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Embryotoxicity of TiO_2 nanoparticles to *Mytilus galloprovincialis* (Lmk)

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ABSTRACT

Few data exist on the ecotoxicological effects of nanosized titanium dioxide ($nTiO_2$) towards marine species with specific reference to bivalve molluscs and their relative life stages. *Mytilus galloprovincialis* Lamarck was selected to assess the potential adverse effects of $nTiO_2$ (0–64 mg/L) on its early larval development stages (pre-D shell stage, malformed D-shell stage and normal D-shell stage larvae) considering two exposure scenarios characterised by total darkness (ASTM protocol) and natural photoperiod (light/dark). This approach was considered to check the presence of potential effects associated to the photocatalytic properties of $nTiO_2$. Parallel experiments were carried on with the bulk reference $TiCl_4$. The toxicity of $nTiO_2$ showed to be mainly related to its "nano" condition and to be influenced by the exposure to light that supported the increase in the number of pre-D shell stage (retarded) larvae compared to the malformed ones especially at the maximum effect concentrations (4 and 8 mg $nTiO_2$/L). The non-linear regression toxicity data analysis showed the presence of two EC50 values per exposure scenario: a) $EC(50)_1 = 1.23$ mg/L (0.00–4.15 mg/L) and $EC(50)_2 = 38.56$ mg/L (35.64–41.47 mg/L) for the dark exposure conditions; b) $EC(50)_1 = 1.65$ mg/L (0.00–4.74 mg/L) and $EC(50)_2 = 16.39$ mg/L (13.31–19.48 mg/L) for the light/dark exposure conditions. The potential implication of agglomeration and sedimentation phenomena on ecotoxicological data was discussed.

1. Introduction

Engineered nanomaterials are at the forefront of ecotoxicologist agendas due to their increasing use in a broad range of industrial and consumer products. Actually, they are manufactured in increasing amounts year-by-year (Ward and Kach, 2009). Particularly, nanosized titanium dioxide ($nTiO_2$) is used in a variety of industries mainly for catalysis and photocatalysis applications and as an additive in paints, papers, inks, plastics and various consumer products (Menard et al., 2011). These extensive applications have led to a rapid increase in its production rate, which is estimated to reach in the United States $2.5 \cdot 10^6$ tons per year by 2025 (Jemec et al., 2008; Robichaud et al., 2009). It is reasonable to assume that such widespread use of $nTiO_2$ will result in an increased environmental exposure to titania (Hall et al., 2009) that may reach concentrations in surface waters posing a potential threat to aquatic ecosystems (Kaegi et al., 2008; Gottschalk et al., 2009; Scown et al., 2010; Wise and Brasuel, 2011).

Most literature on the ecotoxicity of $nTiO_2$ deals with aquatic organisms such as bacteria, algae, invertebrates, and fish, but there are also case studies considering cell lines and rodents (Zhu et al., 2011a). Mostly, testing species are from freshwaters (Cattaneo et al., 2009). Little is known concerning salt water species such as mollusc bivalves. Indeed, amongst all environmental quality status sentinels, marine bivalves are considered a promising group of bioindicators (OECD, 2010) and may represent a unique target group for nanoparticle toxicity with a special focus on *Mytilus* spp. (Canesi et al., 2012; Kadar et al., 2011, 2012). Essentially, they have most of the characteristics that a good bioindicator should have. They are substantially widespread in all fresh, brackish and salt water environments, having a key ecological role both as filter and deposit feeders according to various species and habitats (His et al., 1997; Kadar et al., 2012). All stages of their life cycles (gametes, embryos, larvae and adults) have been used to define an endpoint for a specific monitoring purpose (acute, sub-chronic or chronic toxicity). Bivalve molluscs toxicity tests are generally easy to perform, highly sensitive, cost-effective and not time consuming, ranging from 24 h to 48 h to obtain a first meaningful result, or even

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lower when some biomarker endpoints are taken into consideration. The main disadvantage is related to the reproductive period, that could be quite short for some species sampled from the wild for the performance of embryotoxicity tests. However, conditioning techniques exist and may help to provide reproductive organisms throughout all the year (Libralato et al., 2010; Mamindy-Pajany et al., 2010; OECD, 2010; Canesi et al., 2012).

In vivo experiments with *Mytilus galloprovincialis* Lamarck were carried on considering various biomarker endpoints after 24-h exposure to $nTiO_2$. Results showed that $nTiO_2$ (22 nm average particle size, 51 m^2/g) induced lysosomal membrane destabilisation in the haemocytes and digestive gland (Canesi et al., 2010). It was also found that $nTiO_2$ stimulated an increase in lysosomal lipofuscin and the lysosomal accumulation of neutral lipids, as well as the enhancement of the activity of catalase and glutathione transferase in the digestive glands. No effect on catalase and glutathione transferase activities in the gills and no mortality were observed (Canesi et al., 2010).

