# Effects of alginate on stability and ecotoxicity of nano-TiO$_2$ in artificial seawater

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ABSTRACT

The large-scale use of titanium dioxide nanoparticles (nano-TiO$_2$) in consumer and industrial applications raised environmental health and safety concerns. Potentially impacted ecosystems include estuarine and coastal organisms. Results from ecotoxicological studies with nano-TiO$_2$ dispersed in salt exposure media are difficult to interpret due to fast flocculation and sedimentation phenomena affecting the dispersion stability. The goal of this study was to investigate the stabilisation effect of alginate on uncoated nano-TiO$_2$ in artificial seawater dispersions used in ecotoxicity bioassays. The most effective stabilisation was obtained at alginate concentration of 0.45 g/L after sonicating dispersions for 20 min (100 W). The size distribution remained constant after re-suspension, indicating that no agglomeration occurred after deposition. Ecotoxicity tests on *Artemia franciscana* and *Phaeodactylum tricornutum* did not show any adverse effects related to the presence of alginate in the exposure media, and provided evidence on possible reduced bioavailability of nano-TiO$_2$. The suitable concentration of alginate is recommended to occur on a case-by-case basis.

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## 1. Introduction

The increasing application of titanium dioxide nanoparticles (nano-TiO$_2$) in consumer products raised environmental, health and safety concerns (Donaldson et al., 2004; Gao et al., 2008; Handy et al., 2008; Ju-Nam and Lead, 2008; Klaine et al., 2008; Nel et al., 2006). The introduction of nano-TiO$_2$ in the environment may occur during its whole life cycle including production, use and disposal (Hagendorfer et al., 2010; Kaegi et al., 2008, 2010; Corsi et al., 2014). High concentrations of nano-TiO$_2$ were detected in surface waters (i.e. 10 mg/L) after a simulated rainfall release from outdoor walls (Kaegi et al., 2008). Rain water can be conveyed and treated in wastewater treatment plants, which do not effectively remove nanoparticles from the effluent discharge (Kiser et al., 2009). Nano-TiO$_2$ entering surface freshwater is likely to be stabilised and transported to the sea under conditions of low ionic strength and high levels of natural organic matter (Domingos et al., 2009; Jiang et al., 2009; Keller et al., 2010; Petryshyn et al., 2010; Zhang et al., 2008). Being coastal water and sediment the

final sink for nano-TiO$_2$ particles (Gottschalk et al., 2009, 2010), marine organisms-like crustaceans and algae-may be exposed to them. As a consequence, it is necessary to assess the potential hazards of nano-TiO$_2$ for these target species.

According to some standard ecotoxicological protocols (e.g. ASTM, 2004), culture and exposure media for marine organisms, i.e. artificial seawater (ASW), must have about 35‰ salinity and 0.7 M ionic strength generated by a mixture of selected salts. The high salt concentration of ASW and the pH of 8.00–8.40 form favourable conditions for nano-TiO$_2$ to agglomerate and rapidly settle (Keller et al., 2010, Petosa et al., 2010). As a result, the tested organisms are exposed to material concentrations and size distributions dynamically changing over time throughout various non-equilibrium states; these variations affect the generation of robust test results (e.g. realistic concentration–response relationships), as well as their proper interpretation (Hund-Rinke et al., 2010; Kahru and Dubourguier, 2010; Pace et al., 2010; Strigul et al., 2009; Velzeboer et al., 2008).

The introduction of a stabilising agent in the exposure media appears acceptable if the selected substance occurs naturally in the environment and is not hazardous for the tested species. It is well known that natural organic matter (NOM) can stabilise

colloids (Tiller and O'melia, 1993). The mechanism was initially thought to be solely an electrostatic repulsion due to the negative charge of the NOM coating (Hunter and Liss, 1982; Beckett and Lee, 1990; Wilkinson et al., 1997). Indeed, it has been shown that there may be a steric component to the stabilisation (Sander et al., 2004). It has also been shown that NOM may also destabilise colloids under certain conditions through bridging flocculation (Wilkinson et al., 1997; Chen et al., 2007).

Other recent investigations suggested the use of organic substances to stabilise nanoparticle dispersions in freshwater media. Several example of stabilisation of carbon based and metal-based nanoparticles by humic substances have been published (Chen and Elimelech, 2007; Diegoli et al., 2008; Abe et al., 2011). Tiraferri et al. (2008) tested guar gum, alginate and potato starch on bare iron nanoparticle (nano-Fe) dispersions. The effect of these polymers was preliminary analysed in the range of 1.3–4.0 g/L in presence of 1 mM NaCl and it was observed that guar gum (0.5 g/L) was more effective in preventing sedimentation of 1.5 g/L nano-Fe under low ionic strength at neutral pH, despite the fact that the other two polymers were not investigated in detail. Liu et al. (2010) reported the effects of Suwannee River humic acid (15 mg/L) and alginate (4.4 mg/L C) on 10–20 nm boron nanoparticles (50 mg/L) in electrolyte solutions. Alginate solutions did not significantly stabilise the dispersions in presence of NaCl (0.05–0.3 M), while in $MgCl_2$ solutions low stabilisation effect was observed (0.5–8 mM), while agglomeration was prevented in $CaCl_2$ solutions in the range 0.5–1.3 mM and enhanced in the range 1.3–10 mM.

