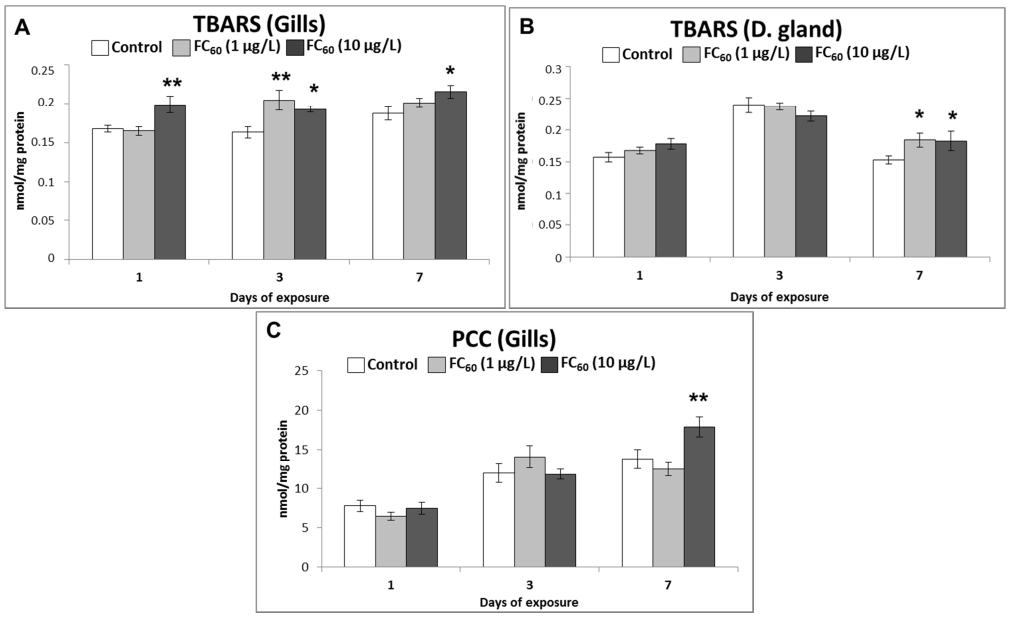

Fig. 4. TBARS (A, B) and PCC (C) levels expressed as nmol/mg protein in *R. philippinarum* after 1, 3 and 7 days of exposure to FC_{60} (1, 10 µg/L). The values are expressed as the means ± SD; n= 6 pools. Asterisks denote significant differences compared to controls at the same time of exposure: *p<0.05, **p<0.01.

PCC values were significantly affected only in gills, in particular by time of exposure ($p<0.001$) and concentration/time interaction ($p=0.007$). The only

significant difference in the pair-wise comparisons was found in FC_{60}-treated clams (10 µg/L) compared to control at T7 (Fig. 4C).
DNA damage was not significantly affected by concentration, time of exposure and their interaction in both gills and digestive gland.

3.2.1. FC_{60} bioaccumulation in gills and digestive gland

The total FC_{60} content in the gills and digestive gland of the clams exposed for 7 days to FC_{60} (1-10 µg/L) is reported in Fig. 5A and B. The results demonstrated significant accumulation of FC_{60} in both the gills ($p=0.001$) and the digestive gland ($p<0.001$).
In gills, FC_{60} content significantly increased respect to control, showing a dose-dependent trend. Moreover, the FC_{60} amount was statistically ($p=0.027$) higher in clams exposed to 10 µg/L respect to 1 µg/L of FC_{60}. In digestive gland, only the lower concentration (1 µg/L) showed a significant FC_{60} increase compared to control, with a statistically higher content ($p=0.001$) also respect to FC_{60}- (10 µg/L) treated clams.

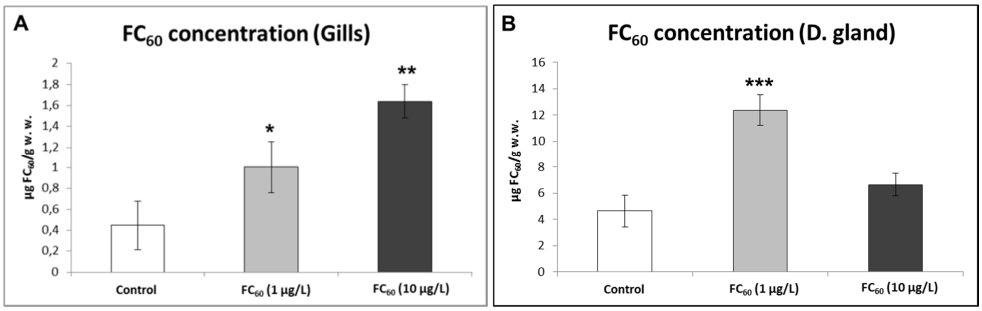

Fig. 5. Levels of FC_{60} expressed as µg FC_{60}/g wet weight, in the gills (A) and the digestive gland (B) of *R. philippinarum* after 7 days of exposure to FC_{60} (1, 10 µg/L). The values are expressed as the means ± SD; n= 4 pools. Asterisks denote significant differences compared to controls: *$p<0.05$, **$p<0.01$, *** $p<0.001$.

3.3. Haemolymph parameters

No evidence of THC modulation was detected during the exposure, instead, the diameter and volume of haemocytes were significantly modulated. The diameter was affected by FC_{60} concentrations ($p<0.001$), time of exposure ($p=0.038$) and concentration/time interaction ($p<0.001$). The volume was affected by concentration ($p<0.001$) and concentration/time interaction ($p<0.001$). For both diameter and volume, increases with respect to controls were significant at T1 in all clams exposed to FC_{60}, but at T3 only in FC_{60} (10 µg/L)- treated clams compared to controls. No significant differences were detected at T7 among all experimental conditions (Fig. 6A, B).
The haemocyte proliferation was statistically influenced by FC60 concentrations ($p<0.001$), time of exposure ($p=0.011$) and concentration/time interaction ($p<0.001$). In particular, the significant differences in the pair-wise comparisons overlapped those observed for haemocyte diameter and volume. Indeed, the haemocyte proliferation significantly increased in clams exposed to FC60 (1 and 10 µg/L) at T1, and to FC60 (10 µg/L) at T3, whereas no differences were detected at T7 (Fig. 6C).

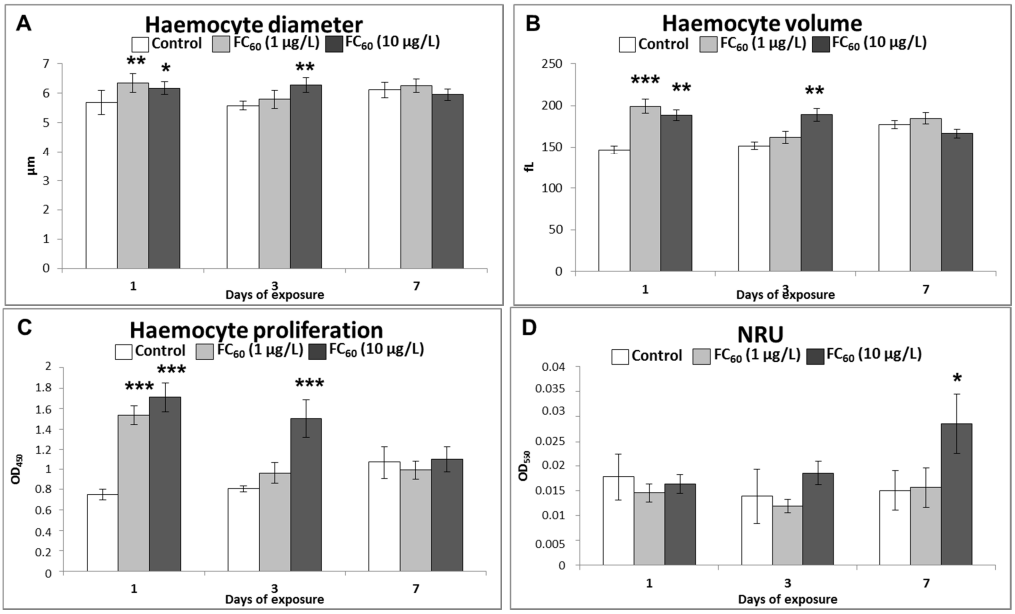

Fig. 6. Diameter of haemocytes (A) expressed in µm, volume of haemocytes (B) expressed in femtolitres (fL), haemocyte proliferation (C) expressed as OD_{450} and NRU (D) expressed as OD_{550} in *R. philippinarum* after 1, 3 and 7 days of exposure to FC_{60} (1, 10 µg/L). The values are reported as the means ± SD (standard deviation); n= 6 pools. Asterisks denote significant differences compared to controls at the same time of exposure: *p<0.05, **p<0.01, *** p<0.001.

NR uptake highlighted significant variations due to concentration in the clam haemocytes (p=0.032), and a significant increase was detected only in clams exposed to the higher FC_{60} concentration compared to controls at T7 (Fig. 6D).
LDH and lysozyme activity were not significantly different in FC_{60} exposed clams compared to controls.

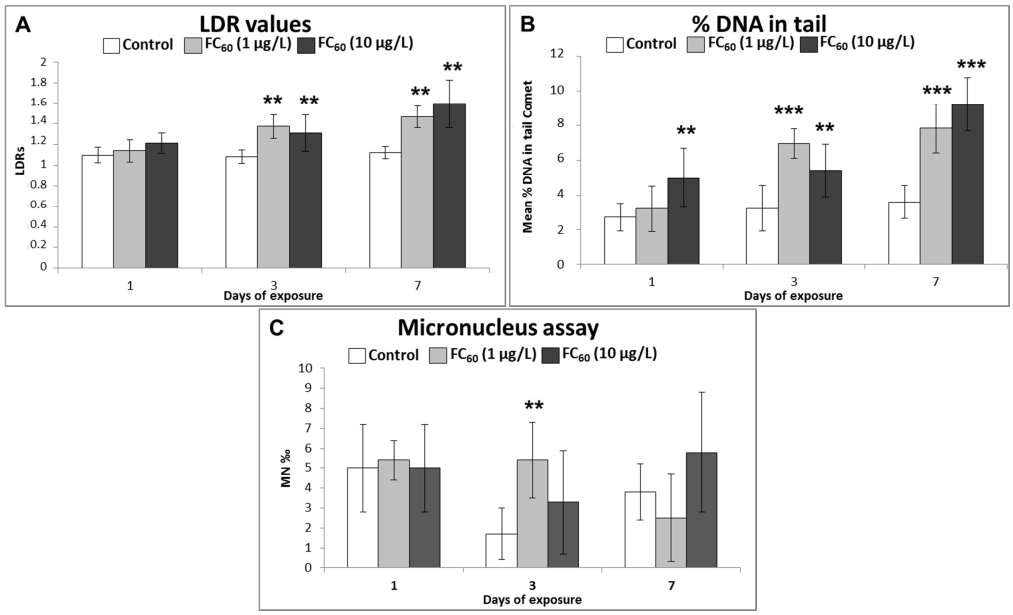

Fig. 7. The SCGE results expressed as length/diameter ratio (A) and the mean percentage of tail DNA (B), and the MN results (C) expressed as frequency (MN‰) in *R. philippinarum* after 1, 3 and 7 days of exposure to FC_{60} (1, 10 µg/L). The values are reported as the means ± SD (standard deviation); n= 6 pools. Asterisks denote significant differences compared to controls at the same time of exposure: **p<0.01, *** p<0.001.

Both SCGE assay endpoints (LDR value and the percentage of DNA in the comet tail) highlighted significant primary genetic damage due to FC_{60} concentrations in the clam haemocytes ($p<0.001$), time of exposure ($p<0.001$), and concentration/time interaction ($p=0.004$ and $p<0.001$, respectively). In particular, the LDR values significantly increased in the presence of both FC_{60} concentrations from T3, reaching maximum values at T7 (Fig. 7A). A statistically significant increase in the percentage of tail DNA was observed just after the first day of exposure onwards at the highest FC_{60} concentration, and from T3 in clams exposed to the lower FC_{60} concentration (Fig. 7B).
The frequency of micronucleus was affected only by concentration/time interaction ($p=0.013$). Difference with respect to control was significant at T3 only in the clams exposed to the lowest FC_{60} concentration (Fig. 7C).

4. Discussion

Manila clams were extensively used in laboratory studies to understand the effects of various pollutants (Blasco and Puppo, 1999; Matozzo et al., 2012a; Santovito et al., 2015), and can be considered a target species to investigate the effects of NPs (Rocha et al., 2015). The widespread use of FC_{60} in a variety of consumer products inevitably causes their release into aquatic environments (Sanchis et al., 2015). Despite this, the potential detrimental effects of FC_{60} on marine species are inadequately studied and therefore information is quite inconclusive in the literature (Ringwood et al., 2009; Canesi et al., 2010; Al-Subiai et al., 2012; Marques et al., 2013).
Moreover, data about the real concentrations in coastal areas are missing, and only PEC values are available (Gottschalk et al., 2009; Ferreira da Silva et al., 2011). In this study, we chose concentrations (1 and 10 µg/L) of FC_{60} that were in the range of PECs. Instead, most of the published studies on NP effects were performed with high concentrations. However, high concentrations tested, as well as acute toxicity assays, and *in vitro* approaches do not provide complete information about the potential interaction of NPs with aquatic organisms. It has to be noted that the behaviour of NPs change depending on the different concentrations in aqueous media (Ringwood et al., 2009; Al-Subiai et al., 2012; Barmo et al., 2013). In addition, the bioavailability, uptake, accumulation and toxicity of NPs in aquatic organisms depend on several physico-chemical properties, such as particle size/shape, surface charge and structure, particle chemistry, solubility, and aggregation state (Baker et al., 2014; Yang et al., 2013; Pakarinen et al., 2014). It is generally accepted that the toxic potential of NPs is not only dependent upon their quality, but also on target cell types and exposure conditions. In the present study, prior to evaluating the potential detrimental biological effects of FC_{60}, its physico-chemical properties were thoroughly investigated as recommended (Handy et al., 2008). The characterisation analysis of FC_{60} confirmed the information from the literature, highlighting FC_{60} high hydrophobic properties.
Despite lacking information about FC_{60} toxicity, some indications useful to assess FC_{60} impact in bivalves can be provided by the general action and fate of NPs in these organisms. Indeed, NPs are known to be filtered by the gills, accumulate in the digestive gland, and transferred to the haemolymph through the epithelium of the digestive gland tubules (Moore et al., 2009; Rocha et al., 2015). For this reason, in our study we investigated the effects in gills, digestive gland and

haemolymph of *R. philippinarum*. A battery of specific biomarkers was selected based on the information of NP toxicity, on the effects determined by FC_{60} in other species and also on the specific characteristics of this NP. The use of a multi-biomarker approach can provide an indication of the sub-lethal impacts of stressors, as well as the biochemical mechanisms that may be affected by them, and it gives an "early warning" of organism level impacts (Handy and Depledge, 1999). Few reports are available on the *in vivo* effects of FC_{60}.

Fullerene toxicity is a controversial issue. Kahru and Dubourguier (2010) compiled fullerene toxicological data for fourteen organisms and classified this nanomaterial as very toxic, taking into account the lowest median lethal concentration values for all test organisms. However, some studies indicated the absence of fullerene toxicity (Xia et al., 2010). Nonetheless, as previously mentioned, there is evidence that carbon NPs in aquatic environments are biologically active, promoting oxidative stress (Oberdörster, 2004; Nel et al., 2006). The toxicity mechanism of FC_{60} has generally been attributed to its potential ability to generate ROS (Marques et al., 2013; Al-Subiai et al., 2012), although, other studies considered the ROS generation by aqueous fullerene suspension as the minimal effect of the contaminant (Henry et al., 2011). In a *in vivo* study of Usenko et al. (2008), FC_{60}- induced oxidative stress was evaluated in the embryonic stage of zebrafish. The authors suggested that this NP acts as a pro-oxidant and enhance toxic response by interacting with biomolecules such as DNA, proteins and lipids.

Considering all the above information, the potential induction of oxidative stress determined by FC_{60} action in clam tissues was investigated in this study, by analysing variations in i) antioxidant enzyme activity ii) detoxification enzyme activity and ii) lipid, protein and DNA damage.

FC_{60} determined various effects in clam gills. The clam exposed to FC_{60} highlighted a modulation of gill antioxidant enzymes, with an increase in SOD activity indicating an increased protection from oxidative stress. Instead, CAT activity showed a decrease in treated clams compared to controls, determined by the direct or indirect action of FC_{60} on the enzyme. Inhibition of enzyme activity could occur either through direct action of chemical on the enzyme or indirectly via the regulation of enzyme genes through different mechanisms. In this scenario, considering the substrate of CAT enzyme, more H_2O_2 can be present and act in the gill cells, and consequently more oxidative stress could be induced (Livingstone, 2001; Lesser, 2006). The antioxidant CAT and SOD are important components of intracellular and antioxidant defences of organisms, and they could work together to convert O^{2-} and H_2O_2 into harmless H_2O and O_2, reduce the formation of hydroxyl free radical -OH, a toxic and active oxidant, as well as lower the overall free radical content of cells (Hu et al., 2010; Jamil, 2001). Low protection, from SOD enzyme only, highlighted in gills damage to lipids and proteins, more relevant at the higher concentration tested. These findings evidenced the high potential FC_{60} toxic action and the oxidative stress as the main mechanism, in spite of the short time of exposure in our experiment. In a long-term exposure, chronic problems to gill functions and moreover to individual health could be hypothesized.

Indeed, in Al-Subiai et al. (2012) mussels (*Mytilus* sp.) exposed to FC_{60} (0.1-1 mg/L) for 3 days showed an hypoplasia in gills and necrosis in digestive gland compared to controls. High oxidative stress and LPO were revealed also in gills of

fish *Cyprinus carpio* after an *in vitro* exposure to FC_{60} (1 mg/L) (Britto et al., 2012).

In our experiment, the FC_{60} content in gills of treated clams followed a dose-dependent increase, with a higher value in clams exposed to FC_{60} (10 µg/L) respect to the lower concentration of FC_{60} (1 µg/L), and bioaccumulation matched the biomarker results. In another study (Wang et al., 2014), increased CAT activity was reported in the worm *Lumbriculus variegatus* exposed for 14 days to FC_{60} (5 mg/kg per day), but a low bioaccumulation of FC_{60} was detected in worm specimens. Nonetheless, the relationship between FC_{60} body residue and CAT activity followed a linear regression. Considering their data, Wang et al. (2014) suggested the need for a more in-depth study of FC_{60} agglomerate accumulation and corresponding oxidative stress in living organisms.

In clam digestive gland slight variations in antioxidant enzyme activities and damage to molecules were detected respect to those observed in gills. At the end of the exposure, only an increase in CAT activity and LPO levels were shown in treated clams compared to controls.

In the digestive gland, FC_{60} content did not show a dose-dependent pattern, and the only significant increase was found in clams exposed to the lower FC_{60} concentration compared to controls. Similarly to what observed by Wang et al. (2014) in *L. variegatus*, at lower FC_{60} concentration, the formation of smaller NP agglomerates could enhance their bioaccumulation. Instead, at higher FC_{60} concentrations, larger NP aggregates could be retained in the gills, thus allowing the observed pattern of accumulation, but they did not reach the digestive gland. Disaggregation of large size NP aggregates might occur (Rocha et al., 2015), even though it probably require time span longer than 7 days, as in our exposure. On the other hand, it is generally recognized that small size NP aggregates are more likely to undergo a process of disaggregation and infiltrate biological systems compared to the larger aggregates that cannot infiltrate (Moore, 2006) and diffuse through cell membranes (Lin et al., 2010; Rocha et al., 2015). In this case lower concentrations in short exposure could be considered more toxic than higher concentrations.

In the oyster *Crassostrea virginica* exposed to FC_{60} (1, 10, 100, 500 µg/L) for 4 days, there was no significant increase in LPO in hepatopancreas. Moreover, confocal microscopy analysis indicated the presence of FC_{60} in oyster hepatopancreas cells within 4 h. In particular, FC_{60} aggregates tended to be localized and concentrated into lysosomes (Ringwood et al., 2009).

Marques et al. (2013) analysed the polychaete *Laeonereis acuta* and the bacteria that colonized the worm mucus after FC_{60} (0.01, 0.10 and 1 mg/L) exposure for 24 h. FC_{60} reduced total antioxidant capacity of bacteria from worms exposed to 0.1 mg/L. In worms lower antioxidant capacity and LPO reduction were observed after exposure to 1 mg/L (Marques et al., 2013).

In our study, DNA damage in gills and digestive gland was not detected in treated clams, the low damage to molecules is consistent with low concentrations of the contaminant and short *in vivo* exposure.

Respect to our data, more accumulation of FC_{60} was detected in digestive gland compared to gills in *Mytilus* sp. exposed to FC_{60} (Al-Subiai et al., 2012), even though the concentrations of NPs tested in that study were thousands of times higher than the concentration considered here.