Recently, it has been reported that $nTiO_2$ produced no significant changes in viability of *M. galloprovincialis* hemocytes under various experimental conditions (1, 5 and 10 mg/L) (Ciacci et al., 2012), whereas lysosomal destabilisation occurred at 5 and 10 mg/mL with values of −18 and −39%, respectively. Moreover, it was stimulated the lysozyme release at both concentrations, increasing phagocytosis of Neutral Red-conjugated zymosan particles at 5 mg/mL and strongly decreasing the phagocytic activity at 10 mg/mL. The N-oxides related effects evaluated as *cyt c* reduction were comparatively higher with nZnO than with $nTiO_2$. The assessment of nitric oxide (NO) production indicated that 1 mg $nTiO_2$/ml was ineffective, whereas 5 mg $nTiO_2$/ml produced a time-dependent increase in NO production. The transmission electronic microscopy analysis (TEM) showed that $nTiO_2$ did not apparently affect the hemocytes morphology, even though, after 60 min, nanoparticles were observed within the nucleus (Ciacci et al., 2012). Other authors (Zhu et al., 2011a, 2011b) assessed the acute and subchronic toxicity and oxidative stress on $nTiO_2$ in marine abalone *Haliotis diversicolor supertexta* Reeve. In particular, no developmental effects of $nTiO_2$ were observed at 2 mg/L, but hatching inhibition and malformations were observed at 10, 50 and 250 mg/L (Zhu et al., 2011a). It was observed that 2 mg/L $nTiO_2$ seem to potentiate the toxicity effect of tributyltin (TBT) compared to the action that it may exert alone (Zhu et al., 2011a). The hatching inhibition effective concentration inducing a 50% effect (EC50) after 10 h exposure was set at 56.9 mg/L (28.0–126.3 mg/L) and the malformation EC50 (8 h) at 345.8 mg/L (155.0–1592.6 mg/L), whereas the no observed effect concentration (NOEC) value for both endpoints was about 2 mg/L (Zhu et al., 2011a). Moreover, it was highlighted that after 96 h exposure of adults in semi-static conditions (daily water change with redosing) no acute toxicity was detected within 0.1–10 mg/L (Zhu et al., 2011b). However, the activity of superoxide dismutase significantly increased in the group exposed to 1.0 mg/L $nTiO_2$, whereas glutathione decreased in treatments presenting ≥1.0 mg/L of $nTiO_2$. Furthermore, reactive oxidative species (ROS) generated by illumination of $nTiO_2$ (Jiang et al., 2008) may contribute to disturb the anti-oxidant system (Brown et al., 2004), potentially damaging lipids, carbohydrates, proteins and DNA (Kelly et al., 1998) and disruption of intra-cellular metabolic activities (Long et al., 2006).

The aim of this work was to assess the potential toxicity of $nTiO_2$ compared to its bulk reference (titanium tetrachloride, $TiCl_4$) through the embryotoxicity on *M. galloprovincialis* (48 h-old larvae). A two-way experimental design was used considering a scenario including not only the natural photoperiod, but also total darkness (ASTM, 2004). Thus, data interpretation could take into account the effects associated to both the nano-dimension as well as the photocatalytic properties of $nTiO_2$ (*i.e.* ROS generation in presence of light) mimicking a more environmentally realistic response.

2. Materials and methods

Several sets of experiments were performed to explore the agglomeration and sedimentation behaviour of the test nanomaterial in artificial seawater (ASTM, 2004) within a 50 h monitoring period at 0.01, 0.1, 1 and 10 mg/L of $nTiO_2$. Data are shown in the dedicated paper of Brunelli et al. (2013).

2.1. Preparation of $nTiO_2$ stock suspensions

Nano-Titanium Dioxide (P25, declared purity >99%) from Degussa Evonik (Darmstadt, Germany) was selected for the experimental activities. Bulk titanium ($TiCl_4$) from Fluka-Sigma-Adrich (CAS: 7550-45-0) was considered as the non-nano control within our experiments.

Stock suspensions for toxicity testing were prepared in artificial seawater (ASTM, 2004) from a primary suspension of 1 g $nTiO_2$/L and 1.73 g$TiCl_4$/L. Due to the fact that the bulk titanium standard was dispersed in concentrated HCl, treatment solutions were buffered with NaOH 3 M to a pH value of about 8.30. All suspensions/solutions were sonicated singly (UP–100H, Hielscher Ultrasound Technology, Teltow, Germany) for 20 min at 100 W cooling at the same time the preparing dispersion in an ice bath. Stock suspensions/solutions were arranged at least 1 h before testing to allow them to equilibrate and stored in amber flasks in the dark and thermostated to the target temperature. Suspensions/solutions were resuspended via 10 s vortex before transferring to the exposure vessels. The experimental design considered the following nominal concentrations: 0.5, 1, 4, 8, 16, 32 and 64 mg/L for both $nTiO_2$ and $TiCl_4$.

2.2. $nTiO_2$ characterization

The nanoparticles characterisation (specific surface area, shape, average dimensions and hydrodynamic radius) considered a combination of analytical techniques such as Brunauer-Emmett-Teller (BET) particle sizer (Autosorb®-iQ, QuantaChrome Instruments), Transmission Electron Microscopy (Fei Tecnai T12), Dynamic Light Scattering (DLS) (ZetaSizer Nano ZEN3600, Malvern).

