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Corresponding Author: Dr. Laura Canesi, PhD

Corresponding Author's Institution: University of Genoa

First Author: Laura Canesi, PhD

Order of Authors: Laura Canesi, PhD; Ilaria Corsi

Abstract: The development of nanotechnology will inevitably lead to the release of consistent amounts of nanomaterials (NMs) and nanoparticles (NPs) into marine ecosystems, with potential adverse effects for marine biota. Up to date, ecotoxicological studies reported a wide variety of biological injuries in marine species belonging to different trophic levels, with the general aim of identifying potential biological targets of NPs, as well as suitable models for risk assessment and prediction of their impact on the health of the marine environment. From these data, different invertebrate species emerged as significant targets for the impact of NPs, with bivalves representing by large the most studied group, whereas less attention has been focused on sediment dwelling species. However, despite the development of fate models and analytical and biological tools for assessment of marine ecosafety of NPs, still major scientific gaps need to be filled. In this work, information on the factors affecting the behaviour of NPs released in the marine environment, and consequent uptake/accumulation/toxicity by representative invertebrate groups will be summarized. Available data on the effects of different NPs in marine invertebrates will be reviewed, in particular with regards to those reported on immune function, oxidative stress and development. Moreover, the possibility that the effects of NPs in marine invertebrates may be influenced by their interactions with biomolecules both in the external environment and within the body fluids, forming and eco-bio-corona, will be introduced. In the framework of the general need to classify NPs into groups and predict the implications of their release into the marine environment, information on their intrinsic properties is clearly insufficient, and a deeper understanding of NP-eco/bio-interactions is therefore urgently required.

Suggested Reviewers: Maria Joao Bebianno
University of Algarve, Campus de Gambelas, 8000 Faro, Portugal
mbebian@ualg.pt
Marine Ecotoxicology, effects of NPs in mussels

Jerome Labille

CEREGE, Centre de Recherche et d'Enseignement de Géosciences de l'Environnement
labille@cerege.fr
expert of interactions of nanomaterials with environmental media

Julian Blasco
Instituto de Ciencias Marinas de Andalucia (CSIC)
julian.blasco@icman.csic.es
Expert in responses of marine organisms to environmental stressors, including NPs

Marco Monopoli
CBNI, UCD
marco.monopoli@cbni.ucd.ie
expert of interactions of NPs with biological components (NP corona)

David Sheehan
University college Cork
d.sheehan@ucc.ie
expert on effects of NPs in marine invertebrates

Opposed Reviewers:

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Laura Canesi

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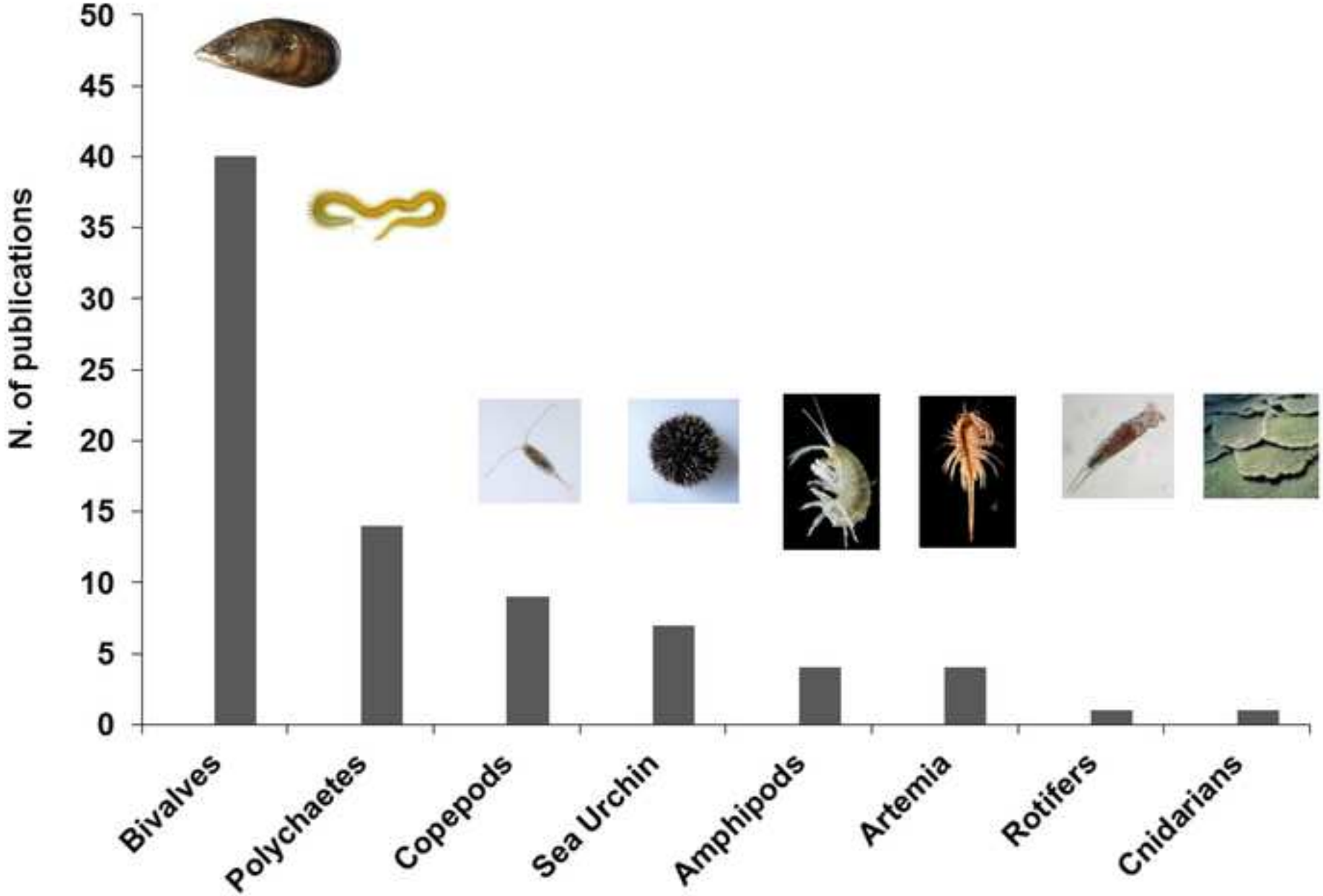