In natural waters, the major classes of Natural Organic Matter (NOM) biopolymers are seasonally varying with higher concentrations during and after plankton blooms (Buffle et al., 1998). It is believed that most biopolymeric NOM are exudates from algae and bacteria and are rich in protein and acid polysaccharide components (Aluwihare and Repeta, 1999). The structure of these exudates have been shown to be fibrillar in both freshwater and seawater (Wilkinson et al., 1999; Santschi et al., 1998; Stolpe and Hassellöv, 2009). This rigid fibrillar characteristic in combination with the charge distribution is believed as important both for steric stabilisation and bridging flocs depending on specific conditions. Although the natural exudates are complex mixtures, different model macromolecules have been used in experiments such as alginate, xanthan, gum Arabic and guar gum.

Among natural polysaccharides, alginate occurs as a structural component in marine brown algae (*Phaeophyceae*) and capsular polysaccharides in soil bacteria; it is a linear chain copolymer consisting of $(1\rightarrow4)$-linked $\alpha$-L-guluronic acid and $\beta$-D-mannuronic acid repeating units that are randomly arranged (Andriamanantoanina and Rinaudo, 2010). Alginate is commonly applied as additive in food, pharmacy, agriculture and environmental industries (Ertesvåg and Valla, 1998; Fayaz et al., 2009). The concentration of dissolved organic carbon is well known in seawater and range from as low as 40 $\mu$M in the open ocean to several hundred $\mu$M in coastal waters with high freshwater input (Pettine et al., 1999). The bulk chemical composition of NOM in seawater revealed that total polysaccharides accounted for approximately 50% of the NOM in surface seawater (Benner et al., 1992), but the different fractions of unaltered macromolecules are much less well quantified due to lack of methods. Another significant source of NOM is the direct effluent discharge into seawater from the sewage treatment plants (STPs) that is one of the major routes for the entry of manufactured nanoparticles in the marine environment being extremely rich in OM both from the sewage and from the exudates of the microbial treatments. It is therefore plausible that the nanomaterials will be exposed to a very high NOM concentration within and shortly after the wastewater treatment before excessive dilution in the receiving water. Recently, about the

use of stabilisers in ecotoxicity testing, OECD (2014) stated that the inclusion of NOM into test dilutions and not within stocks is still open to further discussion due to the batch-to-batch required approach; indeed, depending on the type of NOM, engineered nanomaterials (ENMs) can be stabilized or destabilised after NOM adsorption. Currently, no harmonisation of NOM management procedure is available.

The objective of this work is to assess the effectiveness of alginate in stabilising nano-$TiO_2$ in crustacean (ASW-1) and algae exposure media (ASW-2), used in ecotoxicity testing. In particular, the size distribution, agglomeration, and sedimentation behaviour under centrifugal forces of nano-$TiO_2$ particles in the presence of alginate were determined using different polymer concentrations and sonication dispersion periods. The most effective alginate solution, that means the lowest treatment solution to obtain an adequate nano-$TiO_2$ stabilisation, was studied in great detail in order to assess the possibility to introduce some modifications to the toxicity testing exposure media. Ecotoxicity tests on the anostracan crustacean *Artemia franciscana* and on the unicellular diatom alga *Phaeodactylum tricornutum* were carried out according to the standard methods APAT and IRSA-CNR (2003) and UNI EN ISO 10253 (2006), respectively. Bioassays assessed the potential ecotoxicological effects of nano-$TiO_2$ dispersed both in the standard media ( < 5 mg/L total organic carbon) and alginate rich suspensions.

## 2. Materials and methods

### 2.1. Nanoparticles

Aeroxide® P25 uncoated titanium dioxide powder (declared primary particle size of 21 nm) was obtained from Evonik Degussa Corporation. The material consists of a crystalline state with phase composition of approximately 80% anatase and 20% rutile. The pristine material was stored at room temperature in the dark.

### 2.2. Dispersion media

Ultrahigh-purity water (18.2 M$\Omega$ cm minimum resistivity) was produced by a Milli-Q water purification system (Millipore). Two artificial seawater solutions (ASW-1, ASW-2) were used as dispersion media and prepared according to the standard protocol E 724-98 (ASTM, 2004), using analytical grade salts (purity > 99.8%) (Sigma-Aldrich). Their exact compositions are reported in Table S1. The solutions were prepared by dissolving salts in deionised water under magnetic stirring at room temperature. After completing the salt dissolution, they were aerated with a stream of laboratory air over 12 h, in order to stabilise the pH to 8.2 ± 0.2. The solutions were filtered using 0.45 $\mu$m glass fibre filters (Whatman International Ltd.) and stored at 4 ± 1 °C in the dark.

