Marine Environmental Research 111 (2015) 128–134

Contents lists available at ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

Interactive effects of nanoparticles with other contaminants in aquatic organisms: Friend or foe?

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ARTICLE INFO

Article history:
Received 9 January 2015
Received in revised form
25 March 2015
Accepted 29 March 2015
Available online 31 March 2015

Keywords:
Aquatic organisms
Nanoparticles
Contaminants
Interactive effects
Mytilus

ABSTRACT

The increasing production and use of nanoparticles (NPs) will lead to their release into the aquatic environment, posing a potential threat to the health of aquatic organisms. Both in the water phase and in the sediments NPs could mix and interact with other pollutants, such as organic xenobiotics and heavy metals, leading to possible changes in their bioavailability/bioconcentration/toxicity. However, whether these interactive effects may lead to increased harmful effects in marine organisms is largely unknown. In this work, available data mainly obtained on carbon based NPs and n-TiO_2, as examples of widespread NPs, in aquatic organisms are reviewed. Moreover, data are summarized on the interactive effects of n-TiO_2 with 2,3,7,8-TCDD and Cd^{2+}, chosen as examples of common and persistent organic and inorganic contaminants, respectively, in the model marine bivalve *Mytilus*. The results reveal complex and often unexpected interactive responses of NPs with other pollutants, depending on type of contaminant and the endpoint measured, as well as differences in bioaccumulation. The results are discussed in relation with data obtained in freshwater organisms. Overall, information available so far indicate that interactive effects of NPs with other contaminants do not necessarily lead to increased toxicity or harmful effects in aquatic organisms.

1. Introduction

The increasing production and usage in various fields of different types of manufactured nanomaterials, 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 compartment. This in particular applies to those types of nanoparticles (NPs) produced in higher amounts, such as metal-oxides (silver, titanium dioxide, zinc oxide, silica), and carbon-based NPs (fullerenes including single- and multi-walled carbon nanotubes), thus raising considerable concern on their environmental behavior (aggregation/agglomeration, redox reactions, dissolution, exchange of surface moieties, and reactions with biomacromolecules) and consequent impact on aquatic organisms (Delay and Frimmel, 2012; Matranga and Corsi, 2012; Gottschalk et al., 2013; Baker et al., 2014; Corsi et al., 2014).

Due to their extremely small size, NPs exhibit unique physicochemical properties such as high specific surface area and increased reactivity, which accounts for their widespread use in a number of industrial and biomedical applications. Among these, certain NPs are utilized for environmental remediation (wastewater treatment), due to the high adsorption capacity for metals and organic compounds (Sanchez et al., 2011). However, the inherent properties of NPs can represent a double-edged sword in an environmental context when released into the aquatic compartment. Both in the water phase and in the sediments, NPs will mix and interact with other components, not only dissolved and particulate organic matter, but also hydrophobic organic contaminants and inorganic ions, including heavy metals (Maurer-Jones at al., 2013; Baker et al., 2014; Corsi et al., 2014; Grillo et al., 2014). How these interactions may affect the biological impact of NPs *per se* or that of other pre-existing contaminants is largely unknown, and is therefore the subject of considerable debate.

Limbach et al. (2007) first postulated the '*Trojan horse*' effect, that implies facilitated entry of toxic molecules adsorbed to NPs into the cells. Baun et al. (2008) extended this concept in an environmental context, recommending that risk assessment of NPs should not only focus on their inherent toxicity, but also consider

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http://dx.doi.org/10.1016/j.marenvres.2015.03.010
0141-1136/

Table 1
Studies of co-exposure to carbon-based NPs and other contaminants in aquatic organisms.