For BET analysis, a sample of 0.7514 g of $nTiO_2$ was outgassed considering a defined heating profile (target temperature (°C) − rate of degree/minute − soak time in minutes) (50/5.00/15–100/5.00/15–150/5.00/15–200/5.00/15–250/5.00/15–300/5.00/600).

For TEM analysis (120 kV), nanoparticles were resuspended in ultrapure water at a nominal concentration of 1440 mg/L, deposited on copper grids (10 μL) with a continuous carbon film coating and observed only once the dispersant was completely evaporated overnight at room temperature.

For DLS analysis, $nTiO_2$ reconstituted seawater (ASTM, 2004) dispersions were assessed at 18.0 ± 0.1 °C (*i.e.* toxicity testing temperature) after 48 h ageing and 1 min hand shaking.

2.3. Toxicity test

Batches of *M. galloprovincialis* were collected along the Adriatic coast (Venice, Italy) during the breeding season (April) and used just few hours after sampling. The embryotoxicity test was performed according to ASTM (2004) and Libralato et al. (2010). Adults were induced to spawn by alternated thermal stimulation cycles (18 ± 1 °C and 28 ± 1 °C). Artificial seawater prepared with analytical grade reagents for gametes collection and embryos

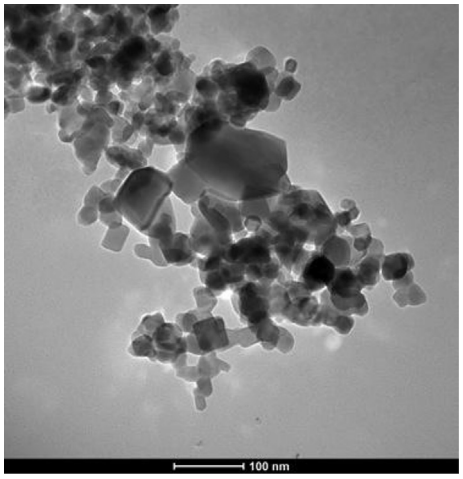

Fig. 1. TEM picture of $nTiO_2$ (P25, Degussa Evonik, Germany) (150000x).

testing was the ASTM one (ASTM, 2004) characterised by a salinity of 34 psu. During each test, gametes derived from a batch of three males and three females were separately filtered at 32 μm (sperm cells) and 100 μm (eggs) to remove impurities. Eggs suspended in 500 mL artificial seawater were fertilized injecting an aliquot of sperm suspension yealding a ratio of approximately 1: 1×10^6 egg/sperm in the final mixture; fertilization success was qualitatively checked by microscopy. Egg density was determined by counting four subsamples of known volume. Fertilized eggs, added to the test solutions in order to obtain a density of about 70 eggs/mL within a 3 mL final volume, were incubated for 48 h at 18 ± 1 °C. At the end of the test, samples were fixed with buffered formalin (4%) and 100 larvae were counted per any replicate suspension, distinguishing between normal larvae (D-shell stage) and abnormalities (malformed larvae – concave, malformed or damaged shell, protruding mantle – and pre-D stages – trochophore larvae or earlier stages). Sample analysis was carried on via an inverted microscope (Leica, Germany) provided with an image acquisition toolbox at 400x enabling to take representative light microscopic pictures.

Sterile capped polystyrene 3 mL 24-well microplates (Iwaki brand, Asahi Techno Glass Corporation, Tokyo, Japan) were used as test chambers for the toxicity test. The acceptability of test results was based on negative controls (ASTM dilution water) for a percentage of normal D-shell stage larvae >70% (ASTM, 2004). A copper solution prepared from copper nitrate standard for atomic absorption spectroscopy in ASTM dilution water was used as positive control considering as reference the following acceptability range 9.47 μg/L ≤ EC50 ≤ 21.72 μg/L (Libralato et al., 2009).

Stock and testing suspensions of $nTiO_2$ and solutions of $TiCl_4$ were prepared within the test day. All suspensions were sonicated for 20 min at 100 W with a UP200S Hielscher Ultrasonic Technology (Teltow, Germany) cooling the preparing dispersions in an ice bath. Once required, light was produced by a set of fluorescent lamps (Philips longlife, 1200 lumen, six lamps in series) to simulate more likely natural levels of sunlight (6000–10000 lux) considering 16 h light/8 h darkness.

Table 2
Results of ANOVA comparison between the ecotoxicological effects from dark and light/dark exposure scenarios; concentrations are in mg/L.

dark \ light	*	0.5	1	4	8	16	32	64
	0.5							
	1							
	4							
	8							
	16							
	32							
	64							

* exposure Concentration
effects do not significatly vary (α = 0.05)

2.4. Data analysis

Toxicity effect data were determined as percentages of abnormal larvae (number of retarded and malformed larvae on 100 counts). EC50 on the exposed populations have been provided as well as the relative confidence limit values at 95%. The responses for each treatment were corrected for effects in the negative control by applying Abbott's formula (ASTM, 2004). The hypothesis test was verified using Analysis of Variance (ANOVA) and Tukey's test to check any difference among the groups after lognormal transformation of concentration data. When ANOVA revealed significant differences among treatments, *post-hoc* tests were carried on with Dunnett's method testing the pairwise difference between each treatment and the control. Parametric or non-parametric methods were considered for points' estimation. All results are presented as means ± standard error using for all statistical analysis the default 5% rejection level. Statistical analyses were performed using XLSTAT (Addinsoft).