The haemocytes of the clam *R. philippinarum* play a key role in internal defence and have various functions (Cima et al., 2000; Donaghy et al., 2009). Several studies have demonstrated the adverse effects of contaminants on haemocyte functionality in bivalves, as well as for NPs (Renault et al., 2015).
In this experiment, only a change in NRU assay results was shown in the haemocytes, together with a low haemocyte DNA damage during the exposure. In particular, a significant increase in NRU values was observed in clams at the higher FC_{60} concentration at the end of the exposure, but this finding was not confirmed by an increased cytotoxicity. Uptake of NR cationic dye by haemocytes occurs by pinocytosis or passive diffusion across cell membranes (Coles et al., 1995). Therefore, alterations in dye uptake may reflect damage to cell membranes (including lysosomal membranes) and/or weakening of haemocyte pinocytotic capabilities. Our results demonstrated that FC_{60} significantly increased NR uptake by haemocytes, suggesting that exposure of clams to FC_{60} induced changes in the membrane permeability of haemocytes and increased the number and/or the volume of lysosomes, which probably contain higher levels of NR than those of haemocytes from control clams.
Only a low DNA damage was determined by SCGE assay in FC_{60} exposed clams, indicating a potential action of the contaminant directly through interaction with DNA inside the nucleus or indirectly through various mechanisms, for example the generation of ROS (Moore, 2006; Handy et al., 2008). Low damage to DNA in haemocytes could be related to the short time of exposure and also to the low concentrations tested. In the study of Al-Subiai et al. (2012), where the concentrations were really higher than ours, FC_{60} determined high genotoxicity in mussel haemocytes.
Various biomarkers in gills (GST activity), digestive gland (SOD and GST activity) and haemolymph (volume and diameter of haemocytes, haemocyte proliferation and MN frequency) responded only in the first phases of the exposure, but these changes in treated clams compared to controls were not maintained until the end of the exposure. In the first phase of the exposure, in gills and digestive gland an increase of GST activity indicated a potential reaction of cells to FC_{60}. GST is an enzyme that participates in the detoxification process due to conjugation reaction between its substrate glutathione (GSH) and xenobiotics, assisting in the elimination of external materials (Cummins et al., 2011). In digestive gland of FC_{60} treated clams, SOD activity showed an increase at T3, indicating more protection to oxidative stress. In haemolymph, the modulation of some haemocyte parameters at T1 and T3 suggested a transient reaction to FC_{60} and overall increased stress conditions possibly responsible for other more persistent effects, such as the increasing DNA damage observed throughout the exposure.
It is possible to hypothesize that clams take advantage from differing pathways, according to differing tissues or organs, to cope with NP impact. This may lead to dissimilar patterns of bioaccumulation and effects depending on the tissue or organ considered.

In conclusion, our experimental results indicated the sensitivity of *R. philippinarum* to FC_{60} under 7 days of exposure. Although, the digestive gland is generally considered the target tissue of NP toxicity in bivalves (Rocha et al., 2015), in this study the gills demonstrated to be more affected by the experimental

conditions tested. As confirmed by our findings, oxidative stress could be recognized as the primary effect of FC_{60}, under a *in vivo* short-term exposure at low concentrations similar to PECs. Instead, unless other NPs, FC_{60} scarcely affected haemocytes in treated clams.

In this study, the potential bioaccumulation of FC_{60} content in gills and digestive gland was also highlighted, in agreement with similar evidence from the literature. To our knowledge, this is the first study that investigated the effects of FC_{60} in Manila clams, thus providing a new topic of discussion for this NP pollution in marine species.

In future studies the toxicity of FC_{60} needs to be investigated for chronic effects to confirm the effects detected here in clams.

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PAPER: V

Ilaria Marisa, Valerio Matozzo, Nicola Brianese, Maria Fedorova, Ralf Hoffman, David Sheehan, Maria Gabriella Marin

Can zinc oxide, titanium dioxide and C_{60} fullerene nanoparticles, alone and as a mixture, differently affect biomarkers and proteome in the clam *Ruditapes philippinarum*?

Can zinc oxide, titanium dioxide and C_{60} fullerene nanoparticles, alone and as a mixture, differently affect biomarkers and proteome in the clam *Ruditapes philippinarum*?

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Abstract
Continuous release of NPs into marine coastal environments, recognized as ultimate sink for these contaminants, results in increased risk of exposure to complex NP mixtures for marine organisms.
To assess mixture effects, the clam *R. philippinarum* was exposed for 7 days to i) 1 μg/L nZnO ii) 1 μg/L $nTiO_2$ iii) 1 μg/L FC_{60} fullerene and iv) all three NPs as a mixture. Haemolymph, gills and digestive gland were used to understand the action of a mixture of NPs which can interact in different ways (i.e. additively, synergistically, or antagonistically) causing different effects respect to the single NPs. In gills and digestive gland, anti-oxidant and detoxification enzymatic activities, lipid peroxidation, DNA damage were measured at three time intervals (after the first, T1, third, T3, and last ,T7, day of exposure); only at T7, protein damage and its modulation were investigated using redox proteomics (one and two dimensional electrophoresis; 1-DE and 2-DE). Moreover, the bioaccumulation of NPs in all experimental conditions were investigated in gills and digestive gland. In haemolymph, DNA damage was evaluated at three time intervals during exposure; only at T7, the production of intracellular superoxide anion was evaluated and the protein damage was investigated with 1-DE redox proteomic analysis.
The 1-DE redox proteomics analysis showed an increase of damage to proteins (redox-thiol and carbonylated) in gills and digestive gland of clams exposed to NP mixture. From the results of 2-DE redox proteomics analysis, 16 proteins in gills and 18 proteins in digestive gland were modulated (in terms of damage or abundance) under the experimental conditions tested. The modulated proteins can be ascribed to two principal groups i) cytoskeleton proteins and ii) energetic metabolism proteins. Moreover, the changes in enzyme activities indicated an

high oxidative stress underway in both tissues; only in digestive gland, a significant increase in damage to lipids and DNA was shown. Bioaccumulation of Zn, Ti and $FC_{60,}$ both as single NPs and as a mixture, was found in both gills and digestive gland. In haemolymph, an increase in superoxide anion production and damage to DNA and proteins was recorded in mixture-exposed clams.
Respect to single NP treatments, all findings indicated higher oxidative stress in act during the exposure to the mixture, with damage to proteins, lipids and DNA. In all biomarkers measured, additive effects were observed in the mixture respect to single NP exposures. The digestive gland was the tissue more affected by NP mixture toxicity.

Keywords: nanoparticles, zinc oxide nanoparticles, titanium dioxide nanoparticles, C_{60} fullerene nanoparticles, mixture, redox proteomics, biomarkers

1. Introduction

Organisms in their environment are exposed to complex mixtures of contaminants rather than to individual chemicals. Thus, the challenge emerges on the importance of understanding the potential combined effects of such mixture exposures. Many studies have investigated the action of various contaminant mixtures to aquatic species. Recently, attention has been given to mixtures of pharmaceutical compounds (González-Ortegón et al., 2013), pharmaceutical and personal care products (DeLorenzo and Fleming, 2008), metals (Mebane et al., 2012), metals and polycyclic aromatic hydrocarbons (Giuliani et al., 2013), herbicides and insecticides (Choung et al., 2013), and pesticides (Hasenbein et al., 2015).
The increasing production and usage in various fields of different types of manufactured nanomaterials (NPs) can lead to their release in substantial amounts in the environment, including the aquatic compartments (Corsi et al., 2014). This in particular applies to those types of nanoparticles (NPs) that are produced in higher amounts, such as metal-oxides and carbon-based NPs, thus raising considerable concern on their environmental behaviour and consequent impact on aquatic organisms (Canesi et al., 2015), not only as single compounds but most of all as a mixture.
Marine coastal areas are contaminated continuously by various types of pollutants, and this could occur also for NPs (Corsi et al., 2014). Most studies on NP toxicity are related to freshwater environments, and relatively little is known regarding the potential biological risks of NPs on marine species (Exbrayat et al., 2015). Generally, these studies have investigated the action of single NPs and/or the combined effects of NPs and other types of contaminants, both organic and metallic, to evaluate a potential 'Trojan horse' effect, that implies facilitated entry of toxic molecules adsorbed to NPs into the cells (Canesi et al., 2015). The exposure to a mixture of pollutants, as in our case the NPs, may result in differing mode of action respect to the single components, since these may interact in many ways (i.e. additively, synergistically, or antagonistically) to induce biological responses at different levels of biological organisation. However, investigations on the combined toxic effects of multiple chemicals in organisms are much more challenging than of those regarding single compounds and are therefore scarce. In addition, the number of possible combinations of pollutants is extremely large, and the combination that is likely to be most important is unknown.

NPs from commercial products having similar patterns of discharge into marine environments may arrive, deposit and occur together, as a mixture, mostly in coastal areas. In this experiment, two metal oxide NPs, zinc oxide (nZnO) and titanium dioxide ($nTiO_2$), and carbon NPs C_{60} fullerene (FC_{60}) were considered as a mixture. The selected NPs are the most produced and used in common products worldwide (Piccinno et al. 2012; Sanchis et al., 2015).
The information regarding the concentrations in marine areas of these three NPs is still lacking, only the predicted environmental concentrations (PECs) can be used as a reference. They provide an estimate of nZnO, $nTiO_2$ and FC_{60} concentrations in environment, that are at low µg/L concentrations (Gottschalk et al., 2013). Although, these NPs are present in aquatic environments at relatively low concentrations, their mixture could determine different effects compared to each single NP. The exploration into co-toxic effects of metal mixtures has revealed that most co-toxic outcomes are not simple additions of individual toxicities (Norwood et al., 2003). Non-additive co-toxic outcomes, either less- or more-than-additive, are common and complicate attempts to address the ecological risk posed by environmental contamination by mixtures.
Many studies were conducted to understand the action of single nZnO, $nTiO_2$ and FC_{60} to aquatic species (Ma et al., 2013; Menard et al., 2011; Minetto et al., 2014; Oberdörster et al., 2006; Ringwood et al., 2009), but to our knowledge no investigation has been conducted to detected the effects of a mixture of these three NPs.
The goal of this study was the investigation of the sub-lethal effects of the NP mixture on the marine clams *Ruditapes philippinarum*. Primarily due to their filter-feeding habit, bivalve molluscs may represent a unique target group for NP toxicity (Moore, 2006; Canesi et al. 2012). Among bivalves, infaunal clams may experience more severe impacts due to exposure to NPs (Rocha et al., 2015).
Based on the physico-chemical characteristics of the three NPs and on the primary mechanisms of NP toxicity recognised in the previous researches on the single NPs (Marisa et al., paper I, III, IV), the oxidative stress and damage were the main objective of the investigation.
To get a better insight into NP effects, the multi-biomarker approach was matched with the redox proteomic approach, aimed at detecting and analysing redox based changes within the proteome as a result of oxidative stress in tissues (Sheehan, 2006). Specifically, in clam gills and digestive gland, superoxide dismutase, catalase, and glutathione S-transferase activities, lipid peroxidation, DNA damage were measured three times throughout a 7-day exposure, whereas protein damage and its modulation were assessed using one dimensional electrophoresis (1-DE) and two dimensional electrophoresis (2-DE) redox proteomics at the end of the exposure. Similarly, in haemolymph, DNA damage was evaluated during the exposure, whereas the production of intracellular superoxide anion and the protein damage using 1-DE redox proteomics were assessed at the end of the exposure.
Lastly, to assess potential differences in bioaccumulation of NPs alone or as a mixture, Zn, Ti and FC_{60} contents were measured in both clam gills and digestive gland at the end of exposure.

2. Materials and methods

2.1. nZnO, $nTiO_2$ and FC_{60} characterisation

nZnO (declared size of <100 nm, percentage of zinc 79.1 - 81.5%, surface area 15 -25 m^2/g), $nTiO_2$ (P25, declared size of 21 nm, percentage of titanium >99.5%, surface area 35 - 65 m^2/g) and

FC_{60} (purity>97.5 %) were purchased from Sigma-Aldrich (Milano, Italy). NPs were characterised via a combination of analytical techniques, already described and used. Details about the characterisation procedure for the three NPs are reported in Marisa et al. (paper I, III, IV), respectively.

2.2. Clams and NP exposure

Specimens of *R. philippinarum* were collected from a reference site located within a licensed clam culture area in the southern part of the Lagoon of Venice (Italy). These specimens were then acclimatised in the laboratory for 5 days before exposure to contaminants. Clams were maintained in large aquaria, which contained a sandy bottom and aerated natural seawater (salinity of 35 ± 1 psu, temperature of 16 ± 0.5 °C) and were fed daily with microalgae (*Isochrysis galbana*).

Stock solutions (0.1 g/L) of nZnO, $nTiO_2$ and FC_{60} were prepared in Milli-Q water and sonicated at 4 °C using a Braun Labsonic U sonifier at 50% duty cycles for 30 min. Clams (35 per tank) were exposed for 7 days to i) 1 μg/L nZnO ii) 1 μg/L $nTiO_2$ iii) 1 μg/L FC_{60} fullerene and iv) all three NPs as a mixture. For each experimental condition tested, three replicate tanks were prepared. The nominal concentration was chosen similar to the PEC values found in the literature. During exposure, the clams were maintained in glass aquaria (without sediment) containing aerated seawater (1 L per animal) in the same thermo-haline conditions used during the acclimatisation period. A movement pump (Hydor, Koralia nano 900, USA) was positioned in every aquarium (both for control and treated clams) to facilitate the water circulation and to prevent NP sedimentation (Marisa et al., paper I). The seawater was renewed daily, NPs and microalgae (at an initial concentration of approximately 150,000 cells/L) were supplied in the experimental tanks. Before adding contaminants, the stock solutions were sonicated, as reported above.

2.3. Collection of tissues for biomarkers analysis

During exposure, clam haemolymph, gills and digestive gland were collected after the first (T1), third (T3), and last (T7) day of exposure. For each tissue, five pools (5 animals per pool) from each experimental condition were prepared. Aliquots of each pooled tissue were frozen in liquid nitrogen and stored at -80 °C until analyses or immediately processed, depending on the various biological responses measured. All assays performed in this study had previously been validated (Marisa et al., paper I; Marisa et al., paper III, Matozzo et al., 2012a; Matozzo et al., 2012b; Matozzo et al., 2013; Parolini et al., 2010; Parolini et al., 2013).

2.4. Gill and digestive gland preparation and biochemical assays

Pooled gills and digestive glands were homogenised at 4 °C using an Ultra-Turrax homogeniser (model T8 basic, IKA) in four volumes of 50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M KCl, 0.5 M sucrose, and Protease Inhibitor Cocktail (P2714, Sigma–Aldrich) and then centrifuged at 12,000 × *g* for 40 min at 4 °C. Supernatants (SN) were collected for the analyses. SN protein concentrations were quantified according to Bradford (1976) using bovine serum albumin (BSA) as the standard.

Total superoxide dismutase (SOD) activity was measured in the SN of both tissues using the xanthine oxidase/cytochrome c method proposed by Crapo et al. (1978). Enzyme activity is expressed as U SOD/mg protein, where one unit of SOD was defined as the amount of sample producing 50% inhibition in the assay conditions. Gill and digestive gland catalase (CAT) activity was measured according to the method of Aebi (1984). The results are expressed in U CAT/mg protein, where one unit of CAT was defined as the amount of enzyme that catalysed the dismutation of 1 μmol of H_2O_2/min.

Glutathione S-transferase (GST) activity was measured spectrophotometrically according to the method described in Habig et al. (1974). GST activity is expressed as nmol/min/mg protein. Lipid peroxidation (LPO) was quantified in both tissues SN using the malondialdehyde (MDA) assay, according to the method of Buege and Aust (1978). The results are expressed as nmoles of thiobarbituric reactive substances (TBARS)/mg protein. TBARS, considered as ‘‘MDA-like peroxide products’’, were quantified by reference to MDA absorbance (Damiens et al., 2007). The results were not expressed as MDA levels because TBA can react with a range of chemical compounds (Csallany et al., 1984). DNA strand breaks were quantified using a fluorescence technique adapted from the alkaline precipitation assay (Olive, 1988). Samples of both gills and digestive gland were weighed (Mettler Toledo, XS105 Dual Range analytical balance, 0.01 mg readability) before tissue preparation (see above), and the wet weight was recorded. Salmon sperm

genomic DNA standards were added for DNA calibration, and the results are expressed as µg/g wet weight.

2.5. Bioaccumulation of NPs in gills and digestive gland

At the end of the exposure (T7), 4 pools of gills and digestive glands per experimental condition (6 animals each) were collected to quantify zinc, titanium and FC_{60} bioaccumulation.

2.5.1. nZnO and $nTiO_2$ bioaccumulation in gills and digestive gland

Tissue samples were freeze-dried, and approximately 150 mg were weighed and digested in TFM vessels with 4 mL of 69% nitric acid, 1.5 mL of 30% hydrogen peroxide and 0.4 mL of 47% hydrofluoric acid. Digestion was performed in a Milestone MLS 1200 MEGA microwave oven. The heating programme consisted of five stages (2 min, 250 W - 2 min, 0 W - 6 min, 250 W - 5 min, 400 W and 5 min, 650 W). After cooling, 5 mL of saturated boric acid solution were added, and the heating programme performed again (20 min, 400 W). Samples were then transferred into graduated flasks and diluted to 25 mL with Millipore Milli-Q water. The sample solutions were analysed via inductively coupled plasma optic emission spectroscopy (ICP-OES) using a Thermo Fischer Scientific iCAP 6300 DUO. Five calibration solutions (0, 0.5, 1, 3 and 6 ppm of Zn and Ti) were prepared by conventional dilution of Carlo Erba 1000 µg/mL mono-elemental standard solution of the analyte as nitrate. The same amount of reagents used for the digestion procedure was added to each calibration solution. Measurements were made at 323,45 nm for Ti and at 202.55 nm for Zn, each sample was analysed in five replicas. The results are expressed as µg Zn/g dry weight and µg Ti/g dry weight. The detection limits of Zn and Ti were 0.9 µg/L and 0.3 µg/L, respectively.

2.5.2. FC_{60} bioaccumulation in gills and digestive gland

Tissue pools were carefully washed with pure toluene to remove surface adsorbed FC_{60} particles. Then, tissues were extracted into toluene (1:6, w:v ratio) using ultra-sonication for 30 min (35 kHz). Extracts were centrifuged (9000 ×g) and the SN was used in HPLC (High Performance Liquid Chromatography) analysis. FC_{60} was separated using Eclipse XDB-C18 4.6 mm ID x 250 mm 5µm 80 Å column. The mobile phase for columns was toluene Sigma-Aldrich HPLC grade, at a flow-rate of 1.0 mL/min. Sample injections were performed manually with volumes of 100 µL. The eluent was monitored at 335 nm using a HPLC Agilent 1100, Chemstation rev. A10.02. For external calibration, a standard curve was generated for FC_{60} concentrations ranging from 0.001 to 0.8 mg/mL. The concentration of each sample was calculated by comparison to the standard curve, and the results were expressed as µg FC_{60}/g wet weight.

2.6. Haemocyte assays

The SCGE assay was performed using the alkaline (pH>13) version of the assay developed by Singh et al. (1988) with some modifications (for more details see Marisa et al., paper I). Imaging was performed using a fluorescence microscope (Leica 5000B, Germany) equipped with an FITC filter (I3, excitation BP 450-490, emission LP 515) at 10× magnification. All steps were performed in the dark to minimise additional UV-induced DNA damage. One hundred cells per slide for a total of 500 cells per condition were analysed using an image analysis system (Comet Score®). The ratio between the migration length and the diameter of the comet head (LDR) and the percentage of tail DNA were chosen to represent DNA damage.

At the end of the exposure (T7), intracellular superoxide anion (O^{-}_{2}) assay was determined in haemocyte samples, according to Matozzo et al. (2008). The results are expressed as optical density per mg protein (OD mg/protein), protein concentrations were quantified according to the Bradford method (1976).

2.7. Collection and preparation of tissues for redox proteomic analysis

At the end of the exposure (T7), 5 pools of haemolymph, gills and digestive glands per experimental condition (4 animals each) were collected to be analysed with redox proteomics. The samples of gills and digestive gland were homogenized in a motor-driven Teflon Potter-Elvejhem homogenizer in Tris-HCl buffer containing 10 mM Tris-HCl pH 7.2, 0.5 M sucrose, 0.15 M KCl, 1 mM EDTA and 1 mM PMSF at a weight:volume ratio of 1:3. The pools of haemolymph were sonicated for 10 minutes in ice with 1 mM EDTA and 1 mM PMSF.

The homogenate tissues were centrifuged at 15,000 xg for 1 h at 4 °C and the SN was collected and stored in -80 °C for further analysis.

In particular, the cytosolic proteins of gills and digestive gland were considered together for 1-DE and 2-DE redox proteomics analysis. The cytosolic proteins of haemocytes and those present in haemolymph were considered together for 1-DE redox proteomics analysis. The protein content was estimated by the Bradford method (1976).

2.7.1. Fluorescent protein labelling and 1-DE redox proteomics

Thiol groups in protein SN (300 μg protein) were labelled by adding 5-(Iodoacetamido)fluorescein (IAF, 0.2 mM in dimethyl sulfoxide) and incubating in ice for 2 h in the dark. IAF is a thiol-specific reagent which reacts only with free thiols but not with oxidized variants such as sulfenic/sulfinic/sulfonic acids, disulfides or nitrosothiols. The fluorescein moiety of IAF provides a ready means for detecting proteins containing free thiols in electrophoretic separations.