Species/Time of exposure	Type of Particle/ Concentration	Type of contaminant/ Concentration	Target cell/ Tissue/Organism	Endpoint/Interactive effect	Contaminant accumulation	Reference
Freshwater						
C. vulgaris	**MWNCTs**	**Diuron**	whole organism			Schwab et al., 2013
3–6–15–24 h	10 mg/L	0.73–2990 μg/L		Increased toxicity (inhibition of photosynthetic activity) due to increased diuron bioavailability		
O. latipes	**SWNCTs**	**Phenanthrene**	digestive tract/tissues			Su et al., 2013
1–4–12–24–72 h	15 mg/L	60 μg/L			72 h: Facilitated the accumulation of Phe in the digestive tract and increased concentrations in liver and brain	
C. auratus	**OH-MWNCTs**	**Cd^{2+}**	liver			Qu et al., 2014
3-12 d	0.5 mg/L	0.1 mg/L		12 d: Inhibition of SOD, CAT, GPx activities and decrease in GSH level (synergistic effect); Increase in MDA content (3–12 d)	12 d: Increased Cd^{2+} concentration	
D. rerio	**C60 fullerene**	**As(III)**	hepatocytes			Azevedo Costa et al., 2012
4 h	1 mg/L	2.5 μM		Antagonistic effects on GSH and TBARS concentration	Increased As concentration	
		100 μM		Decreased intracellular ROS concentration and GST Ω activity		
D. rerio	**C60 fullerene**	**B[a]P**	hepatocytes			Ferreira et al., 2014
4 h	1 mg/L	0.01–0.1–1 μg/L		Lowered cell viability; Reduced intracellular ROS concentration		
		0.1–1 μg/L		Impaired Phase II detoxification response to B[a]P (GST activity)		
		1 μg/L			Increased accumulation of B[a]P	
D. magna	**C60 fullerene**	**Phenanthrene**	whole organism			Baun et al., 2008
P. subcapitata						
48 h	3 mg/L	49 μg/L			Faster Phe uptake	
	6 mg/L	μg/L range		Increased toxicity in *P. subcapitata*; Decreased toxicity in *D. magna*		
	C60 fullerene	**Pentachlorophenol**	whole organism			
	5–8 mg/L	μg/L range		Decreased toxicity (EC50) for both organisms		
	C60 fullerene	**Methyl Parathion**	whole organism			
	6–8 mg/L	μg/L range		No changes in toxicity		
	C60 fullerene	**Atrazine**	whole organism			
	10 mg/L	μg/L range		No statistically significant changes in toxicity in algae		
D. magna	**C60 fullerene**	**Fluoranthene**	whole organism			Yang et al., 2010
1 d	3 mg/L	5 μg/L		Significant decrease in toxicity (LT50 immobilization test)		
1 d	22 mg/L + UV	5 μg/L		Antagonistic effect on fluoranthene photo-induced toxicity		
D. rerio	**C60 fullerene**	**17α-ethinylestradiol**	whole organism/liver			Park et al., 2010
5 d	mg/L range	1 μg/L		Sorbption to C60 resulted in 100% reduced toxicity of the compound; Antagonistic effect on bioavailability of EE2 (reduced induction og vtg1A/B)		
Marine						
M. edulis	**C60 fullerene**	**Fluoranthene**				Al-Subiai et al., 2012
72 h	0.1 mg/L	32 μg/L	hemocyte	Additive effect on genotoxic biomarkers (Comet assay)		
			adductor muscle	Synergistic increase in Glutathione content		
			gills/digestive gland/ adductor muscle		Reduced accumulation of C60	

possible interactions with existing environmental contaminants, that may lead to changes in their bioavailability/bioconcentration/toxicity. This in turn may result in different types of interactive effects within organisms to induce a wide range of responses (i.e. additive, synergistic, or antagonistic) at different levels of biological organization. The biological effects of mixtures of pollutants are usually hard to predict. This is even more challenging when dealing with nanomaterials' peculiar properties, behavior in different media and possible interactions with other contaminants under realistic environmental conditions (Hartmann and Baun, 2010). However, in recent years, many attempts have been made in order to understand whether the predicted increase in NP discharge into the aquatic environment in the presence of other chemicals, including 'classical ' or other 'emerging' contaminants, may pose a further risk to ecosystem health. In this mini-review we will try to briefly summarize available data obtained in co-exposure experiments with different types of NPs and other contaminants, utilizing different experimental models of freshwater and marine organisms. In particular, data obtained in the marine invertebrate, the bivalve *Mytilus*, on the interactive effects of n-TiO_2, one of the most widespread NP type, with Cd^{2+} and 2,3,7,8-TCDD, chosen as models of common and persistent inorganic and organic contaminants, will be discussed.

