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Ecotoxicity of engineered TiO_2 nanoparticles to saltwater organisms: An overview

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ABSTRACT

The innovative properties of nanomaterials make them suitable for various applications in many fields. In particular, TiO_2 nanoparticles ($nTiO_2$) are widely used in paints, in cosmetics and in sunscreens that are products accessible to the mass market. Despite the great increase in the use of such nanomaterials, there is a paucity of general information about their potential effects to the aquatic species, especially to saltwater ones. Moreover, the difficulties of determining the effective exposure scenario make the acquired information low comparable. In this work, questions about the complexity of the real exposure scenario determination are discussed. The state of the art, concerning the experimental activities with $nTiO_2$ toward the saltwater organisms is firstly illustrated, providing statistical information about the different matrices, organisms and nanoparticles employed. A comparison of the $nTiO_2$ ecotoxicity effects, grouped by taxonomic classes, is provided illustrating their relative experimental conditions. Findings show the need to develop specific protocols for toxicity tests with ENPs to control the variability of experimental conditions. Some advices are finally proposed for the future experimental activities.

1. Introduction

The innovative properties showed by engineered nanomaterials (ENMs) respect to bulk materials make nanotechnologies growing faster in many different fields attracting huge investments. Indeed, it is estimated that it will become a trillion dollar business in the next ten years (Wiesenthal et al., 2011).

TiO_2 nanoparticles ($nTiO_2$) are widely used because they show photocatalytic properties, mainly under ultra-violet (UV) radiation, generating reactive oxygen species (ROS) (Bundschuh et al., 2011). They are applied in self-cleaning paints and self-sterilizing surfaces (Clemente et al., 2012; Fujishima et al., 2008), in cements and asphalts for building, road, pavements and tunnel structures also for air cleaning and NOx abating (Chen and Poon, 2009). Moreover, $nTiO_2$ is present in cosmetics and sunscreens in order to stabilize ingredients, enhance the penetration of vitamins and anti-oxidants into the skin, improve the effectiveness of UV filters and make the product more esthetically pleasing (Mu and Sprando, 2010). Frequently, the various market applications are a mixture of two $nTiO_2$ crystalline polymorphic phases such as the anatase mineral, exhibiting the highest photocatalytic properties (Choi et al., 2004; Uchino et al., 2002) and the rutile one, used as flat white covering pigment (Menard et al., 2011) with a yearly four million tons consumption worldwide (Shi et al., 2013).

About the background concentration of $nTiO_2$ in the environment, nowadays, no data are available, due to the difficulties of measuring their values. As a consequence, there is no definite information concerning the ending up of $nTiO_2$ in the ocean. Anyway, it is commonly accepted, by various authors, that $nTiO_2$ may enter the aquatic compound. For example, according to Canesi et al. (2009), it is generally assumed that NPs will enter the aquatic environment. Moreover, Zhu et al. (2011a) declared that $nTiO_2$ could pose an exposure risk to aquatic environments, particularly freshwater and coastal environments. Finally, the release of $nTiO_2$ might be expected having the aquatic environment as the final sink like for many other contaminants (Buffet et al., 2011).

At the present day, the determination of the $nTiO_2$ exposure limits (*e.g.* LC/EC50) and the definition of the effect ranges, for the different groups of saltwater organisms, are not established, yet. The assessment of $nTiO_2$ toxicity with saltwater biological models is challenging mainly due to its salinity conditions (~35‰) and ionic strength (~0.7 M) (French et al., 2009). Indeed, they could limit the stability of $nTiO_2$ dispersions, affecting the size of their aggregates speeding up, potentially, their sedimentation processes (Brunelli et al., 2013; Keller et al., 2010). As a consequence, the ENPs bioavailability may change affecting the reliability of exposure scenarios making the analysis and interpretation of results really hard.

Although the toxicity effects on seawater organisms are a crucial topic, still a general lack of information exists about it both quantitatively and qualitatively. Indeed, the use of not standardized experimental conditions and the absence of specific quantification analysis may also influence the determination of the exposure scenario, making the results little reproducible and comparable.

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This review paper is aimed at highlighting and discussing the issues concerning $nTiO_2$ toxicity data with saltwater species evidencing (i) the role of testing matrices, (ii) the exposure conditions and (iii) the consequent ecotoxicological effects on various biological models.

2. Ecotoxicity tests with ENP: the state of the art

2.1. General trends

Nowadays, the scientific production concerning the ENPs toxicity is growing rapidly, as it can be seen in S1. A research on Science Direct, using "nanoparticles" and "toxicity" as keywords, showed that the publication number, in the last seven years, was enhanced by more than five-fold, from 628 in 2007 to 3508 in 2013. For $nTiO_2$, the specific growth trend is even further increased. Indeed, in the same period, the paper production resulted more than ten-fold enhanced (from 68 to 851) as shown in S2.

2.1.1. Testing matrices

The analysis of 217 articles, concerning the ecotoxicity effects of ENPs toward aquatic organisms, evidenced that most of them were carried on with freshwater species as reported in Fig. 1. Nevertheless, various testing matrices were taken into account such as fresh, brackish and saltwater and their relative sediments, soils and other materials (*i.e.* interior paints and ceramic pellets) with both *in vivo* and *in vitro* tests. Most publications (64%) concern freshwater organisms, while seawater and brackish water focused ones represent only 17%, including sediment as testing matrix as well. Cattaneo et al. (2009) found a similar distribution comparing the ecotoxicity tests performed with invertebrate organisms.

Data distribution about $nTiO_2$ was summarized in Fig. 2, where the gap between freshwater and saltwater scientific production appears broad. Indeed, only 20% (129 publications) are about sea and brackish water species (21% including seawater sediment) as reported in Table 1 *versus* 74% concerning freshwater species. This could be mainly due to the great complexity of saltwater as testing medium. In fact, the relatively high salt content influences the ionic strength of water that, generally, reach values around 0.7 mM (Dyrssen and Hansson, 1973). This may lead to the enhanced ENP instability, promoting the formation of agglomerates and precipitates in a short time compared to freshwater (Brant et al., 2005; French et al., 2009).