For fluorometric determination of protein carbonyl groups in oxidized proteins, carbonyls in protein SN were labelled with fluorescin 5'-thiosemicarbazide (FTSC, 0.2 mM in dimethyl sulfoxide) and treated identically as for IAF. Before electrophoresis, proteins were precipitated using trichloroacetic acid (TCA) and acetone for samples labelled with IAF and TCA and ethyl acetate:ethanol (1:1) for those labelled with FTSC.

After precipitation, fluorescently labelled proteins were resolved using one-dimensional electrophoresis in 12% polyacrylamide gels, at a loading of 50 μg per lane, with four replicate lanes per sample. Briefly, proteins were dissolved in sample buffer (0.5M TRIS-HCl, pH 6.8; glycerol; 10% SDS; 0.5% bromophenol blue), and electrophoresed at 4 °C in running buffer (Tris 19 mM, glycine 193 mM, SDS 0.1%), using an AE-6450 mini PAGE system (Atto, Tokyo, Japan) at 80 V for 1 h and 120 V until the electrophoresis was complete. After electrophoresis, gels were scanned in a Typhoon scanner, model 9410 (Amersham Biosciences) with excitation wavelength set at 488 nm and emission wavelength at 530 ± 20 nm filter. For each 1-DE gel, all bands detected by the Typhoon scanner were subsequently analyzed by Quantity One image analysis software (BioRad, Hercules, CA, USA) measuring the total intensity for each lane. They were quantified as arbitrary units (A.U.).

The gels were then stained with colloidal Coomassie G250. All 1-DE gels stained with Coomassie were scanned in a GS-800 calibrated densitometer (BioRad Hercules, CA, USA) and the optical density from each lane was measured by Quantity One image analysis software as described above. Total optical densities for each lane were normalized with those from Coomassie staining from the same lane in each gel. Four replicates (technical replicates) from 5 different pools (biological replicates) for each treatment were performed in 1-DE.

2.8. 2-DE redox proteomics in gills and digestive gland samples

2-DE analysis was performed on gills and digestive gland protein SN (550 μg protein) incubated with both IAF and FTSC, and precipitated as stated in the previous section for 1-DE analysis. After protein labelling, protein pellets (3 replicates per pool, 180 μg protein) were re-suspended in rehydration buffer (7M urea, 2M thiourea, 2% CHAPS, 1.2% bis (2-hydroxyethyl)-disulfide, 4% ampholytes (3–10 for IEF, GE Healthcare) and a trace of bromophenol blue. The re-suspended proteins was then loaded into an Immobiline DryStrip (pH 3-10 NL, 7cm, GE Healthcare), which was rehydrated overnight in the dark and followed by isoelectric focusing (IEF) using a PROTEAN IEF system (BioRad, Hercules, CA, USA), according to the strip manufacturer's recommendations. Strips were reduced in equilibration buffer (6M urea, 0.375 M Tris, pH 8.8, 2% SDS, 20% glycerol) containing 2% dithiothreitol (DTT) for 20 min and thiols were then blocked with equilibration buffer containing 2.5% iodoacetamide for 20 min. Equilibrated strips were then applied to 12% polyacrylamide gels for SDS PAGE separation. Strips were embedded in molten agarose (0.5%) containing trace bromophenol blue atop 12% SDS PAGE gels, and electrophoresed at 90 V for 30 min followed by a constant voltage (120 V) using a mini PAGE system until the dye front reached the end of the gel. After electrophoresis, gels were scanned for fluorescence and then stained with colloidal Coomassie as for 1-DE analysis.

2.8.1. Protein analysis and identification

Image analysis was performed using Progenesis SameSpots software (Nonlinear Dynamics Limited, UK) to identify significantly altered spots in each treatment group in response to the NPs. This software employs an alignment-based analysis approach which maps the same number of spots across all gels in a single analysis. The data obtained from protein gel images were tested for normality prior to any analysis and significance testing level was set at 0.05. Principal component analysis and analysis of variance (one-way ANOVA) were performed for comparison and

assessment of statistically significant expression and redox variation between treatments and control. The spots were considered significantly different when $p<0.05$ according to ANOVA and a fold change >1.5 were evident. Significant and well-resolved spots of sufficient intensity were selected for identification analysis. These spots were manually excised using sterile pipette tips.

2.8.2. In-gel trypsin digestion

The spots were excised and the gel pieces were destained twice with 200 µL of 25 mM ammoniumbicarbonate in 50% acetonitrile for 30 min at 37 °C. After destaining, they were dehydrated in 100 µL of acetonitrile for 5 min and completely dried in a speed-vac (Thermo Savant, Savant Instruments,USA) after solvent removal. The obtained proteins were digested with trypsin (175 ng of trypsin in 3 mM ammonium bicarbonate) for 4 h (37 °C; 550 rpm), and peptides were extracted via the addition of acetonitrile followed by 15 min of sonication. The collected supernatants were dried and dissolved in 5 µL of 60% acetonitrile containing 0.5% formic acid.

2.8.3. Protein identification

The protein digests were separated in a nanoAcquity UPLC (Waters GmbH, Eschborn, Germany) coupled on-line to an LTQ Orbitrap XL ETD mass spectrometer equipped with a nanoESI source (Thermo Fisher Scientific GmbH, Bremen, Germany). The temperature of the transfer capillary was set to 200 °C and the tube lens voltage to 120 V. The ion spray voltage (1.5 kV) was applied to a PicoTip™ on-line nano-ESI emitter (standard coating) for the nano UPLC (outer diameter 360/20 µm and tip internal diameter 10 µm; New Objective, USA). Eluent A was 0.1% aqueous formic acid, and eluent B was 0.1% formic acid in acetonitrile. Tryptic peptides were dissolved in eluent A (20 µL) and injected via the autosampler at a flow rate of 10 µL/min into a trap column (nanoAcquity UPLC™ Symmetry® C_{18}, 180 µm × 20 mm, particle diameter 5 µm); then, they were separated in a nanoAcquity UPLC® BEH130 C_{18} (100 µm × 100 mm, particle diameter 1.7 µm) using a two-step gradient (from 3% to 50% acetonitrile over 30 min, which was then increased to 85% over 3 min – at a flow rate of 0.4 µL/min).The precursor ion survey scans were recorded in the Orbitrap with a resolution of 60,000. Within the same time span, the tandem mass spectra of the top six peaks with charge states of two or higher were acquired via CID activation in a linear ion trap (isolation width 2, normalised collision energy 35%, activation Q 0.25, activation time 30 ms) using Xcalibur (version 2.0.7). The acquired tandem mass spectra were analysed automatically with Sequest (Proteome Discover, Thermo Fisher) in the Swiss-Prot database, allowing up to two missed cleavage sites and a mass tolerance of 10 ppm for precursor ion scans and a mass tolerance of 0.8 µ for the product ion scans.

2.9. Statistical analysis

The normal distribution (Shapiro-Wilk test) and the homogeneity of the variance (Bartlett test) of the data were assessed. The data of biomarkers with T1, T3 and T7 collection times of each tissue were statistically compared using a two-way ANOVA test, with concentration of contaminant and time of exposure as variables and biomarkers as cases. The ANOVA was followed by a Fischer LSD post-hoc test to evaluate significant differences between treated samples and related controls (time to time) and between exposure times. The results are expressed as the mean ± standard deviation. The STATISTICA 10 software package (StatSoft, Tulsa, OK) was used for statistical analyses.

The data regarding superoxide anion production, NP bioaccumulation in gills and digestive gland, and the redox proteomics results (1-DE) were statistically compared using a one-way ANOVA test followed by Tukey's HSD test, to evaluate significant differences between treated samples and related controls.

3. Results

3.1. nZnO, $nTiO_2$ and FC_{60} characterisation

A TEM image of nZnO, $nTiO_2$ and FC_{60} are provided in Fig. 1A, B, and C respectively. The complete results of the characterisation for the three NPs were reported in Marisa et al. (paper I, III, IV).

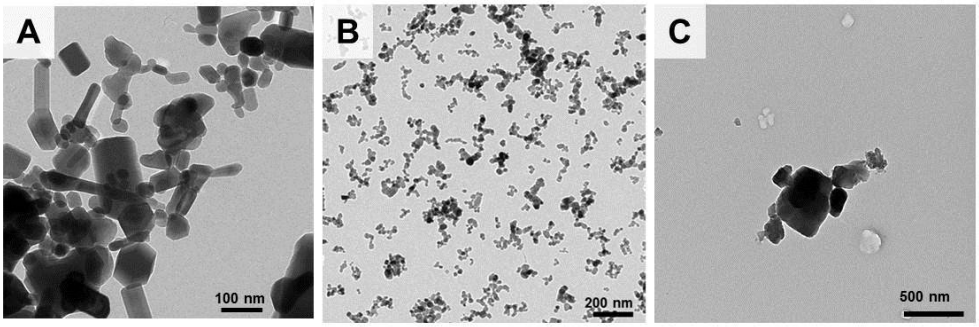

Fig. 1. A, B, C: TEM images of nZnO, $nTiO_2$ and FC_{60}.

3.2. Gill and digestive gland assays

In gills, SOD activity showed a significant modulation due to time of exposure ($p<0.001$) only, with no significant differences in the pair-wise comparisons. In digestive gland, SOD activity was significantly affected by exposure to concentration ($p=0.002$), time of exposure ($p<0.001$) and concentration/time interaction ($p<0.001$). A significant increase of SOD activity was found at T7 in clams exposed to the NP mixture respect to control (Fig. 2A).

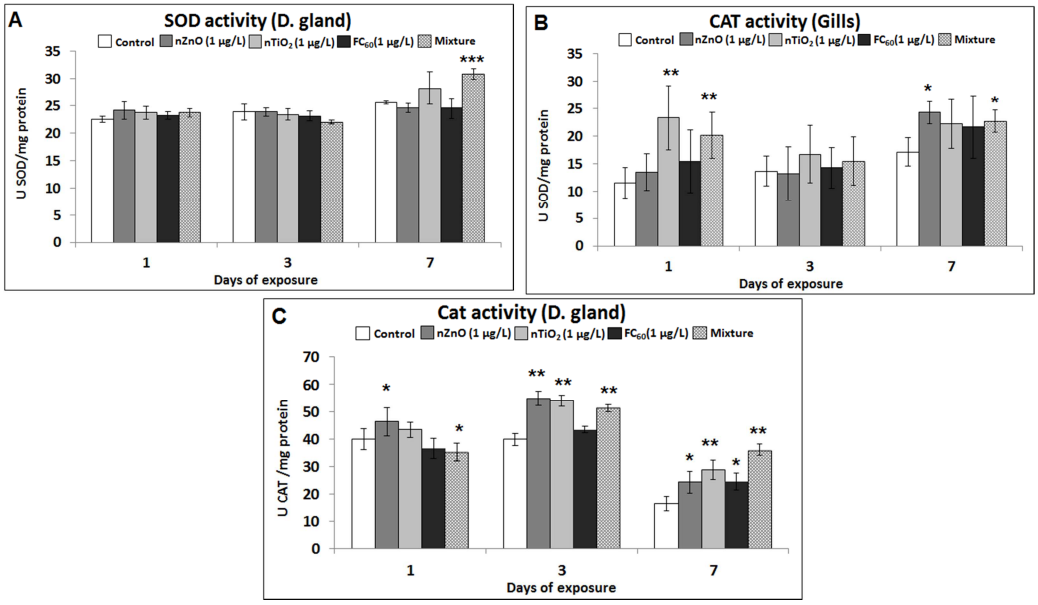

Fig. 2. SOD activity (A) expressed as U SOD/mg protein, and CAT activity (B, C) expressed as U CAT/mg protein, in *R. philippinarum* after 1, 3 and 7 days of exposure to nZnO (1 µg/L), $nTiO_2$ (1 µg/L), FC_{60} (1 µg/L) and mixture. The values are reported as the means ± SD (standard deviation); n= 5 pools. Asterisks denote significant differences compared to controls at the same time of exposure: *$p<0.05$, **$p<0.01$, *** $p<0.001$.

The two-way ANOVA results highlighted a modulation of CAT activity determined by concentration ($p<0.001$) and time of exposure ($p<0.001$) in gills; and by concentration ($p<0.001$), time of exposure ($p<0.001$) and concentration/time interaction ($p<0.001$) in digestive gland. In gills, the post-hoc test showed a significant increase of CAT activity in clams exposed to $nTiO_2$ (1 µg/L) and NP mixture compared to controls at T1, and at T7 in clams exposed to nZnO (1 µg/L) and NP mixture compared to controls (Fig. 2B). In digestive gland, differences in CAT activity with respect to controls were significant from T1 in

clams exposed to nZnO and NP mixture, from T3 in clams exposed to $nTiO_2$ and at T7 in clams exposed to FC_{60} (Fig. 2C).
GST activity in gills and digestive gland was affected by time of exposure ($p<0.001$) and concentration/time interaction ($p<0.001$ and $p=0.003$, respectively). Only in digestive gland, the GST activity was affected by concentration ($p=0.014$). In gills, differences with respect to controls were significant in clams exposed to FC_{60} and NP mixture from T3 (Fig. 3A). In digestive gland, GST activity significantly increased at T1 and decreased at T7 in $nTiO_2$-, FC_{60}- and NP mixture-treated clams compared to control (Fig. 3B).

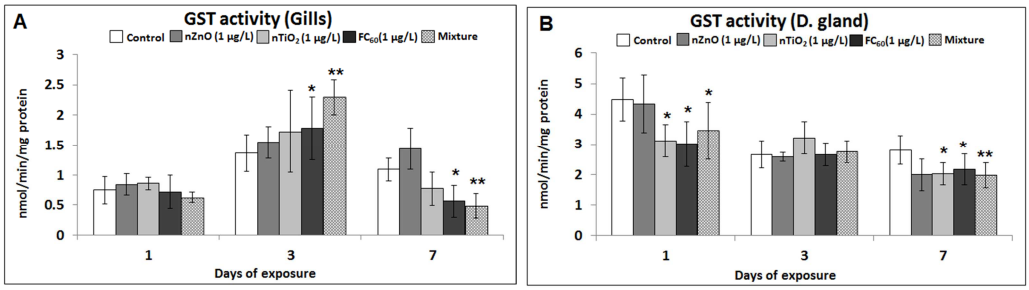

Fig. 3. GST activity (A, B) expressed as nmol/min/mg protein in *R. philippinarum* after 1, 3 and 7 days of exposure to nZnO (1 µg/L), $nTiO_2$ (1 µg/L), FC_{60} (1 µg/L) and mixture. The values are reported as the means ± SD (standard deviation); n= 5 pools. Asterisks denote significant differences compared to controls at the same time of exposure: *p<0.05, **p<0.01.

The TBARS levels revealed significant lipid damage due to time of exposure ($p<0.001$) in gills, and due to time of exposure ($p<0.001$) and concentration/time interaction ($p<0.001$) in digestive gland. No significant differences were detected in pair-wise comparisons in gills, and only at T7 in digestive gland a significant increase in LPO was detected in clams exposed to NP mixture compared to control (Fig. 4A).

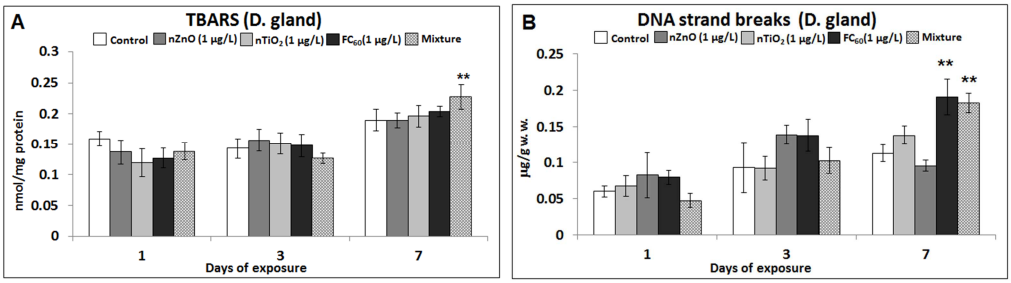

Fig. 4. TBARS levels (A) expressed as nmol/mg protein, and DNA strand breaks (B) expressed as µg/g wet weight, in *R. philippinarum* after 1, 3 and 7 days of exposure to nZnO (1 µg/L), $nTiO_2$ (1 µg/L), FC_{60} (1 µg/L) and mixture. The values are reported as the means ± SD (standard deviation); n= 5 pools. Asterisks denote significant differences compared to controls at the same time of exposure: **p<0.01.

DNA strand breaks were affected by exposure to concentration ($p=0.034$), time of exposure ($p<0.001$) and concentration/time interaction ($p=0.027$) in digestive gland only. The post-hoc test highlighted a significant increase in FC_{60}- and NP mixture-treated clams compared to control at T7 (Fig. 4B). No DNA damage modulations were detected in clam gills.

3.3. nZnO, $nTiO_2$ and FC_{60} bioaccumulation in gills and digestive gland

The total zinc, titanium and FC_{60} content in the gills and digestive gland of the clams exposed for 7 days to single NPs and NP mixture are reported in Table 1.

Zn content was higher in gills ($p<0.001$) and digestive gland ($p<0.001$) of treated animals (nZnO and NP mixture) compared to controls. In digestive gland, Zn content resulted higher in nZnO exposed clams than in NP mixture ($p=0.001$), whereas no significant difference was shown between the two treatments in gills.

Ti content was significantly different in both tissues ($p<0.001$ in gills and $p=0.002$ in digestive gland), but showed also different patterns of variation. In gills, the concentrations of Ti increased in treated animals ($p<0.001$, $nTiO_2$ and NP mixture) with respect to control, with no significant differences between treatments. In digestive gland, Ti content significantly increased only in mixture-exposed clams compared to controls. Moreover, a significant difference was also found between the two treatments, $nTiO_2$ and mixture ($p=0.012$).

In the various experimental conditions, FC_{60} content significantly differed in the two tissues ($p=0.002$ in gills and $p<0.001$ in digestive gland). In both gills and digestive gland, the content of FC_{60} significantly increased in treated conditions (FC_{60} and mixture) respect to control. In addition, in digestive gland was detected an higher content of FC_{60} in mixture-treated clams than in FC_{60}-treated clams ($p=0.044$).

Tab. 1. Levels of Zn, Ti expressed as µg/g dry weight and FC_{60} expressed as µg/g wet weight in the gills and the digestive gland of *R. philippinarum* after 7 days of exposure to exposure to nZnO (1 µg/L), $nTiO_2$ (1 µg/L), FC_{60} (1 µg/L) and mixture. The values are reported as the means ± SD (standard deviation); n=4 pools. Asterisks denote significant differences compared to controls: *$p<0.05$.

Gills	Control	nZnO	$nTiO_2$	FC_{60}	Mixture
Zn	76.30 (± 2.02)	82.68 * (± 2.23)	78.60 (± 1.55)	75.10 (± 4.54)	81.04 * (± 1.05)
Ti	2.00 (± 0.98)	1.98 (± 0.85)	3.92 * (± 1.47)	2.50 (± 1,02)	4.24 * (± 0.5)
FC_{60}	0.37 (± 0.11)	0.40 (± 0.85)	0.64 (± 0.26)	1.32 * (± 0.53)	1.98 * (± 0.16)
D. gland					
Zn	85.54 (± 0.23)	94.86 * (± 0.39)	82.97 (±1.05)	86.89 (± 0.58)	89.52 * (± 0.43)
Ti	1.54 (± 0.11)	1.34 (± 0.22)	1.62 (± 0.17)	1.60 (± 0.08)	1.98 * (± 0.19)
FC_{60}	3.25 (± 1.68)	1.99 (± 2.76)	5.49 (± 1.44)	12.04 * (± 1.75)	16.41 * (± 1.62)

3.4. Haemocyte assays

Both SCGE assay endpoints (LDR value and the percentage of DNA in the comet tail) highlighted significant primary genetic damage due to concentration ($p<0.001$), time of exposure ($p<0.001$) and concentration/time interaction in the clam haemocytes ($p<0.001$). In particular, the LDR values significantly increased in the presence of nZnO, $nTiO_2$ and NP mixture at T1. At T3, LDR values were significantly higher in the $nTiO_2$-, FC_{60}- and NP mixture-treated clams compared to controls. All experimental condition showed a significant increase compared to control at the end of exposure (Fig. 5A).

The % of tail DNA significantly increased in the presence of all single NPs and NP mixture compared to controls from the beginning of exposure, with an increase until the end, when it was significantly higher in the NP mixture-treated clams with respect to the nZnO-, $nTiO_2$- and FC_{60}- treated clams ($p<0.001$) (Fig. 5B).

Intracellular superoxide anion (O^{-2}) was affected by concentration (p=0.037) at the end of exposure. Significant differences were recorded in clams exposed to NP mixture compared to control (p=0.036) (Fig. 5C).