2. Interactive effects of carbon-based NPs with other contaminants

Carbon nanotubes (CNTs) are NPs consisting of a variable number of graphene layers rolled into cylindrical tubes, that can be classified as single-or multi-walled carbon nano-tubes (SWCNTs or MWCNTs). CNTs exhibit a very strong affinity for organic contaminants, and due to their excellent sorption capacity they have potential applications in many areas, including wastewater treatment (Dervishi et al., 2009). Understanding the interactions between CNTs and organic contaminants is therefore essential for evaluating the potential environmental impact of these NPs when discharged into the aquatic compartment. Although CNTs seem to be relatively nontoxic for aquatic organisms, few studies investigated their interactive effects with other pollutants. In the green alga *Chlorella vulgaris* co-exposure with industrial MWCNT (10 mg/L) reduced the adverse effect of the pesticide diuron on photosynthetic activity; however, taking into account the measured dissolved instead of the nominal diuron concentration, the toxic effect of diuron was equal or stronger in the presence of MWCNTs, indicating that sorbed diuron remained partially bioavailable (Schwab et al., 2013). In the Japanese medaka *Oryzias latipes* whole-body accumulation and tissue distribution of phenanthrene were examined in the presence of SWCNTs (15 mg/L) and the potential release of phenanthrene was investigated (Su et al., 2013). The results showed that co-exposure with SWCNTs facilitated the accumulation of phenanthrene in the digestive tract, leading to increased concentrations in the liver and brain. In the goldfish *Carassius auratus* co-exposed to different combinations of Cd^{2+} and OH-MWCNTs (0.5 mg/L), the metal concentration in the liver was significantly higher than those in fish exposed to either single chemical after 12 days; moreover, the mixture induced severe oxidative stress conditions (Qu et al., 2014).

Other studies have focused on fullerenes. C60 is a nanomaterial produced worldwide with a unique cage-like molecular structure made solely of carbon. For this reason, chemico–physical interactions of C60 with several molecules have been widely investigated in search of potential industrial (Nakashima et al., 1998; Cho et al., 2005; Goyal et al., 2005) and medical (Partha et al., 2008; Pinteala et al., 2009; Ganji et al., 2010; Tarabukina et al., 2010; Santos et al., 2011) applications.

Henry et al. (2011) highlighted the potential environmental risk of fullerene exposure due to its capacity to act as a carrier for other contaminants, according to the *Trojan horse* concept. Evidence has been provided that co-exposure with fullerene can affect the uptake rate and toxicity of other environmental contaminants. *In vitro*, in zebrafish hepatocytes, C60 increased uptake of arsenic (AsIII) (Azevedo Costa et al., 2012) and of benzopyrene, decreasing cell viability and impairing Phase II detoxification response (glutathione transferase-GST) (Ferreira et al., 2014). *In vivo*, C60 enhanced the toxicity of phenanthrene to the microcrustacean *Daphnia magna* and to the algae *Pseudokirchneriella subcapitata* (Baun et al., 2008).

These data suggest that the effects of CNTs and fullerenes in

Table 2
Studies of co-exposure to n-TiO_2 and Cd^{2+} in aquatic organisms.

Species/Time of exposure	Type of particle/ Concentration	Type of contaminant/ Concentration	Target cell/ Tissue/Organism	Endpoint/Interactive effect	Contaminant accumulation	Reference
Freshwater						
C. carpio	**n-TiO_2**	**Cd^{2+}**				Zhang et al., 2007
25 d	10 mg/L	0.1 mg/L	visceral mass		Increased Cd accumulation	
B. rerio	**n-TiO_2 ± HA**	**Cd^{2+}**	whole fish, head, muscle, viscera			Hu et al., 2012
20 d	5 and 10 mg/L	50 μg/L			Increased Cd bioaccumulation; Reduced in the presence of humic acid	
D. magna *L. variegatus*	**n-TiO_2**	**Cd^{2+}**	whole organism			Hartmann et al., 2012
24 h and 48 h	2 mg/L	100 μg/L			Increased Cd accumulation in *D. magna* (24 h); No effect in *L. variegatus* (48 h)	
48 h and 96 h	2 mg/L	μg/L range		No increase in toxicity for both the organisms (immobilization test at 48 h in *D. magna* and at 96 h in *L. variegatus*)		
C. reinhardtii	**polyacrylate-coated n-TiO_2**	**Cd^{2+}**	whole organism			Yang et al., 2012
2 d	1–100 mg/L range	1 mg/L		TiO_2 reduced Cd toxicity	Reduced Cd accumulation	
C. fluminea	**n-TiO_2**	**Cd^{2+}**	soft tissues			Vale et al., 2014
3–10 d	0.1 and 1 mg/L	112 μg/L		Antioxidant biomarkers: increased CAT activity, no effects on SOD and GST	No effect on Cd accumulation	

