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Potential effects of TiO_2 nanoparticles and $TiCl_4$ in saltwater to *Phaeodactylum tricornutum* and *Artemia franciscana*

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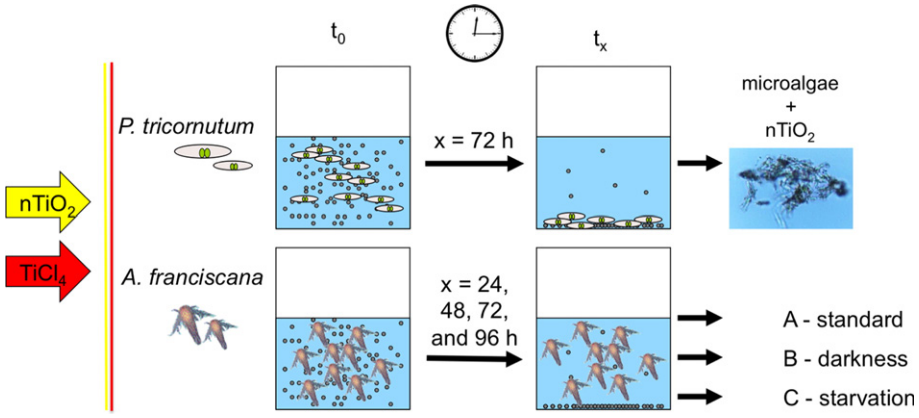
HIGHLIGHTS

- *P. tricornutum* and *A. franciscana* were used to investigate $nTiO_2$ and $TiCl_4$ toxicity.
- Brine shrimps exposure included standard and alternative scenarios.
- Toxicity for microalgae was: $TiCl_4 > nTiO_2$.
- Toxicity for brine shrimps was: $TiCl_4 < nTiO_2$.
- Dark conditions minimized adverse effects of $nTiO_2$ conversely to starvation.

GRAPHICAL ABSTRACT



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ABSTRACT

Nanosized titanium dioxide ($nTiO_2$) is widespread in many commercial products and several authors investigated its ecotoxicity effects focusing mainly on freshwater environments. Data on saltwater species are still lacking or present contradicting results. We compared for the first time the toxicity of $TiCl_4$ and $nTiO_2$ considering standard toxicity tests with microalgae *Phaeodactylum tricornutum* (growth inhibition test, 1.8–90 mg/L) and crustacean *Artemia franciscana* (mortality test, 0.5–64 mg/L). For *A. franciscana*, two alternative scenarios were considered beside standard protocol: i) darkness; and ii) starvation. About microalgae, results evidenced that effects of $TiCl_4$ (EC50 = 63 mg/L) were greater than $nTiO_2$ (no EC50), but IC10 and IC20 were significantly lower suggesting that $nTiO_2$ is more harmful than $TiCl_4$ at lower concentrations. The effects of $TiCl_4$ to crustaceans larvae in all exposure scenarios were lower compared to $nTiO_2$ (EC50(96 h) = 15 mg/L - standard protocol). During toxicity testing, the absence of light generally lowered $nTiO_2$ effects while starvation increased the toxicity of both $TiCl_4$ and $nTiO_2$.

1. Introduction

Nanosized titanium dioxide ($nTiO_2$) is present in various products for its antibacterial and depolluting properties like the generation of

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hydroxyl radical (·OH) when exposed to ultraviolet (UV) light (Hochmannova and Vytrasova, 2010; Bundschuh et al., 2011). Thus $nTiO_2$ can be found in paints (Fujishima et al., 2008; Clemente et al., 2012), road and pavement structures for NO_x abatement (Chen and Poon, 2009), cosmetics and sunscreens stabilizing ingredients (Mu and Sprando, 2010).

Release of $nTiO_2$ can occur during industrial production, use, and disposal of consumer products having as final sink the aquatic environment (Krishnakumar, 2005; Buffet et al., 2011; Wang et al., 2016).

Saltwater media can specifically condition $nTiO_2$ dispersion and stability due to salinity and ionic strength (Brant et al., 2005; French et al., 2009; Corsi et al., 2014) influencing aggregation dynamic and sedimentation processes (Keller et al., 2010; Brunelli et al., 2013). Effective scenarios could drastically change during the exposure time due to nanoparticles (NPs) sedimentation making the match between primary and secondary characterizations, and ecotoxicological results very difficult to interpret according to the considered biological model. Sedimentation may underexpose planktonic and nektonic organisms and overexpose benthic ones.

Currently, $nTiO_2$ effect data are available for bacteria and molluscs (Minetto et al., 2014, 2016), while several gaps are still present for algae and crustaceans (Libralato et al., 2013; Libralato, 2014; Minetto et al., 2016).