2.1.2. Test organisms

Information about the general distribution within various *in vivo* studies on the basis of taxonomical grouping (bacteria, algae, rotifers, annelids, molluscs, crustaceans, echinoderms and fishes) are provided in S3 for freshwater organisms and in Fig. 3 for saltwater ones. As it may be seen, the distribution of these studies is quite similar. The main differences are about researches on molluscs and crustaceans. Indeed, about molluscs, saltwater species publications (Fig. 3) are relatively more abundant (29%) than the freshwater ones (3%) (S3). On the contrary, the papers about saltwater and freshwater crustaceans species are 8% and 25%, respectively. Moreover, $nTiO_2$ *in vivo* studies have been reported in S4 and in Fig. 4, for freshwater and saltwater species, respectively. The comparison between S3 and S4 reveals that the relative distribution of the publications is quite similar. About the $nTiO_2$ and the saltwater species (Fig. 4), bacteria and molluscs result as the most studied bioindicator groups, 30% of the total works for both. Respect to the freshwater species (S4), it can be noted a decrease in the number of publications for the other taxonomic groups such as algae, fish and crustacean. The same trend can be observed comparing these data (Fig. 4) with the data about seawater species and generic ENPs (Fig. 3), with two exceptions for bacteria (30% and 16%, respectively) and fishes (5% and 19%, respectively).

For the sake of completeness, statistical information about the number of the ecotoxicity studies (*in vivo* and *in vitro*) performed with different ENPs toward different taxonomic groups of organisms has been displayed in some tables (A, B and C) reported in S5. As it may be seen in Table A, Ag seems to be the most employed metal ENP for the ecotoxicity tests, followed by Au and Cu. About the ENP metal oxide, the analysis of the data (Table B) suggests that, not only $nTiO_2$ is the most studied ENP (*i.e.* ecotoxicity), but also the ENP to which the widest number of testing organisms was applied. Finally, Table C indicates C_{60} as the most used organic ENP in ecotoxicity experimental activities.

2.2. Determining the real exposure scenario

One of the crucial issues of working with $nTiO_2$ is the scarce knowledge about the real exposure scenarios. This affects the reproducibility and comparability of results that is mainly determined by factors such as (a) the lack of common experimental conditions in the toxicity testing strategy, (b) the availability of ENPs data on primary, secondary characterization and exposure media quantification and (c) the use of not suitable metrics to describe their ecotoxic potential. Frequently, more than one of these conditions occurs at the same time.

Moreover, there is no really quantitative information concerning the ending up of $nTiO_2$ in the ocean. It is assumed that the ENPs enter the aquatic environment (Canesi et al., 2009), being discharged in waterways and thus reaching the sea (Matranga and Corsi, 2012). Indeed, $nTiO_2$ could pose a risk to aquatic environment, particularly, fresh and coastal water (Zhu et al., 2011a) affecting not only the water column, but also the sediment and their relative sentinel species (Klaine et al., 2008). The only exception is reported in Kaegi et al. (2008) that measured the $nTiO_2$ content in the runoff of a model and real facade, as well as in the urban runoff. Results showed $nTiO_2$ concentrations of about 550, 9 and 8 $\mu g\ L^{-1}$ for model facade runoff, real facade runoff and urban runoff, respectively. As the collected samples were centrifuged, the particle size range was between 20 and 300 nm.

In general, about the background concentration of $nTiO_2$ in the environment, no data are available, due to the difficulties of measuring their values and to the complexity to discern the natural high background level of NP from the newest ones (Nanosafe, 2008). Anyway, at the present day, some evaluations and prediction models are available, which were performed by different authors (*e.g.* Gottschalk et al., 2009; Müller and Nowack, 2008; Tiede et al., 2009). So, according to Müller and Nowack (2008), the predicted environmental concentrations (PEC) of $nTiO_2$, in a realistic scenario, are 0.0015 $\mu g\ m^{-3}$, 0.7 $\mu g\ L^{-1}$ and 0.4 $\mu g\ kg^{-1}$, for air, water and soil, respectively. On the basis of the model of Gottschalk et al. (2009), the predicted annual increase of $nTiO_2$ is 0.00017 $\mu g\ m^{-3}$, 0.55 $\mu g\ L^{-1}$, 0.78 and 45 $\mu g\ kg^{-1}$, for air, surface water, sediments and soil, in that order. Moreover, Tiede et al. (2009) estimated that the $nTiO_2$ PECs, arising from use in consumer products, are 24.5 $\mu g\ L^{-1}$ and 1030 $\mu g\ kg^{-1}$, for water and soil, respectively.

2.2.1. Test experimental condition

Various standard protocols (ISO, ASTM and OECD) exist for aquatic toxicity testing with a huge range of target species. All these guidelines were produced for operating with bulk substances and not with nano-sized ones. Specific guidelines for ENM are still missing. As a consequence, traditional protocols may not guarantee the operational uniformity with these new and still little known materials lacking the consideration of specific parameters.

One of the main problem results in a great level of variability, not only due to the use of different ENPs, but also with the same ENP and the same group of testing organisms (Kahru and Dubourguier, 2010), even because of coatings. Actually, Scown et al. (2010) displayed that the effects of ENPs within the tested bacteria, algae and invertebrates varied prominently even using the same ENP thus indicating that there is a lack of consistence about the observed effects. Moreover, there are some evidences that the same ENP having various coatings

Table 1
Ecotoxicity effects of $nTiO_2$ toward the saltwater species. Studies are divided according to the taxonomic classes of the employed organisms. For every reference, the following fields are considered: species, exposure conditions, effects, employed TiO_2 NP, its primary and secondary characterization, the dispersion method and, if performed, the analytical survey.