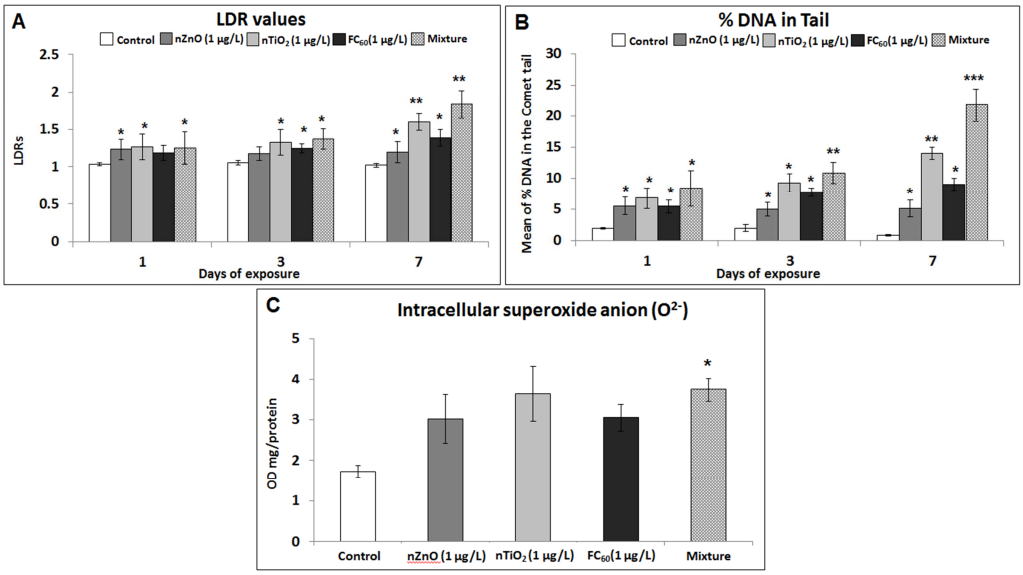

Fig. 5. The SCGE results, expressed as length/diameter ratio (A) and the mean percentage of tail DNA (B), in *R. philippinarum* after 1, 3 and 7 days of exposure to nZnO (1 µg/L), $nTiO_2$ (1 µg/L), FC_{60} (1 µg/L) and mixture, and the intracellular superoxide anion (C) expressed as OD mg/protein in *R. philippinarum* after 7 days of exposure. The values are reported as the means ± SD (standard deviation); n= 5 pools. Asterisks denote significant differences compared to controls, and for SCGE results also at the same time of exposure: *p<0.05, **p<0.01, *** p<0.001.

3.5. 1-DE redox proteomics

1-DE redox proteomics revealed that NPs caused modulations on the oxidation of thiol-containing proteins in gills (p=0.026), digestive gland (p<0.001) and haemolymph (p<0.001). In particular, the pair-wise comparisons showed an increase in gills (Fig. 6A) and a decrease in digestive gland (Fig. 6B) of thiol-containing proteins in clams exposed to NP mixture compared to control. In haemolymph, all exposure condition (nZnO, $nTiO_2$, FC_{60} and NP mixture) decreased the content of thiol proteins respect to control (Fig. 7A).

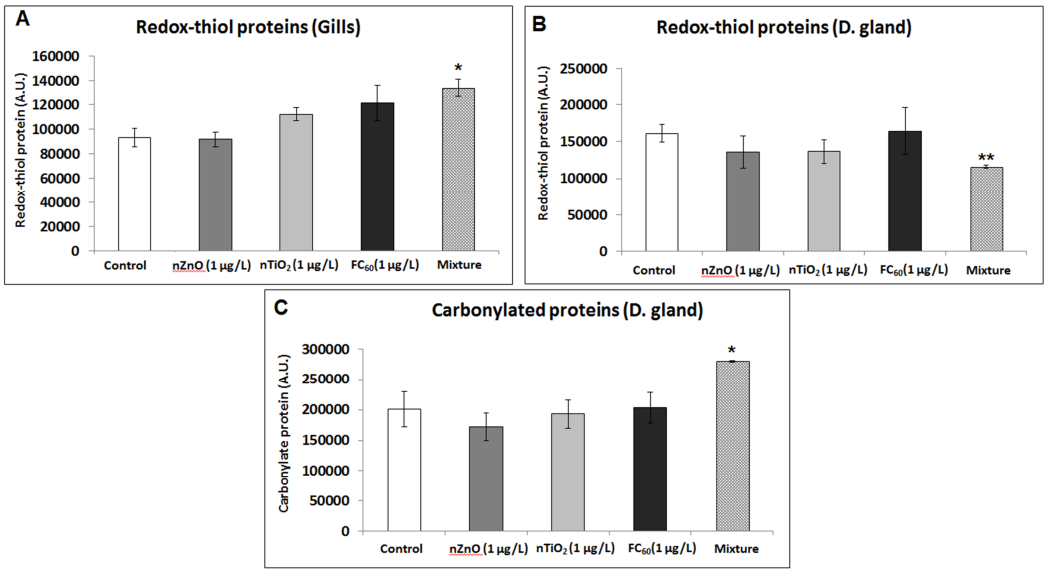

Fig. 6. Levels of proteins containing thiol groups in gills (A) and in digestive gland (B), and levels of proteins containing carbonyls in digestive gland (C), expressed as arbitrary units (A.U.) in *R. philippinarum* after 7 days of exposure to nZnO (1 µg/L), $nTiO_2$ (1 µg/L), FC_{60} (1 µg/L) and mixture. The values are reported as the means ± SD (standard deviation); n= 5 pools. Asterisks denote significant differences compared to controls: *p<0.05, **p<0.01.

After 7 days of exposure, a significant increase in proteins with carbonyl group content was observed in digestive gland (p=0.001) and in haemolymph (p<0.001), and significant changes were detected in NP mixture clams respect control clams (Fig. 6C and 7B, respectively). No significant differences of carbonyl group content were detected in gills of exposed clams.

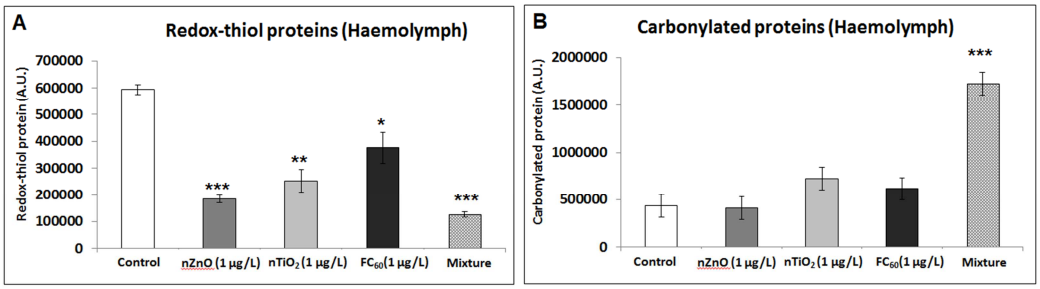

Fig. 7. Levels of proteins containing thiol groups (A) and levels of proteins containing carbonyls (B) expressed as arbitrary units (A.U.) in haemolymph of *R. philippinarum* after 7 days of exposure to nZnO (1 µg/L), $nTiO_2$ (1 µg/L), FC_{60} (1 µg/L) and mixture. The values are reported as the means ± SD (standard deviation); n= 5 pools. Asterisks denote significant differences compared to controls: *p<0.05, **p<0.01, *** p<0.001.

3.6. Protein oxidation patterns and protein identification in 2-DE redox proteomics

Representative 2-DE Coomassie images of cytosolic proteomes from *R. philippinarum* gill and digestive gland tissues are shown in Fig. 8A and B, respectively. Approximately 350 protein spots per gel were visualized and detected, both in Coomassie and fluorescently-labelled proteins.

Firstly, the Coomassie staining gels were analysed to detect the different protein expression profiles among control and treated clams. Only in gills, comparison of control and exposed clams highlighted a total of 5 differentially expressed protein spots in terms of the normalized volume (fold>1.5; p<0.05). Indeed, 4 spots

showed higher volumes (1.7-2.5-fold increase) and 1 spot showed a lower volume (1.9-fold decrease) in NP mixture samples than in control (see Tab. 2). Besides, among these 5 spots, 3 spots (190, 194, 313) highlighted lower volumes (2.2-2.4-fold decrease) in nZnO proteins compared to control. The 310 spot showed an increased volume in $nTiO_2$- and FC_{60}- treated clams (1.8 and 2.2, respectively) (Tab. 2).

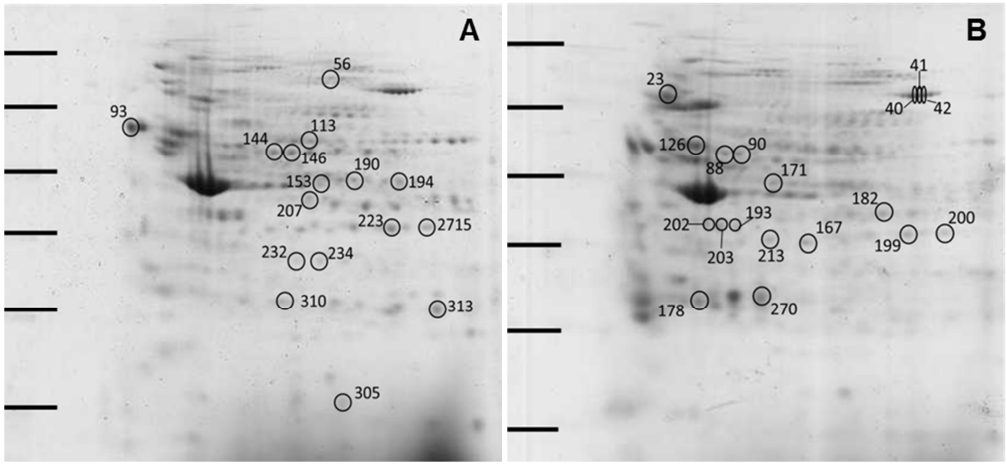

Fig. 8. In representative gel images all spots of the entire proteome are shown in gills (A) and digestive gland (B); those from changed proteins are circled, based on different i) expression or ii) redox status, IAF and FTSC.

No changes in the expression protein profiles were shown in digestive gland.
Secondly, the comparison among fluorescently-labelled proteins normalised by Coomassie data revealed a total of 8 and 11 differentially expressed thiol-containing protein spots (fold>1.5; $p<0.05$) in gills and digestive gland, respectively (Tab. 2 and 3). In particular, the proteins with higher thiol content in gills were detected for all spots in NP mixture condition, for 3 spots in nZnO condition and for 1 spot in $nTiO_2$ conditions compared to control (Tab. 2). Instead, the thiol proteins in digestive gland showed a higher content in all spots of NP mixture-treated clams, in 2 spots of $nTiO_2$-treated clams and in 2 spots of FC_{60}-treated clams compared to control clams (Tab. 3).
In carbonyl-labelled patterns differentially expressed spots (fold>1.5; $p<0.05$) were revealed: 3 in gills and 7 in digestive gland (Tab. 2 and 3, respectively). The proteins with higher carbonyl content in gills were detected for all spots in NP mixture condition respect to control. In digestive gland, the carbonyl content was shown in all NP mixture spots and in 1 spot of $nTiO_2$ exposed clams compared to control.
All of the 34 different spots from gills (16 spots) and digestive gland (18 spots) were suitable for mass spectrometry analysis (RP-UPLC ESI-LTQ-Orbitrap). Although the genome of *R. philippinarum* has not yet been sequenced, 11 different proteins of gills and 14 different proteins of digestive gland were identified based on homology, the details of which are listed in Table 2 and 3. Protein spots 232, 234, 305, 310, 313 in gills and 40, 41, 42, 178 in digestive gland were not identified. We note that the theoretical and experimental values obtained for the mass weight and isoelectric point were highly similar, even though slight differences were detected, most likely due to post-translational modifications and/or the homology identification (Tomanek, 2011).

Tab. 2. Identified proteins differentially expressed or damaged in *R. philippinarum* gill cytosolic fraction after 7 days of exposure to nZnO (1 µg/L), $nTiO_2$ (1 µg/L), FC_{60} (1 µg/L) and mixture.

SPOT n° (type of change)[a]	Fold change (experimental condition)[b]	Homologous protein (species)[c]	Putative protein identification[d]	Biological function/role	Sequest score	Sequence Coverage[e]	Peptide Xcorr (mean)[f]
G56 (IAF)	↑ 1.8 (M)	Q7KN90 SYCC_DROME (Drosophila melanogaster)	Cysteine-tRNA ligase, cytoplasmic	Protein metabolism	10.82	4.86%	2.19
G93 (FTSC)	↑ 2.1 (M)	Q13748 TBA3C_HUMAN (Homo sapiens)	Tubulin alpha-3C/D chain	Cytoskeleton	73.75	48.67%	2.89
G113 (FTSC)	↑ 2.5 (M)	P35381 ATPA_DROME (Drosophila melanogaster)	ATP synthase subunit alpha, mitochondrial	Energetic metabolism	90.06	22.28%	3.24
G144 (IAF)	↑ 2.2 (M)	O02654 ENO_DORPE (Doryteuthis pealeii)	Enolase	Glycolysis	25.28	14.98%	3.11
G146 (IAF)	↑ 2.3 (Z); ↓ 1.9 (M)	O02654 ENO_DORPE (Doryteuthis pealeii)	Enolase	Glycolysis	50.67	20.28%	3.33
G153 (FTSC)	↑ 1.9 (M)	P10990 ACT1_STRFN (Strongylocentrotus franciscanus)	Actin-15A	Cytoskeleton	15.37	21.81%	2.1
G190 (coo)	↓2.2 (Z); ↑ 1.7 (M)	P91427 PGK_CAEEL (Caenorhabditis elegans)	Probable phosphoglycerate kinase	Glycolysis	11.61	13.91%	2.06
G194 (coo)	↓ 1.9 (M)	O18413 PRS8_DROME (Drosophila melanogaster)	26S protease regulatory subunit 8	Protein metabolism	23.56	22.72%	2.57
G207 (IAF)	↑ 1.6 (Z); ↑ 2.5 (M)	O18499 ACT1_SACKO (Saccoglossus kowalevskii)	Actin-1	Cytoskeleton	217.64	59.31%	3.21
G223 (IAF)	↑ 2.3 (M)	P51469 G3P_XENLA (Xenopus laevis)	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis	18.84	9.91%	2.44
G232 (IAF)	↓ 2.1 (Z); ↑ 1.9 (T); ↓ 1.7 (M)	not identified	-	-	-	-	-
G234 (IAF)	↑ 2.1 (M)	not identified	-	-	-	-	-
G305 (IAF)	↑ 2.4 (M)	not identified	-	-	-	-	-
G310 (coo)	↑ 1.8 (T); ↑ 2.2 (F); ↓ 1.9 (M)	not identified	-	-	-	-	-
G313 (coo)	↓ 1.6 (Z)	not identified	-	-	-	-	-
G2715 (coo)	↓ 2.4 (Z);↑ 2.3 (M)	P46563 ALF2_CAEEL (Caenorhabditis elegans)	Fructose-bisphosphate aldolase 2	Glycolysis	9.22	6.28%	2.71

[a]Spot number on 2-DE map (Fig. 8A)(G=gills).
[b]Fold change increase (↑) or decrease (↓) (in terms of relative spot volume) in treated clams compared to control (experimental condition: Z=nZnO, T=$nTiO_2$, F=FC_{60} and M=mixture).
[c]Accession number in SwissProt database (name of the species regarding identified protein).
[d]Name of the identified protein.
[e]Sequence coverage: percentage of aminoacidic sequence coverage of matched peptides in the identified proteins.
[f]Xcorr= value of cross-correlation obtained by the SEQUEST algorithm.

Tab. 3. Identified proteins differentially damaged in *R. philippinarum* digestive gland cytosolic fraction after 7 days of exposure to nZnO (1 µg/L), $nTiO_2$ (1 µg/L), FC_{60} (1 µg/L) and mixture.

SPOT n° (type of change)[a]	Fold change (experimental condition)[b]	Homologous protein (species)[c]	Putative protein identification[d]	Biological function / Role	Sequest score	Sequence Coverage[e]	Peptide Xcorr (mean)[f]
D23 (IAF)	↓ 1.7 (F); ↓ 2.3 (M)	Q16956 GRP78_APLCA (Aplysia californica)	78 kDa glucose-regulated protein	Energetic metabolism	75.77	13.94%	3.47
D40 (FTSC)	↑ 2.6 (M)	not ideitifed	-	-	-	-	-
D41 (FTSC)	↑ 1.9 (M)	not ideitifed	-	-	-	-	-
D42 (FTSC)	↑ 2.4 (M)	not ideitifed	-	-	-	-	-
D88 (FTSC)	↑ 1.6 (M)	Q8T6A5 TBA1_APLCA (Aplysia californica)	Tubulin alpha-1 chain	Cytoskeleton	120	53.44%	3.53
D90 (FTSC)	↑ 1.9 (M)	P06603 TBA1_DROME (Drosophila melanogaster)	Tubulin alpha-1 chain	Cytoskeleton	138.48	53.56%	3.42
D126 (IAF)	↓ 2.6 (M)	P11833 TBB_PARLI (Paracentrotus lividus)	Tubulin beta chain	Cytoskeleton	51.92	42.06%	3.6
D167 (FTSC)	↑ 2.2 (M)	Q3SWW9 PP1B_BOVIN (Bos taurus)	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	Protein metabolism	11.89	14.07%	2.97
D171 (IAF)	↑ 2.4 (T); ↑ 2.5 (M)	P69004 ACT2_STRFN (Strongylocentrotus franciscanus)	Actin-15B	Cytoskeleton	124.08	62.50%	2.84
D178 (FTSC)	↓ 2,1 (T); ↑ 2.5 (M)	not identified	-	-	-	-	-
D182 (IAF)	↑ 1.9 (M)	O18413 PRS8_DROME (Drosophila melanogaster)	26S protease regulatory subunit 8	Protein metabolism	40.93	33.58%	2.64
D193 (IAF)	↓ 2.2 (M)	P69004 ACT2_STRFN (Strongylocentrotus franciscanus)	Actin-15B	Cytoskeleton	198.7	57.98%	3.01
D199 (IAF)	↓ 2.6 (F); ↓ 1.9 (M)	P30883 TBB4_XENLA (Xenopus laevis)	Tubulin beta-4 chain	Cytoskeleton	31.49	25.39%	2.59
D200 (IAF)	↓ 1.7 (M)	P35381 ATPA_DROME (Drosophila melanogaster)	ATP synthase subunit alpha, mitochondrial	Energetic metabolism	25.57	16.30%	2.64
D202 (IAF)	↓ 2.1 (M)	P69004 ACT2_STRFN (Strongylocentrotus franciscanus)	Actin-15B	Cytoskeleton	182.88	55.32%	3.28
D203 (IAF)	↓ 2.5 (M)	O18499 ACT1_SACKO (Saccoglossus kowalevskii)	Actin-1	Cytoskeleton	74.71	39.10%	2.67
D213 (IAF)	↓ 2.4 (M)	P10990 ACT1_STRFN (Strongylocentrotus franciscanus)	Actin-15A	Cytoskeleton	35.46	17.82%	3.03
D270 (IAF)	↑ 1.8 (M)	Q2YDE4 PSA6_BOVIN (Bos taurus)	Proteasome subunit alpha type-6	Protein metabolism	19.41	16.26%	3.04

[a]Spot number on 2-DE map (Fig. 8B)(D=digestive gland).
[b]Fold change increase (↑) or decrease (↓) (in terms of relative spot volume) in treated clams compared to control (experimental condition: Z=nZnO, T=$nTiO_2$, F=FC_{60} and M=mixture).
[c]Accession number in SwissProt database (name of the species regarding identified protein).
[d]Name of the identified protein.
[e]Sequence coverage: percentage of aminoacidic sequence coverage of matched peptides in the identified proteins.
[f]Xcorr= value of cross-correlation obtained by the SEQUEST algorithm.

4. Discussion

The increasing production and usage in various fields of different types of manufactured NPs, estimated to grow to over half a million tons by 2020, would lead to their release in substantial amounts in the environment, including the aquatic compartments (Canesi et al., 2015). In the environment, organisms are generally exposed to mixtures of different contaminants. These mixtures include combinations of organics, trace metals and also NPs, which can interact in many ways (i.e. additively, synergistically, or antagonistically) to induce biological responses at different levels of biological organisation (Al-Subiai et al., 2012). To the best of our knowledge, information about the effects of NP mixture is currently lacking. The effects of mixtures of pollutants in the environment are usually hard to predict due to many factors. In any case, once released into the environment, NPs will interact with each other and with their surrounding environment (Wiesner et al., 2009).

In this study, nZnO, $nTiO_2$ and FC_{60} were chosen following extensive studies on their effects in *R. philippinarum* (Marisa et al., paper I, III, IV). To obtain more useful data about NP mixture toxicity, the common multi-biomarker approach was associated with the redox proteomics approach.

In bivalves, most of the NP studies mainly focused on immunotoxicity, oxidative stress, DNA damage, subcellular accumulation and lysosomal damage, and also on protein damage and protein expression changes (Rocha et al., 2015). Overall, oxidative stress, injuries to cell proteins and membranes, and DNA damage are recognized as the major modes of action of NPs in bivalves. NPs can determine, with direct or indirect action, an increased production of ROS, possibly resulting in different damage to molecules. However, the oxidative stress and damage induced by NPs depends on the size, composition and concentration, mode and time of exposure, bivalve species and target organ analysed (Moore, 2006; Rocha et al., 2015).

Oxidative stress was also confirmed as the main mechanism of action of single nZnO, $nTiO_2$ and FC_{60} in treated clams (Marisa et al., paper I, III, IV). Moreover, when compared with the results obtained in exposures to single NPs, the effects observed in the NP mixture-exposed clams in this study were more severe than those previously found at the higher single NP concentration (10 µg/L) .