### 2.3. Alginate solutions

The alginate (Fluka Code 71240, Fluka – Milan, Italy) is characterised by an average molecular weight of 345 KDa (Serp et al., 2000). The alginate solutions were prepared by dispersing the polymer in ASW with initial concentrations of 0.1, 1, and 10 g/L sodium alginate. The solutions were vigorously shaken for 60 s and left at 4 ± 1 °C for two days in order to achieve complete dissolution of the alginate powder. The undissolved alginate was removed by sequential filtration through 1 $\mu$m, 0.45 $\mu$m, and 0.22 $\mu$m glass fibre filters (Whatman International Ltd.). Once prepared, the alginate solutions were stored at 4 ± 1 °C in the dark. Prior to use, they were kept at room temperature for at least 2 h and then vigorously shaken.

## 2.4. Preparation of nano-TiO₂ dispersions

An exact amount of nano-$TiO_2$ powder (1, 10 and 100 mg) was weighed with a micro-analytical balance (Sartorius Mechatronics Italy S.r.l.), using tin disposable supports. The powder was transferred into a measured volume of dispersion medium (1 L), which was then probe-sonicated (100 W) (sonic probe UP-200H – Hielscher) in ice water bath to avoid uncontrolled heating. Preliminarily, the screening dispersions were sonicated only for 20 min, while during the assessment of the best dispersion procedure various sonication periods were investigated (5, 10, 15, 20, and 25 min) in order to study the effect of sonication time on the dispersion stability. After sonication, the sonic probe was washed three times in ultrahigh-pure water (5 min per wash, 100 W). Probe-sonication (20 min at 100 W) and hand-shaking (60 s) were investigated in the preliminary tests as appropriate re-suspension procedures. The solution pH was adjusted by titration with aliquots of 1 M NaOH (Romil) in order to obtain the ASW target pH.

## 2.5. Characterisation of pristine nano-powder, dispersion media and suspended particles

The nano-$TiO_2$ surface area was obtained by nitrogen adsorption at $-196\ °C$ according to the B.E.T. method (Micromeritics ASAP 2000, Micromeritics). Particle sizes were determined by transmission electron microscopy (TEM) (Jeol JEM-3010, Oxford Instruments), operating with an accelerating voltage of 300 keV. TEM samples were prepared by deposition of 5 μL of nano-$TiO_2$ dispersion in ultrahigh-purity water on a carbon-coated copper grid, followed by solvent evaporation prior to analysis.

The carbon content in the alginate powder was determined by using a CHN Elemental Analyser Fisons (Italy) (NA 1500 NCS), calibrated with acetanilide as standard. The pH of the solutions and the nano-$TiO_2$ dispersions was measured 0, 3, 6, 24, and 48 h after sonication by a pH-metre (GLP 21+, Crison Instruments S.p. A.). The non-purgeable organic carbon was determined in salt and alginate solutions after filtration by a Total Organic Carbon analyser (TOC-5050A, Shimadzu). Each sample (9 mL) was acidified with 1 mL $H_3PO_4$ (25% w/w) to reach pH < 2, then sparged with laboratory air for 5 min in order to remove the inorganic carbon. Analyses were performed in triplicate; the relative standard deviation (RDS) was < 5%. The calibration curve (linear regression coefficient $R^2 = 0.999$) was built by using solutions of potassium hydrogen phthalate (purity > 99.95%) (Sigma-Aldrich). The salt and alginate solutions conductance was measured with an electrical conductivity metre (HD 8706, DeltaOhm S.r.l.) at $22\ °C$ in triplicate. The salinity of the solutions was measured with a salinometer (PCE-0100, Atago) at $22\ °C$.

The size distribution of the suspended material was measured at $22\ °C$ by means of a dynamic light scattering (DLS) device (Nicomp DLS PSS 370, Nicomp), with a 362.8 nm laser wavelength and a 90° scattered light detector. Collected data (6 min per measurement) were processed following the NICOMP algorithm that automatically selects the best fitting distribution and recognises from one to three particle populations. The dimensional analyses are identified by hydrodynamic diameter ($d_H$) and standard deviation, which is an index of the width ($w$) of the population of agglomerates. The DLS analyses were carried out on freshly prepared as well as aged dispersions, which had been kept at $4 \pm 1\ °C$ in the dark for four weeks and re-suspended by hand-shaking after reaching room temperature. Size analyses were performed at given intervals after the dispersion preparation: 0, 24 and 48 h for the screening of the alginate solution; 0 and 24 h for the development of the dispersion method; 0, 24, 48 and 72 h for the reproducibility and repeatability of experiments. The electrophoretic mobility of nano-$TiO_2$ was determined in ultra-high pure water at $22\ °C$ by using the Zeta-potential function (Nicomp DLS PSS 370, Nicomp).

The long-term stability of nano-$TiO_2$ dispersions in the alginate solutions was evaluated by a stability analyser (LUMifuge® 116, L. U.M. Gmbh, Qi srl) (Krause et al., 2009; Kuentz and Rothlisberger, 2003), consisting of an analytical centrifuge with an optoelectronic sensor system that measures variations in the transmitted near infra-red radiations along horizontally inserted sample tubes ($1 \times 1$ cm rectangular polycarbonate cells) caused by sedimentation of suspended material. The experiments were performed in duplicate at 1800 rpm rotational velocity (light factor: 0.25) for 50 min at room temperature. The integration of transmission profiles was calculated between the positions 96 and 110 mm, within the detection region of 89–114 mm, by using SEPView 4.0 (LUMiFuge software).