freshwater organisms can be partly ascribed to potential adsorption and consequent increased accumulation of other contaminants, thus resulting in increased toxicity; however, this is not always the case. For example, C60 fullerene did not influence the toxicity of atrazine and methyl parathion in *P. subcapitata* and *D. magna* (Baun et al., 2008). In *D. magna*, C60 even protected against short-term UV and fluoranthene photo-induced toxicity (Yang et al., 2010). In adult male zebrafish, sorption of 17α-ethinylestradiol (EE_2) to C60 reduced bioavailability of EE_2 and resulted in 100% reduced toxicity of the compound, antagonizing the induction of Vitellogenin (Vtg) synthesis in the liver (Park et al., 2010).

In Table 1 studies on co-exposure to carbon-based NPs and other contaminants in aquatic organisms are summarized. It should be underlined that increased bioaccumulation and/or adverse effects in different experimental models were observed only for certain combinations of NPs and other contaminants (in terms of both concentrations and times of exposure). Moreover, most data were obtained at concentrations of CNTs or fullerenes ≥1 mg/L, that are many orders of magnitude higher than the predicted environmental concentrations (PECs) for these types of NPs in surface waters (few ng/L) (Gottschalk et al., 2013). Further work should be carried out at realistic, or at least closer to, environmental concentrations of NPs. Finally, information is essentially limited to freshwater species, and studies are needed in order to give an insight on the possible effects on marine organisms. With regards to this, the only available data so far are those obtained with the bivalve *Mytilus edulis* exposed *in vivo* to C60 fullerene (0.1 mg/L) and fluoranthene, alone and in combination; exposure to the mixture increased genotoxicity in the immune cells, hemocytes, and the glutathione content in the adductor muscle; moreover, reduced tissue accumulation of C60 was observed in the presence of phenanthrene (Al-Subiai et al., 2012).

3. Interactive effects of metal-oxide NPs with other contaminants: the example of n-TiO_2

Nanosized titanium dioxide (n-TiO_2) is one of the most widespread NPs in use (Robichaud et al., 2009; Menard et al., 2011). Due to its large production and growing application, n-TiO_2 is released in huge amounts in urban and industrial sewage and occurs in the aquatic environment (Menard et al., 2011; Gondikas et al., 2014) and eventually will end up in the sea, where it may represent a risk to the health of marine organisms (Matranga and Corsi, 2012). Photocatalytic interactions of n-TiO_2 with different organic compounds and metals have been investigated, in view of their application in water treatment (Huang et al., 2013; Zou et al., 2014).