3. Results and discussion

3.1. NPs characterisation

The mean average size of primary particles (anatase/rutile 3:1) in artificial seawater (ASTM, 2004) after 48 h ageing in the darkness was 24.00 ± 9.55 nm (n = 724, TEM) (Fig. 1) presenting a shape partly irregular and semi-spherical. The average size of aggregates measured in artificial seawater per every single treatment was in the range 217–590 nm at 18.0 ± 0.1 °C (DLS) as reported in details per every single treatment in Table 1. The $nTiO_2$ specific surface area was 4.478 m^2/g (BET) (intercept = 0.2919, r = 0.9999, constant = 2664.24); it was classified as microporous.

3.2. Embryotoxic effects of $TiCl_4$

The negative controls for both dark (11% ± 0% of effect) and light/dark (12% ± 2% of effect) scenarios complied with the standard value (<30% of effect) (ASTM, 2004) and showed to be not significantly different ($p < 0.05$). Moreover, the reference toxicant

Table 1
Trend of the size of $nTiO_2$ (P25, Degussa Evonik, Germany) agglomerates in artificial seawater (ASTM, 2004).

$nTiO_2$ (mg/L)	0	0.5	1	4	8	16	32	64
Average size of agglomerates (nm)– 18.0 ± 0.1 °C (n = 3)	–	217 ± 24	252 ± 54	303 ± 106	473 ± 105	469 ± 66	426 ± 72	590 ± 59

exhibited the expected effect generating an EC50 = 10.31 μg/L (9.35–11.43 μg/L) (Probit analysis) falling within the range reported in Libralato et al. (2009). According to Fig. 2, all tested concentrations in both experimental conditions presented a percentage of normally developed D-shell stage larvae averagely equal or greater than 79%. Toxicity effects are mainly related to the presence of retarded larvae (6–17%), while the amount of malformed D-shell stage larvae is rather small (2–6%). This suggests that toxicity effects occurred at an early stage of the embryo larval development after the zygote formation. No EC50 values were determined and the maximum effects were evidenced at 64 mg/L in both scenarios as showed in Fig. 2. In particular, experiments carried on in total darkness showed a maximum effect of 20% ± 4% ($n = 3$), whereas the light/dark scenario generated a maximum effect of 21% ± 5% ($n = 3$). They are significantly different from the negative controls ($p > 0.05$), but the difference between them is not considered significant ($p < 0.05$). Thus, the presence of light during the 48 h test with mussel embryos exposed to $TiCl_4$ treatments does not significantly affect toxicity compared to its total absence. Moreover, considering the adjustment for the effects within the negative controls according to Abbott's formula, the net maximum adverse effects are 12% ± 4% (dark) and 14% ± 5% (light/dark), respectively.

3.3. Embryotoxic effects of TiO_2 nanoparticles

The effects in the negative controls for both dark (12% ± 4%, $n = 3$) and light/dark (16% ± 2%, $n = 3$) scenarios complied with the standard value (<30% of effect) (ASTM, 2004) and were not significantly different ($p < 0.05$). Moreover, the reference toxicant showed the expected effect generating an EC50 = 11.35 μg/L (10.57–12.19 μg/L) (Probit analysis) falling within the range reported in Libralato et al. (2009).

Toxicity data for both scenarios were presented in Fig. 3 including details about the contributions to the final toxicity due to the amount of malformed D-shell stage larvae and retarded pre-D shell stage larvae compared to normally developed ones. The trend of adverse effects is similar in both experimental conditions evidencing that the maximum effects occurred at 4 and 8 mg/L. These concentrations presented a prevailing amount of malformed D-shell stage larvae rather than retarded larvae (pre-D shell stage). Nevertheless toxicity effects might have occurred since the beginning of the exposure, they did not substantially impaired the embryo larval development, but induced the generation of malformed larvae after the first metamorphosis from the trochophore stage larva to the D-shell one. Particularly, the amount of retarded larvae