Highlights

- Marine invertebrates are significant targets for NPs released in the environment
- Most data on bivalves and echinoderms, much less in sediment dwelling species
- Main biological responses: immunomodulation, oxidative stress, embryotoxicity
- NPs interactions with environmental/biological components affect their fate/toxicity
- More information on eco/bio-interactions of NPs in the marine environment is needed

Effects of nanomaterials in marine invertebrates

Laura Canesi[a*] and Ilaria Corsi[b]

[a] *Department of Earth, Environment and Life Sciences (DISTAV), University of Genoa, Italy*

[b] *Department of Physical, Earth and Environmental Sciences (DSFTA), University of Siena, Italy*

*Corresponding Author

Laura Canesi

DISTAV, University of Genoa

Corpo Europa 26

16132 Genoa, Italy.

Laura.Canesi@unige.it

Abstract

The development of nanotechnology will inevitably lead to the release of consistent amounts of nanomaterials (NMs) and nanoparticles (NPs) into marine ecosystems, with potential adverse effects for marine biota. Up to date, ecotoxicological studies reported a wide variety of biological injuries in marine species belonging to different trophic levels, with the general aim of identifying potential biological targets of NPs, as well as suitable models for risk assessment and prediction of their impact on the health of the marine environment. From these data, different invertebrate species emerged as significant targets for the impact of NPs, with bivalves representing by large the most studied group, whereas less attention has been focused on sediment-dwelling invertebrates (benthic infauna). However, despite the development of fate models and analytical and biological tools for assessment of marine ecosafety of NPs, still major scientific gaps need to be filled. In this work, factors affecting NP fate in the marine environment, and consequent uptake/accumulation/toxicity in representative invertebrate groups will be summarized. Available data on the effects of different NPs in marine invertebrates will be reviewed, with particular regard to those reported on immune function, oxidative stress and embryo development. Moreover, the possibility that such effects may be influenced by NP interactions with biomolecules both in the external environment and within the body fluids, forming *eco-bio-coronas*, will be introduced. In the framework of the general need to classify NPs into groups and predict the implications of their release into the marine environment, information on their intrinsic properties is clearly insufficient, and a deeper understanding of NP eco/bio-interactions is therefore urgently required.

1. Introduction

Recent environmental fate models underline that *nanowastes* will end up in the aquatic environment, thus potentially affecting natural ecosystems and human health (Bystrzejewska-Piotrowska et al., 2009; Brar et al., 2010; Liu et al., 2014). Marine ecosystems, historically seen as a major sink of anthropogenic contaminants, will receive consistent amounts of nanomaterials (NMs) and nanoparticles (NPs), whose intrinsic and extrinsic complexity are making the prediction and assessment of their fate, exposure and biological effects a major challenge (Klaine et al, 2008). As recently discussed in our overview on fate models and tools (analytical and biological) for *ecosafety* assessment and design of nanomaterials entering the marine environment (Corsi et al., 2014), still major scientific gaps need to be filled. The development for instance of quantitative approaches for integrating more realistic NP exposure scenarios and predicting ecosystem impacts are urgently needed for a proper risk assessment (Gottschalk et al., 2013). Up to date, ecotoxicological studies focusing on marine organisms reported a wide variety of biological injuries in species belonging to different trophic levels, from planktonic to benthic species, including fish, with the general aim to identify potential biological targets as well as suitable models for both risk assessment and prediction of marine environmental implications (Minetto et al., 2014; Baker et al., 2014; Matranga and Corsi, 2012). In order to achieve this goal, basic information on the factors affecting the behaviour of NPs released in the marine environment, and consequent uptake/accumulation/toxicity by representative species must be taken into account. The importance of evaluating the potential impact of NP exposure on aquatic invertebrates has been underlined (Baun et al., 2008; Canesi et al., 2012; Baker et al., 2014; Corsi et al., 2014; Matranga and Corsi, 2014; Canesi et al., 2015; Rocha et al., 2015). The first studies were mainly carried out in freshwater species utilizing standard ecotoxicity tests, with less than 20% of published papers on marine species (Cattaneo et al., 2009). However, the number of publications on NP ecotoxicity in invertebrates has exponentially grown in the last few years, with a strong contribution of those

focused on NP accumulation, sub-lethal effects and mechanisms of action. Among these, the percentage of studies on marine species has risen considerably (up to 38% of the total) (Fig. 1A). Bivalve molluscs are so far the most studied marine invertebrate group (50%), whereas less attention has been focused on sediment-dwelling invertebrate (benthic infauna) (about 20%) (Fig. 1B).

Mytilus spp. represents the most utilized bivalve model: special emphasis has been given to the effects of different types of NPs on the immune system and on the main tissues involved in NP uptake and accumulation, i.e. the gills and the hepatopancreas (reviewed in Canesi et al., 2012, 2015; Rocha et al., 2015). Scattered information is available on other bivalve species and marine invertebrate groups (echinoderms, anellids). In this work, those aspects related to the effects of NPs on immune function and oxidative stress in different invertebrate species will be analyzed in more detail. Moreover, whether such effects may be influenced by NP interactions with biomolecules both in the external environment and within the body fluids, forming *eco-bio-coronas*, will be introduced. Finally, available data on the effects of NPs on development of marine invertebrate species will be summarized.