Group of organisms	Species	Exposure conditions		Effects	$nTiO_2$ producer, origin, crystal phases (%), particle size (as declared by producer)	Primary and secondary characterisation (analytical method)	Dispersion method	Analytical survey	References
Bacteria ($n = 5$)	*Vibrio fischeri*	*In vivo*	18 h (MARA test); 15 min (Microtox® test)	At 18 h EC50 > 100 mg L^{-1}; at 15 min EC50 > 100 mg L^{-1}	Sigma-Aldrich, <100 nm	n.d.	75 mg diluted in 150 mL of ASTM, then placed in a rotator (VELP Scientifica, Overhead mixter, Rotax 6.8) and mixed for 24 h at room temperature; then filtered at 0.22 μm (retained 54% of the ENP with filtering) ; pH = 6.0–6.8	n.p.	Blaise et al. (2008)
		In vivo	Till 20,000 mg L^{-1} for 30 min (Microtox® test)	At 30 min EC50 > 20000 mg L^{-1}; EC20 > 20,000 mg L^{-1}; NOEC > 20,000 mg L^{-1}; MIC > 20,000 mg L^{-1}	Sigma-Aldrich, 25–70 nm	n.d.	sonication in Milli-Q ultrapure water for 30 min, then storage in the dark at 4 °C; vortexed before the tests; pH = 6.5	n.p.	Heinlaan et al. (2008)
		In vivo	Till 1000 mg L^{-1} for 15 min (Microtox® test); pH = 7.8–8.1	For SW, BSTE, AW n.d., 651, 941 mg L^{-1}; NOEC = 500, 250, 250 mg L^{-1}; LOEC = 1000, 500, 500 mg L^{-1}	from dried sludge of 3 different water sources (AW, BTSE, SW); Evonik-Degussa P25	for AW, BTSE, SW: 65% A, 25% R (XRD); 6, 15, 40 nm (SEM); 76, 104, 68 m^2 g^{-1} (BET); for P25: 65% A, 25% R (XRD); 25 nm (SEM), 42 m^2 g^{-1} (BET)	n.d.	n.p.	Lee et al. (2008)
		In vivo	At 0.4395–112.5 mg L^{-1} for 5, 10, 15 min; pH 7.5–7.7	No inhibition effects	Prepared by hydrolysis of titanium sulfate solution, 6 nm	Aggl.: 0.5–2 mm (DLS)	Ultrasonication in D.I. water for 30 min	n.p.	Strigul et al. (2009)
		In vivo	At 1, 10, 100 mg L^{-1} for 30 min (Microtox® test)	At 15 min EC50 > 100 mg L^{-1}	Sigma-Aldrich, 10% dispersion in water, <40 nm	Aggl.: 0.5–70 μm (AFM, X-Ray)	n.d.	ICP-MS, UV spectrophotometer, flow cytometry	Velzeboer et al. (2008)
Algae ($n = 4$)	*Dunaliella tertiolecta*, *Isochrysis galbana*, *Skeletonema marinoi*, *Thalassiosira pseudonana*	*In vivo*	At 10, 100, 500, 1000 μg L^{-1} for 96 h; 14:10 h light:dark; T = 15 °C	No effect on growth of the phytoplankton	Evonik-Degussa	81% A, 19% R, 15–20 nm	Sonication in filtered natural seawater for 60 s, briefly vortexed and diluted with filtered natural seawater	n.p.	Miller et al. (2010)
	Dunaliella tertiolecta, *Isochrysis galbana*, *Skeletonema costatum*, *Thalassiosira pseudonana*	*In vivo*	At 1, 3, 5, 7 mg L^{-1} with or without UV light for 96 h	No effect on growth rates of any species in the blocked-UV treatment, except for *I. galbana* at 7 mg L^{-1}. Under UV light, significant suppression of population growth at 1 mg L^{-1} for *I. galbana* and *D. tertiolecta*, at 3 mg L^{-1} for *S. costatum* and *T. pseudonana*; adhesion of ENP on the cell surface (10 and 100 nm aggregates)				n.p.	Miller et al. (2012)
	Phaeodactylum tricornutum	*In vivo*	At 1.8–54 mg L^{-1} for 72 h , 14:10 h light: dark, at 25 °C, pH = 8.25–8.28	Low toxicity (IC20 = 57 mg L^{-1}); presence of cell agglomerates for $nTiO_2$ concentrations ≥ 5 mg L^{-1}; the agglomerate number and the dispersion concentrations and also between the cell numbers composing the agglomerates and the dispersion concentrations	Evonik-Degussa P25	15–60 nm (TEM), 65 m^2 g^{-1} (BET), aggl.: 180–3800 nm (DLS)	sonication (UP-100H, Hielscher Ultrasound Tecnology) in test media for 15 min at 100 W, cooling with ice bath	n.p.	Minetto (2012)