A fluorescence spectrophotometer (LS55, Perkin-Elmer) was used in nephelometric turbidity mode to semi-quantitatively determine (Sene et al., 2009) dilution of nano-$TiO_2$ dispersions. The experiments were carried out with a nephelometric setup: incident ray and scattered light detector at $\lambda = 625$ nm; 2.5 nm excitation slit width; 7.0 nm emission slit width; and 1 cm thick quartz cuvette. The calibration curve ($R^2 = 0.998$) was built by using five dispersions at known mass concentrations, which were prepared by adding a weighted amount of pristine powder to 1 g/L alginate solution and probe sonicating for 20 min.

## 2.6. Ecotoxicity tests

Test organisms were exposed to nano-$TiO_2$ dispersed in reconstructed seawater with and without the addition of alginate (ASW-2 for algae, ASW-1 for crustaceans). The experimental design considered a series of negative and positive controls with and without the presence of alginate in order to prevent any false negative and positive results.

The P. tricornutum short-term chronic toxicity tests were carried on according to UNI EN ISO 10253 (2006). The algal culture (lot PT070509, SAG strain, Gent, Belgium) was kept at $20 \pm 2\ °C$ and 6000–10000 lx, in order to obtain $> 10^6$ cell/mL after $3 \pm 1$ days. The cell density was measured with a Bürker counting chamber by microscopy and the relative growth inhibition determined according to the protocol. The algal sub-population was exposed to nano-$TiO_2$ dispersions (nominal tested concentrations 0.9, 9, 45, and 90 mg/L) into sterile 3 mL 24-well plates with lid (Iwaki, Japan) ($2 \cdot 10^3$–$10^4$ cell/mL per well, obtained by dilution) for $72 \pm 2$ h at $20 \pm 2\ °C$ with a 14 h photoperiod (6000–10000 lx).

The A. franciscana acute immobilisation tests were carried on according to APAT and IRSA-CNR (2003). The cysts (lot AF/F2006, Gent, Belgium) were incubated in Instant Ocean® and maintained at $25 \pm 1\ °C$ with a 1 h photoperiod (3000–4000 lx); after 24 h, the hatched Instar-I nauplii were collected and kept under the same conditions for 24 h in order to allow them to reach the Instar-II stage. To carry on the test, the organisms were placed in 3 mL sterile 24-well plates with lid (Iwaki, Japan) (10 nauplii per well) with a working volume of 2 mL. The crustaceans were exposed to nano-$TiO_2$ dispersions (nominal tested concentrations 0.01, 0.1, 1, 5, 10, 30, 50, 70, and 90 mg $L^{-1}$ nano-$TiO_2$) for 48 h at $25 \pm 1\ °C$ with a 12 h photoperiod (3000–4000 lx). The number of immobilised larvae was counted every 24 h.

Positive control tests were carried on for P. tricornutum with $K_2Cr_2O_7$ (Baker) (EC50 = $20.1 \pm 5.3$ mg $L^{-1}$) and with $CuSO_4\ 5H_2O$ (Baker) (EC50 < 6.5 mg $L^{-1}$) for A. franciscana both with and without the presence of alginate.

The experimental design was set up considering 2 and 5 experiments in triplicate for P. tricornutum and A. franciscana, respectively.

## 2.7. Data analysis and statistics

All data were statistically elaborated with XLSTAT. DLS-derived data were tested for significance using the Student $t$-test and the one-way analysis-of-variance (ANOVA) method setting a $\alpha$-level at 0.05. The ANOVA null hypothesis is that the means of the groups are equal and the variations in the measurement are no greater than those caused by normal variation of samples' characteristics and error in their measurement. The ANOVA tests are based on the F-ratio. Dimensional data to use in reproducibility tests showed normal distribution (Shapiro–Wilk's test) and homogeneity of variance (Bartlett's test), matching with ANOVA assumptions. Moreover, the same approach was taken into consideration to compare the ecotoxicological results obtained from the various exposure scenarios experienced.

Whenever possible, ecotoxicity data were expressed as the median (50% effect) effect concentration (EC50). Various parametric statistical assessment methods were taken into account for both *P. tricornutum* (semi-log linear model) and *A. franciscana* (logistic and polynomial). Otherwise, ecotoxicity was expressed as percentage (%) of effect normalised to negative controls with Abbott's formula.

## 3. Results and discussion

### 3.1. Pristine material and exposure media characterisation

Characterisation of the uncoated nano-TiO$_2$ by TEM and selected area electron diffraction (SAED) revealed that the size of the pristine particles ranged between 15 and 60 nm and that the main crystallographic phases were anatase and rutile. A TEM picture of nano-TiO$_2$ from the same batch was already reported by Brunelli et al. (2013). The B.E.T. analysis estimated a specific surface area of 65 m$^2$/g. The nano-TiO$_2$ electrophoretic mobility, determined in ultrahigh-purity basified water, was $-10.5$ mV at pH 8 (equivalent to pH of ASW). The CHN analysis showed that the carbon content in the alginate powder was 31.1% w/w.

The values of pH (over 2 days monitoring), total organic carbon, conductance and salinity of ASW-1 and ASW-2 are reported in Table S2.