2. Behavior of different particles in SW

Environmental implications of NPs are strongly linked to their peculiar features such as particle surface charge, size, shape, functionalization and coating, all properties that affect their interaction with the surrounding media and the resulting fate and toxicity (Klaine et al., 2008). For instance, dispersion and aggregation or agglomeration in aqueous solutions are driven by size and surface charges of the NPs, but also by several other parameters of the receiving media such as pH, ionic strength (osmolarity) and presence of natural organic matter (NOM) (Corsi et al., 2014; Keller et al., 2010; Petosa et al., 2010). Natural environmental scenarios are currently barely represented in nanoecotoxicological studies, this being probably mainly related to substantial lack of information on interactions between specific NPs and the exposure media as, for instance, natural sea water

(NSW) *vs* artificial sea water (ASW). The high ionic strength which also affect pH, as well as organic matter content, have been reported to play a significant role on NP aggregation and thus affecting their fate in the water column and sediment (Chen et al., 2011). In our recent work with marine mussels *Mytilus galloprovincialis*, co-exposure to titanium dioxide NPs (nano-TiO_2) and $CdCl_2$ in ASW resulted in reduced Cd toxicity and increases Ti accumulation in selected tissues (i.e. gills) (Della Torre et al., 2015). On the contrary, in the digestive gland such effect was not observed, suggesting that the fate of NPs within the organism may be also related to interactions with biomolecules which affect NP state and effects. Bioavailability, as well as biodistribution and consequent biological responses, are highly dependent on exposure mechanisms, but also on fate of NPs inside the body of the organism itself. Further studies on exposure assessment and fate into the aquatic environment, as well as into the internal environment of exposed organisms are strongly needed.

Both heteroaggregation and homoaggregation are driven by NP surface charges and the chemical composition of the surrounding media, due to the presence of elevated concentrations of inorganic ions as well as organic molecules such as polysaccharides, proteins and colloids (Praetorius et al., 2014; Zhou et al., 2013). Moreover, the presence of functional groups on the surface of NPs, more than their core composition (metal-based, carbon-based, etc) is now being considered to play a major role in sorption to biofilms in high ionic strength media such as in NSW. Single NPs may barely adsorb NOM, while "bridging effects" due to colloids interacting with multiple particles can increase aggregation. For instance, colloidal polymers produced by algae known as exopolymeric substances (EPS) can influence aggregation and transformation of NPs in aqueous media, mainly as a function on the hydrophobic and electrostatic interactions between NPs and EPS (Nevius et al., 2012; Therezien et al., 2014; Adeleye et al., 2014a,b; Kadar et al., 2014).

Interactions of NPs with biomolecules have been described within biological fluids of mammalian models, where plasma proteins originate a coating known as protein *corona*, which affect cellular uptake (biokinetics) and ultimate toxicity (Lundqvist et al., 2011; Monopoli et al.,

2011; Salvati et al., 2011). The formation of a NP corona has been recently reported also in terrestrial and freshwater invertebrate species, i.e. secreted proteins from the coelomic fluid of the rugworm *Eisena fetida* and from *Daphnia magna* neonates (Hayashi et al., 2015; Nasser and Linch, 2015), and may also occur in marine invertebrates (see below).

Whether these processes could take place also in the external environment, for example in NSW as consequence of interactions with NOM, as well as within biological fluids of marine organisms, deserves further investigation. NP extrinsic properties related to the formation of an *eco-corona* or bio (protein) *corona* will affect consistently their ultimate fate in the water column and therefore biological activity and toxicity (Fig. 2). NP surface charge and size, irrespective to core composition, have been reported to play a significant role in cellular adhesion, particle recognition as well as efficiency of uptake. We recently showed that polystyrene NP (PS NPs) having cationic ($-NH_2$) or anionic (-COOH) surface groups undergo different aggregation in NSW affecting their biodistribution and ultimate toxicity both in larvae of brine shrimp *Artemia franciscana*, as well as sea urchin embryos *Paracentrotus lividus* (Della Torre et al., 2014; Bergami et al., 2015). Anionic PS NPs undergo significant aggregation in NSW and retention in the gut lumen of exposed organisms, while cationic PS NPs showed far less aggregation in NSW but clear signs of toxicity.

Several mechanisms can be involved in NP uptake but how they are dispersed in exposure media may result in the absence or presence of a eco/bio-*corona*, like it may occur in artificial *vs* natural sea water or in different body fluids (Salvati et al., 2011; Liu et al., 2011; Rossi et al., 2014). Moreover, in marine coastal areas significant changes in NOM and ion concentrations are influenced by the season and river flows, which might consistently affect NP interactions and ultimate fate towards both planktonic and benthic invertebrates (Corsi et al., 2014).

In the framework of a general need to classify NPs into groups and predict environmental implications once they are released into the marine environment, information on their intrinsic properties is clearly insufficient, and a deeper understanding of eco/bio-interactions is therefore

urgently needed. Moreover, ecotoxicity studies based on realistic environmental scenarios could be valuable tools for both screening and categorizing NPs for risk assessment purposes.

3. Uptake and accumulation

NPs surface charges (positive or negative), size and shape, irrespective to core composition, have been recently showed to drive cellular adhesion, particle recognition but more important uptake efficiency (Lunov et al., 2011; Varela et al., 2012). Moreover, surface charge, more than size, has an important role on cellular uptake in human cells, as for instance for polymeric NPs, which are consequently more efficiently internalized (Bramini, 2014; Lesniak et al., 2010; Liu et al., 2011; Lunov, 2011; Rossi et al., 2014). Cationic polystyrene NPs (PS-NH_2) have been shown to be cytotoxic for mammalian cells *in vitro*, while anionic PS NPs (PS-COOH) exhibited greater and rapid accumulation in selected organs such as lung (Bexiga et al., 2011; Wang et al., 2013a,b). Recent findings on sea urchin embryos and brine shrimp larvae confirmed such behavior; a significant gut retention is observed for anionic PS-COOH NPs while cationic PS-NH_2 NPs showed respectively developmental toxicity and increase of molts (Della Torre et al., 2014; Bergami et al., 2015). More contributions on other marine invertebrate species confirm a significant accumulation of anionic PS NPs in the gut of rotifers (Snell and Hicks, 2011) and in bivalves (Ward and Kach, 2009; Wegner et al., 2012). Based on our past findings, nanoscale aggregates of PS-NH_2 (~ 100 nm) are still present in NSW media while PS-COOH NPs originated microscale aggregates (>900 nm). The formation of aggregates as a consequence of interaction with dispersing media has a strong influence on their route of uptake and biodistribution. Bioaccumulation seems in general not affecting mortality, but it has been associated with several sub-lethal effects (behavioral, physiological and biochemical) able to affect species survival in prolonged exposure scenarios. Accumulation of NP aggregates in the digestive tract, as observed for PS-COOH NPs, can significantly limit food intake and disrupt growth and development of planktonic invertebrate larvae