		In vivo	At 0.01–100 mg L^{-1} for 72 h, under continuous light (10,000 lx), at 20 °C	Concentration-dependent cell growth inhibition for all ENP. EC50 = 10.91, 11.30 and 14.30 mg L^{-1} respectively for 15, 25 and 32 nm; cell growth stimulation at very low concentrations	Alfa-Aesar, 15 nm; 32 nm; 25 nm	100% A for all three ENP (XRD)	Magnetic stirring and ultrasonication bath in test media for 4 min at 100 W	n.p.	Clément et al. (2013)
Rotifers ($n = 1$)	*Brachionus plicatilis*	*In vivo*	At 0.005–1000 mg L^{-1} for 48 h, in the dark, at 25 °C	Lethal effects: EC50 = 5.37, 10.43 and 267.3 mg L^{-1}, respectively for 15 nm, 25 nm and 32 nm	Alfa-Aesar, 15, 32, 25 nm	100% A for all three ENP (XRD)	Magnetic stirring and ultrasonication bath in test media for 4 min at 100 W	n.p.	Clément et al. (2013)
Annelids ($n = 1$)	*Arenicola marina*	*In vivo*	10 d toward the sediment (1–3 g kg^{-1}), 8:16 h light:dark, at 15 °C	No behavioral effect on burrowing ability; significant decrease of LMS for the highest concentration; coelomocyte DNA damage; presence of ENP aggregates (>200 nm) around the microvilli of the gut epithelium cells	Sigma-Aldrich, mixed A and R	23.2 nm (TEM), 46.3 m^2 g^{-1} (XRD)	Sonication (Sonicor TS9045) in Milli-Q ultrapure water for 30 min, once in the beacker, hand stirring in ASW for 15 min, at 15 °C	ICP-OES (sediment and organisms)	Galloway et al. (2010)
Molluscs ($n = 7$)	*Mytilus galloprovincialis*	*In vitro*	Hemocytes, at 1, 5, 10 mg L^{-1}, from 30 min to 4 h, at 16 °C	No significant decrease in LMS;	Evonik-Degussa P25	22 nm (TEM), 51 m^2 g^{-1} (BET), aggl.: 150–1600 nm (DLS)	Sonication (UP200S Hielscher Ultrasonic Technology) in ASW for 15 min at 100 W, cooling with ice bath	n.p.	Canesi et al. (2009)
		In vivo	Adults, at 0.05, 0.2, 1, 5 mg L^{-1}, for 24 h at 16 °C	Concentration-dependent extracellular oxyradical production at 5 mg L^{-1}; high NO production, maximum for 2 h; rapid (after 5 min) increase in phosphorylation of p38 MAPK and significant decrease at 60 min	Evonik-Degussa P25	22 nm (TEM), 51 m^2 g^{-1} (BET), aggl.: 150–1600 nm (DLS)	Sonication (UP200S Hielscher Ultrasonic Technology) in ASW for 15 min at 100 W, cooling with ice bath	n.p.	Canesi et al. (2010)
		In vivo	Adults, at 1, 5, 10 mg L^{-1}, from 30 min to 4 h	Effects on LMS of both the hemocytes and the DG, at the higher concentrations; lysosomal lipofuscin accumulation; GST and CAT activities stimulated in the DG, but not in the gills; no mortality effects	Evonik-Degussa P25, 20 nm	15–60 nm (TEM), 61 m^2 g^{-1} (BET); aggl.: 4195 nm at 15 min, 5514 at 1 h, 5846 at 24 h (DLS)	Sonication (UP200S Hielscher Ultrasonic Technology) in ASW for 15 min at 100 W, cooling with ice bath	n.p.	Ciacci et al. (2012)
		In vivo	Adults, at 1, 10, 100 μg L^{-1}, for 96 h, at 16 °C	*In DG*: dose-dependant decrease in LMS and increase of lypofuscin at lower concentration (+80% at 1 μg L^{-1}); decrease of GSR, GST, GPX activity and concentration-dependant increase of CAT activity (+90% at 100 μg L^{-1}); decrease of antimicrobial peptides transcription; no morphological alteration of the tubules/cells, but ENP visible on the microvilli and within lysosomal vacuoles *in hemocytes*: LMS decrease; phagocytic activity induced; increasing of extracellular O_2^- production and of nitrite accumulation; hemocyte count decrease at highest concentrations (−40%) and observation of necrosis processes; defensing gene transcription increase (10-fold at the highest concentrations)	Evonik-Degussa P25	A and R (TEM, TEM-EDX, SAED), 15–60 nm (TEM, TEM-EDX, SAED), 61 m^2 g^{-1} (BET); aggl.: at 15 min, 900, 2659, 4195 nm for 10, 50 and 200 mg L^{-1} (BET)	Sonication (UP200S Hielscher Ultrasonic Technology) in ASW for 15 min at 100 W, cooling with ice bath	n.p.	Barmo et al. (2013)

(continued on next page)

Table 1 (*continued*)

Group of organisms	Species	Exposure conditions		Effects	$nTiO_2$ producer, origin, crystal phases (%), particle size (as declared by producer)	Primary and secondary characterisation (analytical method)	Dispersion method	Analytical survey	References
Molluscs (n =7)	*Mytilus galloprovincialis*	*In vivo*	Embryos, at 0.5, 1, 4, 8, 16, 32, 64 mg L^{-1}, for 48 h in the dark and dark/light (16/8 h), at 18 °C	*dark scenario*: non linear responses; EC50(1) = 1.23 mg L^{-1}; EC50(2) = 38.56 mg L^{-1} *dark/light scenario*: non linear responses with significant effects at 4 and 8 mg L^{-1}; EC50(1) = 1.65 mg L^{-1}; EC50(2) = 16.39 mg L^{-1} For both scenarios, presence of ring like bidimensional structures surrounding the larvae	Evonik-Degussa P25	33 nm (TEM); aggl.: 217–590 nm (DLS)	Sonication (UP-100H, Hielscher Ultrasound Tecnology) in test media for 20 min at 100 W, cooling with ice bath, then vortexed for 10 s	n.p.	Libralato et al. (2013)
	Haliotis diversicolor supertexta	*In vivo*	adults, at 0.1, 1, 10 mg L^{-1}, for 96 h, at 24 °C, pH = 7.9–8.1	no mortality effects detected; for ≥ 1 mg L^{-1} increase of SOD enzyme, decrease of GSH and increase LPO level; excessive NO production for 1 and 10 mg L^{-1} treatments	Nanjing High Technology Nano Material Co., 100% A, <10 nm, 150 m^2 g^{-1}	n.d.	Sonication in ultrapure water for 10 min at 50 W L^{-1} at 40 kHz	n.p.	Zhu et al. (2011a)
		In vivo	embryos exposed till hatching at 2–1000 ng L^{-1} of TBT, in presence of nTiO2 (2, 10, 50, 250 mg L^{-1}), for 9 h; to determine hatching rates the exposure was 12–13 h	*direct toxicity of ENP*: no effect at 2 mg L^{-1}; at 10 mg L^{-1} developmental delay and 30% of malformed embryos; NOEC = 2 mg L^{-1}; for hatching inhibition (10 h) and embryo malformation (8 h) EC50 were = 56.9 and 345.8 mg L^{-1}, respectively *enhanced TBT toxicity*: in presence of nTiO2 (2 mg L^{-1}) the EC50 for hatching inhibition (10 h) and embryo malformation (8 h) decreased from 46.5 to 2.1 ng L^{-1} and from 13.4 to 1.9 ng L^{-1}, respectively; in presence of ENP-TBT complex SOD and GSH activity decreased and LPO increased	Nanjing High Technology Nano Material Co., 100% A, <10 nm, 150 m^2 g^{-1}	Aggl.: 562 nm–22.7 μm (DLS)	Sonication in ultrapure water for 10 min at 50 W L^{-1} at 40 kHz	n.p.	Zhu et al. (2011b)