Although in all tissues analysed the oxidative stress was the major mechanism of NP effect, different levels and patterns of response were observed depending on tissue and biomarker considered. In particular, the anti-oxidant enzyme activities showed an increase, with more changes in digestive gland respect to gills. Indeed, in digestive gland an increase in LPO and DNA damage were observed. In haemolymph, the increase in superoxide anion production highlighted an oxidative stress underway, which was confirmed by the high DNA damage level in haemocytes. Overall, all the biomarkers measured in the three tissues were more affected in NP mixture-exposed clams than in single NP-exposed ones.

In gills and digestive gland, Zn, Ti and FC_{60} content was higher in treated clams compared to control, thus confirming the bioaccumulation of NPs already observed in our previous studies (Marisa et al., paper I, III, IV). Bioaccumulation of Zn did not change if nZnO was present alone or as a mixture in gills, but in digestive gland was found an higher bioaccumulation in clams exposed to nZnO respect to NP mixture. No differences were obtained for Ti accumulation in gills.

Instead, in the digestive gland Ti was more accumulated as a mixture than as a single NP. This finding suggested that the co-exposure to other NPs facilitated the process of $nTiO_2$ bioaccumulation in digestive gland. The same hypothesis can be made for FC_{60} bioaccumulation in both tissues, since no differences in FC_{60} content were found between clams exposed to the single NP and those exposed to the mixture.

In other studies, where nZnO, $nTiO_2$ and FC_{60} were used in co-exposure with other types of contaminants, the NP accumulation depended on species, tissues and type of NPs. For example, in mussels, *Mytilus edulis*, exposed to FC_{60} and fluoranthene as a mixture (0.1 mg/L and 32 mg/L, respectively) for 72 h, the FC_{60} content decreased compared to clams exposed only to FC_{60} (Al-Subiai et al., 2012).

It is known that ROS can modify and inactivate proteins in a wide variety of ways. Sulphur-containing molecules are notoriously susceptible to oxidation. Cysteine thiols (-SH) can be irreversibly oxidised to sulphinic ($-SO_2H$) and cysteic ($-SO_3H$) acids or reversibly oxidised to sulphenic acid (-SOH), thiyl radicals (-S.) or nitrosothiols (-SNO). Methionines can be oxidised to sulphoxides and sulphones. Amino acid side-chains can be irreversibly converted to aldehyde/ketone groups collectively called protein carbonyls (Pedriali et al., 2013). Also the redox proteomic analysis highlighted an increase of ROS, resulting in a high level of damaged proteins. Both types of protein damage (redox-thiol and carbonylated protein) showed an increase under NP exposure in both 1-DE and 2-DE redox proteomic results.

In gills, only an increase of IAF-labelled proteins was detected in NP mixture-treated clams. This result did not exclude the increase of ROS production and consequently damage to proteins. Indeed, other redox lesions of proteins, including glutathionylation and/or formation of disulphides, can protect cysteine residues from oxidation. Moreover, a high detection of –SH groups could be determined by the break of tertiary and quaternary structure of proteins under stress conditions (Schafer and Buettner, 2001).

In digestive gland and haemolymph, high protein damage was shown, both in FTSC-labelled proteins and in IAF-labelled proteins. A decrease in protein thiol groups could be considered as damage to proteins, but also an implementation of anti-oxidant defence and absorption of ROS. Indeed, the roles of proteins in signal transduction pathways depend strongly on the redox properties of cysteine thiol groups which occur both in proteins and in low-molecular mass thiols (Winterbourn and Hampton, 2008). Thiols react significantly faster than other amino acid side-chains with oxidizing species and thus contribute to antioxidant defence (Hansen et al., 2009).

All the 1-DE redox proteomic results highlighted more effects in clams exposed to NP mixture than to the single NPs.

Gold NPs and menadione caused significant decrease of total protein thiols in digestive gland, but not in gills or mantles of *Mytilus edulis* (Tedesco et al., 2010), and similar results were shown also in the freshwater mussel *Dreissena polymorpha* exposed to benzoylecgonine (Pedriali et al., 2013).

Among differentially labelled protein spots (2-DE) we selected only the most intense and well-defined for subsequent identification analysis. Some spots were either too low in abundance and/or too close to each other to be isolated and excised with certainty. In the 2-DE redox proteomic results, due to the incomplete

genome sequence of *R. philippinarum*, not all proteins were identified. However, identification occurred for more than 75% proteins, a percentage higher than that reported in other studies conducted on the proteome of other species (Liska and Shevchenko, 2003; Waridel et al., 2007).
In gills, 5 proteins showed a variation of their abundance in treated clams compared to control clams. Among these, only 3 proteins were identified: probable phosphoglycerate kinase (spot G190), 26S protease regulatory subunit 8 (spot G194) and fructose-bisphosphate aldolase 2 (spot G2715) (see Table 2). In particular, phosphoglycerate kinase is found in all living organisms and its sequence has been highly conserved throughout evolution. It is an enzyme that catalyses the formation of ATP in glycolysis pathway. The fructose-bisphosphate aldolase 2 is a glycolytic enzyme that catalyses the reversible aldol cleavage or condensation of fructose-1,6-bisphosphate into dihydroxyacetone-phosphate and glyceraldehyde 3-phosphate (http://www.uniprot.org/). These two proteins are involved in energetic metabolism and an increase of their abundance could highlight a stimulation of energetic pathway in NP mixture treated clams. Instead, a decrease of these two proteins in nZnO exposed clams could show a potential lower energetic metabolism with loss of functions in the cells. Muller et al. (2014) showed that nZnO affected the energy budget in the mussel *Mytilus galloprovincialis*, through a reduction in feeding capacity and an increase in maintenance requirements, and that these two effects lead to a reduction in the expected lifetime production of reproductive matter.
The 26S protease regulatory is involved in the ATP-dependent degradation of ubiquitinated proteins. A decrease of this protein, in NP mixture-treated clams compared to controls, could determine wicked effects on the protein degradation pathway to molecules that should be excreted (Götze et al., 2014).
The other spots resulted in redox proteomic analysis were IAF- or FTSC-labelled. In gills, 8 proteins revealed changes in thiol containing proteins, but only 5 proteins were identified. Cysteine-tRNA ligase is required for decoding cysteine codons in all known organisms. It catalyses the attachment of cysteine to its cognate transfer RNA molecule. The change of the redox status of this protein could negative change the protein metabolism (Lüchmann et al., 2012). Instead, enolase and glyceraldehyde-3-phosphate dehydrogenase variations could determine problems in energetic pathways. They are abundantly expressed cytosolic proteins which are widespread in many tissues and are essential glycolytic enzymes, the former catalyses the conversion of 2-phosphoglycerate and phosphoenol pyruvate and the latter is responsible for the conversion of 1,3-diphosphoglycerate and glyceraldehyde-3-phosphate (Díaz-Ramos et al., 2012). Glyceraldehyde-3-phosphate dehydrogenase is known as a redox sensitive protein; Schmidt et al. (2014) reported a down-regulation of this protein in digestive gland of *Mytilus* sp. after exposure to two pharmaceuticals.
The last identified IAF-labelled protein is actin. Conventional actin is one of the principal components of the eukaryotic cytoskeleton, and it has a central role in cellular processes ranging from cell motility to intracellular transport and cell organisation (Goodson and Hawse, 2002). Actin is expressed in different isoforms that are generally obtained in eukaryotes by alternative splicing of duplicated and highly conserved genes (Jonsson et al., 2006). A modification of the actin redox status could determine various problems in the cell structure and functions.

In gills, the carbonylated proteins changed respect to controls were 3. One is the mitochondrial membrane ATP synthase that produces ATP from ADP in the presence of a proton gradient across the mitochondrial membrane which is generated by electron transport complexes of the respiratory chain (Kang et al., 2006). In other hand, two of them are cytoskeleton proteins: actin and tubulin. Tubulin, like actin, is one of the major components of the eukaryotic cytoskeleton (Venier et al., 2003).

The abundance of cytoskeletal proteins may have made their identification easier, although they are also implicated in oxidative stress. Besides, the cytoskeleton proteins are one of the protein group that change more the expression or the redox status under contaminant exposure (Dowling and Sheehan, 2006). As we summarised above, NP mixture exposure highlighted a modulation of three main groups of proteins i) protein metabolism, ii) energetic metabolism and iii) cytoskeleton protein. All these proteins have important functions in cells and their variation could determine serious damage and deficiency.

The proteins changed in digestive gland showed modulation in thiol and carbonyl group content only, the modulation of protein abundance did not change. Eighteen proteins were changed in their redox status, in particular eleven were IAF-labelled proteins and seven were FTSC-labelled proteins. Among these, three were not identified (one in IAF and two in FTSC). The biological role of the identified proteins determines two big functional groups i) protein metabolism and ii) cytoskeleton proteins. Moreover, the 26S protease regulatory and ATP synthase, identified also in gills, in digestive gland were both damaged in redox-thiol status. The identified spots with protein metabolism function were three common eukaryotic proteins. In particular, the glucose-regulated protein (D23 spot) plays a role in facilitating the assembly of multimeric protein complexes inside the endoplasmic reticulum (http://www.uniprot.org/) and showed a damage in redox-thiol status. Another damaged protein was serine/threonine-protein phosphatase, that is known to be involved in the regulation of a variety of cellular processes, such as cell division, glycogen metabolism, muscle contractility and protein synthesis (Boutet et al., 2008). Proteasome subunit (D270 spot) is a part of eukaryotic proteasome complex that recognized degradable proteins and it is characterized by its ability to cleave peptides (http://www.uniprot.org/).

Three identified proteins were different isoforms of tubulin and other five proteins were different isoform of actin, main elements of the cytoskeleton. The most common proteins changed in pollutant-stress responses include oxidative stress proteins, cytoskeletal proteins, chaperones, proteases, and proteins involved in the detoxification of xenobiotics (Tedesco et al., 2010). Also in this study, the cytoskeleton proteins were one of the most represented group of changed proteins.

Variations in enzymes involved in energetic pathways were also observed, but more in gills than in digestive gland. This is of interest since up- or down-regulation of metabolic enzymes are often related to stress status after exposure to chemical contaminants or oxidative stress. Thus, the alteration of redox status could further confirm the ability of NPs to trigger oxidative stress in *R. philippinarum* with implications for energy metabolism. This result could be due to an increased requirement for both energy and protein synthesis/degradation pathways (Zhang et al., 2005).

Key antioxidant systems, such as thioredoxin, glutaredoxin, glutathione and glutathione-ascorbate cycle, depend heavily on NADPH+ rather than NADH+ for

reducing equivalents. Under exposure to pro-oxidants, cells need to shift rapidly from pathways producing NADH+ to others, such as the pentose phosphate pathway, that produce NADPH+. In this regard, it is interesting to note that glyceraldehyde 3-phosphate dehydrogenase is a well known redox sensor in cells, and a glycolytic enzyme (Morigasaki et al., 2008).

Some protein spots highlighted, both in gills and digestive gland tissue, an increase in thiol groups or a decrease in carbonyl groups. This situation could be due to glutathionylation, which may be an adaptation of tissues to allow animals to survive in the presence of high oxidative stress. Previous studies conducted in rats and humans showed that highly-abundant proteins, such as cytoskeletal proteins, were glutathionylated during diamide stress thus preventing irreversible oxidation (Brennan et al., 2004; Cumming et al., 2004).

In all the 1-DE and 2-DE redox proteomic results the NP mixture-exposed clams showed more changes compare to controls, although in some cases there were changed spots in single NP-exposed clams. Respect to single NP treatments, both in biomarker and redox proteomic approaches, all findings indicated higher oxidative stress in act during the exposure to the NP mixture, with damage to proteins, lipids and DNA in all clam tissues. Moreover, additive effects were observed in the mixture respect to single NP exposures.

The effects of a contaminant mixture could arise, as in this study, with an additive response, but also with a synergistic and/or antagonist response. For example, *R. philippinarum* was used to investigate the water/sediment pollution in Venice Lagoon and despite a high bioaccumulation was shown to different contaminants in soft tissues, no similar pattern was observed in the other biomarkers considered, possibly due to antagonistic effects of the complex contaminant mixture (Nasci et al., 2000). In another study, although the freshwater crustaceans *Simocephalus sp.* abundance was significantly different in single and pesticide mixture treatments, and the results suggested at the most an additive effect rather than a synergistic interaction (Belden et al., 2007). Moreover, Verbruggen and van den Brink, (2010) in their review, concerning mixture toxicity of pesticide to aquatic organisms, concluded that the effects of pesticide mixtures are unlikely to produce large synergistic interactions and that the overall impact may be explained by the effects from the individual pesticides. Instead, synergistic interactions of chemicals in mixture conditions was shown in metal and antifoulant mixtures in different experimental exposure and species (Cedergreen, 2014).

In this study, the findings indicated that the digestive gland was the tissue more affected by NP mixture toxicity. This result has been highlighted by the integrated use of multi-biomarker and redox proteomic approaches, which gave evidence of oxidative stress and damage, providing a more in depth insight into impacted molecules and most of all into their major physiological functions.

To our knowledge, this is the first study that investigated the effects of a mixture of NPs, thus providing a new topic for discussion in NP ecotoxicological studies, under a more environmentally realistic experimental context.

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PAPER: VI

Ilaria Marisa, Valerio Matozzo, Paula Costa, Marinella Farré, Josep Sanchis, Nicola Brianese, Maria Gabriella Marin

Nanoparticle mixture under different salinity values: investigating the effects to clams *Ruditapes philippinarum* and *Ruditapes decussatus*

Nanoparticle mixture under different salinity values: investigating the effects to clams *Ruditapes philippinarum* and *Ruditapes decussatus*

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Abstract
In coastal areas, the marine organisms are exposed to multiple changes and stressors of different type that have the potential to seriously and adversely affect marine ecosystem functioning. Despite these concerns, the combined stressor studies are poorly addressed. Among predicted changes in environmental parameters under global change scenarios, there is an increasing concern about future alterations in seawater salinity values, which will affect the performance of species. In the other hand, the presence of new emerging contaminants, such as nanoparticles (NPs), and the complex mixtures originated in the environment, are posing new potential risks for marine organisms. In this study, two clams, *Ruditapes philippinarum* and *Ruditapes decussatus* were exposed for 7 days to a mixture of three of the most common NPs worldwide, zinc oxide (nZnO), titanium dioxide ($nTiO_2$) and C_{60} fullerene (FC_{60}), at the concentration of 1 μg/L each, under different salinity values (18-28-38 psu). At various time intervals during the exposure, cellular and biochemical responses were evaluated in clam gills, digestive gland and haemolymph. In addition, the NP content was determined in clam gills and digestive gland after 7 days of exposure.
In both species, at all salinity tested the NP mixture induced oxidative stress, as revealed by a modulation of anti-oxidant enzyme activities in gill and digestive gland tissues. Under NP mixture exposure, the activity of the detoxification enzyme showed a decrease in gills of both species at 18 psu, and an increase in digestive gland of *R. philippinarum* only at 28 psu. Regarding evidence of oxidative stress, an increase in lipid peroxidation was observed in gills and digestive gland of *R. philippinarum* and in digestive gland of *R. decussatus* exposed to NP mixture. Moreover, an increase of protein damage was found in the gills of mixture-exposed *R. decussatus* at 38 psu. Regarding the haemocytes parameters, cytotoxicity was the only response clearly modulated under NP mixture at different salinity values only in *R. decussatus*.
Overall, at all salinity values tested various changes were shown, depending on the tissues, the biomarkers and the species considered. Although, the bioaccumulation results did not clarify the biomarker responses. More variations in both species were shown in NP treated clams exposed to 18 and 38 psu. Moreover, the comparison between the two species did not show a clear pattern of response. Although more in-depth evaluation is needed, it has to be noted that under NP exposure at the three salinities tested, the number of responses

significantly varied respect to controls was slightly higher in *R. decussatus* than in *R. philippinarum*.

Keywords: *Ruditapes philippinarum, Ruditapes decussatuss,* nanoparticle mixture, salinity, biomarkers.

1. Introduction

Since the Rio Convention, environmental changes are central concerns in political and scientific debates. Coastal marine ecosystems are among the most ecologically and socio-economically vital on the planet (Occhipinti-Ambrogi and Savini, 2003). Marine habitats from the intertidal zone out to the continental shelf break are estimated to provide a considerable percentage of ecosystem goods and services per year. However, there is a strong scientific consensus that coastal marine ecosystems, along with the goods and services they provide, are threatened by anthropogenic global changes (Harley et al., 2006). In coastal areas, the marine organisms are exposed to multiple changes and stressors of different type that have the potential to seriously and adversely affect marine ecosystem functioning. Environmental changes include modifications in abiotic parameters, such as temperature, pH, dissolved oxygen, UV radiation, salinity, and also in pollution (Coehlo et al., 2013). The information regarding interactions between changes in environmental parameters and pollutants in marine ecosystems is very scarce. Such interactions can alter chemical speciation and the bioavailability of several pollutants with potentially deleterious effects on the biota. Even without any change in the levels of toxicant exposure, changes in other environmental conditions may affect the sensitivity of organisms to current toxicants (Moe et al., 2013).

Among pollutants the emerging environmental contaminants give cause for increasing concern, and nanoparticles (NPs) in particular. Zinc oxide (nZnO), titanium dioxide ($nTiO_2$) and C_{60} fullerene (FC_{60}) NPs are the most used and produced NPs worldwide (Piccinno et al. 2012; Sanchis et al., 2015), they are inserted in similar commercial products and have common route of discharge into the sea (Corsi et al., 2014; Farrè et al., 2009; Exbrayat et al., 2015). NP mixture effects on organisms under changing environmental parameters are still unknown.

Salinity is an important environmental parameter, and its changes can determine different effects on the species (Lee et al., 1998; Boeuf and Payan, 2001; Correia et al., 2016). Furthermore, it is known that salinity can influence the NP physicochemical properties and consequently could change the action and toxicity of NPs. High salinity values determined a major aggregation and sedimentation of single NPs in simulation laboratory studies aimed at comparing NP features in freshwater and seawater environments (Keller et al., 2010).

Some recent researches have investigated the combined effects of NPs and different salinity values, with some similar results, indicating lower toxicity at higher salinity. In Yung et al. (2015), for example, the influences of salinity (12, 17, 22, 27, 32 psu) on nZnO characteristics and the effects of nZnO (0.5, 1, 5, 10, 50 mg/L) exposure in the marine diatom *Thalassiosira pseudonana* were investigated during 96 h experiments. The authors found that the aggregate sizes of nZnO significantly increased with increasing salinity, but generally decreased with increasing exposure concentrations. Ion release decreased with increasing salinity, whereas the surface charge of the particles was not affected by salinity.

The growth inhibition (IC50) of *T. pseudonana* decreased under nZnO exposure to higher salinity. Overall, nZnO-treated diatom showed less effects than the Zn^{2+}-treated ones. The results highlighted the importance of salinity as an influential environmental factor governing the aggregation, dissolution and the toxicity of nZnO.

The sheepshead minnow *Cyprinodon variegatus* was exposed to copper oxide NPs (5 and 50 mg/L, nCuO) at different salinity regimes (about half and full strength seawater, 1.5 and 3.0% respectively) for 7 days (Ates et al., 2014). The results indicated that nCuO could cause behavioural changes in the fish, such as increased mucus secretion, less general activity and loss of equilibrium. However, higher oxidative stress was recorded at half strength seawater respect to seawater exposure medium, which can be associated with decreasing toxicity of nCuO as salinity increases. In addition, Cu contents in the tissues of the fish were significantly higher at low salinity (Ates et al., 2014).

In other studies, the effects of salinity (5‰, 15‰, 25‰ and 35‰) on metal ion (Cu and Zn) and on nCuO and nZnO toxicity to the copepods *Tigriopus japonicus* (Park et al., 2014) were investigated. Increasing salinity decreased the dissolved concentrations of Cu and Zn ions due to the precipitation of the metal ions, consequently reducing the acute toxicity to *T. japonicus*. The effect of salinity on acute nCuO and nZnO toxicity was similar to that on metal ion toxicity. Since the aggregation of NPs generally enhanced at higher salinities, both the dissolution and aggregation of nCuO and nZnO may control the effect of salinity on acute toxicity to *T. japonicas* (Park et al., 2014).

In order to better understand the effects of NP mixture on marine bivalves, in the present work two edible clams from the Venice Lagoon (Italy), the native species *Ruditapes decussatus* (Linnaeus, 1758) and the alien species *Ruditapes philippinarum* (Adams and Reeve, 1850), were used. Some studies developed under laboratory conditions showed that both species respond differently to other contaminants, despite their phylogenetic closeness (Figueira et al., 2012; Velez et al., 2015). Moreover, the two species were chosen as model organisms considering their different recent history in the Lagoon of Venice (Italy) (Pranovi et al., 2006). As infaunal filter-feeders, both species are particularly exposed to the impact of contaminants having in sediments their ultimate sink, such as NPs.