(Bergami et al., 2015; Besseling et al., 2014). In addition, the adsorption of hydrophobic aquatic pollutants by nano-debris may increase their bioavailability and then cellular uptake once accumulated in the gut (Rochman et al., 2013; Velzeboer et al., 2014). As for inorganic NPs, a general mechanism of uptake of aggregates by endocytosis in the digestive system and individual NPs or ionic forms in the gills, respectively, has been hypothesized, despite some recent evidence of an higher accumulation of nano- compared to micro-metric sized ones has been reported for soluble metals as gold and copper (both ions and NPs) (Canesi et al., 2010; Gomes et al., 2011, 2012; Dai et al., 2013). Therefore, the ability to act as carriers of toxic contaminants seems affected by their dispersion in exposure media and linked also to absence or presence of NP-eco/biomolecular *coronas* (Monopoli et al., 2011; Lesniak et al., 2013; Salvati et al., 2011). Moreover, how both type and fate of the corona (hard and soft corona) will affect uptake and bioaccumulation, as reported in mammalian systems, still represents a major challenge and deserve further investigation in both adults and larval/embryo stages of marine invertebrate species.

In bivalves for instance mucus, fecal pellets and gametes can have a significant role in bioavailability of the NPs but also on uptake by different organs (i.e. gills or digestive gland) (Della Torre et al., 2015; Rocha et al., 2015). At the level of the cell membrane, ATP-binding cassette transporters belonging to the ABC family have been shown to have a significant a role in NP biodistribution. As recently reported in Caco-2 gut epithelial cells, nano-TiO_2 (anatase) significantly increases the functionality of the cells, then forming a protective barrier against exogenous toxicants and on nutrient absorption (Dorier et al., 2015). Our recent studies on mussels exposed to nano-TiO_2 showed similar results with a significant increase in efflux of RhB, a P-glycoprotein substrate, from the gills, suggesting that this protective role could be also shared by selected barrier organs as gills in marine invertebrates (Canesi et al., 2014; Della Torre et al., 2015). Further studies are thus needed in order to clarify the role of efflux activity of ABC transporters in marine invertebrates upon exposure to selected NP and therefore on their toxicity.

4. Effects and mode of action of NPs in marine invertebrate models

4.1 Effects on immune function

Increasing evidence supports the hypothesis that in marine invertebrates the immune system represents a significant target for the effects of NPss. Invertebrates lack an adaptive immunity: however, they are endowed with a potent and complex innate immune system (humoral and cellular defenses) similar to that of vertebrates. In most invertebrates, free circulating cells (hemocytes/coelomocytes) are responsible for cell-mediated immunity; when activated, different hemocyte types are capable of phagocytosis, Reactive Oxygen Species (ROS) and NO production, release of hydrolytic enzymes and antimicrobial peptides. Conservation of the general mechanisms of innate immunity from invertebrates to mammals is a key feature that represents an useful basis for evaluating the environmental impact of NPs (Canesi and Procházková, 2013).

In *M. galloprovincialis, in vitro* studies showed that different NP types (carbon black, fullerenes, and different nanosized metal-oxides) are rapidly taken up by mussel hemocytes, affecting different functional parameters, from lysosomal function to phagocytic activity and oxyradical production, and also inducing pro-apoptotic processes; the effects were mediated by stress-activated mitogen activated protein kinase (MAPK) signaling, as in mammalian phagocytes, and were observed in the low μg/ml range (Canesi et al., 2012; Canesi and Procházková, 2013). Recent studies also showed that amino-modified PS NPs (PS-NH_2) induced significant activation of immune parameters at lower concentrations (1-5 μg/ml), whereas lysosomal damage and apoptotic process were observed at higher concentrations (50 μg/ml) (Canesi et al., 2015). Different NPs were shown to target the phagocytic activity also in the hemocytes of the oyster *Crassostrea gigas* (Abbott Chalew et al., 2012) and of the clam *Ruditapes decussatus* (Marisa et al., 2015). Interestingly, induction of functional responses, as well as of cellular damage and apoptotic

processes, were particularly rapid, occurring within 1 h of exposure. This is in line with the physiological role of bivalve hemocytes, that represent the first line of defence against non-self material (Canesi et al., 2002; Canesi and Procházková, 2013).

Another *in vitro* approach has been successfully utilized to evaluate NP-mediated cytoxicity in *Mytilus* hemocytes after prolonged exposure (24 h) to different types of NPs (CdS quantum dots, nanosized Au and Ag NPs, n-ZnO, n-SiO_2, n-TiO_2) in a wide concentration range; the effects were compared with those observed in isolated gill cells (Katsumiti et al., 2014, 2015a,b,c). These studies underlined different cytotoxic effects in terms cell viability (EC_{50}), and parameters related to oxidative stress, genotoxicity, cytoskeletal alterations, and membrane transport activities. Moreover, various degrees of toxicities were observed in hemocytes and gill cells that were related to the NP mode of synthesis, crystalline structure and size, presence of additives in the suspensions, metal ion release in high ionic strength medium, etc.

Overall, the application of a battery of functional/toxicity tests on bivalve hemocytes proved as a powerful tool for the screening of the immunomodulatory/immunotoxic effects of different types of NPs in cell models of marine organisms, as well as a robust alternative method for testing the toxicity of NPs and a possible basis for a 'safety by design' approach (reviewed in Canesi et al., 2012; Canesi and Procházková, 2013).