Crustaceans ($n = 2$)	*Artemia salina*	*In vivo*	adults and larvae exposed at 10, 50, 100 mg L^{-1} for 24 and 96 h 16:8 h light:dark, at 24 °C, pH = 8.1–8.7	accumulation of ENP into the nauplii and adult guts *nauplii*: 0.47, 2.65, 3.19 mg g^{-1} for 24 h exposure, and 1.29, 3.87, 4.43 mg g^{-1} for 96 h exposure to 10, 50, 100 mg L^{-1}; maximum mortality = 18% at 100 mg L^{-1} for 96 h; at 96 h increase of MDA level *adults*: 2.30, 3.88, 4.19 mg g^{-1} for 24 h exposure, and 4.38, 5.63, 6.20 mg g^{-1} for 96 h exposure to 10, 50, 100 mg L^{-1}; maximum mortality = 14% at 100 mg L^{-1} for 96 h; at 96 h increase of MDA level	Skyspring Nanomaterial Inc., 100% R, 10–30 nm, 50 m^2 g^{-1}	8–40 nm (TEM), aggl.: 371, 498, 589 nm at 10, 50, 100 mg L^{-1} respectively (DLS)	Vortex for 20 s and sonication (ultrasonic bath) for 10 min, in D.I. water	ICP-MS (organisms)	Ates et al. (2013)
	Artemia franciscana	*In vivo*	48 h old larvae exposed at 0.5–64 mg L^{-1} for 96 h, at 25 °C, pH = 8.27–8.31, considering the effects of light, feed and starvation on toxicity	1) "light and feed" scenario: minimum effect at ; maximum effect at; EC50(24 h) = 26.52 mg L^{-1}; 17.74 mg L^{-1}; 13.40 mg L^{-1} 2) "dark and feed" scenario: minimum effect at ; maximum effect at 3) "light but no feed" scenario: minimum effect at ; maximum effect at; EC50(24 h) = 27.13 mg L^{-1};	Evonik-Degussa P25	15–60 nm (TEM), 65 m^2 g^{-1} (BET), aggl.: 180–3800 nm (DLS)	Sonication (UP-100H, Hielscher Ultrasound Tecnology) in test media for 15 min at 100 W, cooling with ice bath	n.p.	Minetto et al. (2012)
Echinoderms (n = 1)	*Lytechinus pictus*	*In vivo*	embryos exposed at 0.5, 1, 5, 10 mg L^{-1} for 96 h, at 15 °C, pH = 8.0–8.2	No adverse effect on embryo development	Evonik-Degussa	82% A, 18% R (XRD), 27 nm (TEM), 51,5 m^2 g^{-1} (BET), aggl.: 194 nm (DLS)	Sonication (Branson model 2510 sonic bath, Dan-bury, CT) in D.I. water for 30 min at 100 W (max); solution was then diluted into 0.45 μm filtered seawater containing 10 mg L^{-1} of alginic acid	n.p.	Fairbairn et al. (2011)
Fishes ($n = 1$)	*Oryzias latipes*	*In vivo*	Embryos exposed till 17 d at 0.03, 0.07, 0.3, 0.7, 3.0, 7.0, 14.0 mg L^{-1}, 12:12 light:dark, at 25 °C; hatched embryos exposed for additional 5 d	Hatching time was up to 50% shorter respect to the control; prematurely hatched embryos exhibited altered swimming activity, due to the large size of the yolk sac; no significant mortality effect at any concentration, but at 0.7 mg L^{-1} occurrence of pericardial edema; moribund behavior exhibited by approximately 15 d of exposure for all treatment	Evonik-Degussa P25, A 80%, R 20%, 21 nm	91 nm (SEM), 84 nm (DLS), aggl.: 5–100 μm (SEM, FFF)	Sonication (Fisher Sonic Dismembrator 500, Ottawa, Canada) in ultrapure water for 1.5 min at 375 W L^{-1}, cooling with ice bath, then centrifuge for 10 min at 1000 g	UV-spectrophotometry (stock solutions)	Paterson et al. (2011)
Cetaceans ($n = 1$)	*Tursiups truncatus*	*In vitro*	Leukocytes exposed at 25, 50, 100 mg L^{-1}, for 4, 24 and 48 h	DNA basis fragmentation and the increase of DNA migration at 50 and 100 mg L^{-1}, for 24 and 48 h (Comet Assay); leukocyte viability slightly decreased after 48 h at 100 mg L^{-1}	Sigma-Aldrich 100% A, <25 nm; Sigma-Aldrich 100% R, <5000 nm	Aggl.: from few to several μm (TEM)	Sonication (Transonic 460/H, Elma) in RPMI for 30 min at 35 kHz	n.p.	Bernardeschi et al. (2010)

A = anatase; AFM = atomic force microscopy; aggl. = agglomerates; AW = artificial water; BET = Brunauer Elmet Teller; BTSE = biological treated sewage effluent; CAT = catalase; D.I. = de ionized; DG = digestive gland; DLS = dynamic light scattering; EC50 = median effective concentration; EDX = energy dispersive X-ray spectroscopy; FFF = field flow fractionation; GPX = glutathione peroxidase; GSH = glutathione; GSR = glutathione reductase; GST = glutathione S-transferase; ICP = inductively coupled plasma; LMS = lysosomal membrane stability; LOEC = lowest observed effect concentration; LPO = lipidic peroxidation; MAPK = mitogen-activated protein kinases; MARA = microbial array for risk; MDA = malondialdehyde; MS = mass spectrometry; n.d. = not declared; n.p. = not performed; OES = optical emission spectrometry; R = rutile; RPMI = Roswell Park Memorial Institute (culture medium); SEM = scanning electron microscope; SAED = selected area electron diffraction; SOD = superoxide dismutase; SW = seawater; TBT = tributyl tin; TEM = transmission electron microscopy; UV = ultra violet; XRD = X-ray diffraction.

may exert different ecotoxicological effects (Strigul et al., 2009; personal communications).