To assess NP mixture effects under different salinity values, clams were exposed for 7 days to 18, 28 and 38 psu of salinity, both in the absence and in the presence of NP mixture (1 μg/L nZnO, 1 μg/L $nTiO_2$, and 1 μg/L FC_{60}). The values of salinity were chosen based on minimum, medium and maximum values measured in the Southern Venice lagoon from 2000 to 2009 (Zirino et al., 2014). The NP concentration was chosen in the range of the predicted environmental concentrations (PECs), the only available data to have indications on NP concentrations in marine areas (Gottschalk et al., 2009; Ferreira da Silva et al., 2011).

Thus, the present study aimed to i) investigate various sub-lethal effects of NP mixture/salinity combinations on gills, digestive gland and haemolymph of clams (*R. decussatus* and *R. philippinarum*), with a multi-biomarker approach (antioxidant enzyme activities, levels of damage to molecules and haemocyte parameters) and ii) assess potential effects of salinity on the bioaccumulation of nZnO, $nTiO_2$ and FC_{60} in clam gills and digestive glands after 7 days of exposure.

2. Materials and Methods

2.1. nZnO, $nTiO_2$ and FC_{60} characterisation

nZnO (declared size of <100 nm, percentage of zinc 79.1 - 81.5%, surface area 15 -25 m^2/g), $nTiO_2$ (P25, declared size of 21 nm, percentage of titanium >99.5%, surface area 35 - 65 m^2/g) and FC_{60} (purity>97.5 %) were purchased from Sigma-Aldrich (Milano, Italy). NPs were characterised via a combination of analytical techniques, already described and used. Details about the characterisation procedure for the three NPs are reported in Marisa et al. (paper I, III, IV), respectively.

2.2. Clams and NP exposure

Specimens of *R. philippinarum* and *R. decussatus* were collected from a reference site in the southern part of the Lagoon of Venice (Chioggia, Italy).

For each species, three groups of animals were selected, placed in large aquaria and then acclimated to the three experimental salinity values (18, 28 and 38 psu), which were gradually achieved in 12 days. Clams were maintained in aquaria, which contained a sandy bottom and aerated natural seawater (temperature of 16 ± 0.5 °C) and were fed daily with microalgae (*Isochrysis galbana*).

Stock solutions (0.1 g/L) of nZnO, $nTiO_2$ and FC_{60} were prepared in Milli-Q water and sonicated at 4 °C using a Braun Labsonic U sonifier at 50% duty cycles for 30 min. Clams (35 per tank) were exposed for 7 days to 18, 28 and 38 psu of salinity, both in the absence and in the presence of NP mixture (1 μg/L nZnO, 1 μg/L $nTiO_2$, and 1 μg/L FC_{60}). The various experimental conditions were abbreviated as C-MIN (control at 18 psu), C-MED (control at 28 psu), C-MAX (control at 38 psu), NP-MIN (NP mixture at 18 psu), NP-MED (NP mixture at 28 psu) and C-MAX (NP mixture at 38 psu). For each experimental condition tested, two replicate tanks were prepared. The nominal concentrations were chosen similar to the PEC values found in the literature.

During exposure, both clam species were maintained in glass aquaria (without sediment) containing aerated seawater (1 L per animal) in the same thermo-haline conditions used during the acclimatisation period. A movement pump (Hydor, Koralia nano 900, USA) was positioned in every aquarium (both for control and treated clams) to facilitate the water circulation and to prevent NP sedimentation (Marisa et al., paper I). The seawater was renewed daily, NP mixture and microalgae (at an initial concentration of approximately 150,000 cells/L) were supplied in the experimental tanks. Before adding contaminants, the stock solutions were sonicated, as reported above.

2.3. Collection of tissues for biomarker analyses

During exposure, the haemolymph, gills and digestive gland of e both clam species were collected after first (T1), third (T3), and last (T7) days of exposure. For each tissue, five pools (5 animals per pool, 2 or 3 from each replicate tank) from each experimental condition were prepared. Aliquots of each pooled tissue were frozen in liquid nitrogen and stored at -80 °C until analyses or immediately processed, depending on the various biological responses measured. All assays performed in this study had previously been validated (Marisa et al., paper I; Matozzo et al., 2012a; Matozzo et al., 2012b; Matozzo et al., 2013; Parolini et al., 2010; Parolini et al., 2013).

2.4. Gill and digestive gland preparation and biochemical assays

Pooled gills and digestive glands were homogenised at 4 °C using an Ultra-Turrax homogeniser (model T8 basic, IKA) in four volumes of 50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M KCl, 0.5 M sucrose, and Protease Inhibitor Cocktail (P2714, Sigma–Aldrich) and then centrifuged at 12,000 × *g* for 40 min at 4 °C. Supernatants (SN) were collected for the analyses. SN protein concentrations were quantified according to Bradford (1976) using bovine serum albumin (BSA) as the standard.

Total superoxide dismutase (SOD) activity was measured in the SN of both tissues using the xanthine oxidase/cytochrome c method proposed by Crapo et al. (1978). Enzyme activity is expressed as U SOD/mg protein, where one unit of SOD was defined as the amount of sample producing 50% inhibition in the assay conditions. Gill and digestive gland catalase (CAT) activity was measured according to the method of Aebi (1984). The results are expressed in U CAT/mg protein, where one unit of CAT was defined as the amount of enzyme that catalysed the dismutation of 1 μmol of H_2O_2/min.

Glutathione S-transferase (GST) activity was measured spectrophotometrically according to the method described in Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) and reduced

glutathione as substrates. GST activity is expressed as nmol/min/mg protein. Lipid peroxidation (LPO) was quantified in both tissues' SN using the malondialdehyde (MDA) assay, according to the method of Buege and Aust (1978). Absorbance was read spectrophotometrically at 532 nm, and the results are expressed as nmoles of thiobarbituric reactive substances (TBARS)/mg protein. TBARS, considered as ''MDA-like peroxide products'', were quantified by reference to MDA absorbance (Damiens et al. 2007). The results were not expressed as MDA levels because TBA can react with a range of chemical compounds (Csallany et al. 1984). Protein carbonyl content (PCC) was measured via the formation of labelled protein hydrazone derivatives, after 2,4-dinitrophenylhydrazide (DNPH) reaction, which were then quantified spectrophotometrically (Dalle-Donne et al. 2003; Mecocci et al. 1999). The carbonyl content was calculated from the SN absorbance via the molar absorption coefficient of 22,000 mol/cm and expressed as nmol/mg protein.

2.5 Bioaccumulation of NPs in gills and digestive gland

At the end of the exposure (T7), 4 pools of gills and digestive glands per experimental condition (4 animals each) were collected to quantify zinc, titanium and FC_{60} bioaccumulation.

2.5.1 nZnO and $nTiO_2$ bioaccumulation in gills and digestive gland

Tissue samples were freeze–dried, and approximately 150 mg were weighed and digested in TFM vessels with 4 mL of 69% nitric acid and 1.5 mL of 30% hydrogen peroxide and 0.4 mL of 47% hydrofluoric acid. Digestion was performed in a Milestone MLS 1200 MEGA microwave oven. The heating programme consisted of five stages (2 min, 250 W - 2 min, 0 W - 6 min, 250 W - 5 min, 400 W and 5 min, 650 W). After cooling, 5 mL of saturated boric acid solution were added, and the heating programme performed again (20 min, 400 W). Samples were then transferred into graduated flasks and diluted to 25 mL with Millipore Milli-Q water. The sample solutions were analysed via inductively coupled plasma optic emission spectroscopy (ICP-OES) using a Thermo Fischer Scientific iCAP 6300 DUO. Five calibration solutions (0, 0.5, 1, 3 and 6 ppm of Zn and Ti) were prepared by conventional dilution of Carlo Erba 1000 µg/mL mono-elemental standard solution of the analyte as nitrate. The same amount of reagents used for the digestion procedure was added to each calibration solution. Measurements were made at Ti 323,45 nm and at Zn 202.55 nm, each sample was analysed in five replicas. The results are expressed as µg Zn/g dry weight and µg Ti/g dry weight. The detection limits of Zn and Ti were 0.9 µg/L and 0.3 µg/L, respectively.

2.5.2. FC_{60} bioaccumulation in gills and digestive gland

At the end of the exposure (T7), 4 pools of gills and digestive glands per experimental condition (6 animals each) were collected to quantify FC_{60} bioaccumulation. Tissue samples were carefully washed with pure toluene to remove surface adsorbed FC_{60} particles. Then, tissues were extracted into toluene (1:6, w:v ratio) using ultra-sonication for 30 min (35 kHz). Extracts were centrifuged (9000 ×*g*) and the SN was used in ACQUITY UPLC (Ultra Performance Liquid Chromatography, Waters, Milford, MA, USA) analysis. FC_{60} was separated using Buckyprep (150×2.0 mm, particle size 5 ìm) analytical column (Nacalai Tesque, Kyoto, Japan). The FC_{60} content was detected and analysed with a mass spectrometer Q-Exactive (Thermo Fisher Scientific, San Jose, CA, USA).

2.6. Haemolymph parameters

Cytotoxicity was evaluated using a colorimetric assay based on the measurement of lactate dehydrogenase (LDH) activity in cell-free haemolymph. A commercial kit (Cytotoxicity Detection Kit, Roche) was used to assess cell damage. The results, normalised to total haemocyte count values, are expressed as the optical density (OD) at 490 nm.

The Neutral Red uptake assay (NRU) provides a quantitative estimation of viable cells, and it was performed according to the modified method of Cajaraville et al. (1996). This test is based on the ability of cells to incorporate and bind the vital dye neutral red;, it was used to evaluate the capability of haemocytes to perform pinocytosis. The results, normalised to total haemocyte count values, are expressed as the optical density (OD) at 550 nm.

The Micronucleus (MN) test was performed according to the method of Pavlica et al. (2000). The slides were kept in the dark at 4 °C prior to examination under the microscope. Using a pre-arranged pathway, slides were scored under the fluorescent microscope Leica 5000B equipped with a submerged lens at 100× magnification. Four hundred nucleus were counted for each slide for a total of 2000 nucleus/treatment. Only intact and non-overlapping haemocytes were

considered. Micronuclei were identified according to the criteria proposed by Kirsch-Volders et al. (2000), and the MN frequency (MN‰) was calculated.

2.7. Statistical analysis

All biomarker data of the three tissues (gills, digestive gland and haemolymph) from both species were statistically evaluated using a PERMANOVA analysis (PRIMER 6 PERMANOVA plus software package). The variables considered were i) species ii) concentration of contaminants (absence or presence of NP mixture) iii) time of exposure (T1, T3, T7) and iv) salinity (18-28-38 psu).

After that, all biomarker data of the three tissues, immunological data (NRU, LDH activity, Micronucleus test), biochemical data of gills and digestive gland (SOD, CAT and GST activity; LPO and PCC) were statistically evaluated using a PERMANOVA analysis. The variables considered were i) concentration of contaminants (absence or presence of NP mixture) ii) time of exposure (T1, T3, T7) and iii) salinity (18-28-38 psu).

The normal distribution (Shapiro-Wilk test) and the homogeneity of the variance (Bartlett test) of the data were assessed. For each biomarker measured, a multifactorial ANOVA (STATISTICA 10 software package, StatSoft, Tulsa, OK) was set with factors i) concentration of contaminants, ii) time of exposure and iii) salinity. The ANOVA was followed by a Fischer LSD post-hoc test to evaluate significant differences between NP treated samples and related controls (time to time), among exposure times at the same experimental condition, and among salinities in both treated samples and controls.

The data regarding the bioaccumulation of NP mixture (Zn, Ti and FC_{60}) in gills and digestive gland were statistically compared using a two-way ANOVA test with factors i) concentration of contaminants and ii) salinity; followed by a Fischer LSD post-hoc test to evaluate significant differences between treated samples (NP-MIN, NP-MED and NP-MAX) and related controls (C-MIN, C-MED and C-MAX), and among salinities in both treated samples and controls.

3. Results

3.1. nZnO, $nTiO_2$ and FC_{60} characterisation

TEM images of nZnO, $nTiO_2$ and FC_{60} are provided in Fig. 1 A, B and C, respectively. The complete results concerning the characterisation of the three NPs were reported in Marisa et al. (paper I, III, IV).

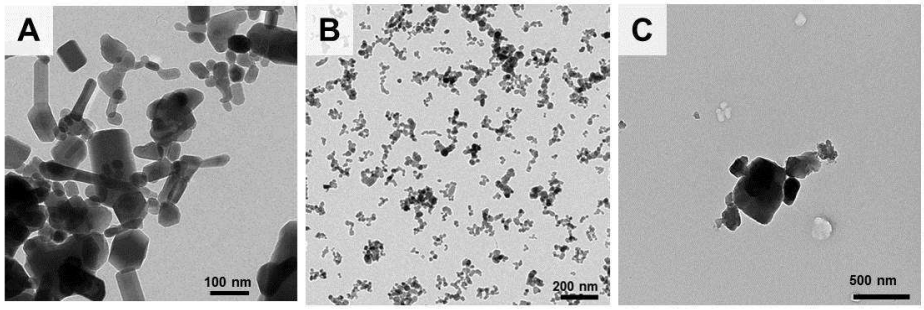

Fig. 1. A, B, C: TEM images of nZnO, $nTiO_2$ and FC_{60}.

3.2. *Ruditapes philippinarum* and *decussatus* PERMANOVA results

PERMANOVA highlighted significant differences in the response of biomarkers in clam tissues due to the species (Pseudo-F=45.429 and p=0.001), concentration (Pseudo-F=23.673 and p=0.001), time of exposure (Pseudo-F=13.436 and p=0.001), salinity (Pseudo-F=14.507 and p=0.001), species/time interaction (Pseudo-F=18.801 and p=0.001), and species/salinity interaction (Pseudo-F=37.841 and p=0.001).

3.3. *Ruditapes philippinarum* results

PERMANOVA results

PERMANOVA highlighted significant differences in the response of biomarkers in gills, digestive gland and haemolymph due to concentration (Pseudo-F=3.006 and p=0.025), time of exposure (Psuedo-F=6.714 and p=0.001), salinity (Psuedo-F=23.557 and p=0.001), concentration/time interaction (Psuedo-F=2.673, p=0.009), salinity/concentration (Psuedo-F=2.281 and p=0.016), and salinity/time interaction (Psuedo-F=1.796 and p=0.032).

In gills and digestive gland, the PERMANOVA analysis showed a significant variations of the biomarker responses determined by concentration (Pseudo-F=8.623 and p<0.001), time of exposure (Pseudo-F=15.913 and p<0.001), salinity (Pseudo-F=22.158 and p<0.001), concentration/time interaction (Pseudo-F=2.635 and p=0.013), concentration/salinity interaction (Pseudo-F=3.8104 and p=0.002), and salinity/time interaction (Pseudo-F=2.586 and p=0.002).

In haemolymph, biomarker responses were statistically different by time of exposure (Pseudo-F=2.3667 and p=0.048) and salinity (Pseudo-F=24.218 and p<0.001).

Gills and digestive gland assays

A significant time-dependent (p=0.024), concentration/time interaction-dependent (p=0.002) and concentration/salinity interaction-dependent (p=0.031) variation in the activity of SOD was found in the gills: NP-MIN-treated clams exhibited significantly lower value of SOD activity with respect to its corresponding control at T1 and an higher value at T3. At the end of exposure NP-MAX showed an increase of SOD activity in exposed clams compared to C-MAX (Fig. 2A).

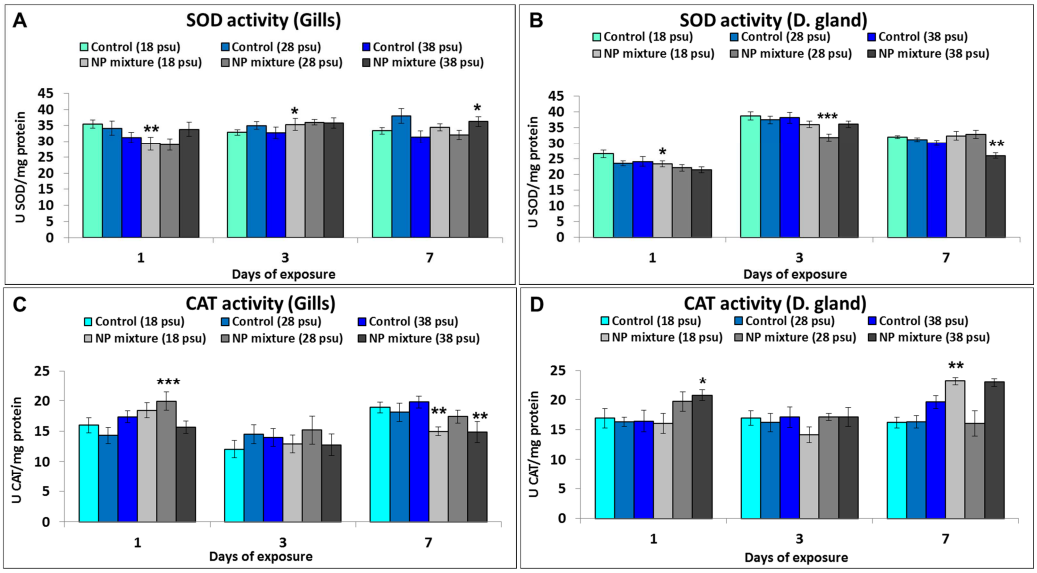

Fig. 2. SOD activity (A-B) expressed as U SOD/mg protein and CAT activity (C-D) expressed as U CAT/mg protein in *R. philippinarum* after 1, 3 and 7 days of exposure to 18, 28 and 38 psu of salinity, both in the absence and in the presence of NP mixture (1 µg/L nZnO, 1 µg/L $nTiO_2$, and 1 µg/L FC_{60}). The values are reported as the means ± SD (standard deviation); n= 5 pools. Asterisks denote significant differences in NP treated clams compared to their respective controls (C-MIN *vs* NP-MIN, C-MED *vs* NP-MED and C-MAX *vs* NP-MAX) at the same time of exposure: *p<0.05, **p<0.01, *** p<0.001.

SOD activity was significantly affected by the concentration in the digestive gland ($p<0.001$), time of exposure ($p<0.001$), salinity ($p=0.002$), time/salinity interaction ($p=0.003$) and concentration/time/salinity interaction ($p=0.043$). A significant decrease of SOD activity was found at T1 in clams exposed to NP-MIN, at T3 in clams exposed to NP-MED and at T7 in clams exposed to NP-MAX compared to their corresponding controls (Fig. 2B).
CAT activity was affected significantly by salinity ($p<0.001$), concentration/time interaction ($p=0.021$) and concentration/salinity interaction ($p=0.004$) in gills. The pair-wise comparisons highlighted a significant increase of CAT activity in NP-MED-treated clams at T1, but it was not maintained during the exposure. Other variations were found at T7, where at NP-MIN and NP-MAX the CAT activity decreased compared to their controls (Fig. 2C).
In digestive gland, CAT activity was affected by the concentration ($p=0.009$), time of exposure ($p=0.004$), salinity ($p=0.020$), concentration/time interaction ($p=0.034$), time/salinity interaction ($p=0.033$) and concentration/time/salinity interaction ($p=0.018$). At the beginning of exposure, only NP-MAX showed a significant increase of CAT activity compared to C-MAX, but the change was not maintained until the end of the exposure. At T7 NP-MIN showed an increase of the activity compared to its control (Fig. 2D).

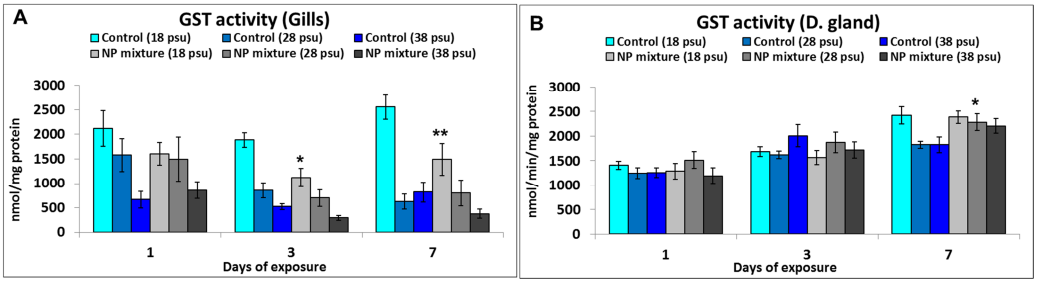

Fig. 3. GST activity (A, B) expressed as nmol/min/mg protein in *R. philippinarum* after 1, 3 and 7 days of exposure to 18, 28 and 38 psu of salinity, both in the absence and in the presence of NP mixture (1 µg/L nZnO, 1 µg/L $nTiO_2$, and 1 µg/L FC_{60}). The values are reported as the means ± SD (standard deviation); n= 5 pools. Asterisks denote significant differences in NP treated clams compared to their respective controls (C-MIN *vs* NP-MIN, C-MED *vs* NP-MED and C-MAX *vs* NP-MAX) at the same time of exposure: *p<0.05, **p<0.01, *** p<0.001.

The GST activity exhibited a modulation due to concentration ($p=0.004$), time of exposure ($p=0.002$), salinity ($p<0.001$) and concentration/salinity interaction ($p=0.013$) in gills. Only in clams exposed to NP-MIN a decrease of GST activity was shown compared to C-MIN exposed clams from T3 (Fig. 3A).