### 3.2. Development of the dispersion procedure

#### 3.2.1. Screening of alginate solutions for nanoparticle stabilisation

Toxicity tests (only with ASW-1) were carried on to evaluate the stabilisation effect of alginate on nano-TiO$_2$ dispersions in saltwater media.

Alginate solutions in ASW-1 were prepared at the following exposure concentrations: 0.1 g/L (A0.1_ASW-1), 1 g/L (A1_ASW-1), and 10 g/L (A10_ASW-1). Large flocs of alginate were immediately formed at the bottom of the vessels mainly at 1 g/L and 10 g/L. This effect was likely caused by the high concentrations of Ca$^{++}$ (0.01 M) and Mg$^{++}$ (0.05 M) in solution (Table S1), promoting gel formation/polymer aggregation (Pamies et al. 2010). After two days, the solutions were filtered sequentially through 1, 0.45, and 0.20 μm glass fibre filters in order to remove the not-dissolved residues. The filtration process of 10 g/L alginate solution determined the saturation of the 1 μm membrane and was thus excluded from further testing. Moreover the resulting high viscosity was recognised as inappropriate for ecotoxicological testing.

No data was recorded from DLS analyses of filtered solutions since the light scattered intensity was lower than 5 KHz. Dispersions at 100 mg/L were prepared by adding nano-TiO$_2$ powder to the filtered solutions A01_ASW-1, A1_ASW-1 as well as to ASW-1, prior to 20 min probe-sonication (100 W). DLS analyses of

dispersions were performed to estimate the size distribution of suspended material 0, 24, and 48 h after the preparation, showing a bimodal distribution in A01_ASW-1 and ASW-1, and a Gaussian distribution in A1_ASW-1 (Table S3). The size distribution in A1_ASW-1 remained relatively constant over time (RDS < 6%), while in A01_ASW-1 and ASW-1 significant variations in $d_H$ and $w$ were observed (RDS > 25%). For this reason, A1_ASW-1 (1 g/L initial alginate concentration) was selected for the subsequent tests.

The 100 mg/L nano-TiO$_2$ dispersion in A1_ASW-1 was diluted with ASW-1 at ratio 1:10. The obtained diluted suspensions were dispersed by probe-sonication (20 min at 100 W) or handshaking (60 s). The sonication increased the $d_H$ of agglomerates by 35%, while both the $d_H$ and $w$ in hand-shaken dispersions remained stable with time (RDS < 6%). The plausible reason behind this phenomenon is that the sonication partially removed the alginate coating off the nano-TiO$_2$ agglomerates, thus reducing the steric hindrance effect of the substance or inducing additional collisions. Therefore, the sonication was abandoned and all the diluted dispersions were suspended by 60 s handshaking.

#### 3.2.2. Characterisation of dispersion media

Filtered alginate solutions (1 g/L alginate in ASW-1 and ASW-2, in triplicate per culture medium) showed TOC levels of $138.9 \pm 31.1$ mg/L ($11.6 \pm 2.6$ mM) in A1_ASW-1, and $149.5 \pm 16.6$ mg/L ($12.4 \pm 1.4$ mM) in A1_ASW-2. The analyses indicated that the filtration process removed $57 \pm 9\%$ of the initial alginate content from A1_ASW-1 and $53 \pm 7\%$ from A1_ASW-2. Conductivity, salinity and pH were measured in pure culture media and in alginate solutions after filtration in order to assess whether the coagulation of alginate modified the media main properties. The measurements evidenced no variation in salinity, 0.4% increase in conductivity and an average of 0.3 pH unit decrease.

#### 3.2.3. Time of probe-sonication and sedimentation behaviour

The effect of the probe-sonication period (5, 10, 15, 20, and 25 min at 100 W) on the stability of 100 mg/L nano-TiO$_2$ A1_ASW-1 dispersions was investigated by dimensional and sedimentation analyses. The size distribution of aggregates was measured 0 and 24 h after sonication. The variation in integral transmission profiles (correlated with sedimentation) was calculated in the initial part of the analysis (140–1250 s), where the sedimentation rate was linear. The results are reported in Fig. 1.

The dispersions with the smallest sedimentation rate and size of agglomerates ($d_H = 252 \pm 2$ nm and $w = 86 \pm 9$ nm after 0 h; $d_H = 277 \pm 1$ nm and $w = 89 \pm 1$ nm after 24 h) were formed by 20 min sonication. No significant difference in sedimentation profiles and agglomerates size distributions was observed increasing the sonication time over about 20 min.

Although gravitational sedimentation occurred in dispersions 24 h after preparation (as predicted with the analytical centrifuge), a 60 s hand-shaking procedure was sufficient to re-suspend the sediment with no effect on the size distribution ($t$-test: $p < 0.01$), which indicates that no major additional agglomeration occurred during the sedimentation of suspended particles.