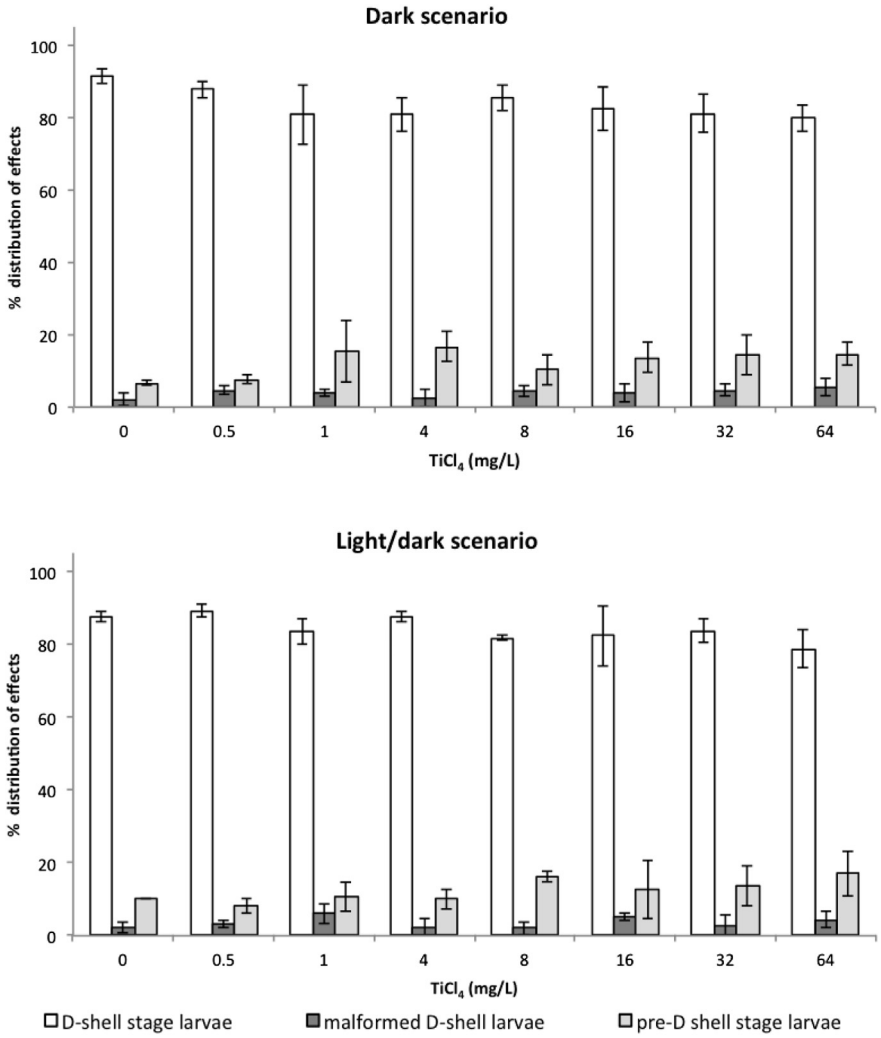

Fig. 2. Trend of $TiCl_4$ toxicity data in both dark and light/dark exposure scenarios showing in details the contributions as percentage of normal D-shell stage, malformed D-shell stage and pre-D shell stage (retarded) larvae to the final toxicity.