Increasing evidence suggests that in different biological systems interactions of n-TiO_2 with other chemico/physical factors may result in an increase in toxicity or adverse effects, this increasing concerns on its ecotoxicity and toxicity on the aquatic environment as well as to humans (Liu et al., 2013). With regards to the interactive effects of n-TiO_2 and heavy metals, the most studied example so far is that of co-exposure of freshwater species to n-TiO_2 and the heavy metal Cd^{2+}: these studies showed that Cd^{2+} bioavailability, bioaccumulation and toxicity can be affected by the presence of n-TiO_2, at mg/L levels, with contrasting results obtained in algae, daphnids and fish. In Table 2 studies on n-TiO_2 and Cd^{2+} in aquatic organisms are summarized. In the carp, enhanced bioaccumulation of Cd^{2+} was observed in the presence of n-TiO_2 (Zhang et al., 2007). In *Danio rerio*, n-TiO_2 enhanced Cd^{2+} bioaccumulation, but this effect was reduced in the presence of humic acid (Hu et al., 2011). In *D. magna* increased Cd^{2+} bioavailability has been reported in the presence of n-TiO_2 (Hartmann et al., 2012). However, in the green algae *Chlamydomonas reinhardtii*, Cd^{2+} toxicity as well as accumulation seemed to be reduced by n-TiO_2 (Yang et al., 2012), and no influence of n-TiO_2 was observed on Cd^{2+} uptake on *Lumbriculus variegatus* (Hartmann et al., 2012). In the freshwater bivalve *Corbicula fluminea*, Cd^{2+} uptake and antioxidant biomarker responses were unaffected by the presence of n-TiO_2, despite n-TiO_2 significantly reducing the free Cd^{2+} content in the medium (Vale et al., 2014). Overall, although available data indicate changes in bioavailability/uptake/bioaccumulation of Cd^{2+} in the presence of n-TiO_2, probably due to sorption of the metal to n-TiO_2 in freshwater media, no clear evidence of increased toxicity is provided. These results were obtained with different n-TiO_2 types, model organisms and experimental conditions, utilizing concentrations of n-TiO_2 ≥1 mg/L. These concentrations, although higher than the PECs for n-TiO_2 in surface waters (μg/L) (Gottschalk et al., 2013) are however closer to realistic environmental conditions than those utilized for experimental studies with carbon based- NPs.

The combined effects of n-TiO_2 and organic contaminants have been investigated in the developing zebrafish. Co-exposure to n-TiO_2 (0.1 mg/L) and the polybrominated diphenyl ether BDE-209, increased BDE-209 bioavailability and metabolism, altered thyroid hormones levels, gene and protein expression levels and larval locomotor behavior, indicating thyroid endocrine disruption and developmental neurotoxicity (Wang et al., 2014). Adsorptive interactions between n-TiO_2 and Bisphenol A (BPA) (both at mg/L concentrations) have been demonstrated, leading to increased toxicity in the embryo only at certain combinations of the two contaminants (Yan et al., 2014). With regards to marine organisms, in the ark shell *Scapharca subcrenata*, enhanced uptake of phenanthrene by clams was recently observed in the presence of n-TiO_2 (Tian et al., 2014). Actually, the utilization of bivalve models seems to be particularly useful for evaluating the interactive effects of NPs with other contaminants.

4. Interactions between NPs and other contaminant in marine invertebrates: the example of the bivalve *Mytilus*

Among aquatic organisms, suspension-feeders have been shown to represent important test organisms in ecotoxicological tests with respect to NPs (Baun et al., 2008), as well as in studies aimed at evaluating the possible interactive effects of NPs with other pollutants (Hartmann et al., 2012). With regards to marine organisms, interactive effects of different types of NPs and organic chemicals have been reported in the model bivalve, the mussel *M. edulis*. Apart from the aforementioned work of co-exposure to fullerene and phenenatrene (Al-Subiai et al., 2012), it was previously demonstrated that co exposure to AuNPs and the redox cycling compound menadione reduced Au accumulation in the digestive gland, with no interactive effects on oxidative stress parameters (Tedesco et al., 2010).

In *Mytilus galloprovincialis*, exposure to n-TiO_2 alone, in a wide concentration range (from 1 μg/L to 5 mg/L), has been shown to induce significant changes in different biomarkers, from molecular to tissue level, in particular those related to immune and digestive gland function (reviewed in Canesi et al., 2012; Barmo et al., 2013). Interestingly, the *in vivo* effects of n-TiO_2 were observed at concentrations (μg/L) much lower than those usually utilized in ecotoxicity tests on aquatic species (mg/L), and closer to PECs for n-TiO_2 (Barmo et al., 2013). These results allowed formulating an hypothesis on the possible pathways leading to NP-induced immunomodulation (Canesi and Procházková, 2013; Corsi et al., 2014). Due to the physiological mechanisms involved in the feeding process, n-TiO_2 agglomerates formed in sea water are taken up by the gills and partly directed to the digestive gland, where intracellular uptake of NPs induces lysosomal perturbations and changes in the expression of antioxidant and immune-related genes. NPs can be then potentially translocated from the digestive

system to the hemolymph, and to circulating hemocytes, where n-TiO_2 induces changes in functional parameters (lysosomal integrity, phagocytosis, ROS and NO production, induction of pre-apoptotic processes), as well as changes in transcription of antimicrobial peptides.