Focusing on titania, $nTiO_2$ is normally used as a mix of anatase and rutile in different proportion but, sometimes, just only one crystal phase is used (Ates et al., 2012; Bernardeschi et al., 2010; Zhu et al., 2011a,b). In particular, Choi et al. (2004) confirmed that the anatase crystal phase exhibits the highest photocatalytic activity. This aspect suggests that a great content of anatase may lead to a great radical oxygen species (ROS) production and, as a consequence, that different mix of crystal phases may cause different effects toward the testing organisms, especially, when bioassays includes a photoperiod.

Moreover, no standardized methods exist for the preparation of ENPs' dispersions. Presently, most of the authors seem to prefer using a sonication procedure (*e.g.* by means of a sonic probe), but, even if the technical approach is sometimes the same, the operating parameters such as sonication time and power can vary significantly as reported in Table 4 where it is evident how some authors tried various combinations of dispersion techniques. For example, Blaise et al. (2008) diluted the ENPs in ASTM artificial seawater (2004) then filtering it at 0.22 μm; Galloway et al. (2010) sonicated the ENPs for 30 min in ultrapure water concluding with hand stirring them for 15 min; Fairbairn et al. (2011) sonicated $nTiO_2$ for 30 min at 100 W in de-ionized water and then diluting the ENP in seawater amended with 10 mg L^{-1} of alginic acid; Ates et al. (2012) vortexed the ENP for 20 s and, afterwards, sonicated them for 10 min in deionized water before the final dilution in seawater. All these dispersing methods and their further variations may plausibly be a source of ENPs within a series of different size ranges, contributing to a general increase in the variability of the exposure scenarios.

Finally, in most of the cases, no information is available about the time passing between the end of the sonication procedure and the start of the exposure to testing species or physico-chemical characterisation of ENPs. This is of high relevance, considering that the agglomerates formation, which may influence the ENPs bioavailability and potentially the exposure scenario, has been observed as a time-dependent process (Miller et al., 2010).

2.2.2. The analytical quantification of ENPs

At present, this review highlighted that most papers did not report the real or analytical concentrations of ENPs dispersions, but just the nominal ones. In general, until 2011, in most of the papers, the ENPs concentration values were simply measured after the preparation of the dispersions but scarce attention was paid to the exposure scenario evolution, frequently related to the deposition of the ENPs. So, the authors worked without making a real analytical quantification of the ENPs, neither in the media (during and/or at the end of the test) nor in exposed organisms.

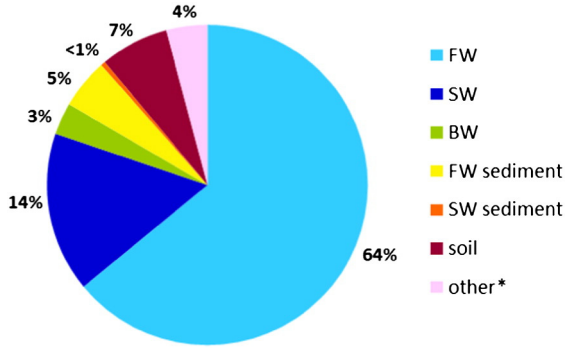

Fig. 1. Publication distribution about ENP toxicity, depending on the different matrices used in the test for the organism exposure. The publication are related to both *in vivo* and *in vitro* tests ($n = 217$). FW = freshwater, SW = saltwater, BW = brackish water. *other: cotton tissues, interior paints, ceramic pellets, orange juice and products for which the authors assessed the antimicrobial properties of some ENP such as nAg or $nTiO_2$.

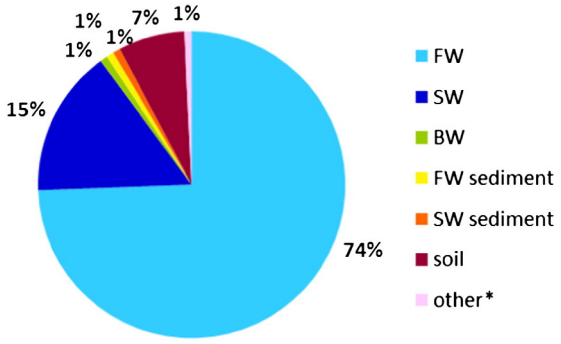

Fig. 2. Publication distribution about $nTiO_2$ toxicity, depending on the different matrices used in the test for the organism exposure. The publication are related to both *in vivo* and *in vitro* tests ($n = 129$). FW = freshwater, SW = saltwater, BW = brackish water. *other: interior paints and ceramic pellets.

About $nTiO_2$ and seawater organisms (Table 1), the quantification analyses were performed in four studies (Ates et al., 2012; Galloway et al., 2010; Paterson et al., 2011; Velzeboer et al., 2008). Indeed, as reported in Table 4, Velzeboer et al. (2008) in their study with *Vibrio fischeri*, *Pseudokirchneriella subcapitata* and *Chydorus sphaericus* employed ICP-MS, UV-visible light spectrophotometry and flow cytometry for measuring the total Ti content, the TiO_2 nanoparticles and both numbers and sizes of larger particles (>0.5 μm) in the used dispersions, respectively. Galloway et al. (2010) for their experiments with the annelid *Arenicola marina*, quantified the $nTiO_2$ in the sediment by means of ICP-OES. Paterson et al. (2011) used the UV-visible spectrophotometer to assess the concentration of $nTiO_2$ within the stock suspension. Finally, Ates et al. (2012) detected the content of Ti with ICP-MS to determine the bioaccumulation in both nauplii and adult of *Artemia salina*.

2.2.3. Metric for result interpretation

The classic toxicology was funded on the concept that the dose makes the poison, but now with ENPs other variables showed to play a role conditioning apart from concentration the final output of the evaluation process. Oberdorster et al. (2005) argued about rat lung inflammation effects induced by $nTiO_2$. In this case, the dose–response relationship fitted better when expressed as percentage of neutrophils *versus* $nTiO_2$ surface area (cm^2) rather than *versus* its mass (μg). Savolainen et al. (2010) confirmed this issue concluding that the surface area of ENPs might be a more relevant metric for correlating its appropriate dose. This could be due to the wider reactive surface area of finer particles, thus determining a potential increase of toxicity in the exposed organisms compared to coarser particles. Moreover, their reduced dimensions could facilitate uptake events. Anyway, as summarized in Table 4, even if most of the authors provided the agglomerate dimensional analysis, generally, it was not used for the interpretation of results.