The results obtained *in vitro* on mussel immune cells were confirmed by *in vivo* exposure to different NPs, and in particular to n-TiO_2 chosen as a model NP type (Canesi et al., 2010b; Barmo et al., 2013). These data allowed formulating an hypothesis on the possible pathways leading to nano-induced immunomodulation (Canesi and Procházková, 2013; Corsi et al., 2014). Due to the physiological mechanisms involved in the feeding process, n-TiO_2 agglomerates/aggregates formed in sea water are taken up by the gills and partly directed to the digestive gland. NPs can be then potentially translocated from the digestive system to the hemolymph, and to circulating hemocytes, where n-TiO_2 induced changes in functional parameters, as well as in transcription of different antimicrobial peptides and downregulation of Toll-like receptors (Barmo et al., 2013; Balbi et al.,

2014). Interestingly, these effects were observed at concentrations much lower than those usually utilized in ecotoxicity tests on aquatic species (1-100 μg/L), and closer to predicted environmental concentrations (PEC) (Gottschalk et al., 2013).

Sea urchin immune cells represent another suitable model for testing the effects of NPs on the immune system of marine invertebrates . Well established as sensors of environmental hazards, coelomocytes of the mediterranean species *Paracentrotus lividus* have been recently showed to activate/express specific cell stress proteins upon n-TiO_2 exposure both in *in vitro* and *ex vivo* (Pinsino and Matranga, 2015; Pinsino et al., 2015). Moreover, *in vivo* exposure elicited a phagocytic mechanism, involving the TLR/p38 MAPK signaling pathway (Pinsino et al., 2015). This species has been used to study the *in vivo* potential toxicity of a few selected metal oxide NPs (i. e. SnO_2, CeO_2, Fe_3O_4), introduced by forced ingestion (Falugi et al., 2012). After 5 days, nano-aggregates/agglomerates were found inside the sea urchin immune cells (coelomocytes), causing modifications of the trans-Golgi and ER subcellular compartments, inhibiting the activity of the three ChE isoforms and affecting the expression levels of the stress proteins HSC70 and GRP78 (Falugi et al., 2012).. In contrast to what expected by analogy with bivalve molluscs, injection of n-TiO_2 into the coelomic cavity did not cause a stress response in the sea urchin, nor immune cell death or autophagy and lysosomal dysfunction (Pinsino et al., 2015).

Phenoloxidase PO and lysozyme activities were induced by AgNP aggregates in the clam *Scrobicularia plana* and in the polychaete *Nereis (Hediste) diversicolor*, respectively (Mouneyrac et al., 2014). In the coelomocytes of *H. diversicolor*, AgNPs induced lysosomal and DNA damage (Cong et al., 2014). These results indicate that NPs may affect the function of immune cells also in marine worms.

The availability of the sea urchin genome, shown to be in close phylogenetic relationship to the humans (Sea Urchin Genome Sequencing Consortium, 2006), offers the possibility to analyze its complex and sophisticated immune system and compare the biological effects observed in sea urchins and human phagocytes. Although the *Mytilus* genome is not available yet, the rapidly

expanding application of DNA microarrays and Next Generation Sequencing (NGS) is leading to the identification of an increasing number of immune-related genes that could represent the target for different NPs also in mussels (Phillip et al., 2012; Gerdol and Venier, 2015). In this light, both the mussel and the sea urchin represent promising models for evaluating the effects of NPs on the immune system of marine invertebrates.

4.2 Effects of NPs at tissue level

Oxidative stress and cell injury in proteins, membrane and DNA damage have been so far identified as the major modes of action of NPs in bivalve tissues (reviewed in Rocha et al., 2015). However, the paradigm proposed to explain most of the adverse effects exerted by NP exposure in mussels, that is all the effects are directly or indirectly mediated by reactive oxygen species (ROS) and free radical production, cannot be applied to all NP types. Moreover, oxidative damage induced by NPs in bivalves depends on particle size, composition and concentration, mode and time of exposure, as well as the target organ analyzed.

In *Mytilus* gills, short-term exposure (24 h) to both carbon based NPs (Nano carbon black-nNCB, C60 fullerene), and n-oxides (n-TiO_2 and n-SiO_2) (0.05, 0.2, 1, 5 mg/L) induced changes in catalase and GST activities, with both increases and decreases depending on NP type and concentration; however, antioxidant enzymes did not seem to represent a significant target for these types of NPs (Canesi et al., 2010b). Longer exposure to n-TiO_2 (0.1 mg/L, 96 h) increased P-gp efflux activity and NO production; this latter effect may be mainly due to infiltrating hemocytes, since no changes in antioxidant enzyme activities were observed (Della Torre et al., 2014). Although the gills of *C. gigas* and *M. galloprovincialis* are susceptible to oxidative stress induced by ZnO and CuO NPs, this was not the case with other metal-based NPs (reviewed in Rocha et al., 2015). Gene transcription profiles were not significantly altered in the gills of *M. galloprovincialis*

exposed to Ag NPs after 15 days, although changes in expression may occur at shorter times of exposure (Bebianno et al., 2015).

As described above, NP behavior (aggregation/agglomeration) in both ASW and NSW is a key factor in their accumulation and effects in different tissues. Although mussel gills are the first organ/barrier to surrounding water, being more vulnerable to particle interactions, uptake of individual NPs s or ionic metal forms seems to mainly occur in this tissue, while NP agglomerates may enter in the digestive system of the organism mainly by endocytosis (Corsi et al. 2014; Rocha et al., 2015).