#### 3.2.4. Effectiveness of dispersions dilution

The relevance of dilution, widely applied to homogenous solutions, was investigated in the context of colloidal systems. Diluted nano-TiO$_2$ dispersions underwent dimensional and quantitative analyses and the results were compared with those from dispersions with known mass concentrations. The nano-TiO$_2$ dispersions at 200 mg/L were diluted at ratio 1:2 in ASW-1, obtaining 100 mg/L dispersions, which were then re-suspended by 60 s handshaking. The dimensional analysis showed that diluted
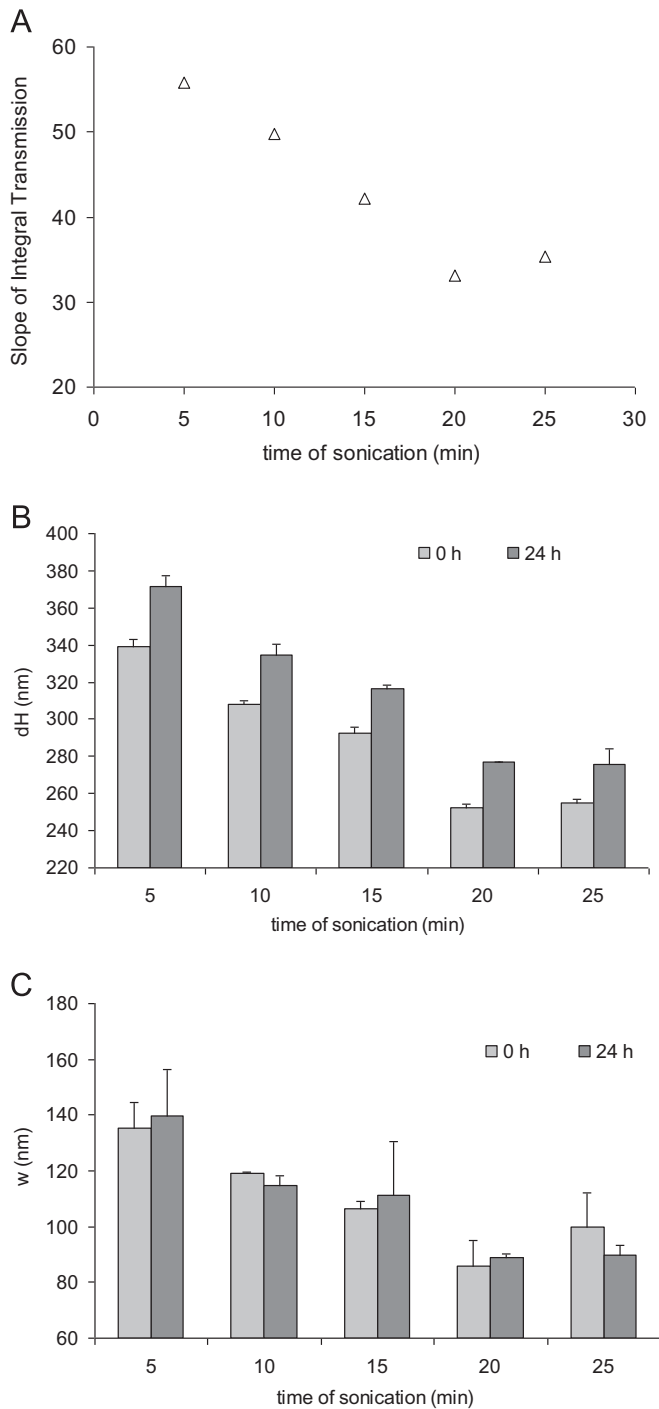
error) and a wider spread in their $w$ (38% error). The samples used to evaluate the effectiveness of the dilution process were obtained by diluting in ratio 1:2 and 1:10 the 10, 50, and 100 mg/L nano-TiO$_2$ dispersions in A1_ASW-1. The differences between expected and measured concentration, derived by applying the linear regression equation, were in the range of $-0.90\%$ and $3.63\%$. The dimensional analyses of diluted samples revealed no variation in $d_H$ and $w$ of samples diluted 1:2 (error $< 5\%$); an increase $> 90\%$ in both parameters was observed in the sample diluted 1:10, probably due to the too high reduction in alginate concentration.