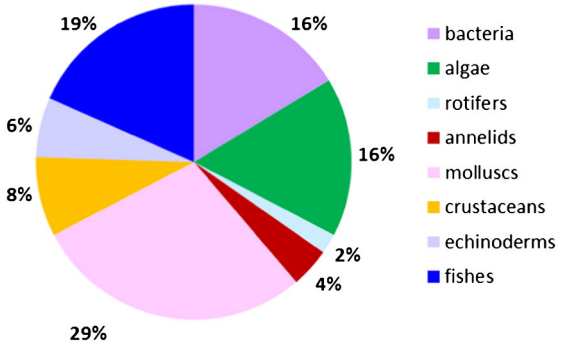

Fig. 3. Distribution of the different *in vivo* studies about ENP toxicity, depending on the taxonomic group of the used saltwater species ($n = 49$).

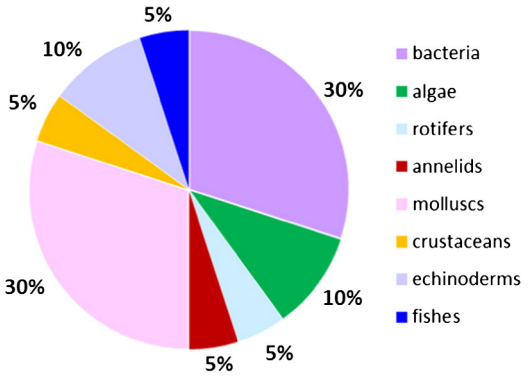

Fig. 4. Distribution of the different *in vivo* studies about $nTiO_2$ toxicity, depending on the taxonomic group of the used salt and brackish water species ($n = 20$).

3. Ecotoxicological evidences about $nTiO_2$

3.1. Ecotoxicity results and testing approaches

An overview about research studies carried on the $nTiO_2$ effects toward saltwater organisms is proposed in Table 1. Toxicity data have been ranked according to organisms' taxonomic classes and summarized in Table 5. Moreover, the analysis included the assessment of various testing approaches according to Losso and Volpi Ghirardini (2010), distinguishing between three groups of bioindicators:

1. Test organisms, deliberately exposed to contaminants or contaminated materials (Hahn, 2002);
2. Biomarkers, which are biochemical, physiological or behavioral variations measured in a cell, tissue, biological fluid or the whole organism (individual or population) evidencing exposure and/or effect to one or more contaminants (Depledge and Fossi, 1994);
3. Bioconcentration/bioaccumulation indicators providing information on pollutants not detectable on abiotic matrices, thus measuring the real bioavailability of pollutants and their potential transferability within the trophic web (Losso and Volpi Ghirardini, 2010).

Thus, the kind of the information related to the testing activities has a different ecological representativeness providing various contributions within hazard toxicity assessment.

3.1.1. Bacteria

Most results about bacteria come from the bioluminescence inhibition bioassays. To the best of our knowledge, *V. fischeri* is the only marine bacterium used for assessing the $nTiO_2$ toxicity. Most authors used the Microtox® test looking for the inhibition of the natural bioluminescence that is directly correlated to organisms' level of stress.

From Table 5, it seems that $nTiO_2$ has no effect toward the marine bacterium *V. fischeri* within the tested concentration range (from >100 to >20,000 mg L^{-1}), that are surely not representative of the real environmental concentrations (Müller and Nowack, 2008; Tiede et al., 2009). Certainly, this result cannot be generalized to all bacteria, as only one species was employed for the assessment. Surely, more studies are needed; in particular, it could be interesting to examine the potential direct uptake of $nTiO_2$ from the bacteria as a function of their cell wall nature, as some authors already done with other ENPs, using freshwater species (Balusamy et al., 2012; Jain et al., 2013; Riding et al., 2012).

Only Blaise et al. (2008) carried on the microbial assay for risk assessment (MARA) with *V. fischeri* (*i.e.* colorimetric assay). The more the organisms are stressed, the lower their ability to reduce the dye. Even in this case, no toxicity was found (Blaise et al., 2008).

3.1.2. Algae

The cell growth inhibition bioassay was the only kind of saltwater microalgae test used for assessing $nTiO_2$ considering various testing species as *Dunaliella tertiolecta*, *Isochrysis galbana*, *Phaeodactylum tricornutum*, *Thalassiosira pseudonana* and *Skeletonema* spp.

From these studies, it appears hard to establish whether $nTiO_2$ may represent a hazard because results originated from different species, ENP concentrations and experimental designs. As it can be seen in Table 5, for example, Miller et al. (2010) could not observe any toxicity effect till a maximum tested concentration of 1 mg L^{-1}, thus supposing that $nTiO_2$ is not soluble in water. But, in this case, could just the low solubility of the $nTiO_2$ be the reason of the effect absence? Indeed, it is known that the $nTiO_2$ dispersed in saltwater are quite unstable and tend to form agglomerates within few hours after water contact time, sinking to the bottom of the container (Keller et al., 2010). The algal species taken in consideration by Miller et al. (2010) were all planktonic meaning that their exposure could be reduced along time due to $nTiO_2$ sedimentation.

The experiment of Clément et al. (2013), instead, displayed concentration-dependent inhibitory effects with no significant differences amongst the sizes of the employed ENPs. Finally, from his tests Minetto (2012) did not observe traditional growth inhibition effects but the presence of concentration-dependent cell agglomerates. These could represent a kind of effects that, actually, for the traditional chemicals tests, are not considered valid toxicity endpoints but that in the future, if recurring, they could be proposed like new peculiar endpoints to assay the ENP toxicity, together the traditional ones.