In bivalves, the digestive gland is therefore considered the main organ for accumulation of NPs, where their cellular fate and effect differ according to the NP type and experimental conditions. The endosomal-lysosomal system of digestive cells, due to its role in intracellular digestion of food particles, represents the main subcellular target for NPs in bivalves (Moore, 2006; Canesi et al., 2012). In *Mytilus* digestive gland, acute exposure (24 h) to NP suspensions (NCB, C60, n-TiO_2, n-SiO_2) induced lysosomal membrane destabilization, decreases in antioxidant defenses and lipofuscin accumulation, indicating oxidative stress conditions only at high concentrations (> 1 mg/L) (Canesi et al., 2010). At lower concentrations (1-100 μg/ L) and longer times of exposure (96 h), tissue antioxidant defenses were apparently able to cope with potential oxidative stress conditions induced by n-TiO_2 (Barmo et al., 2013). In whole tissues of the endobenthic bivalve *S. plana*, the response of oxidative stress biomarkers was more important after dietary than waterborne exposure to AgNPs; however, defenses were relatively efficient since no biomarkers of damage were observed. Moreover, labile Ag released from AgNPs was mainly responsible for the observed effects (Buffet et al., 2012). Mesocosm exposure to low concentrations of n-CuO resulted in induction of oxidative stress biomarkers but not in lipid peroxidation in both *S. plana* and *H. diversicolor* (Buffet et al., 2013). In *R. decussatus* digestive gland, exposure to predicted environmental concentrations (0.75 μg/L) of AuNPs induced oxidative stress and inflammatory response markers, as measured by phase II antioxidant enzymes and q-PCR gene expression

analysis; however, the overall magnitude of responses was low, and oxidative damage was not observed (Volland et al., 2015).

Application of proteomics in mussel tissues revealed oxidative changes in response to different metal-based NPs (reviewed in Rocha et al., 2015). In particular, tissue redox proteomics indicated that NP size is a key factor in determining biological responses in mussels exposed to AuNPs (Tedesco et al., 2010).

Overall, the results suggest that, although the mechanisms of action of NPs in marine invertebrates can be ascribed to increased radical production/changes in antioxidant defences, exposure to different types of NPs, at concentrations compatible with predicted environmental levels, does not seem to elicit significant oxidative stress at the tissue level. Oxidative stress conditions observed with mostly metal-based NPs (namely containing Ag, Cu, Zn, Au, Fe) could be mainly related to dissolution of free metal ions from the particles (Baker et al., 2014).

4.3 NP interactions with biological fluids: the protein corona

As described above, NPs undergo considerable environmental transformations before reaching the target biological system: however, the evaluation of the biological effects of NPs requires additional understanding of how, once within the organism, NPs interact at the molecular level with cells in a physiological environment.

In mammalian systems, extracellular serum proteins adsorb onto the NP surface, forming a protein *corona* which affects particle interactions with target cells (Lundvquist et al., 2008; Fleischer and Payne, 2014 and refs. quoted therein). The *corona* proteins control the specific cellular receptors used by protein-NP complex, the cellular internalization pathways, and the immune response. Cells recognize the biomolecular *corona* around a NP, but the biological identity of the complex may be considerably different among mammalian species.

In this light, there is the intriguing possibility that NPs may interact with extracellular medium or plasma proteins in lower organisms as marine invertebrates. The formation of a NP protein

corona has been described so far only in two invertebrate species. In the earthworm *Eisenia foetida*, soluble coelomic proteins (EfCP) secreted by coelomocytes, form a long-lived *corona* around AgNPs (Hayashi et al., 2013). Incubation of AgNPs with coelomic proteins lead to formation over time of AgNP-EfCP corona complexes that induced significantly greater NP accumulation in coelomocytes. The effects were species specific and NP-specific. In *D. magna* neonates, proteins secreted in the growing medium formed a coating around PS NPs; such an eco-bio-*corona* lead to particle agglomeration and increase in uptake and toxicity (Nasser and Linch, 2015).

Data on formation of a NP-protein *corona* in marine invertebrates are still lacking: however, in *Mytilus* hemocytes exposed to PS-NH_2 in the presence of hemolymph serum, an increase in lysosomal membrane damage was observed in comparison with only sea water as incubation medium (Canesi et al., 2015). Such an effect seems driven by specific type of NP, since it was not observed for instance in n-TiO_2 exposed hemocytes (Canesi, data not shown), suggesting that a NP-protein *corona* might also be formed in biological fluids of marine invertebrates that may affect NP-cell interactions.

In general, the presence of proteins reduces NP surface energy by nonspecific adsorption, leading to lowered membrane adhesion and uptake efficiency (Lesniak et al., 2013). Therefore, formation of the protein *corona* in mammalian serum is considered as a general protective effect from the potential cytotoxicity of NPs. In contrast, in earthworms, it was hypothesized that long-lived proteins present on the NPs (hard *corona*) function as recognizable molecular patterns for coelomocytes, making the NP-protein complexes "visible" for clearance by these phagocytic cells responsible for innate immunity (Hayashi et al., 2013). Moreover, the results obtained in *D. magna*, a filter feeder that can selectively take up food particles based on size, texture and consistency, suggested that the larger size of NPs caused by the destabilization of the proteins in the eco-*corona* could make the NPs a more attractive size food source and therefore cause *D. magna* to take up protein-coated NPs more readily than bare and monodisperse NPs (Nasser and Linch, 2015). The results obtained in the hemocytes of marine mussels in the presence of hemolymph serum, although

preliminary, seem to support this hypothesis. In this light, research is in progress on NP-*corona* proteins in mussels as suitable model marine invertebrates.

Overall, these data point out the determinant role of the recognizable biological identity during *in vitro* testing of NPs with invertebrate immune cells; however, the results obtained so far suggest that in invertebrate species the formation of a protein *corona* may increase the potential for NP toxicity, rather than play a protective role as in mammalian systems However, the protein composition of extracellular fluids of marine invertebrates is largely unknown, given the large diversity of phyla and species, and different proteins may be involved in the formation of a stable corona around different NPs in different invertebrate groups. In earthworms, lysenin was identified as the major corona protein for AgNP (Hayashi et al., 2013), and different secreted proteins related to cell-to-cell signalling were identified in *D. magna* (Nasser and Linch, 2015). In *Mytilus*, soluble hemolymph proteins have been recently characterized by proteomics (Oliveri et al., 2104). Among these the major serum protein, Extrapallial Protein (EP) plays a crucial role in non-self recognition (Pezzati et al., 2015) and represents a possible candidate for NP corona formation.