Miller et al. (2010) assessed the effects of $nTiO_2$ (anatase and rutile; 10, 100, 500 and 1000 μg/L nominal concentrations) on *Dunaliella tertiolecta, Isochrysis galbana, Skeletonema marinoi* and *Thalassiosira pseudonana*. After 96 h, no cell growth inhibition was observed due to its low solubility in seawater. Miller et al. (2012) investigated the potential effects of $nTiO_2$ with and without UV-light on *D. tertiolecta, I. galbana, S. costatum* and *T. pseudonana* (1, 3, 5 and 7 mg/L nominal concentrations). No effect was observed when UV-light was switched off. In presence of UV-light, *S. costatum* showed no adverse effect, but all others evidenced a range of effects at various exposure concentrations. Growth inhibition was set at 1 mg/L for *I. galbana,* and at 3 mg/L for *D. tertiolecta* and for *T. pseudonana*. Han et al. (2011) investigated the effects of $nTiO_2$ on Cd^{2+} uptake by *D. salina*. In presence of 10 μg/L, results showed that Cd^{2+} was uptaken over 50% decreasing its median inhibition concentration (IC50) from 682 to 366 μg/L. Clèment et al. (2013) exposed for 72 h under constant illumination *Phaeodactylum tricornutum* to $nTiO_2$ (0.01–100 mg/L nominal concentrations) using anatase NPs with various dimensional ranges. Results highlighted concentration-dependent inhibitory effects and an inverse proportionality between toxicity effects and the size of NPs. Li et al. (2015) investigated the effects of $nTiO_2$ (5, 10, 20 and 30 mg/L nominal concentrations) on *Karenia brevis* and *S. costatum* for 72 h (14:10 h light dark). Cell growth inhibition was concentration dependent with IC50s equal to 11 and 7 mg/L for *K. brevis* and *S. costatum*, respectively. Changes in stress related biomarkers were observed as well including chlorophyll *a* decrease. Xia et al. (2015) exposed *Nitzschia closterium* to $nTiO_2$ (5–500 mg/L and size: 21, 60 and 400 nm) determining a direct dependency of cell growth inhibition to NPs concentration. After 96 h, IC50s were 89, 119 and 179 mg/L for 21, 60 and 400 nm, respectively. Reactive oxygen species (ROS) level, and superoxide dismutase (SOD), catalase (CAT) and defence-related peroxidase (POD) activities increased from 1.1 to 1.7 times. NPs adhesion to algae cell walls could have probably caused stress events (Callegaro et al., 2015). Wang et al. (2016) exposed *P. tricornutum* to uncoated $nTiO_2$ (Emperor Nanomaterial Corporation, Nanjing, China) (50–200 mg/L) in f/2 media checking for growth inhibition (up to 13 days), chlorophyll *a*, cell integrity and ROS. Exposed cultures were resuspended every 8 h. Growth inhibition of $nTiO_2$ showed EC50 values of 168, 204 and 168 after 72, 96 and 120, respectively, but no confidence intervals are available. Chlorophyll *a* trend like ROS showed $nTiO_2$ low toxicity. About ROS, the strong antioxidative defence system of this microalgae was able to overcome the weak stress induced by $nTiO_2$.

Effects of $nTiO_2$ on saltwater crustacean are mainly focused on *Artemia salina*. Most data were already summarised in Libralato (2014), Callegaro et al. (2015) and Libralato et al. (2016). Rajasree et al. (2011) exposed *A. salina* larvae at five developmental stages and adults after $nTiO_2$ suspension sonication (0.2–10 mg/L nominal concentrations) or filtration (50–500 mg/L). Sonication produced concentration and time dependent median lethal concentration in adults (LC50(96 h) = 98 mg/L) with little mortality in larvae. Filtration showed a similar trend but with higher toxicity levels. The Instar I larvae showed 43% mortality at 10 mg/L, and 86% for Instar II. At the other developmental stages, 100% effect was observed at 10 mg/L. Ates et al. (2013) investigated $nTiO_2$ effects on both *A. salina* nauplii and adults (96 h) considering a multi-endpoint approach (i.e. biomarker, lethality and bioaccumulation) (10, 50 and 100 mg/L nominal concentrations) keeping organisms in starvation. Instar I nauplii and adults showed $nTiO_2$ accumulation increasing with exposure concentration and time; adults accumulated more than nauplii. Mortality presented a maximum effect of 18% for nauplii at 100 mg/L. After 24 h exposure, lipid peroxidation (LPO) was absent, but malondialdehyde levels (MDA) (96 h) suggested that lethality was caused by oxidative stress for both nauplii and adults.

The effects of anatase (A) (100%), and A (80%) and rutile (R) (20%) mixture (A + R) were investigated by Clemente et al. (2014) considering two exposure scenarios (sunlight and UV-light) on *A. salina* nauplii (48 h). Organisms were exposed at 1, 10 and 100 mg/L of $nTiO_2$. UV-light significantly increased lethality. LC50s (24 h) decreased from 949 (A) and 945 (A + R) mg/L to 14.40 (A) and 16.68 (A + R) mg/L under UV-light mainly due to ROS. Nogueira et al. (2015) investigated $nTiO_2$ effects on *A. salina* exposing 48 h old larvae for 24 h (8.2, 10.2, 12.8, 16.0 and 20.0 mg/L analytical concentrations). No effects were detected, but NPs aggregation was evidenced as the main bias for future assessment. Callegaro et al. (2015) investigated $nTiO_2$ with both *P. tricornutum* (0.9–90 mg/L for 72 h) and *A. franciscana* (0.01–90 mg/L up to 48 h) with and without the use of alginate as a potential exposure stabilizer. Effects within the considered exposure range did not show any adverse effects related to alginate even though it was evidenced a reduced bioavailability of $nTiO_2$ related to its presence.

This research investigated $nTiO_2$ and $TiCl_4$ effects on *P. tricornutum* and *A. franciscana* in order to compare for the first time their effects in saltwater. Standardised methods were taken into consideration. For crustacean, alternative exposure scenarios were checked including total darkness and starvation.

2. Materials and methods

2.1. Chemicals

2.1.1. Nanoparticles characterisation

Aeroxide® P25 $nTiO_2$ powder (Evonik Degussa Corporation, Darmstadt, Germany) is a mixture of approximately 80% anatase and 20% rutile with a declared particle size of about 21 nm. It was stored in the dark at room temperature until use. Particle morphology and size were examined by transmission electron microscopy (TEM) with a Jeol 3010 (Tokyo, Japan) operating at 300 kV. TEM samples were prepared as follows: i) deposition of a drop of $nTiO_2$ dispersion in ultrahigh-purity water on a carbon-coated copper TEM grid; and ii) water evaporation at room temperature prior to analysis. The Brunauer, Emmett and Teller (BET) (Brunauer et al., 1938) method was used to obtain surface area and pore volume by nitrogen adsorption on a Micromeritics (Norcross, GA, USA) ASAP 2000 instrument at an adsorption temperature of −96 °C after pre-treating the sample under high vacuum at 300 °C for 2 h. Details about $nTiO_2$ characterisation are reported in Brunelli et al. (2013).

2.1.2. Media, dispersion and solution preparation

As testing medium and dispersing agent, artificial seawater (ASW) was prepared according to ISO (2006) for algae (ASW1) and ASTM (1998) for crustacean (ASW2) using analytical grade reagents (purity

>99.8%) and ultrapure water (18.2 MΩ cm, Milli-Q, Millipore). One night aeration was useful to stabilise their pH within 8.00–8.40. Prior to storage at 4 ± 1 °C in the dark, ASWs were filtered using 0.45 μm glass fibre filters (Whatman International Ltd).