### 3.2.5. Dimensional analysis: repeatability and reproducibility

The reproducibility among size distribution analyses was evaluated by testing 1, 10, and 100 mg/L nano-TiO$_2$ dispersions in A1_ASW-1 and A1_ASW-2 (three replicates of alginate solutions). Two dispersions were prepared with each solution while three 100 mg/L dispersions were prepared with the A1_ASW-1 solutions. Each suspension was analysed twice 0, 24, 48 and 72 h after sonication (dispersions in A1_ASW-1 were investigated also after 6 h). Further tests were performed at 100 mg/L A1_ASW-1 after aging the dispersions at $4 \pm 1$ °C in the dark for four weeks before starting the survey (0, 24, 48 and 72 h after sonication). Trends of $d_H$ and $w$ average values are shown in Figs. 2 and 3. Average
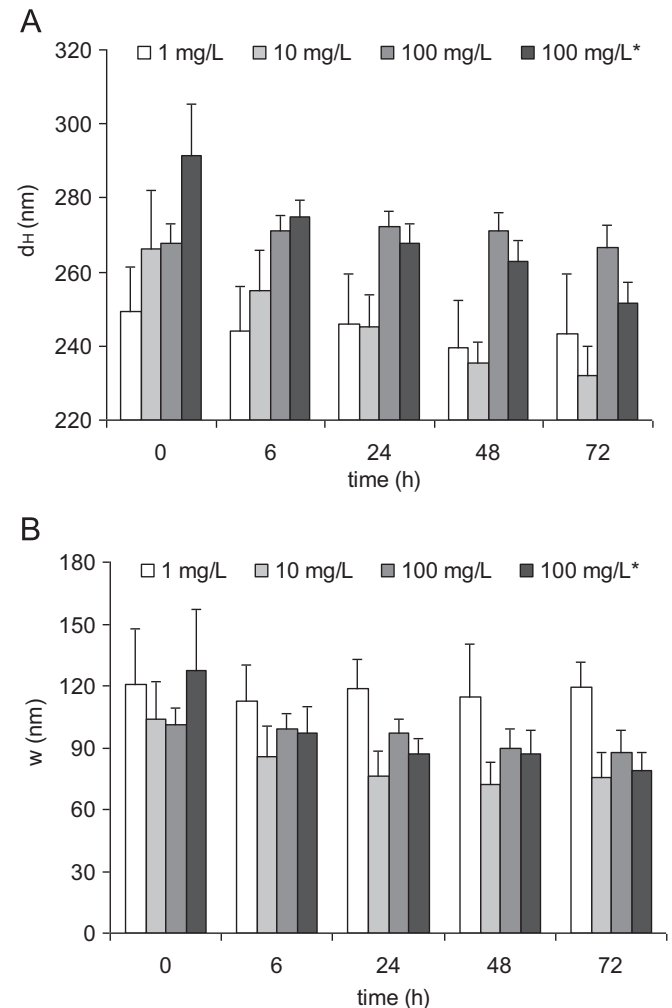
**Fig. 1.** Effect of probe-sonication (5, 10, 15, 20, and 25 min at 100 W) on 100 mg/L nano-TiO$_2$ dispersions in A1_ASW-1: average initial slopes (140–1250 s) of integral transmission (RDS $< 0.5\%$) (A); average hydrodynamic diameter ($d_H$) (B) and population width ($w$) (C) of agglomerates, recorded 0 and 24 h after sonication; no statistically significant effects were detected.



**Fig. 2.** Average hydrodynamic diameters ($d_H$) (A) and distribution widths ($w$) (B) of 1, 10, and 100 mg/L nano-TiO$_2$ dispersions in A1_ASW-1 analysed 0, 6, 24, 48, and 72 h after preparation (20 min probe-sonication at 100 W); standard deviation bars were calculated using all the dataset (three alginate solutions, two or three sub-samples per solution, two analyses per sub-samples); aged dispersions are 100 mg/L*; no statistically significant effects were detected.
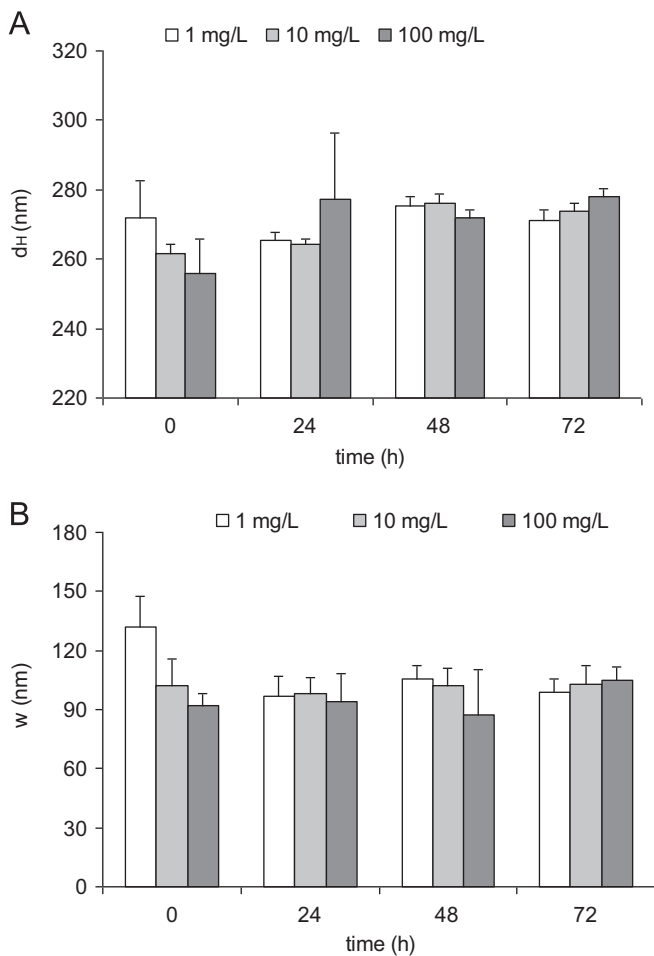
dispersions had similar characteristics compared to the ones prepared by direct inclusion of weighted powder (Table S4). Quantitative analyses were performed by a spectrophotometer set up as nephelometer (i.e. measuring the scattered light intensity). The calibration curve ($R^2 = 0.998$) was built with five dispersions at different mass concentrations (10, 25, 50, 75, and 100 mg/L nano-TiO$_2$ in A1_ASW-1). The dimensional analyses of dispersions used for calibration were recorded immediately after sonication; all samples showed a high homogeneity in $d_H$ of agglomerates (5%

**Fig. 3.** Average hydrodynamic diameters ($d_H$) (A) and distribution widths ($w$) (B) of 1, 10, and 100 mg/L nano-TiO$_2$ dispersions in A1_ASW-2 analysed 0, 24, 48, and 72 h after preparation (20 min probe sonication at 100 W); standard deviation bars were obtained from 12 analyses (three alginate solutions; two sub-samples per solution; two analyses per sub-samples); no statistically significant effects were detected.

dimensional values are reported in Table S5.