Overall, the results obtained so far underline the need of understanding how the formation of a NP protein *corona* may affect the biological outcome of NP exposure in invertebrates in comparison to mammalian systems.

5. Effects on development

The application of early life stage toxicity tests, involving exposure during the most sensitive stages of the organism to environmental stress, would greatly help in the identification of those chemicals that represent a major threat to marine species. This also applies to studies on the biological impact of NPs on marine invertebrates where, complimentary to the use of adult specimens, embryos have emerged as valid tools for studies on developmental perturbations induced by different types of NMs (metal based NPs and metal-oxides, carbon based NPs, nanoplastics etc.).

Echinoderms have been so far the most studied taxonomicgroup of marine invertebrates used for assessing NP developmental toxicity. In the white sea urchin *Lytechinus pictus* no toxicity of n-TiO_2 and n-SiO_2 was observed. In contrast, n-ZnO was highly toxic (EC50 = 99.5 μg/L). However, toxicity of n-ZnO was similar to that of soluble Zn^{2+}, and was shown to be related to particle dissolution in sea water (Fairbairn et al., 2011). In *P. lividus*, exposure to AgNPs caused dose-dependent developmental defects as well as alterations in swimming patterns: however, the cause-effect relationship was not demonstrated (Siller at al 2013). In *Strongylocentrotus droebachiensis*, Ag^{+}, AgNPs and f-SWCNTs, separately and in mixtures, induced different developmental defects in exposed embryos (Magesky and Pelletier, 2015).

Burić et al. (2015) showed that the effects of AgNPs on embryonal development in Mediterranean sea urchin species (*Arbacia lixula*, *Paracentrotus livi*dus and *Sphaerechinus granularis*) were species-specific. The most sensitive species was *A. lixula*, with high numbers of malformed embryos or arrested development observed at the lowest AgNP concentrations tested (1-10 μg/L) .Moreover, the greatest impact on development was noted for those embryos first exposed to NPs at 6 and 24 h post fertilization, this underlying how the timing of exposure to NPs may represent an important factor in the development of abnormalities.

In *P. lividus*, n-SiO_2 did not affect fertilization, whereas undeveloped and anomalous embryos were observed at the gastrula stage (EC_{50}=0.0 6 mg/L), and at the pluteus stage, including skeletal anomalies and delayed larvae (EC_{50}=0.27 mg/L) (Gambardella et al., 2015).

The first data on bivalve molluscs showed that in the oyster *Crassostrea virginica* C_{60} fullerene affected embryonic development and lysosomal membrane stability from concentrations as low as 10 μg/L (Ringwood et al., 2009). AgNP also induced lysosomal membrane destabilization and up-regulation of metallothionein (MT) gene expression; however, is was not established whether these effects were due to the NP themselves or to Ag^{2+} dissociation (Ringwood et al., 2010).

In *M. galloprovincialis*, n-TiO_2 did not affect embryo development unless at high mg/L concentrations (Libralato et al., 2013, Balbi et al., 2014). Similarly, embryos were unaffected by

Fe_2O_3 NPs (Kadar et al., 2010). On the other hand, sperm preincubation with stabilized (i.e., coated with organic polyacrylic stabilizer) and nonstabilized forms of zero-valent nanoiron (nZVI) caused serious disruption of development (30% mortality among spermatozoa, 20% decline in fertilization success, and delay in development) (Kadar et al., 2011). The effects of sperm pre-incubation with coated zero-valent iron (nZVI) were compared on early life stage development of three key marine invertebrate species: *M. galloprovincialis*, *Ciona intestinalis* and *Psammechinus milliaris*. Disruption of embryo development was most severe in sea squirts followed by mussels, while the urchin embryos were not significantly affected. Decreases in fertilisation success and delay in embryo development were observed, and the effect was more severe with the coated form, possibly owing to its better colloidal stability (Kadar et al., 2013). The effects of two coated AgNPs (oleic acid coated AgNPs and polyvinylpyrrolidone coated AgNPs) on marine benthic invertebrate larvae across three phyla (i.e., the barnacle *Balanus amphitrite*, the slipper-limpet *Crepidula onyx*, and the polychaete *Hydroides elegans*) were evaluated in terms of growth, development, and metamorphosis (Chan and Chu, 2015). Chronic exposure to coated AgNPs resulted in a significant delay in growth and development, and reduction of larval settlement rate, in a species-specific manner. Types of surface coatings did not affect the sub-lethal toxicity of AgNPs. However, uptake of coated AgNPs were observed in the vacuoles of epithelial cell in the digestive tract of *C. onyx*, suggesting that coated AgNPs could be taken up through diet and the toxicity of coated AgNPs might be mediated through toxic $Ag^{+,}$ as well as the novel modalities of coated AgNPs.

In the deposit feeder annelid *Platynereis dumerilii*, exposure to various concentrations of citrate (cit-Ag NPs) or humic acid (HA-Ag NPs) capped silver NPs (Ag NPs) induced abnormal development rate, that were higher in younger life stages. Both Ag NPs were more toxic than $AgNO_3$ (Garcia-Alonso et al., 2014).

Overall, the results obtained in different species and experimental conditions with metal based NPs indicate that, although the observed effects can be largely due to metal ion solubilisation in sea water, developmental toxicity may be also due to other mechanisms that are particle-specific.

Moreover, particle coating may contribute to toxicity, possibly owing to its better colloidal stability or differences in uptake routes in different types of embryos.