Stock suspension of $nTiO_2$ (1 g/L) and solution of $TiCl_4$ (3 g/L) (Carlo Erba, Italy) were prepared according to Taurozzi et al. (2012) and Libralato et al. (2013), respectively. Prior to sample suspension aliquots for treatment preparation, the stock suspension was sonicated in an ice bath for 15 min at 100 W by means of a sonic probe (UP-100H, Hielscher, Teltow, Germany). The stock solution of $TiCl_4$ was buffered at pH = 8.41 with 1 M NaOH. Treatment suspensions and solutions were prepared 24 h before the beginning of toxicity tests and sonicated in an ice bath for 15 min at 100 W before transferring them into the testing vessels. Ecotoxicological exposure scenarios were designed according to Libralato et al. (2013). Toxicity tests were static: no agitation or resuspension occurred during the exposure period.

2.2. Ecotoxicity

2.2.1. Growth inhibition test with P. tricornutum

Short-term chronic toxicity tests with the benthic diatom *P. tricornutum* were carried out according to UNI EN ISO (2006) (Libralato et al., 2011). Maintaining the algal culture (lot. PT070509, SAG strain, Gent, Belgium) at 20 ± 2 °C and 6000–10,000 lx, we obtained a cellular density of >106 cells/mL after 3 ± 1 days useful to obtain the initial test algal density of about 10^4 cells/mL. The experimental design included the exposure of *P. tricornutum* for 72 ± 2 h to increasing nominal concentrations of $nTiO_2$ and $TiCl_4$ (1.8–90 mg/L) at 20 ± 2 °C and 14 h light (6000–10,000 lx) using sterile polystyrene micro-plates with lids (3 mL 24 wells) as testing vessels. Negative and positive ($K_2Cr_2O_7$) controls were included in each experiment. Experiments were carried out in triplicate. Cell density was assessed using a Bürker counting chamber.

The point estimation and the calculation of the growth inhibition concentrations after 10%, 20% and 50% population response (IC10, IC20 and IC50) and their relative 95% confidence limit values were carried on by a linear regression model after natural logarithm data transformation of the measured cell density corrected for blank. The significant difference for experimental treatments and controls was investigated by the analysis of variance (ANOVA) ($\alpha = 0.05$). If necessary, post hoc tests were carried on with Dunnett's method and Tukey's test. Statistical analyses were performed using Microsoft® Excel 2013/XLSTAT®-Pro (Version 7.2, 2003, Addinsoft, Inc., Brooklyn, NY, USA).

2.2.2. Mortality test with A. franciscana

The acute mortality tests with *A. franciscana* larvae were carried out according APAT and IRSA-CNR (2003) and Libralato et al. (2007) and Libralato (2014). Prior to toxicity testing, about 100 mg of cysts (lot. AF/F2006, Gent, Belgium) were incubated in a Petri dish in 12 mL of Instant Ocean® (35 ± 1‰) at 25 ± 1 °C (1 h under 100 W light and 23 h in darkness). After 24 h, hatched Instar I nauplii were transferred in 12 mL of Instant Ocean® for 24 h (25 ± 1 °C) to allow their further growth up to Instar II-III. Experimental design included the exposure of *A. franciscana* larvae for 96 ± 1 h at 25 ± 1 °C to increasing concentrations of $nTiO_2$ and $TiCl_4$ (0.5–64 mg/L) counting dead nauplii (Instar II-III) after 24, 48, 72 and 96 h. Nauplii (Instar II-III) were exposed (ten nauplii per treatment) in sterile polystyrene micro-plates with lids (24 wells × 3 mL) as testing vessels with a 2 mL operative volume. Experiments were carried out in triplicate. After 48 h, 100 μL of *P. tricornutum* suspension at 2×10^6 cells/mL was used to feed larvae. Larvae were considered as dead if after mechanical stimulation with a tip any movement was appraised for 10 s. Negative (ASW1) and positive ($CuSO_4$) controls were included in each experiment. Data were analysed as specified for *P. tricornutum*. Point estimation including lethal concentration after 10%, 20% and 50% population response (LC10, LC20 and LC50) occurred via linear and non-linear parametric approaches (e.g. polynomial functions).

The experimental design was iterated three times considering various scenarios: i) APAT and IRSA-CNR (2003) (scenario A) (with 14: 10 h light: dark photoperiod at 4000 lx); ii) APAT and IRSA-CNR (2003) with total darkness (scenario B) like in Libralato et al. (2007); iii) APAT and IRSA-CNR (2003), but with larvae starvation (scenario C). The scenario B investigated how the photocatalytic properties of $nTiO_2$ can impact toxicity, while the scenario C evaluated the lack of feeding in changing the potential effect.

3. Results and discussion

3.1. Behaviour of $nTiO_2$ in artificial seawater

Brunelli et al. (2013) reported detailed characterisation results about how $nTiO_2$ behaves in ASW1 and ASW2. During the present study, salinity (Atago, Japan) and pH were 32 ± 1‰ and 8.25–8.28 for ASW1, and 34 ± 1‰ and 8.27–8.31 for ASW2 as in Brunelli et al. (2013). Results were summarised in S1 for Brunauer Elmet Teller, inductively coupled plasma mass spectrometry, selected area electron diffraction, and transmission electron microscopy analyses.