These studies represented the basis for investigating the possibility that in *M. galloprovincialis* n-TiO_2 may interact with other contaminants to induce responses at different levels of biological organization. TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) and Cd^{2+} were chosen as models of organic and inorganic contaminants, respectively.

The interactive effects of n-TiO_2 and TCDD were investigated both *in vitro* and *in vivo* (Canesi et al., 2014). *In vitro* data obtained in mussel hemocytes and gill biopsies showed antagonistic effects on hemocyte phagocytic activity and on gill efflux activities mediated by ABC transporters. *In vivo*, mussels were exposed to n-TiO_2 (100 μg/L) or to TCDD (0.25 μg/L), alone and in combination, for 96 h. A wide range of biomarkers, from molecular to tissue level, were measured: lysosomal membrane stability and phagocytosis in hemocytes, ATP-binding cassette efflux transporters in gills (gene transcription and efflux activity), several biomarkers of genotoxicity in gill and digestive cells (DNA damage, random amplified polymorphic DNA-RAPD changes), lysosomal biomarkers and transcription of selected genes in the digestive gland. The results showed that co-exposure to n-TiO_2 and TCDD affected immune, gill and digestive gland function. Both synergistic or antagonistic effects were observed, depending on experimental condition, cell/tissue or type of measured response. However, antagonistic effects on different biomarkers seem to prevail. In particular, *in vivo* as well as *in vitro*, co-exposure with TCDD prevented the effects of n-TiO_2 in terms of reduced phagocytic activity of the hemocytes, decreased Multi Xenobiotic Resistance (MXR) activities and increased genotoxicity in gills; moreover, n-TiO_2 blunted TCDD-induced lysosomal lipid accumulation in the digestive gland. With regards to the effects at the molecular level, no interactive effects of n-TiO_2/TCDD were observed on expression of antioxidant, heat-shock response, abcc-like or immune related genes in hemocytes, gills, or digestive gland. Overall, the results indicated that combined exposure to n-TiO_2 and TCDD did not result in increased stressful conditions in *Mytilus*. Interestingly, in the presence of n-TiO_2, a significantly higher accumulation of TCDD was observed in whole soft tissues. However, due to the high solubility of TCDD in lipids, such an increase may be due to higher bioaccumulation in the mantle, the main tissue for lipid accumulation during gametogenesis, that was not considered for evaluation of biomarker responses in this work. Ongoing studies are aimed at investigating whether the interactive effects observed at the tissue level can be related to differences in bioaccumulation of TCDD in the presence of n-TiO_2, and other possible molecular targets of n-TiO_2, TCDD and n-TiO_2/TCDD mixture.

The effects of n-TiO_2 and Cd^{2+} were also investigated in mussels exposed to n-TiO_2 (100 μg/L) or to Cd^{2+} (100 μg/L), alone and in combination, for 96 h (Balbi et al., 2014). In the hemolymph of mussels exposed *in vivo* to the mixture antagonistic effects were observed on functional immune parameters (Fig. 1). Cd^{2+} abolished the increase in lysozyme activity and NO production induced by n-