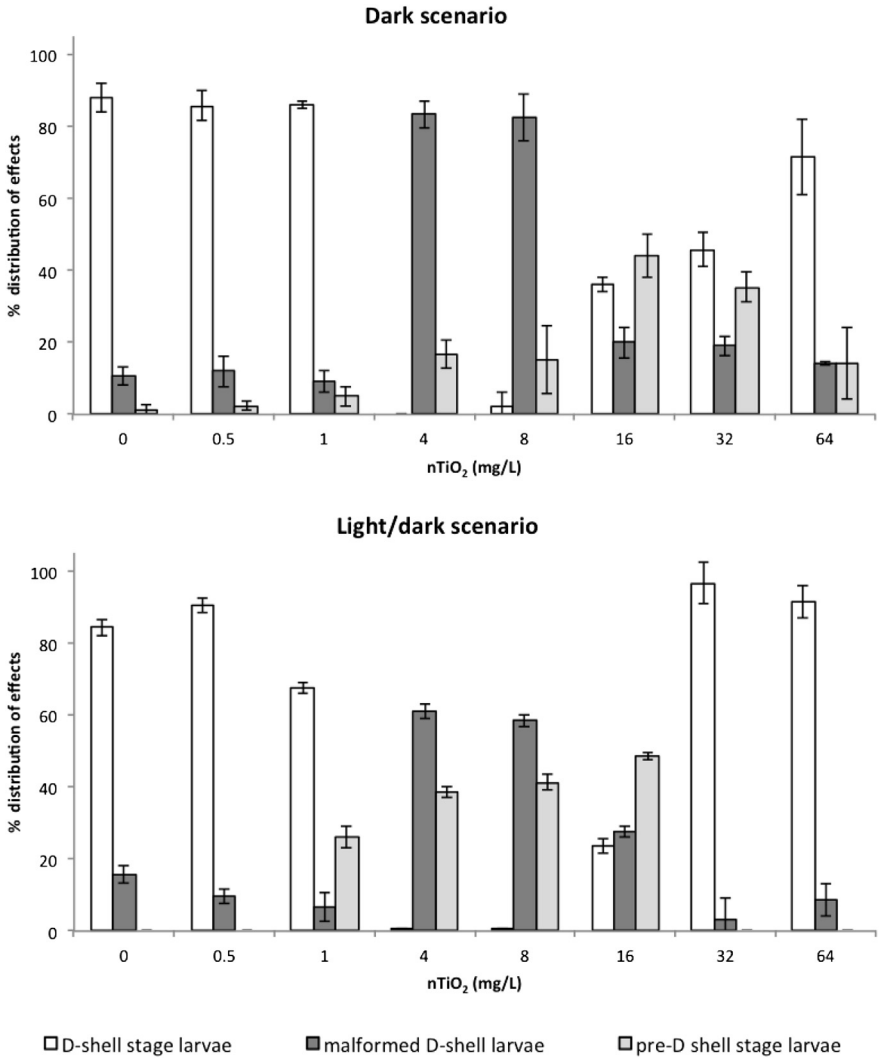

Fig. 3. Trend of $nTiO_2$ toxicity data in both dark and light/dark exposure scenarios showing in details the contributions as percentage of normal D-shell stage, malformed D-shell stage and pre-D shell stage (retarded) larvae to the final toxicity.

at 4 and 8 mg/L is more than double in the light/dark scenario compared to the dark one ($p < 0.05$) suggesting that the exposure to light increased the adverse effects of $nTiO_2$.

The reason why the maximum adverse effects were detected in both scenarios at 4 and 8 mg/L remained unclear. Actually, it could be suspected that the specific dimensional range of $nTiO_2$ aggregates at 4 and 8 mg/L is in some way more bioavailable than those of the other treatments and appeared to be maximised at 303 ± 106 nm and 473 ± 105 nm (Table 1), respectively. Anyway, this is a high speculative explanation that will require more focused experiments due to both the limits of the DLS technique (tendency to data overestimation, need of highly stable suspesions and a relatively high number of particles per unit volume for a representative output) and the fact that $nTiO_2$ agglomerates similar in size (16 and 32 mg/L, Table 1) to the above mentioned ones led to only minor toxicity effects in both scenarios.

In the dark exposure scenario, the ANOVA evidenced that the effects on negative controls always differ from treatments except for 0.5 and 1 mg/L ($p < 0.004$). Considering the differences within treatments, they always differ from each other except for 0.5–1, 4–8 and 16–32 mg/L ($p > 0.05$). Moreover, in the light/dark exposure scenario the ANOVA showed that the effects on negative controls always differ from treatments except for 0.5 and 64 mg/L ($p < 0.001$). Considering the differences within treatments, they always differ from each other except for 0.5–32, 0.5–64, 4–8 and 32–64 mg/L ($p > 0.05$). Considering the comparison between dark and light/dark exposure scenarios, the two groups of negative controls do not significantly differ from each other ($p < 0.01$). Similarly, it occurred for the assessment within treatments as summarised in Table 2 for 0.5–0.5, 0.5–32, 0.5–64, 1–0.5, 1–32, 1–64, 4–4, 4–8, 8–4, 8–8 and 64–1 mg/L ($p < 0.05$) for dark and light/dark exposure scenarios, in that order.

Besides, the maximum effects observed for both scenarios at 4 and 8 mg/L, the lowest effects were detected at the 0.5 mg/L of $nTiO_2$ for both dark and light/dark experiments presenting an average effect of 14.33% ± 4.04% and 9.67% ± 2.08% in that order. Moreover, further low toxicity effects were detected at 32 mg/L of $nTiO_2$ within the light/dark experiments (3.33% ± 5.77%) and at 64 mg/L of $nTiO_2$ within the dark scenario (28.67% ± 10.50%).

Toxicity data have been plotted in Fig. 4 as toxicity frequencies within the 0–1 range against the exposure concentrations after their lognormal transformation in order to highlight the results obtainted at the lowest tested concentrations (0.5 and 1 mg/L). Each data set was analysed for the best fit. Only non-linear regression analysis provided suitable correlation coefficients (R^2) of about 0.80 as shown in Fig. 4. As a consequence, two likely EC50

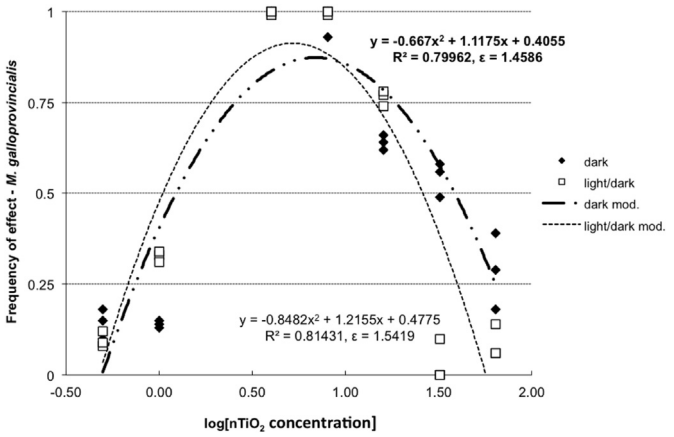

Fig. 4. Toxicity data distribution as percentage of effect (abnormal and retarded larvae) as frequency plotted against $nTiO_2$ concentration after lognormal transformation for both dark and light/dark exposure scenarios. The two types of dashed lines describe the potential modeled trend of the two datasets.