3.1.3. Rotifers

The work of Clément et al. (2013) is the only study reporting results about $nTiO_2$ toxicity toward marine rotifers. It is based on immobilization bioassay with 28 h old *Brachionus plicatilis* juveniles. Results showed an evident inverse proportionality between the toxicity effects and the ENP sizes, as equally seen about the algae by the same authors (Clément et al., 2013).

3.1.4. Annelids

The sandworm *A. marina* is the only marine annelid exposed to $nTiO_2$ (Galloway et al., 2010). Results are relevant and involved various approaches including various levels of biological complexity as well as some histological analysis (*in vivo* toxicity to $nTiO_2$ spiked sediment, behavioral biomarker with the burrowing bioassay, lysosomal membrane stability (LMS) and Comet assay (DNA damage), $nTiO_2$ gut accumulation *via* the analysis of the quantity and the dimensions of ENPs).

The main effects emerged from biomarkers. The quantification of ENPs in organism samples, performed with an ICP-OES, showed the presence of $nTiO_2$ aggregates (>200 nm diameter) around the microvilli of the epithelium cells. Anyway the ENP did not cross the epithelial membrane of the gut lumen in significant quantities (Galloway et al., 2010).

3.1.5. Molluscs

Actually, the more abundant and representative existing information, about $nTiO_2$ effects and seawater organisms, concerns the molluscs. The toxicity tests were performed using two species: *Mytilus galloprovincialis* and *Haliotis diversicolor supertexta*. The information about *M. galloprovincialis* includes both *in vitro* and *in vivo* endpoints. Biochemical and physiological variations were measured by different authors, exposing the whole adult organisms or their parts to $nTiO_2$ (Barmo et al., 2013; Canesi et al., 2009, 2010; Ciacci et al., 2012). Embryotoxicity bioassays were also performed to evaluate the ENP toxicity effects toward 48 h old larvae (Libralato et al., 2013). A double approach was considered to evaluate *H. diversicolor supertexta*, integrating the results of the toxicity bioassay with the measures of various biomarkers (Zhu et al., 2011a,b).

All the authors could observe toxicity effects but, as seen for the algae, it is difficult to compare the data, as the experimental conditions were different.

About the new peculiar endpoints another case must be signaled. Libralato et al. (2013) recorded the existence of ring like mucus structures, surrounding the larvae. It could be suspected that the secretion of mucus may prevent or limit the undesired presence of nanoparticles aggregates around the embryos (Libralato et al., 2013).

3.1.6. Crustaceans

The publications about the marine crustaceans are just related to the genus *Artemia*, more specifically with the anostracan *A. salina* (Ates et al., 2012) and *Artemia franciscana* (Minetto, 2012). However, the overall results are quite representative, because they come from immobilization bioassays, biomarkers measures and bioaccumulation evaluation, performed with both adults and nauplii and diversifying the exposure scenario.

In both the publications, the authors could verify the toxic effects of the $nTiO_2$ in the overall concentration range (0.5–100 mg L^{-1}) (Ates et al., 2012; Minetto, 2012).

3.1.7. Echinoderms

Fairbairn et al. (2011) proposed the only paper about $nTiO_2$ and echinoderms. The authors measured exposed the embryos of *Lytechinus pictus* to various $nTiO_2$ concentrations, observing the effects in the earlier life stages. No induced developmental abnormalities were appreciated (Fairbairn et al., 2011).

3.1.8. Fishes

Paterson et al. (2011) published the only research about $nTiO_2$ toxicity to euryhaline fish *Oryzias latipes*, being able to live in fresh and brackish water. Testing activities included biomarker endpoints and embryotoxicity, after quantifying the ENPs using an UV-spectrophotometer. Although, no mortality effects were seen, the reduction of the hatching time, the altered swimming activity and the different malformations revealed the toxicity of $nTiO_2$ toward the fish embryos (Paterson et al., 2011).

3.1.9. Cetaceans

Only one *in vitro* study exists about the effects of $nTiO_2$ toward cetaceans, referred to *Tursiups truncatus* leukocytes (Bernardeschi et al., 2010). The authors exposed dolphin leukocytes at two crystalline forms of $nTiO_2$ (rutile and anatase). In both crystalline forms, the tests evidenced DNA damages and decreased leukocyte viability (Bernardeschi et al., 2010).

4. Conclusions

From this review paper, it was highlighted the existence of an incomplete database about $nTiO_2$ toxicity on saltwater organisms suggesting that the main challenge to be tackled is still its hazard assessment. In most cases, only one or two papers for just few saltwater testing species are available reporting data obtained mostly in different experimental conditions that are frequently not easy to compare and integrate. Some biological models, such as bacteria and molluscs, were investigated more frequently mainly due to their friendliness (*i.e.* bacteria) or the number of the potentially available endpoints within the same organism (*i.e.* molluscs). In general, the existence of various experimental designs and endpoints, the lack of a real exposure scenarios and the scarcity of the related effect data make the results really hard to approach. Endpoints with invertebrate and vertebrate standardized focus species are missing such as those with sensitive crustaceans (*Acartia spp.*, *Amphibalanus* spp. and *Tigriopus* spp.), sea urchins (*e.g. Paracentrotus* spp., *Strongilocentrotus* spp.) and fish (*e.g. Dicentrarchus labrax*). Particular attention should be paid to sub-lethal effects at various developmental stages taking into consideration phenomena such as bioconcentration and bioaccumulation that are completely absent.

Specifically, it should be important to define methods for toxicity tests with ENPs controlling the variability of experimental conditions and designs. ENPs quantification analysis should be performed both within the test media to know the real organism exposure concentrations and for the test organisms, correlating the potential observed effects with the eventual accumulated ENPs. Actually, a change of the point of view would be desirable. It is increasingly shared the idea of a necessary change in results interpretation, considering parameters and metrics that may be more representative of the ENP behavior, as the agglomeration state and the agglomerate surface area. Great care should be given to the information source, considering the integration of various approaches such as biomarkers, bioassays and bioaccumulation indicators to evaluate a wider number of responses thus increasing the information completeness. Moreover, attention should also be paid on emerging new endpoints besides traditional ones that could start describing real nanoecotoxicological effects.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.envint.2014.01.012.

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