3.2. Toxicity testing results

3.2.1. Effects on P. tricornutum

Results from negative and positive controls were in accordance with ISO (2006) (S2). In Fig. 1, effects as growth inhibition percentage (%) were reported for both $nTiO_2$ and $TiCl_4$. No stimulation effects were detected. For $nTiO_2$, effects ranged between 1% (0.9 mg/L) and 28% (36 mg/L). The 50% effect was not achieved between 1.8 and 90 mg/L after 72 h; only IC10 (14 mg/L) and IC20 (16 mg/L) values were available ($y = 6.44 + 7.41$ x, $R^2 = 0.30$, MSE = 50.25). These results confirmed that the toxicity of $nTiO_2$ to *P. tricornutum* as EC50 could be determined only for exposure periods longer than 72 h (up to 5–13 days) and exposure concentrations (up to 200 mg/L) like in Wang et al. (2016). Results accounted for slightly higher test sensitivity than in Callegaro et al. (2015) (18% growth inhibition effect at 45 mg/L) nevertheless the use of the same protocol and microalgae population. A difference in nanoecotoxicological data for the same NP seems quite common. Kahru and Dubourguier (2010) observed that variability in results could rise not only using different NP, but also considering the same NP exposed to various groups of biological models. Scown et al. (2010) evidenced that effects towards bacteria, algae and invertebrates can change even using the same NP. Nevertheless this benthic microalgae was overexposed to $nTiO_2$ during testing period (72 h) according to Brunelli et al. (2013) because of $nTiO_2$ settling (96% after 50 h at 10 mg/L), only little toxicity effects were detected (Table 1)

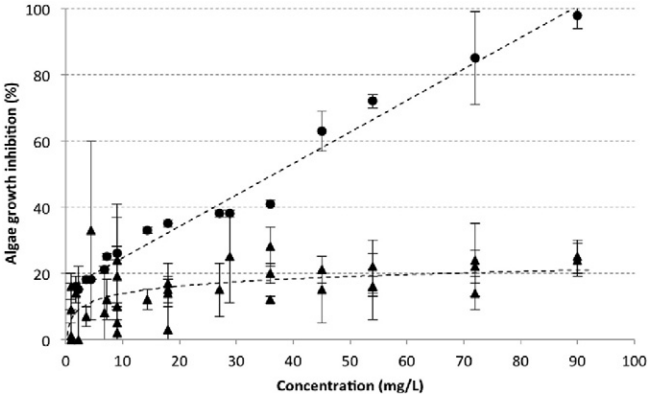

Fig. 1. Growth inhibition of *P. tricornutum* exposed to $nTiO_2$ (▲ = experimental data) and $TiCl_4$ (● = experimental data).

Table 1
Growth inhibition effects in *P. tricornutum* (mg/L) as IC10, IC20 and IC50 as mean values and their relative 95% confidence limit range; n.a. = not available.

	$nTiO_2$	$TiCl_4$
IC10	14 (12–15)	25 (18–22)
IC20	16 (12−20)	34 (31–38)
IC50	n.a.	63 (57–96)

similarly to Keller et al. (2010) and Wang et al. (2016). According to Francius et al. (2008) and Wang et al. (2016), agglomerates of microalgae and $nTiO_2$ were observed at exposure concentrations ≥5 mg/L (Fig. 2) probably due to the release of extracellular glycoproteins as reaction to the stressor. Qualitatively, a positive correlation seems to exist between the number and size of agglomerates and exposure concentrations, as well as the number of cells included within. Speculations about the role of aggregates in toxicity contribution evidenced potential physical effects (e.g. cell wall damage) or indirect physical effects (e.g. shading, limitations in nutrient uptake) (Wang et al., 2016). In the present study, morphologically, no changes or relevant abnormalities were found in microalgae and $nTiO_2$ agglomerates. Difficulties were found in test reading: algae suspensions were heavily shacked separating microalgae aggregates for counting.

Effects of $TiCl_4$ (Fig. 1 and Table 1) were greater than $nTiO_2$ at higher concentrations (>50 mg/L), but at lower concentrations the nano-form showed higher effects. Indeed, $TiCl_4$ IC50 is 63 mg/L ($y = 15.14 + 0.95\times$, $R^2 = 0.98$, MSE = 145.2). This partly confirmed results from other authors (Aruoja et al., 2009; Ji et al., 2011; Clèment et al., 2013; Libralato et al., 2013) about the comparison of bulk and nanosized materials evidencing increased toxicity when NPs were assessed.

3.2.2. Effects on A. franciscana

Negative and positive controls were in accordance with Libralato et al. (2007) (S3). The best fit for concentration-response relationships for $nTiO_2$ and $TiCl_4$ were reported in S4 and S5, respectively, and were used to calculate the parameters reported in Table 2. Details about concentration-response relationships for $nTiO_2$ and $TiCl_4$ were shown in Fig. 3, Fig. 4 and Fig. 5 considering the exposure scenarios A, B and C, in that order. Linear concentration-response trends were observed for $TiCl_4$, while non-linear parametric regressions were used for $nTiO_2$ like in Libralato et al. (2013). In this case, only the lowest LC50 value was provided (Table 2).

The exposure to $TiCl_4$ according to scenario A (Fig. 3) showed only slight effects reporting no LC50s; LC20s were available only after 96 h (54 mg/L) and LC10 values after 72 (33 mg/L) and 96 h (19 mg/L). About $nTiO_2$, the scenario A evidenced concentration-response