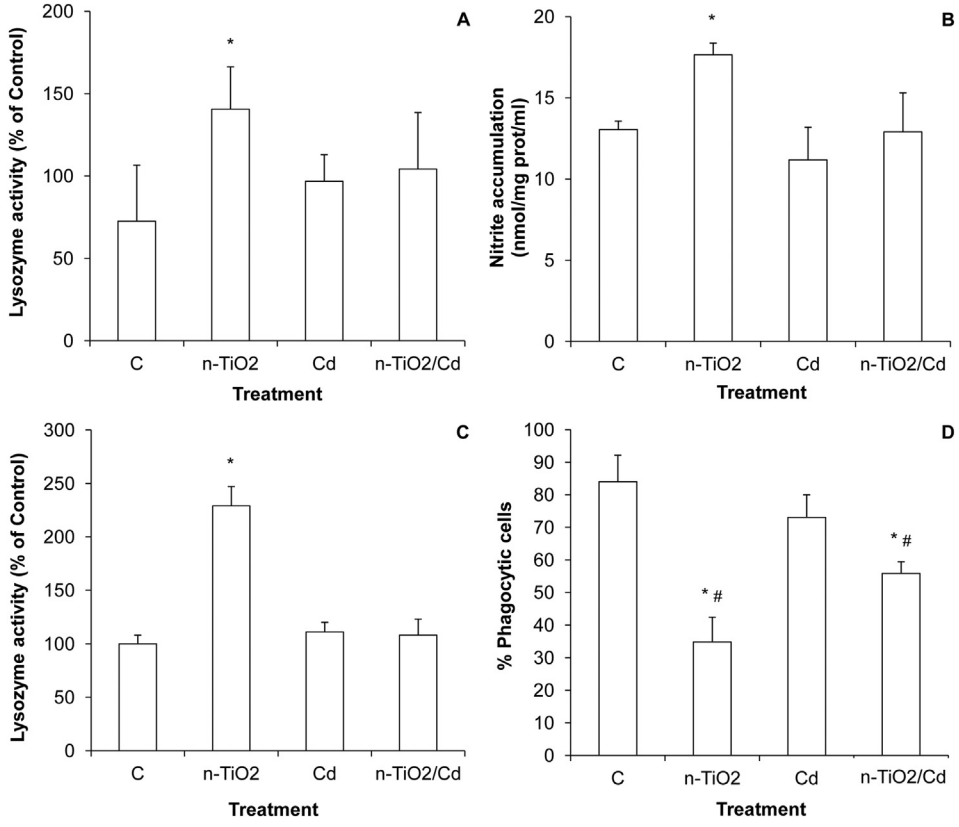

Fig. 1. Interactive effects of n-TiO_2 and Cd^{2+} on functional immune parameters of mussel hemocytes *in vivo* (upper panel) and *in vitro* (lower panel). *In vivo*: mussels were exposed n-TiO_2 (100 μg/L) or to Cd^{2+} (100 μg/L), alone and in combination, for 96 h. A) lysozyme activity; B) NO production (modified from Balbi et al., 2014). *In vitro*: hemocyte monolayers were exposed to n-TiO_2 (10 μg/mL) or Cd^{2+} (0.1 μg/mL), alone and in combination, for 60 min. C) lysozyme activity; D) phagocytosis. For experimental details see Barmo et al., 2013. Data, representing the mean ± SD of 4 experiments in triplicate, were analyzed by ANOVA plus post hoc Tukey's test ($p \leq 0.05$). * = all treatments *vs* controls; # all treatments *vs* Cd^{2+} alone.

TiO_2 (Fig. 1A and B). Interestingly, short-term exposure experiments (60 min) subsequently carried out in hemocytes *in vitro*, although at different concentrations, also showed antagonistic effects of Cd^{2+} towards n-TiO_2: Cd^{2+} protected from the n-TiO_2 induced lysozyme release, and reduced the n-TiO_2- mediated increase in NO production (Fig. 1C and D). These data support the hypothesis that the two contaminants could rapidly act on common targets in the hemocyte.

In the digestive gland, distinct interactive effects of n-TiO_2 and Cd^{2+} were observed *in vivo* on different lysosomal biomarkers. Despite the additive effects on lysosomal membrane stability, indicating stronger stressful conditions induced by the mixture, co-exposure with TiO_2 apparently protected from the effects of Cd^{2+} on other lysosomal parameters, such as increased neutral lipid accumulation and lysosome/cytoplasm volume ratio. Synergistic effects on transcription of the immune genes Lysozyme and Toll-like receptor were observed in the digestive gland, confirming interactive effects of the two contaminants on molecular targets related to the immune function.