Although these studies demonstrated that, in marine invertebrates, embryo development can represent a significant target for different types of NP, in a concentration range of μg/L - low mg/L, information on the mechanisms underlying the observed developmental effects is still extremely scarce. In *P. lividus* SiO_2 NPs caused neurotoxic damage and a decrease in AChE expression in a non-dose-dependent manner (Gambardella et al., 2015). In the same species, amino modified polystyrene NPs (PS-NH_2) caused severe developmental defects at both 24 and 48 h pf , whereas carboxylated particles (PS-COOH) were ineffective. Distinct effects were observed on transcription of selected genes; the Abcb1 transporter was up-regulated at 48 h pf by PS-COOH whereas PS-NH_2 induced cas8 gene at 24 hpf, suggesting an apoptotic pathway (Della Torre et al., 2014). In the larvae of the brine shrimp *Artemia salina*, exposure to SnO_2, CeO_2 and Fe_3O_4 NPs (from 0.01 to 1.0 mg/mL) induced changes in swimming speed and alterations and cholinesterase, glutathione-S-transferase and catalase activities, with different effects depending on the NP type (Gambardella et al., 2015). Further studies are needed in order to identify the molecular and cellular targets involved in the sensitivity of marine invertebrate embryos and larvae to NP exposure.

Conclusions and perspectives

Different invertebrate species are now been recognized as significant biological targets for NPs. Available data show that laboratory exposure to different types of NPs, in a wide concentration range, elicit significant biological responses, from immunomodulation and oxidative stress to developmental defects. However, since the majority of studies have been so far obtained in species living in the water column, more information is needed in those species living in close contact with sediments, where NP settlement/deposition, and consequent concentrations, may be higher.

While recognizing the importance of the studies carried out so far, it is becoming clear that information on the physico-chemical properties of pristine NPs, or on their behavior in low ionic strength aqueous solutions, is insufficient to predict their behavior in sea water and consequent uptake/accumulation/effects in marine organisms. Future studies should address NP fate in complex environmental matrices, such as SW and sediments, as well as their interactions with biomolecules present in both environmental media and biological fluids. These eco-bio-interactions may play a crucial role in particle uptake and accumulation, as well as in their distribution, fate and effects within the organism. In the absence of experimental data on occurrence and concentrations of NPs in the marine environment, especially in coastal areas that represent the major sites of potential discharge, this information may be crucial in understanding whether the biological responses of marine invertebrates to NPs exposure observed in laboratory experiments may be utilized for risk assessment and prediction of their impact on the health of the marine environment.

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Figure Legends

Fig. 1 - Studies on the effects of NPs in aquatic invertebrates. A) Percentage of publications on freshwater and marine invertebrates with respect to total publications in aquatic invertebrates at 31/12/2008 and 31/12/2014 (source http://www.ncbi.nlm.nih.gov/pubmed); B) Number of publications on different marine invertebrate groups and genera at 31/12/2014.

Fig. 2 – Schematic representation of the possible interactions of NPs with different natural organic compounds in the external environment (left) and within the organisms (right), leading to the formation of a NP eco-corona and bio-corona, respectively, in the water column and sediment. A bio-corona can be formed from interactions of NPs with components of internal body fluids or with secreted bio-molecules.

Figure1

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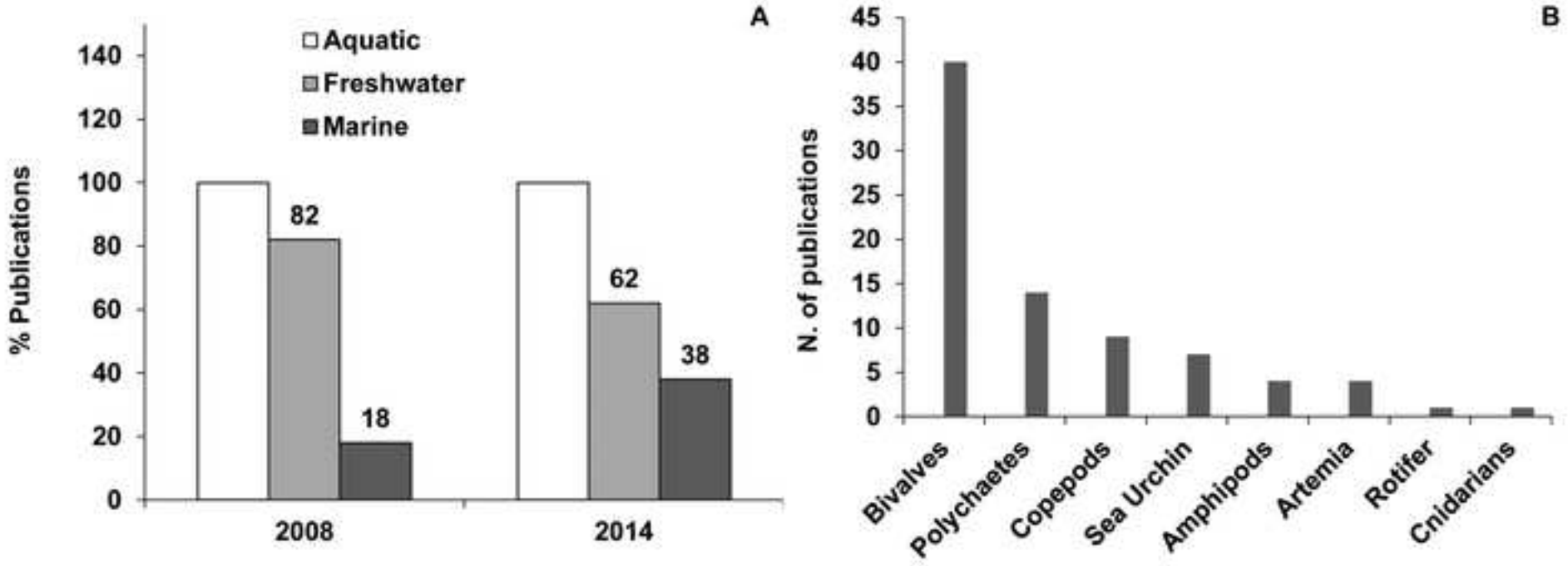

Figure2

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