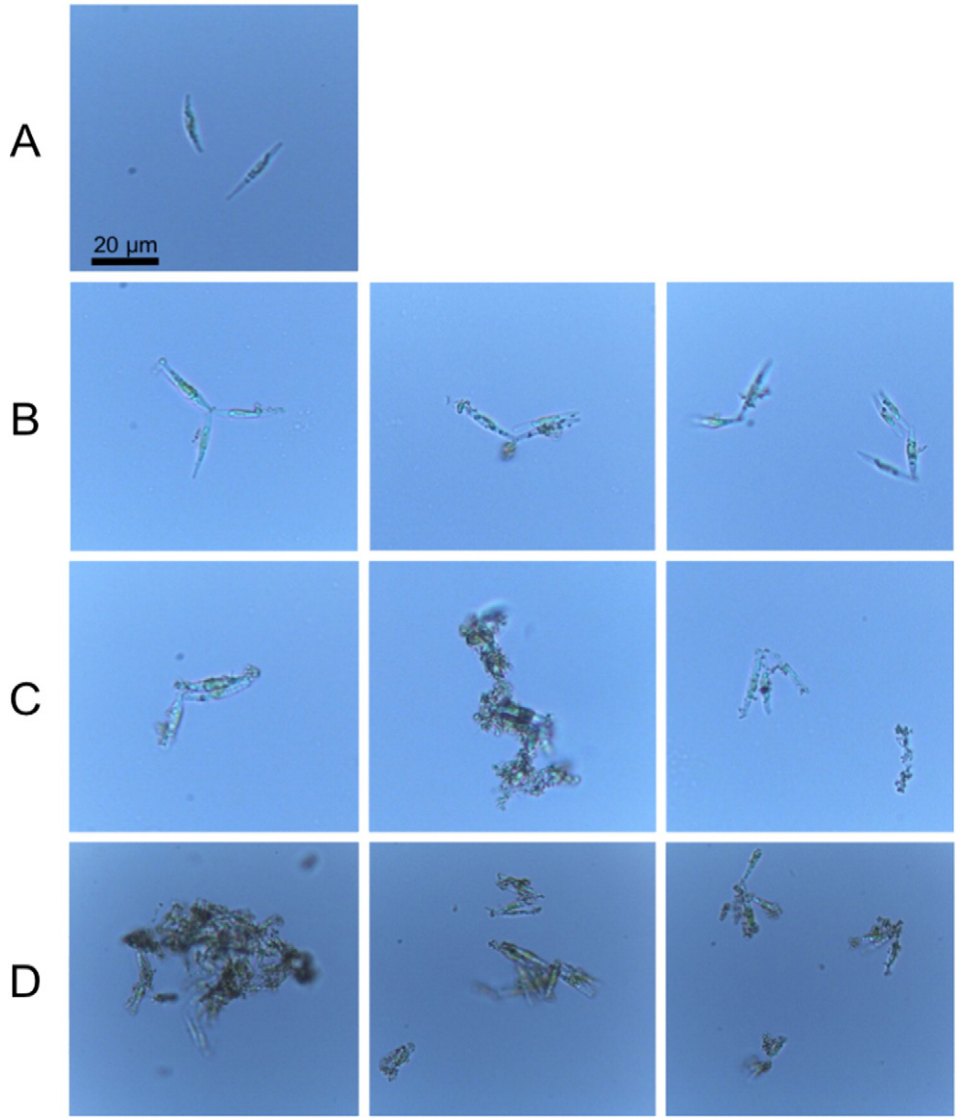

Fig. 2. *P. tricornutum* exposed for 72 h to increasing $nTiO_2$ concentrations (A = 0 mg/L; B = 5 mg/L; C = 7.5 mg/L; D = 10 mg/L).

Table 2
Mean values and their relative 95% confidence limit range for lethal effect concentrations (LC10, LC20 and LC50) in *A. franciscana* according to exposure scenarios (A = APAT and IRSA-CNR (2003), B = APAT and IRSA-CNR (2003) and total darkness and C = APAT and IRSA-CNR (2003) and starvation) and various contact times (24, 48, 72 and 96 h); underlined values indicated a non-monotonous non-decreasing toxicity trend; n.a. = not available.

Exposure scenario		$nTiO_2$			$TiCl_4$		
		LC10 mg/L	LC20	LC50	LC10 mg/L	LC20	LC50
24 h	A	16 (15–17)	19 (17–21)	28 (26–29)	79 (77–80)	n.a.	n.a.
	B	n.a.	n.a.	n.a.	45 (44–47)	n.a.	n.a.
	C	16 (14–18)	19 (17–20)	27 (26–28)	n.a.	n.a.	n.a.
48 h	A	11 (10 − 12)	13 (11–14)	18 (14–19)	58 (56–59)	n.a.	n.a.
	B	6 (5–7)	3 (2–4)	n.a.	36 (35–37)	n.a.	n.a.
	C	1 (1–3)	11 (9–12)	18 (17–20)	67 (65–68)	n.a.	n.a.
72 h	A	4 (3–5)	10 (9–11)	18 (17–20)	33 (32–35)	n.a.	n.a.
	B	26 (25–28)	8 (7–9)	1 (1–2)	22 (21 − 23)	50 (49–52)	n.a.
	C	n.a.	n.a.	3 (1–2)	7 (5–9)	32 (31 − 33)	n.a.
96 h	A	n.a.	6 (4–7)	15 (13–17)	19 (17–22)	54 (50–56)	n.a.
	B	31 (30–37)	18 (16–19)	2 (1–4)	15 (13–16)	41 (39–43)	n.a.
	C	n.a.	n.a.	1 (1–3)	n.a.	4 (2–5)	67 (63–68)

relationship with effects generally increasing with contact time except for concentration values >50 mg/L (Fig. 3). After 24 h, no toxic effects were observed up to 16 mg/L, while between 16 and 50 mg/L mortality was very high (97% at 50 mg/L); no effect was present at 64 mg/L. After 48 h, the toxicity trend was similar to 24 h exposure, but significant lethality effects were anticipated at approximately 20 mg/L. ANOVA evidenced no significant difference ($p < 0.05$) between outputs after 24 and 48 h. After 72 and 96 h, toxicity was already detectable at 2 mg/L (30% and 40%, respectively). At 64 mg/L, mortality decreased in all scenarios and contact times up to 37%. The LC50 after 24, 48, 72 and 96 h resulted 28, 18, 18, and 15 mg/L (Table 2), respectively. Thus compared to $TiCl_4$, $nTiO_2$ showed significant adverse effects displaying a bell-shaped line as in Libralato et al. (2013). Other authors reported lower effects of $nTiO_2$, but considering other exposure scenarios and titania