solutions may be obtained from each equation for both exposure scenarios: a) $EC(50)_1$ = 1.23 mg/L (0.00–4.15 mg/L) and $EC(50)_2$ = 38.56 mg/L (35.64–41.47 mg/L) for the dark exposure scenario; b) $EC(50)_1$ = 1.65 mg/L (0.00–4.74 mg/L) and $EC(50)_2$ = 16.39 mg/L (13.31–19.48 mg/L) for the light/dark exposure scenario. This fact suggests that hazard assessment of $nTiO_2$ should take care of considering primarily the lowest EC50 value according to the precautionary principle. It can be observed that there is apparently no significant difference ($p < 0.05$) between the $EC(50)_1$ values from both scenarios. Conversely, the $EC(50)_2$ of $nTiO_2$ from the light/dark scenario is lower than the $EC(50)_2$ of the dark scenario, meaning that exposure to light might have increased the toxicity effect of $nTiO_2$. Nevertheless, two exceptions were found, apart the EC50 values determined on the basis of the best fit approach. Indeed, the effects detected within the light/dark scenario at 32 and 64 mg/L, the highest tested concentrations, were significantly lower ($p < 0.05$) than the dark one without any clear explanation at the moment. According to the ecotoxicological results obtained for the bulk reference $TiCl_4$ in both exposure scenarios, most part of the adverse effects of $nTiO_2$ can be attributed to it "nano" condition. Indeed, the effects of $TiCl_4$ adjusted for the effects in the control via the Abbott's formula are 4% ± 2% and 11% ± 5% at 4 mg/L and 10% ± 1% and 6% ± 4% at 8 mg/L for the dark and light/dark scenarios, in that order.

The existence for *M. galloprovincialis* of non-linear responses in the recorded toxicity effects may be due to a wide series of events related to the exposure to $nTiO_2$ that cannot be adequately investigated in the present paper starting from direct/indirect cell entrance at an early embryo larval stage to ingestion rate and egestion dynamics of $nTiO_2$ starting from the D-shell larvae stage. Within all these scenarios, the presence of light showed to significantly influence the ecotoxicological effects increasing the number of retarded larvae compared to malformed ones at least in coincidence to the maximum effect concentrations (4 and 8 mg/L).

Moreover, ecotoxicological effects can be potentially related to the level of bioavailability of nanomaterials and thus to their agglomeration and sedimentation behaviour in synthetic seawater (ASTM, 2004). Aggregation may influence the pathway of trophic transfer of nanoparticles as already suggested by Alber and Valiela

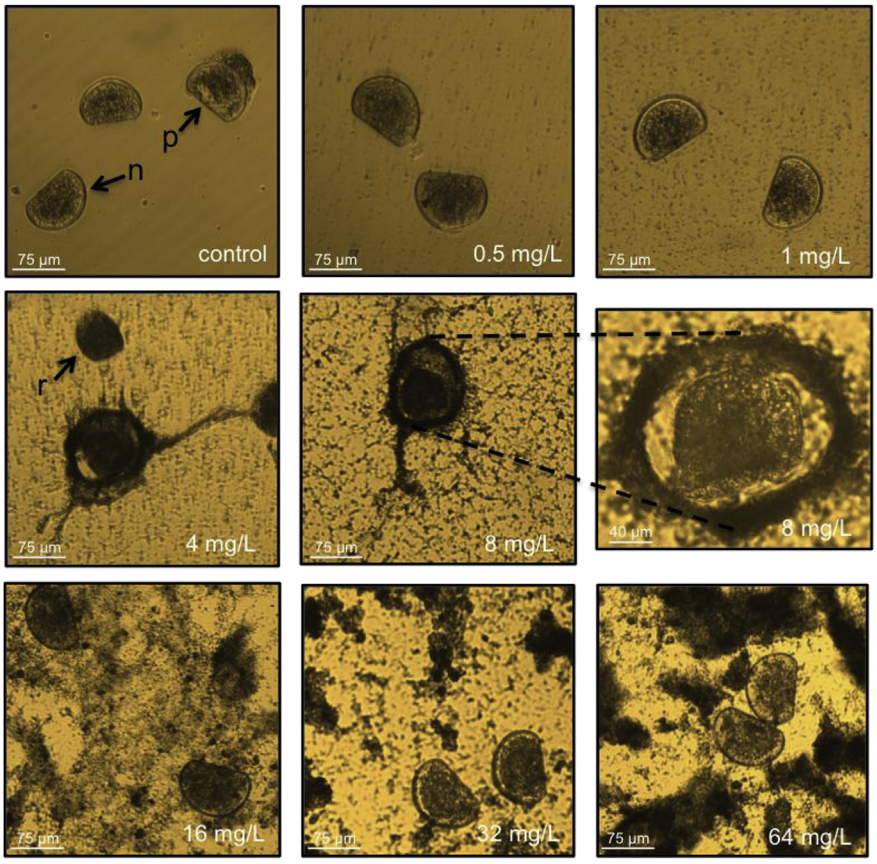

Fig. 5. Normally developed and abnormal (malformed or retarded) *M. galloprovincialis* larvae after 48 h from fertilization exposed to the 0.5, 1, 4, 8, 16, 32 and 64 mg/L $nTiO_2$; n = normal larva; p = protruded-mantle larva; r = retarded larva – trochophore stage.