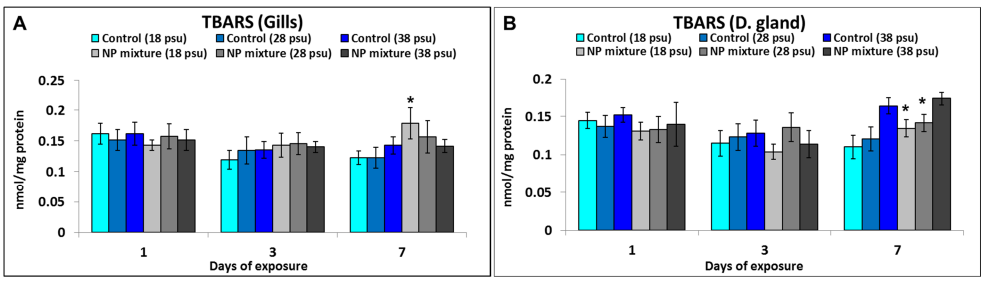

Fig. 4. TBARS (A, B) and PCC (C) levels expressed as nmol/mg protein in R. philippinarum after 1, 3 and 7 days of exposure to 18, 28 and 38 psu of salinity, both in the absence and in the presence of NP mixture (1 µg/L nZnO, 1 µg/L nTiO2, and 1 µg/L FC60). The values are reported as the means ± SD (standard deviation); n= 5 pools. Asterisks denote significant differences in NP

treated clams compared to their respective controls (C-MIN vs NP-MIN, C-MED vs NP-MED and C-MAX vs NP-MAX) at the same time of exposure: *p<0.05, **p<0.01, *** p<0.001.

In digestive gland, GST activity was affected by time of exposure (p<0.001), concentration/salinity interaction (p=0.042) and time/salinity interaction (p=0.035), the pair-wise comparisons showed only an increase of GST activity in NP-MED exposed clams compared to C-MED exposed ones at T7 (Fig. 3B).
In the gills, LPO was significantly affected by concentration (p=0.022), time of exposure (p<0.001), concentration/time interaction (p<0.001), concentration/salinity interaction (p=0.028), and concentration/time/salinity interaction (p=0.040). Pair-wise comparisons highlighted significant increase of LPO in clams exposed to NP-MIN respect to C-MIN only at the end of exposure (Fig. 4A).
LPO was significantly influenced by the time of exposure (p<0.001), salinity (p<0.001), concentration/time interaction (p=0.001) and time/salinity interaction (p<0.001) in digestive gland. In post-hoc analysis results, the TBARS levels showed a significant increase in clams exposed to NP-MIN and NP-MED compare to their specific controls at T7 (Fig. 4B).
PCC values were significantly affected by the exposure to concentration (p<0.001), time of exposure (p<0.001), salinity (p=0.038), and all their interaction (p<0.001) in gills. In the pair-wise comparisons, a significant increase was detected in NP-MIN exposed clams compared to C-MIN at T1 and at T3 (Fig. 4C).
In digestive gland, the PCC values exhibited a modulation due to time of exposure (p<0.01) and salinity (p=0.023). Only a difference was found at T3, where NP-MAX showed an higher PCC value respect NP-MIN (p=0.026).

Zn, Ti and FC_{60} content in gills and digestive gland

The total Zn, Ti and FC_{60} content in the gills and digestive gland of the clams exposed for 7 days to NP mixture under different salinity values are reported in Table 1.

Tab. 1. Levels of Zn, Ti expressed as µg/g dry weight and FC_{60} expressed as ng/g wet weight in the gills and the digestive gland of *R. philippinarum* after 7 days of exposure to 18, 28 and 38 psu of salinity, both in the absence and in the presence of NP mixture (1 µg/L nZnO, 1 µg/L $nTiO_2$, and 1 µg/L FC_{60}). The values are reported as the means ± SD (standard deviation); n= 4 pools. Asterisks denote significant differences in treated clams compared to their specific controls (C-MIN *vs* NP-MIN, C-MED *vs* NP-MED and C-MAX *vs* NP-MAX): *p<0.05. n.d.= not detected.

Gills						
	C-MIN	**C-MED**	**C-MAX**	**NP-MIN**	**NP-MED**	**NP-MAX**
Zn	86.8 (± 7.3)	83.5 (± 2.1)	72.7 (± 3.5)	94.8 (± 3.7)*	89.8 (± 2.4)	75.9 (± 4.5)
Ti	1.2 (± 0.1)	1.7 (± 0.05)	1.7 (± 0.1)	1.4 (± 0.1)	7.3 (± 0.1)*	9.0 (± 0.1)*
FC_{60}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Digestive gland						
Zn	122.4 (± 1.9)	100.9 (± 2.4)	101.5 (± 2.0)	132.0 (±3.3)*	98.7 (± 1.0)	98.9 (±1.1)
Ti	1.5 (± 0.03)	3.4 (± 0.2)	1.5 (± 0.1)	3.5 (± 0.3)*	3.1 (± 0.3)	2.5 (± 0.3)*
FC_{60}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Zn content was affected by concentration (p=0.014) in gills only, salinity (p<0.001) in both gill and digestive gland tissues and concentration/salinity

interaction ($p<0.001$) in digestive gland only. In both gills and digestive gland, the Zn content was higher only in animals exposed to NP-MIN respect to the related controls.
Ti content was significantly affected by concentration ($p<0.001$), salinity ($p<0.001$) and concentration/salinity interaction ($p<0.001$) in both clam tissues. In gills, the concentration of Ti significantly increased in NP-MED and NP-MAX. In digestive gland, Ti content was higher in clams exposed to NP-MIN and NP-MAX compare to their controls.
In the various experimental conditions, FC_{60} content was not detected in both gills and digestive gland.

Haemolymph assays
LDH activity was significantly affected by time of exposure ($p=0.005$), salinity ($p<0.001$) and their interaction ($p=0.002$), but no significant differences were detected in pair-wise comparisons.
NRU assay highlighted significant variation due to time of exposure ($p=0.005$) and concentration/time interaction ($p=0.004$). In post-hoc test, only a difference was shown in clams exposed to NP-MIN respect to C-MIN at T3 ($p=0.029$).
The MN frequency was affected only by the salinity ($p<0.001$), and only an increase was detected in NP-MAX compared to C-MAX at T1 ($p=0.025$).

3.4. *Ruditapes decussatus* results
PERMANOVA results
In gills, digestive gland and haemolymph, the PERMANOVA analysis showed significant variations in the whole set of biomarkers used due to concentration (Pseudo-F=9.817 and $p<0.001$), time of exposure (Pseudo-F=13.973 and $p<0.001$), salinity (Pseudo-F=20.68 and $p<0.001$), concentration/time interaction (Pseudo-F=7.758 and $p<0.001$) and concentration/salinity interaction (Pseudo-F=2.749 and $p=0.014$).
PERMANOVA highlighted significant differences in the response of biomarkers in gills and digestive gland determined by concentration (Pseudo-F=41.807 and $p<0.001$), time of exposure (Pseudo-F=57.908 and $p<0.001$), salinity (Pseudo-F=9.503 and $p<0.001$), concentration/time interaction (Pseudo-F=19.466, $p<0.001$), salinity/concentration (Pseudo-F=4.889 and $p<0.001$), salinity/time interaction (Pseudo-F=3.984 and $p<0.001$), and concentration/time/salinity interaction (Pseudo-F=3.537 and $p<0.001$).
In haemolymph, biomarker responses were significantly affected by salinity (Pseudo-F=23.992 and $p<0.001$) and concentration/time interaction (Pseudo-F=4.289 and $p=0.006$).

Gill and digestive gland assays
SOD activity was significantly affected in both gill and digestive gland tissues by concentration ($p=0.034$ and $p<0.001$, respectively), time of exposure ($p<0.001$), salinity ($p<0.001$), and all their possible interactions ($p<0.001$). In gills, a significant variation of SOD activity was found at T1 in clams exposed to NP-MIN (decrease) and in NP-MAX (increase), whereas at T3 a decrease in SOD activity was shown in all NP treated clams . Instead, only the clams exposed to NP-MED and NP-MAX revealed an increased enzyme activity compared to their controls at T7 (Fig. 5A). Significant variations in the activity of SOD were found

in the digestive gland: NP-MIN treated clams exhibited significantly lower values of SOD activity respect to control at T1. Only NP-MAX showed an increase of SOD activity from T3 until T7. At the end of exposure, also clams exposed to NP-MED exhibited an increase of enzyme activity compared to control (Fig. 5B).
In both gills and digestive gland, CAT activity was affected significantly by concentration ($p=0.009$ and $p<0.001$, respectively), time of exposure ($p<0.001$), salinity ($p=0.001$ and $p<0.001$, respectively) and all their interaction ($p<0.001$). In gills, the pair-wise comparisons highlighted significant increase of CAT activity in NP-MIN exposed clams compared to C-MIN at T1 and T7, also in NP-MAX-treated clams an increase of CAT activity was shown at the end of exposure. At T3, only a decrease of CAT activity was shown in NP-MED treated clams (Fig. 5C). In digestive gland, at the beginning of exposure, only NP-MIN showed a significant increase of CAT activity compared to C-MIN, but the change was not maintained during the exposure. At T3 NP-MED and at T7 NP-MAX exposed clams showed an increase in CAT activity compared to their controls (Fig. 5D).

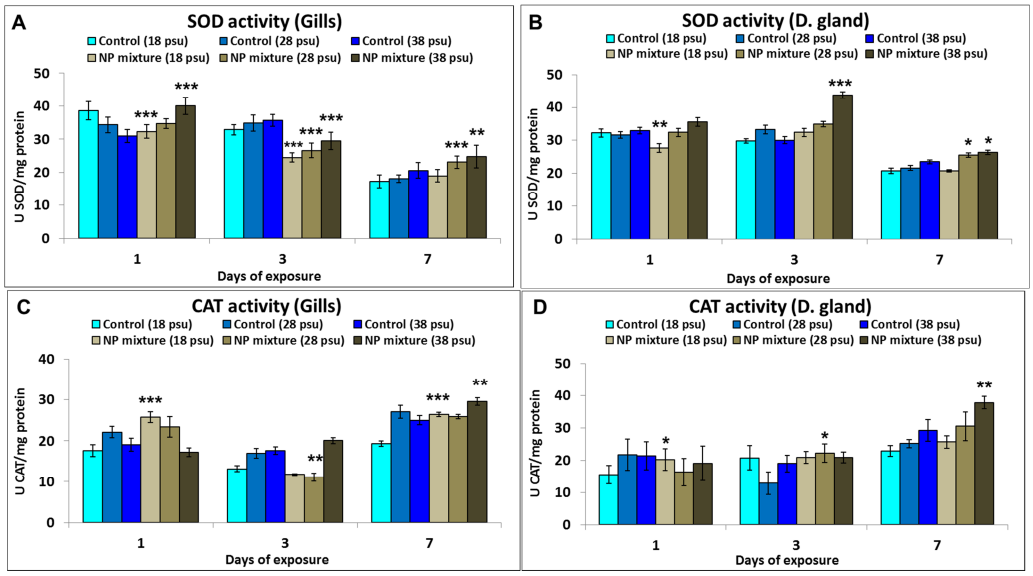

Fig. 5. SOD activity (A, B) expressed as U SOD/mg protein and CAT activity (C, D) expressed as U CAT/mg protein in *R. decussatus* after 1, 3 and 7 days of exposure to 18, 28 and 38 psu of salinity, both in the absence and in the presence of NP mixture (1 µg/L nZnO, 1 µg/L $nTiO_2$, and 1 µg/L FC_{60}). The values are reported as the means ± SD (standard deviation); n= 5 pools. Asterisks denote significant differences in NP treated clams compared to their respective controls (C-MIN *vs* NP-MIN, C-MED *vs* NP-MED and C-MAX *vs* NP-MAX) at the same time of exposure: *p<0.05, **p<0.01, *** p<0.001.

In gills, the GST activity exhibited a modulation due to concentration ($p<0.001$), time of exposure ($p<0.001$), salinity ($p<0.001$) and all their interaction ($p<0.001$).
At T1, a significant decrease of GST activity was shown in clams exposed to NP-MIN and NP-MAX, but only in NP-MIN the variation was maintained until the end of exposure (Fig. 6A). In digestive gland, GST activity was affected by concentration ($p=0.027$) and time of exposure ($p<0.001$), but the pair-wise comparisons did not revealed significant variations in NP treated clams compared to controls.
LPO was significantly affected in both gills and digestive gland by concentration ($p<0.001$), time of exposure ($p<0.001$), concentration/time interaction ($p<0.001$), time/salinity interaction ($p<0.001$ and $p=0.001$, respectively),

concentration/salinity interaction in gills only (p=0.004) and salinity and concentration/time/salinity interaction in digestive gland only (p<0.001). In gills, pair-wise comparisons did not highlight variations of TBARS levels among treated clams and their respective controls. In digestive gland, only at T7 an increase in LPO was found in clams exposed to NP-MIN and NP-MAX compared to their controls (Fig. 6B).

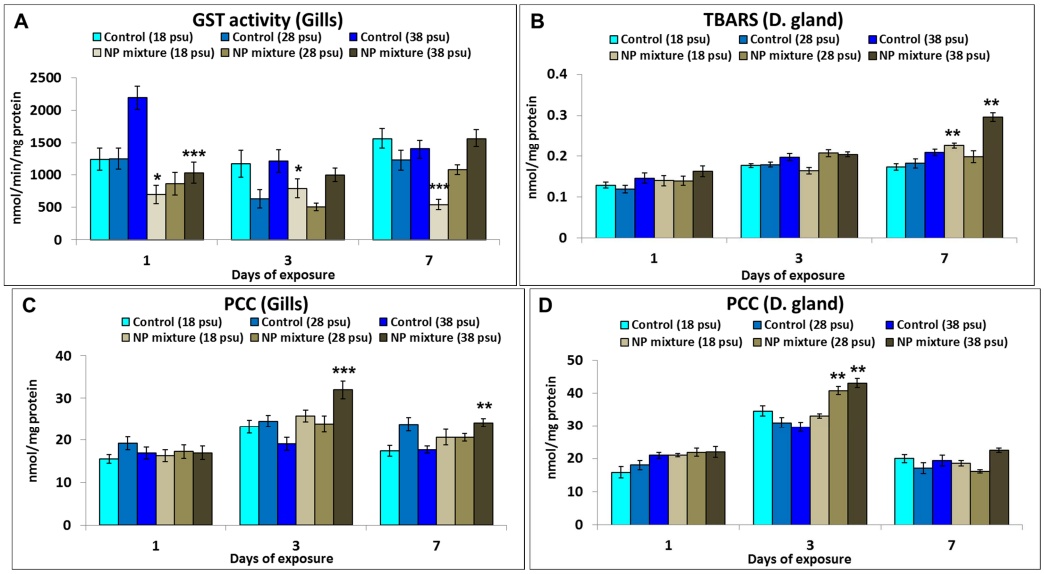

Fig. 6. GST activity (A) expressed as nmol/min/mg protein, TBARS levels (B) expressed as nmol/mg protein, PCC levels (C, D) expressed as nmol/mg protein in *R. decussatus* after 1, 3 and 7 days of exposure to 18, 28 and 38 psu of salinity, both in the absence and in the presence of NP mixture (1 µg/L nZnO, 1 µg/L $nTiO_2$, and 1 µg/L FC_{60}). The values are reported as the means ± SD (standard deviation); n= 5 pools. Asterisks denote significant differences in NP treated clams compared to their respective controls (C-MIN *vs* NP-MIN, C-MED *vs* NP-MED and C-MAX *vs* NP-MAX) at the same time of exposure: *p<0.05, **p<0.01, *** p<0.001.

In gills, PCC values were significantly influenced by the exposure to concentration (p=0.001), time of exposure (p<0.001) and their interaction (p=0.001). The post-hoc results showed an increase of PCC values in clams exposed to NP-MAX from T3 until the end of exposure (Fig. 6C). In digestive gland, the PCC values exhibited a modulation due to concentration (p<0.001), time of exposure (p<0.01), salinity (p=0.003), concentration/time interaction (p<0.001), concentration/salinity interaction (p=0.002) and concentration/time/salinity interaction (p<0.001). Only at T3, a significant increase in PCC value was found in clams exposed to NP-MED and NP-MAX compared to controls (Fig. 6D).

Zn, Ti and FC_{60} content in gills and digestive gland

The total Zn, Ti and FC_{60} content in the gills and digestive gland of the clams exposed for 7 days to NP mixture under different salinity values are reported in Table 2.

In both clam tissues, Zn content was affected by concentration (p=0.012 and p<0.001, respectively), salinity (p<0.001) and concentration/salinity interaction (p<0.001 and p=0.012, respectively). In gills, Zn content was significantly higher in clams exposed to NP-MIN and NP-MED compared to their respective controls.

In digestive gland, an increase of Zn accumulation was shown in NP-MED and NP-MAX.

Tab. 2. Levels of Zn, Ti expressed as µg/g dry weight and FC_{60} expressed as ng/g wet weight in the gills and the digestive gland of *R. decussatus* after 7 days of exposure to 18, 28 and 38 psu of salinity, both in the absence and in the presence of NP mixture (1 µg/L nZnO, 1 µg/L $nTiO_2$, and 1 µg/L FC_{60}). The values are reported as the means ± SD (standard deviation); n= 4 pools. Asterisks denote significant differences in treated clams compared to their respective controls (C-MIN *vs* NP-MIN, C-MED *vs* NP-MED and C-MAX *vs* NP-MAX): *p<0.05.

Gills						
	C-MIN	**C-MED**	**C-MAX**	**NP-MIN**	**NP-MED**	**NP-MAX**
Zn	96.8 (± 6.6)	90 (± 1.5)	85.5 (± 1.1)	103.0 (± 2.9)*	100.3 (± 2.2)*	83.0 (± 2.6)
Ti	1.6 (± 0.1)	1.0 (± 0.05)	1.7 (± 0.1)	1.5 (± 0.1)	1.4 (± 0.1)*	1.5 (± 0.1)
FC_{60}	1.08 (± 0.4)	0.6 (± 0.1)	1.4 (± 0.3)	5.1 (± 0.5)*	2.8 (± 0.8)*	1.7 (± 0.3)
Digestive gland						
Zn	93.4 (± 1.06)	95.5 (± 2.7)	91.4 (± 1.2)	95.5 (± 1.3)	105 (± 2)*	97 (± 1.7)*
Ti	2.6 (± 0.1)	1.5 (± 0.05)	3.3 (± 0.1)	2.9 (± 0.1)	2.8 (± 0.1)*	3.9 (± 0.05)*
FC_{60}	3.6 (± 0.3)	2.5 (± 0.8)	2.3 (± 1.03)	7.2 (± 0.7)*	7.5 (± 1.2)*	2.9 (± 0.9)

Only in digestive gland, Ti content was significantly affected by concentration ($p<0.001$) , whereas in both tissues it was influenced by salinity ($p<0.001$) and concentration/salinity interaction ($p=0.001$ and $p<0.001$, respectively for gills and digestive gland). In gills, the concentrations of Ti increased significantly in NP-MED. In digestive gland, Ti content was significantly higher in clams exposed to NP-MED and NP-MAX respect to their controls.

In the various experimental conditions, FC_{60} content was affected by concentration ($p<0.001$), salinity ($p<0.001$) and concentration/salinity interaction ($p<0.001$ and $p=0.004$, respectively) in both gills and digestive gland. In gills and digestive gland, FC_{60} content increased significantly in clams exposed to NP-MIN and NP-MED compared to the respective controls (Tab. 2).

Haemolymph assays

LDH activity was significantly affected by the concentration ($p<0.001$), time of exposure ($p<0.001$), salinity ($p<0.001$) and all their possible interactions ($p<0.001$). From T3 until the end of exposure, NP-MIN and NP-MED exposed clams showed a significant increase in LDH activity in haemolymph compared to their controls. Moreover, an increase of the enzyme activity was also found in NP-MAX exposed clams at T7.

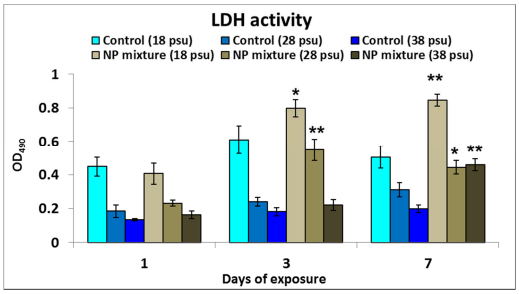

Fig. 7. LDH activity expressed as OD_{490} in *R. decussatus* after 1, 3 and 7 days of exposure to 18, 28 and 38 psu of salinity, both in the absence and in the presence of NP mixture (1µ/L nZnO, 1 µg/L $nTiO_2$, and 1 µg/L FC_{60}). The values are reported as the means ± SD (standard deviation); n= 5 pools. Asterisks denote significant differences in NP treated clams compared to their respective

controls (C-MIN *vs* NP-MIN, C-MED *vs* NP-MED and C-MAX *vs* NP-MAX) at the same time of exposure: *p<0.05, **p<0.01.

NRU assay revealed a significant variation among experimental conditions due to salinity (p=0.001), although no significant difference were obtained using post-hoc tests.
The MN frequency was affected only by the salinity (p<0.001), a significant increase in micronuclei was detected in NP-MAX compare to C-MAX at T3 only (p=0.003).