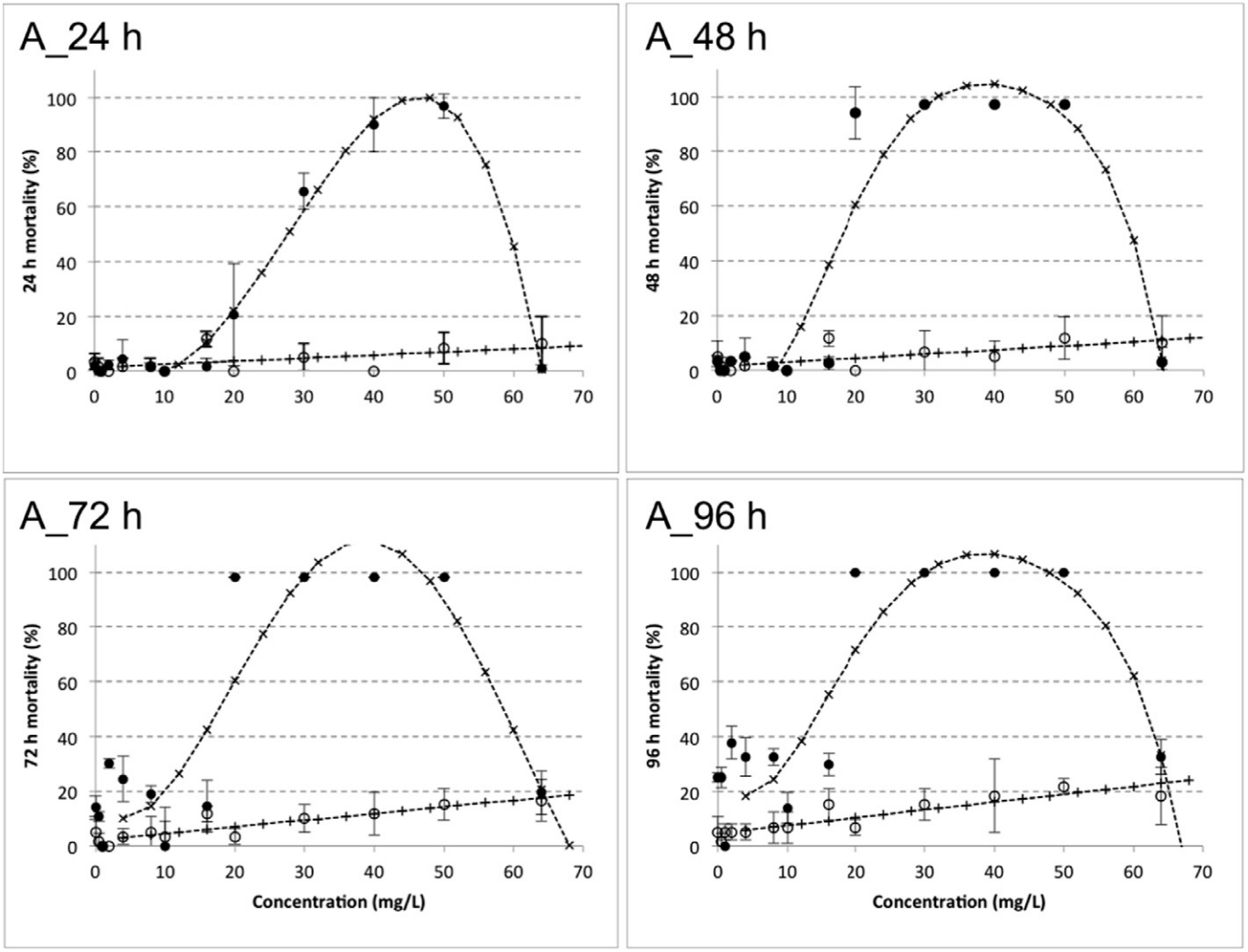

Fig. 3. Scenario A: APAT and IRSA-CNR (2003) - Effects on *A. franciscana* after 24, 48, 72 and 96 h of contact time to nano-TiO_2 (●, ▲, ■) and $TiCl_4$ (○, Δ and □).