(1994) and Kach and Ward (2008) for bivalves in their adult stage. Apparently, no data are available for bivalves ingestion rate and egestion dynamics during their larval stage such as the D-shell stage considered in the present research. A similar trend in toxicity response was observed by Spangenberg and Cherr (1996) on *Mytilus californianus* (Conrad) larvae exposed to $BaSO_4$. Indeed, $BaSO_4$ above certain concentrations occurs as an insoluble precipitate and thus is no more bioavailable in seawater highlighting that lower level of Ba in seawater appear to result in greater toxicity than do higher levels. Similarly, it might occur in relation to the size of $nTiO_2$ aggregates where the rate of aggregation might control its bioavailability. Indeed, Brunelli et al. (2013) explored the agglomeration and sedimentation behaviour of $nTiO_2$ (P25, Degussa Evonik, Darmstadt, Germany) in dispersions prepared according to the same protocol used in this work over an observation time of 50 h. The results showed that a fast agglomeration takes place in the concentration range 0.01, 0.1, 1 and 10 mg/L, followed by sedimentation of agglomerates. Extent and rate of $nTiO_2$ agglomeration and sedimentation depended mostly on its initial concentration and only to a minor extent on salt content, ionic strength, and dissolved organic carbon (Brunelli et al., 2013). Substantially, the actual exposure concentration was lower than the nominal concentration and decreased over time (50 h) by a factor from 2 to 20, approximatively. Although these results did not account for the consequences on $nTiO_2$ behaviour due to the presence of biota, they suggested a potential underestimation of the toxicity effects and the related parameters such as the effective concentration values (*i.e.* the adverse effect is observed at a concentration that is different, *i.e.* lower, than the nominal one) within the simplified model scenario. This could be considered as a first step towards the comprehension of how $nTiO_2$ acts in seawater media, being necessary to understand also how the biotic component might influence the way it is exposed to $nTiO_2$ investigating for example resuspension phenomena due to larvae swimming activities or aggregation events enhanced by extracellular compounds released into the contaminated aqueous media (*i.e.* mucous). Of course, this kind of observation is highly linked to the single testing protocol taken into consideration and to the relative testing species suggesting a potentially species-specific acting behaviour.

Fig. 5 summarised the best pictures available from both experimental conditions presented in Figs. 3 and 4. From Fig. 5, it can be highlighted the presence of a visible amount of sedimented agglomerates (*i.e.* after 48 h of exposure in artificial seawater) increasing from the upper left corner (negative control) to the bottom right one (64 mg/L). It can be appreciated that normal D-shell larvae are also present at very high exposure concentrations (16, 32 and 64 mg $nTiO_2$/L) as also diplayed by the ecotoxicological results of Figs. 3 and 4. Moreover, besides the presence of retarded or malformed larvae, it can be noticed in Fig. 5 the presence of ring-like bidimensional structures surrounding some larvae mostly at the pre-D trochophore stage. In particular, they were found mainly at 4 and 8 mg/L of $nTiO_2$ in both dark and light/dark scenarios accounting for about 1–3% of the total amount of abnormalities. The presence of these ring-like structures might be the result of an increase in the general embryo stress, causing its development delay as well as activating defensive systems such as the secretion of mucus preventing or limiting the undesirable presence of nanoparticles aggregates. Thus, the toxicity effects of $nTiO_2$ might also result in an energy reallocation from growth and development to the support of defensive mechanisms.

4. Conclusions

This paper assessed for the first time the $nTiO_2$ toxicity effects on mussel early larval stages considering dark and light/dark exposure scenarios within the 0–64 mg/L concentration range in artificial seawater. In parallel, the same experiments were carried on with $TiCl_4$ as the bulk reference. The toxicity of $nTiO_2$ showed to be mainly related to its "nano" condition and to be influenced by the exposure to light that amplified the ecotoxicological effects increasing the number of retarded larvae compared to the malformed ones obtained in total darkness especially at 4 and 8 mg/L. A non-linear regression analysis fitted the obtained transformed data showing that the adverse effects are similar or slightly different from the negative controls at the lowest and highest tested concentrations, whereas they were maximised at 4 and 8 mg $nTiO_2$/L. Due to the second order of the curves, two pairs of EC50 were identified per every experimental condition. Even though, the reason why the maximum ecotoxicological effects were detected in both scenarios at 4 and 8 mg/L remained unclear, suggestions were made about how the bioavailability of $nTiO_2$ might be influenced by the dimension of its agglomerates. This is an interesting topic that should be further assessed to provide not only the actual bioavailable exposure to $nTiO_2$ in ecotoxicological tests, but also for understanding its seawater mobility and fate in the biota and the environment.

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