The interactive effects of n-TiO_2 and Cd^{2+} observed at the tissue level could be due to differences in metal bioavailability/accumulation in the presence of n-TiO_2. However, negligible adsorption of Cd^{2+} to n-TiO_2 agglomerates was observed in ASW standard suspensions, and comparable concentrations of soluble Cd^{2+} were detected in exposure media in the absence and presence of n-TiO_2 (Balbi et al., 2014). In addition, no differences in total Cd^{2+} tissue content were observed in the digestive gland of mussels exposed to Cd^{2+}, alone and in combination with n-TiO_2. Moreover, n-TiO_2 did not affect the Cd-induced increase in metallothionein at both transcriptional and protein level, and no interactive effects were observed on oxidative stress-related biomarkers (i.e. catalase and GSH transferase transcription and activities, and lysosomal lipofuscin accumulation) (Balbi et al., unpublished data). Overall, these results clearly showed that the interactive effects of n-TiO_2 and Cd^{2+} observed on digestive gland biomarkers could not be ascribed to changes in bioavailability and/or bioaccumulation of Cd^{2+} in the presence of n-TiO_2, or in the heavy metal detoxication response. This is in contrast to what is observed in freshwater organisms, where n-TiO_2, at higher concentrations (mg/L), could affect Cd^{2+} bioavailability, accumulation and toxicity in various ways in algae, daphnids and fish (see Table 2).

In *Mytilus* the possible impact of n-TiO_2/Cd^{2+} mixtures at the organism level was also evaluated in the 48 h embryo toxicity assay (Balbi et al., 2014). When fertilized eggs were exposed for 48 h to n-TiO_2 and Cd^{2+}, at the same concentrations utilized in adult exposure experiments, significant effects were observed only with Cd^{2+}, in terms of both retarded development and malformed D-larvae. However, the effects of the metal were abolished in the presence of n-TiO_2, further indicating antagonistic effects of the two contaminants.

Overall, the results indicate that in *M. galloprovincialis* co-exposure to the n-TiO_2/Cd^{2+} mixture seemed to mitigate, rather than exacerbate, the adverse effect of each contaminant alone. The observed effects of n-TiO_2 and Cd^{2+} may result from interactions of either contaminant with both common and distinct targets/mechanisms of action at different levels of biological organization.

5. Conclusions

Conflicting data are so far available on the combined effects of NPs and other contaminants in aquatic, let alone marine organisms, indicating that co-exposure with NPs may either amplify or alleviate the toxic effects of other compounds. Overall, exposure to NPs in the presence of other pollutants does not necessarily lead to increased bioavailability/bioaccumulation/toxicity or harmful effects in aquatic organisms. The application of ecotoxicity tests, generally carried out at high concentrations (mg/L) of NPs represents the basis for understanding the risk of exposure to NPs in aquatic species; however, further studies using NP concentrations closer to their PECs may reveal subtler, unexpected actions of NPs, alone and in combination with other contaminants, at different levels of biological organization.

The results so far obtained indicate that such interactions might be affected by particle properties and behavior in water media (i.e. freshwater *vs* sea water), as well as by the type of other contaminants. Although adsorption of different chemicals to NPs may be relevant to these processes in freshwater, no data are available in sea water media. This information may greatly help understanding the physico-chemical variables that can influence the possible impact of NPs on marine biota.

Moreover, available data underline how the effects of NPs and NP mixtures might vary between different aquatic species, although the potential mechanisms behind this variability are largely unknown. However, it is now clear from research in nanomedicine that, in human plasma, NPs interact with a range of biopolymers, especially proteins, organized into the “protein corona”, in rapid dynamical exchange with other proteins in the surrounding medium (Lundqvist et al., 2008). Understanding the protein corona is crucial for understanding how NPs interact with cells, as the corona proteins control the specific cellular receptors used by the protein–NP complex, the cellular internalization pathway, and the immune response (Fleischer and Payne, 2014), with important implications for the safety of NPs used as drug carriers. Although no information on the interactions of NPs with biological fluids of aquatic organisms (fish plasma, invertebrate hemolymph) is available yet, understanding these interactions might represent a future key issue for environmental risk assessment of NPs and NP mixtures.

Acknowledgments

This work was partly supported by the Italian Ministry of Research (PRIN2009, prot. 2009FHHP2W).

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