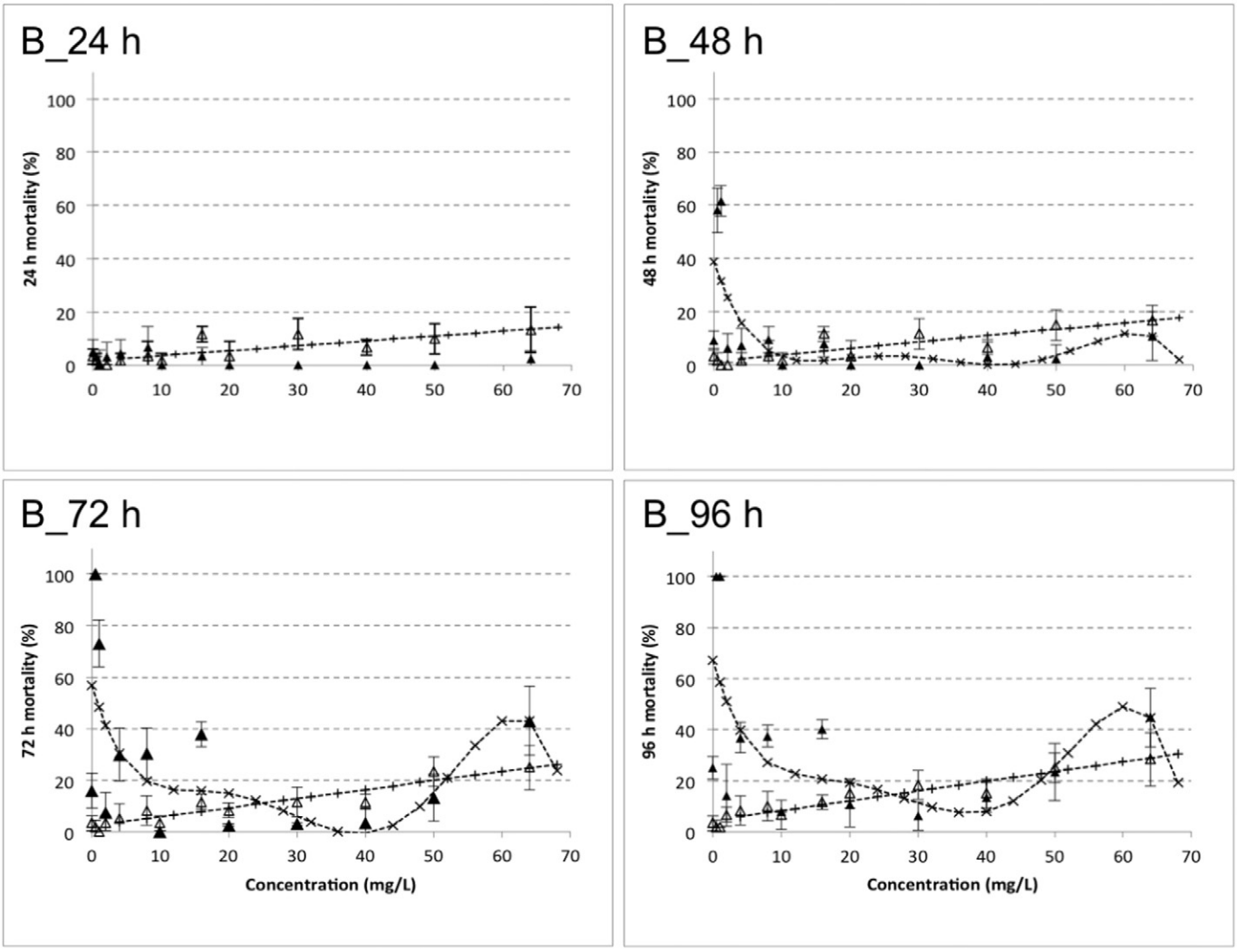

Fig. 4. Scenario B: APAT and IRSA-CNR (2003) and total darkness (Libralato et al., 2007) - Effects on *A. franciscana* after 24, 48, 72 and 96 h of contact time to nano-TiO_2 (●, ▲, ■) and $TiCl_4$ (○, Δ and □).

suppliers. Rajasree et al. (2011) found that $nTiO_2$ LC50 = 98 mg/L (96 h) but using adults instead of nauplii. Ates et al. (2013) observed low mortality events (96 h) on nauplii with a maximum effect of 18% at 100 mg/L. Clemente et al. (2014) obtained EC50(48 h) for $nTiO_2$ equal to 481 (anatase) and 285 mg/L (anatase/rutile mixture) under visible light. When UV radiation was present, EC50(48 h) dropped down drastically to 4 mg/L for both materials, respectively. Callegaro et al. (2015) assessed $nTiO_2$ effects with *A. franciscana* reporting that no significant effects were highlighted after 48 h between 0.01 and 90 mg/L compared to negative controls considering a 12 h light period (3000–4000 lx).

The scenario B (total darkness) evidenced a general decrease in $nTiO_2$ effects (Fig. 4) compared to scenario A. After 24 h, effects were <10%. After 48 h, effects were <10% except for 0.5 mg/L (58% mortality) and 1 mg/L (62%). ANOVA showed no significant differences ($p < 0.05$) between 24 and 48 h data. After 72 and 96 h, concentration-response trends were similar to that after 48 h with increase of toxicity levels suggesting that lower concentrations of $nTiO_2$ (0.5–1 mg/L) are more available that higher ones probably due to limited aggregation phenomena and aggregates' size. ANOVA test revealed no significant differences ($p < 0.05$) between 48, 72 and 96 h data. No EC50 values could be calculated for all the 4 sub-scenarios. According to Table 2, LC50 values were not available after 24 and 48 h, and were not significantly different after 72 and 96 h of exposure (approximately in the range 1–2 mg/L). Total darkness condition decreased the general level of toxicity, but LC50s after 72–96 h were greater than scenario A, even if effects were generally lower as indicated by LC10 and LC20 values that resulted both >LC50s due to the atypical concentration-response trend.

Effects of $TiCl_4$ were very similar to scenario A (Fig. 4) evidencing toxicity increase with contact time. After 24 and 48 h, effects were <20%. LC50s were not available. The LC10 decreased resulting 79, 58, 33 and 19 mg/L after 24, 48, 72 and 96 h, respectively.

The scenario C (starvation) was the most critical for *A. franciscana* (Fig. 5) especially in presence of $nTiO_2$. Up to 48 h, effects of $nTiO_2$ were not significantly different from scenario A. After 72 and 96 h, average mortalities were 50% and 60%, respectively. LC50s decreased with increasing contact times: 24 h (27 mg/L), 48 h (18 mg/L), 72 h (3 mg/L), and 96 h (1 mg/L) (Table 2). After 72 h, mortality significantly increased even at very low exposure concentrations compared to scenario A evidencing the contribution of starvation in toxicity definition. According to scenario C, the exposure to $TiCl_4$ showed effects not significantly different from scenario A up to 48 h (Table 2). After 72 and 96 h, starvation increased the average effects up to 3 and 5 folds, respectively. LC50 for $TiCl_4$ was available only after 96 h (67 mg/L). In general, effects of Ti from $TiCl_4$ were lower than $nTiO_2$.

Considering the sedimentation curves of $nTiO_2$ in artificial seawater from Brunelli et al. (2013), the exposure concentrations for water