3.5. Effects of salinity on biomarker responses in gills and digestive gland from the two bivalve species

In Table 3 are reported the salinity values to which NP mixture exerted significant effects on biomarker responses of the two clam species. The comparison highlighted the absence of a clear variation pattern, depending on species, biomarkers and tissues analysed. Between the two tissues, gills seem to be more responsive at 18 psu.

Tab. 3. Schematisation of the salinity values (**18**-**28**-**38** psu) to which NP mixture affected biomarker responses in gills and digestive gland of *R. philippinarum* (R.p.) and *R. decussatus* (R.d.). (T1-T3-T7= days of exposure; n.a.= not available; n.d.=not detected; /= no significant).

Bivalve species		Gills				D. gland		
		T1	T3	T7		T1	T3	T7
R.p.	SOD	18	18	38	SOD	18	28	38
R.d		18-38	18-28-38	28-38		18	38	28-38
R.p.	CAT	28	/	18-38	CAT	38	/	18
R.d		18	28	18-38		18	28	38
R.p.	GST	/	18	18	GST	/	/	28
R.d		18-38	18	18		/	/	/
R.p.	TBARS	/	/	18	TBARS	/	/	18-28
R.d		/	/	/		/	/	18-38
R.p.	PCC	18	28	/	PCC	/	/	/
R.d		/	38	38		/	28-38	/
R.p.	Zn	n.a.	n.a.	18	Zn	n.a.	n.a.	18
R.d		n.a.	n.a.	18-28		n.a.	n.a.	28-38
R.p.	Ti	n.a.	n.a.	28-38	Ti	n.a.	n.a.	38
R.d		n.a.	n.a.	28		n.a.	n.a.	28-38
R.p.	FC_{60}	n.a.	n.a.	n.d.	FC_{60}	n.a.	n.a.	n.d.
R.d		n.a.	n.a.	18-28		n.a.	n.a.	18-28

4. Discussion

Nowadays, there are many emerging anthropogenic contaminants that are continuously released into the coastal environment and interact each other, such as NPs. In a perspective of global change, there is an increasing need of knowledge about the potential combined effects of these pollutants and environmental parameters which may affect their toxicity, as well as the susceptibility of marine species to pollution. Indeed, such interactions can alter chemical speciation and bioavailability of several pollutants with potentially deleterious effects on the biota. Even without any change in the levels of toxicant exposure, changes in other environmental conditions may affect the sensitivity of organisms to current toxicants (Moe et al., 2013). For example, it is generally accepted that a higher temperature increases the rate of pollutant uptake, via changes in ventilation rate

in response to an increased metabolic rate and decreased oxygen solubility. Additionally, it has been shown that the upper temperature tolerance limits are decreased in the presence of certain organic chemicals (Schiedek et al., 2007).
Combined effects of different parameters of the aqueous media cause changes in the behaviour of NPs, e.g. in either aggregation or stabilization. This also affects NP bioavailability, and the phase (water column or sediments) in which the particles are likely to reside and deposit (Keller et al., 2010). The NP aggregation and sedimentation changed considering different type of water, and more aggregation and quick time of sedimentation were shown in seawater compare to freshwater (Keller et al., 2010; Majedi et al., 2013; Yang et al., 2013). Salinity and its variations are a feature to be considered in NP toxicity investigations. Coastal environments, such as estuaries and lagoons, are subject to wide variations in salinity under the impact of tidal and seasonal changes. The ebb and flood of the tide, combined with freshwater inputs from rivers and climate changes, can dramatically alter the salinity of these aquatic systems (Reid et al., 2003).
Moreover, few ecotoxicological studies have also shown that NP size and aggregation play an important role in determining toxicity (Moore, 2006; Tedesco et al., 2010). Only a few studies have addressed the behaviour of NPs in complex aqueous matrices (Zhu et al., 2008; Keller et al., 2010), and only a few studies investigated the toxicity of single nZnO and nCuO under different salinity values, comparing also with the metal ion action (Ates et al., 2014; Park et al., 2014; Yung et al., 2015).
To our knowledge, this is the first study attempting the assessment of response patterns caused by NP mixture exposure under different salinity values in *R. philippinarum* and *R. decussatus* living in sympatry in the Lagoon of Venice. Despite their phylogenetic closeness, the two species have a recent different story; *R. philippinarum* is an alien species introduced in the 80's and then farmed and fished in the lagoon. *R. decussatus* is the native species and only a scanty natural population inhabits the lagoon. Indeed, *R. philippinarum* proved to be harder and faster-growing than *R. decussatus* and today this clam contributes 91% to European yields of the two species. Although both clam species are phenotypically similar, they constitute two distinct entities with high genetic distance (Donaghy et al., 2010).
In the present study, the three salinity values were chosen based on data recorded in the Venice Lagoon, as reported in Piccino et al. (2012), in particular we choose the minimum, medium and maximum values measured. Although these salinity values already occur in the lagoon, in global scenarios they could approach mean salinity predicted in the next century. Unlike temperature, predictive models for salinity hypothesise both higher or lower values compared to current mean values in the future (Vuorinen et al., 2015).
Various papers in literature have investigated the salinity effects in clams. In particular, it is known that salinity can affected various responses in *R. philippinarum* (Kim et al., 2001; Coughlan et al., 2009: Munari et al., 2011). Moreover, four common heavy metals were shown to affect differently this clam, under different salinities (Riba et al., 2004).
As in our previous study investigating the effects of NP mixture to *R. philippinarum* (Marisa et al., paper V), also under salinity variations, the overall adverse effects of the contaminants was confirmed in this study. In particular, the

present findings highlighted an increase in oxidative stress in treated clams, with a modulation of anti-oxidant enzyme activities in gills and digestive gland tissues. Moreover, in gills a decrease of GST activity in clam exposed to NP-MIN was detected, confirming an oxidative stress. Instead, in digestive gland, the GST activity increased in clam exposed to NP-MED, indicating a potential detoxification process underway. GST is an enzyme that participates in the detoxification process due to conjugation reaction between its substrate glutathione (GSH) and xenobiotics, assisting in the elimination of external materials (Cummins et al., 2011). Moreover, an increase of lipid peroxidation was also detected in clam exposed to NP-MIN in gills, and to NP-MIN and NP-MAX in digestive gland. The haemocyte parameters measured were not affected during the exposure. The LDH activity, NRU assay and MN test are biomarkers useful to identify high toxicity determined by pollutants showing high damage to cells in term of death, cell membrane stability and functionality, and unrepaired DNA damage. These findings are in agreement with the results obtained in the previous studies conducted to assess the nZnO, $nTiO_2$ and FC_{60} toxicity in Manila clam (Marisa et al., paper I, III, IV), where the haemolymph did not show high damage under NP exposure, so that only in $nTiO_2$-treated clam the haemocytes could be considered the cell target of NP toxicity. This is consistent with the role of haemocytes as the ultimate site of NP uptake. Indeed, in bivalves, NPs are known to be filtered by the gills, accumulate in the digestive gland, and transferred to the haemolymph through the epithelium of the digestive gland tubules (Moore et al., 2009; Rocha et al., 2015). Low damage to haemolymph was consistent with low concentrations of contaminants and short *in vivo* exposure. Moreover, in this experiments the salinity values and the NPs as mixture could be important features that can change the NP behaviour respect to the single NP action in the responses of clams.

Reid et al. (2003), studied the effects of salinity (20 to 40‰) on various immune parameters of *R. philippinarum*, and the progression of brown ring disease, a vibriosis affecting clams, in experimentally infected individuals. The results indicated that haemocyte parameters were significantly affected more by salinity than by pathogen. In addition, a low-salinity environment (20‰) was significantly associated with higher disease prevalence.

From literature, the clam *R. decussatus* was not used in other NP toxicity studies, and only in one study to understand the effects of salinity on its immune responses to a pathogen (Casas et al., 2002). Despite these lacking information, *R. decussatus* was often used to monitor marine coastal pollution and to understand the potential effects determined by various groups of pollutants in laboratory experiments (Romeo and Gnassia-Barelli, 1995; Hamza-Chaffai et al., 1998; Chìcharo and Chìcharo, 2001; Geret et al., 2002; Chora et al., 2010; Sellami et al., 2015). In our study, NP mixture under different salinity values exhibited a similar overall effect in both clam species. Indeed, also in *R. decussatus* variations in the anti-oxidant enzyme activities were shown in both gills and digestive gland tissues of NP exposed clams. The decrease of GST activity in gills confirmed in NP-MIN-treated clams an underway oxidative stress in cells. The inhibition of its activity indicated an antioxidant role for GST (Roling and Baldwin, 2006). Nonetheless, also damage to proteins in gills and to lipids in digestive gland was detected. *R. decussatus* revealed an increased LDH activity in haemocytes of NP exposed clams, indicating an high cytotoxic action ascribable to the NP mixture.

Indeed, LDH is a stable cytoplasmic enzyme that can be released by damaged cells (at cell membrane level) into the haemolymph (Matozzo et al., 2012a). The results demonstrated an increase in LDH activity in haemolymph of exposed clam at all salinity values tested, suggesting that the contaminant caused destabilisation of haemocyte membranes. Respect to *R. philippinarum*, the haemolymph of native species could be considered more sensitive to NP toxicity, but more future investigation are needed.

Despite that the two clam species exposed to pathogenic bacteria (Vibrio PI) highlighted a similar pattern of responses, with an increase of haemocytes in both species after 72 h (Oubella et al., 1993). Currently, gaps still persist concerning the information about the differences between the types and roles of the *R. philippinarum* and *R. decussatus* haemocytes (Donaghy et al., 2009).

In both species used, some biomarkers tested showed a variations during the exposure (T1-T3), which was not maintained until the end of exposure. In *R. philippinarum*, this pattern of responses was found in gills for GST activity and PCC levels, and in haemolymph for NRU and MN assays. In *R. decussatus*, early but transient variations were detected in digestive gland for PCC levels and in haemolymph for MN assays. These effects could be determined by a recovery of the clams exposed to NPs at the end of exposure. In this regard, long-term experiments would be useful to define realistic risks due to NP exposure under increased or decreased salinity.

The NP bioaccumulation occurs in the two species, but with a different pattern between Zn and Ti and between the tissues analysed. In particular, metal bioaccumulation occurs in both clams at all salinities, even though to a greater extent at low salinity (in *R. philippinarum*) and at medium-high salinity (in *R. decussatus*). Respect to the findings of Marisa et al. (paper I, III, IV and V) the salinity influences the bioaccumulation pattern in clams *R. philippinarum*.

The FC_{60} content was detected only in gills and digestive gland of *R. decussatus*. This could be related to the higher accumulation rate of contaminants in this species compared to *R. philippinarum*, as already demonstrated in other studies (Figueira et al., 2012; Freitas et al., 2012). In particular, Figueira et al. (2012) found a significant accumulation of cadmium (10, 18, 32, 56 and 100 μM) after 5 days of exposure in both *R. philippinarum* and *R. decussatus*, but the cadmium concentration was 3 to 8 times higher in *R. decussatus*. Similar results were found also by Freitas et al. (2012) for a battery of metals.

Conversely, the measurements of FC_{60} content in Marisa et al. (paper IV and V) highlighted an accumulation of this NP in both gills and digestive gland of *R. philippinarum*. This different bioaccumulation report could depend on differences of analytical methods used and on the salinity values that could change the NP mixture behaviour. Regarding these differences about FC_{60} content in *R. philippinarum*, deeper investigations are needed to better understand the potential bioaccumulation of FC_{60} in clams.

Overall, at all salinity values tested various changes in the biomarkers' responses were shown, depending on the tissues, the biomarkers, the NP accumulation and the species considered. Although the major variations respect to controls were found at the minimum and the maximum value of salinity, the comparison between the two species did not highlight clear and distinct patterns of response (Fig. 3). However, it has to be noted that under NP exposure at the three salinities tested, the number of responses that significantly varied respect to controls was

slightly higher in *R. decussatus* than in *R. philippinarum*. Indeed, the PERMANOVA results, regarding the analysis of the two species together, highlighted that the responses of *R. philippinarum* and *R. decussatus* were different and the concentration, time of exposure and salinity affected the biomarkers in both species. In addition, the two species were influenced differently by time of exposure and salinity, but did not by concentration.

In Velez et al. (2015) *R. philippinarum* and *R. decussatus* from mercury pollution areas were analysed to understand their different responses and accumulation of metal. The authors found a different results in the two clams and *R. philippinarum* is the most impacted species. Instead, in Figueira et al. (2012) exposed to cadmium highlighted an higher accumulation in *R. decuss*atus respect to *R. philippinarum*. Nevertheless, *R. philippinarum* presented higher oxidative stress and higher CAT activity compare to *R. decussatus*. The authors conclude that the paradox observed between the two clams can be explained by the higher capacity of *R. decussatus* to increase the expression of metallothioneins when exposed to cadmium (Figueira et al., 2012). Similar results was also found in Moraga et al. (2002), where two genetic markers (phosphoglucomutase and glucosephosphate isomerase) and a protein marker (metallothionein) were monitored in order to determine the impact of heavy metals in different natural populations of the two clam species.

Although preliminary, results of the present study indicate that environmental parameters, such as salinity, can modulate NP toxicity to clams. This should be take into account under a global change scenario, considering that changes in temperature, salinity and pH are expected in the future. In any case further studies are needed to understand better the combined effects of environmental parameters and emerging contaminants, such as NPs, in aquatic environments.

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General conclusions and remarks

Even if in the last few years nanotechnology is the most promising field of science and technology, the potential NP impact on coastal environments is only partially investigated, since only few aspects of NP environmental fate and biological effects have been addressed. Moreover, it has to be considered that marine organisms are exposed to multiple stressors that have the potential to seriously and adversely affect marine ecosystem functioning. Despite this, the combined effects of both natural and anthropogenic stressors, are still poorly studied, also because of the complexity of the experimental approach .

This PhD thesis, is an attempt to give a more comprehensive view about the NP topic under a global change scenario, where new contaminants and variations in environmental parameters, together, can act as the major drivers. In order to reach this goal, a stepwise *in vivo* approach using a suite of biomarkers has been used on the marine clam *R. philippinarum*.

On the basis of results obtained in the present work, we can conclude that NPs induced remarkable biological responses in clams. Among the single NP tested, the $nTiO_2$ affected more severely clam responses in all tissues (haemolymph, gills and digestive gland) (Fig. 13).

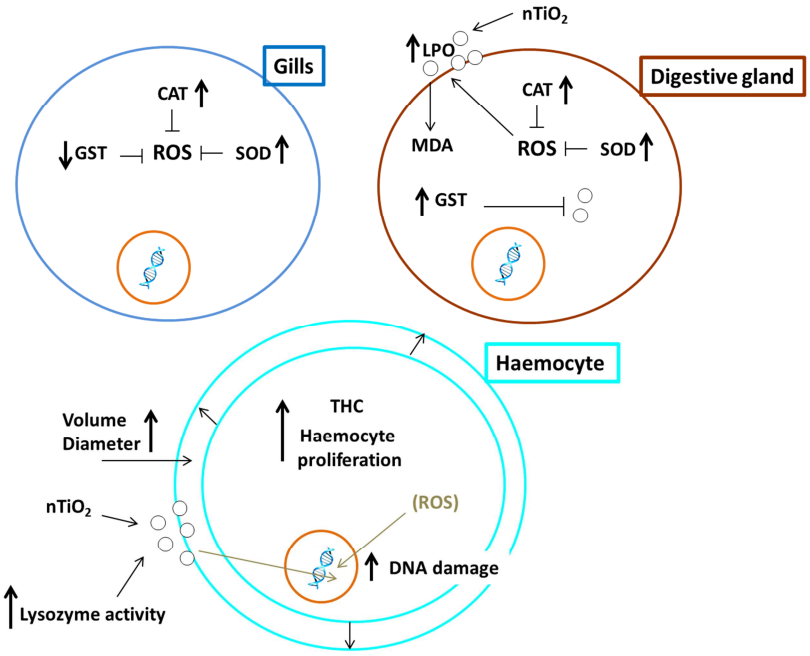

Fig. 13. General scheme summarizing the effects of $nTiO_2$ recorded in gills, digestive gland and haemolymph of *R. philippinarum*, as described in Marisa et al. (paper III).

However, it is important to stress that the three NPs acted in different way based on the analysed tissues, the biomarker measured and the concentration tested. In figure 14, the major effects of nZnO and FC_{60} are illustrated. Based on these findings, not only the digestive gland and haemolymph could be considered as

target tissues of NP action, but also the gills. This information is novel in the NP literature. Moreover, the results obtained allowed us to draft a toxicity scale of the selected NPs ($nZnO>FC_{60}>nTiO_2$), highlighting a potential risk for clams at concentrations similar to PECs.

The NP size and high surface area are key factors in defining uptake and thus induction of biological responses (e.g. oxidative stress). For example, small gold NPs induce greater oxidative stress than larger ones in *M. edulis* after 24 h exposure (Tedesco et al., 2008 and 2010). Thanks to the characterization analysis, the $nTiO_2$ were found to be the smallest NPs tested. This condition could explain the major toxicity of this $nTiO_2$ compared to the other two NPs.

Oxidative stress induced by NPs in bivalves depends on the size, composition and concentration, mode and time of exposure, bivalve species and target organ analysed. However, our results make possible to affirm that the oxidative stress is the main mechanism of NP action in the tissues of clams. Despite the oxidative damage showed was low, it was consistent with the low concentrations of contaminants and the short *in vivo* exposure.

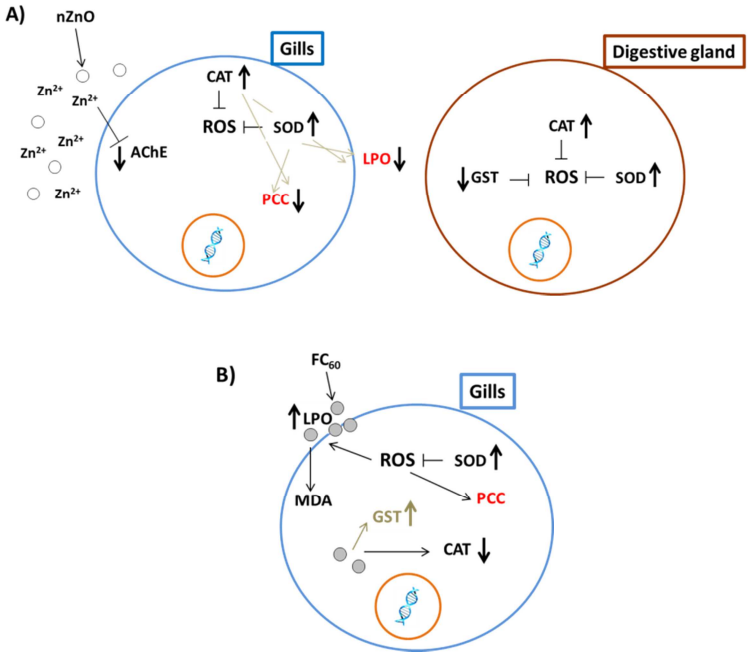

Fig. 14. General scheme summarizing the effects of nZnO (A) and FC_{60} (B) recorded in tissues of *R. philippinarum*, as described in Marisa et al. (paper I and IV, respectively).

The exposure to NP mixture represents a novel approach that can provide better insight into the NP impacts under environmental conditions. The observed additive action of the three NPs could open a new research to understand better the various mechanisms on NP toxicity. The combined NP effects indicated that the digestive gland was the tissue more affected by NP mixture toxicity (Fig. 15). Moreover, the oxidative stress was confirmed as the primary mechanism of action determined by the NP mixture. The redox proteomics applied in this experiment helps to better understand the oxidative damage action of the three NPs, and it

demonstrated to be a useful tool in ecotoxicology studies, also in *R. philippinarum* whose genome has not been fully sequenced.

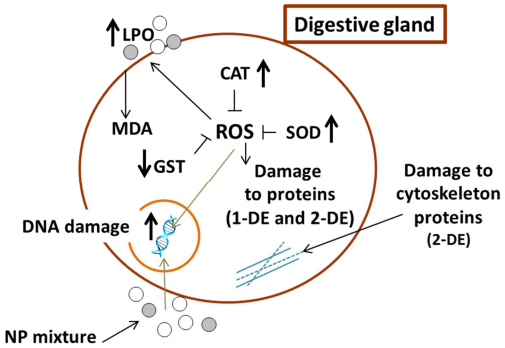

Fig. 15. General scheme summarizing the effects of NP mixture recorded in digestive gland of *R. philippinarum*, as described in Marisa et al. (paper V).

The study of interaction between environmental variables and NP exposure could be useful to define better potential risks for clam species in the environment. Salinity is one of the dominant environmental factors controlling species distribution, influencing physiological processes in marine organisms and changing the behaviour of NPs respect to other type of natural aqueous media. The exposure of *R. decussatus* and *R. philippinarum* to NP mixture under different salinity values confirmed the NP toxicity highlighted in the other studies in both species. Although the comparison between the two species did not show a clear and distinct pattern of response, slightly higher responses in *R. decussatus* than in *R. philippinarum* were highlighted. A modulation in the various biomarkers considered was mostly evident at low and high salinities.

Overall, all the results obtained in this PhD thesis can provide a new topic for discussion in NP toxicity studies, as well as in risk assessment of NPs as emerging environmental pollutants in marine coastal ecosystems.

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