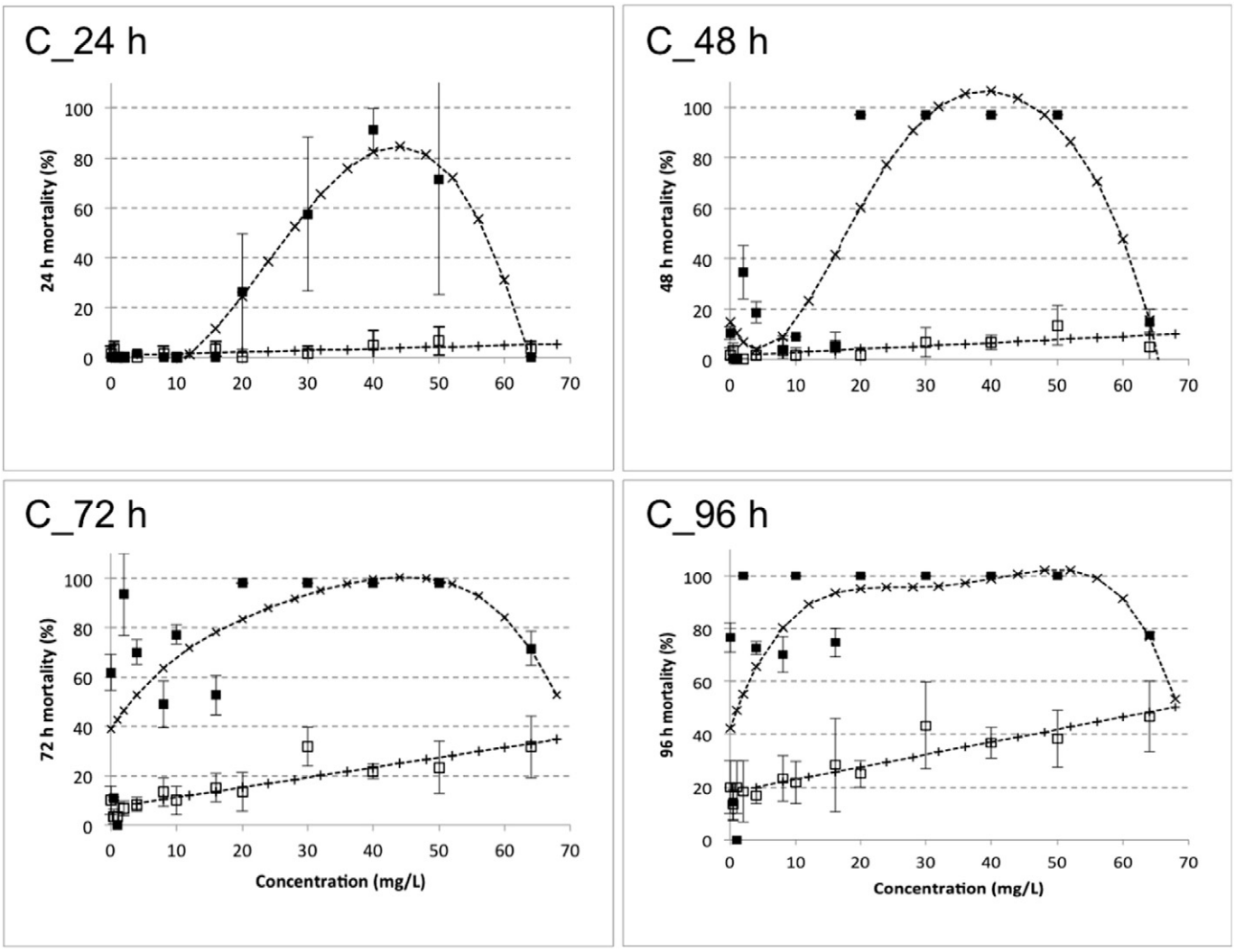

Fig. 5. Scenario C: APAT and IRSA-CNR (2003) and starvation - Effects on *A. franciscana* after 24, 48, 72 and 96 h of contact time to nano-TiO_2 (●, ▲, ■) and $TiCl_4$ (○, Δ and □).

column organisms (i.e. *A. franciscana*) decreased from 40% to 96% considering 0.01 and 10 mg/L of $nTiO_2$ after 50 h, respectively. Moreover, aggregates within tens of minutes were of micrometer size particles in seawater at 10 mg/L of $nTiO_2$ (Keller et al., 2010). Thus water contact time and concentration can both directly influence $nTiO_2$ sedimentation rate and the relative exposure scenarios suggesting that parameters like EC50s could be adjusted for modelled exposure scenarios rather than taking into account just the initial concentration values. Anyhow, sedimentation rates have been scarcely investigated (Keller et al., 2010; Brunelli et al., 2013) and more efforts should be carried out to fill this gap into the knowledge considering medium-term exposure scenarios at least up to 96 h at various exposure concentrations (up to 100 mg/L). If water column organisms could be underexposed to $nTiO_2$ during the experimental phase, conversely benthic organisms could be overexposed to its agglomerates (i.e. *P. tricornutum*).

About *P. tricornutum*, further efforts should be devoted to understand the role of biological adaptation of this species to stress events generated by NPs especially considering low exposure concentrations for prolonged periods than the conventional 72 h test lasting. Values like EC10 or EC20 might be used for hazard assessment rather than EC50, being generally available in presence of more environmentally (low) realistic concentrations.

The effects of $TiCl_4$ to crustaceans larvae in all exposure scenarios were lower compared to $nTiO_2$ (EC50(96 h) = 15 mg/L - standard protocol). During toxicity testing, darkness generally lowered $nTiO_2$ effects while starvation sharpened larval mortality increasing the toxicity of both $TiCl_4$ and $nTiO_2$.

4. Conclusions

The exposure of microalgae (*P. tricornutum*) and crustaceans (*A. franciscana*) to $TiCl_4$ and $nTiO_2$ was carried out considering standardised protocols. Alternative scenarios including potential real stressing events (changes in the photoperiod and starvation) took place only for brine shrimps. For the first time, the main inhibition concentrations (IC10, 20 and 50) are fully available for *P. tricornutum*, which provided other nano-related cues suggesting that growth inhibition can be interpreted, at least from a qualitative viewpoint, along with microalgae aggregation starting from 1 mg/L of $nTiO_2$. At concentrations >10 mg/L of $nTiO_2$, the toxicity test could not be read either by the Bürker counting chamber or spectrophotometrically due to both nanomaterial and microalgae aggregates and their shading effect, respectively. Compared to $nTiO_2$, $TiCl_4$ showed greater inhibitory effects at higher concentrations (IC50), but for $nTiO_2$ IC10 and IC20 were significantly lower suggesting that $nTiO_2$ is more harmful than $TiCl_4$ at lower concentrations.

The effects of $TiCl_4$ to crustaceans larvae in all exposure scenarios were lower compared to $nTiO_2$ (EC50(96 h) = 15 mg/L - standard protocol). During toxicity testing, both light and starvation promoted

crustaceans mortality for both nano- and bulk titania. These results suggest that increasing the complexity of the standard experimental conditions can provide new and more relevant toxicity data meaningful to inform risk assessment of nanomaterials.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.11.135.

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