The aged 100-mg/L dispersions showed more considerable variations in $w$ values compared to the fresh dispersions, although the mean values were similar.

The statistical analysis of dimensional data showed that

dispersions prepared in different alginate solutions or with different mass suspended presented comparable size distributions of agglomerates, which had a not statistically significant difference over time.

### 3.3. Ecotoxicological tests

In the following sections, nano-TiO$_2$ toxicity results are presented on a species basis. Negative and positive control tests were carried on in standard exposure media (ASW-2 for microalgae; ASW-1 for crustaceans) with and without alginate treatments (1 g/L).

#### 3.3.1. Negative and positive controls

Algae negative control data with and without the presence of alginate in ASW-2 are shown in Fig. S1. It can be observed that the presence of alginate did not significantly affect the growth of *P. tricornutum* (ANOVA, $p < 0.05$) (no adverse effects or biostimulation). The same occurred considering the results about positive controls (K$_2$Cr$_2$O$_7$) carried on in ASW-2 with and without alginate as displayed in Fig. S2. The algae growth inhibition was not significantly changed (ANOVA, $p < 0.05$) by the presence of alginate. The K$_2$Cr$_2$O$_7$ EC50s and their relative 95% confidence limits were 13.28 (12.13–14.40) mg/L and 15.25 (10.92–20.38) mg/L with and without alginate, respectively.

Crustaceans negative control data with and without the presence of alginate in ASW-1 showed that the presence of alginate did not significantly affect the *A. franciscana* immobilisation either after 24 or 48 h of exposure. Positive control data (CuSO$_4$ 5H$_2$O) with and without alginate treatment in ASW-1 are reported in Figs. S3 and S4 for the crustacean after 24 and 48 h of cumulative exposure, respectively. After 24 h of contact time, it was observed that the presence of alginate made a significant difference ($p < 0.05$) on the immobilisation of *A. franciscana* between alginate treated and untreated ASW-1. Indeed, no effect was detected in the alginate-enriched solutions compared to the untreated ones, which presented the expected toxicity trend. Thus, the alginate inhibited the ecotoxicological effects of the reference toxicant that should have presented an EC50 value of about 6 mg/L after 24 h of contact time (APAT and IRSA-CNR, 2003). Similarly, it was found after 48 h of cumulative exposure. Actually, the toxicant present in the alginate treated ASW-1 solutions showed toxicity effects significantly (ANOVA, $p < 0.05$) lower than the relative untreated ones. Looking at Figs. S3 and S4, the medium lacking in alginate displayed a rapid increase in the toxicity effects from about 5 mg/L of CuSO$_4$ 5H$_2$O, while the other presented a very slight raise that
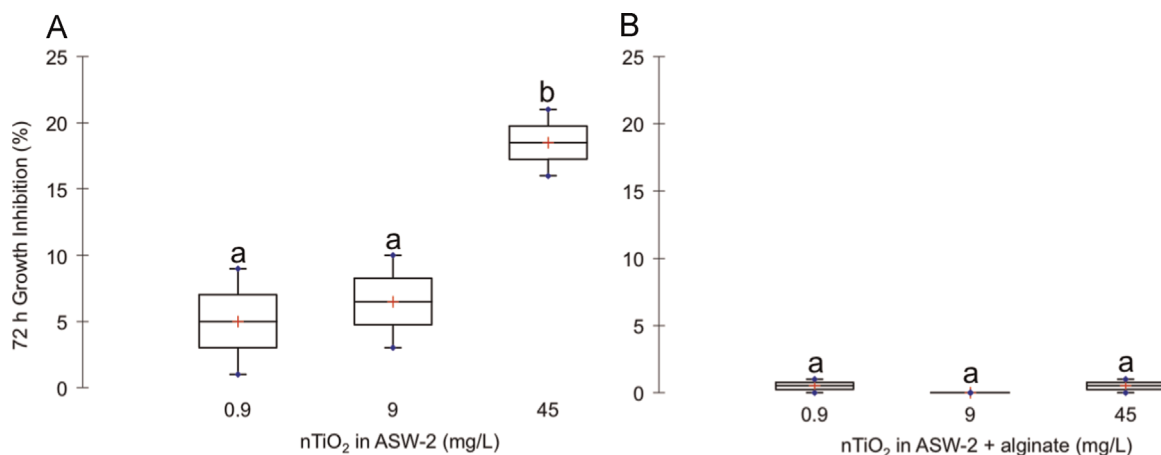


**Fig. 4.** (A) and (B) Effects of nano-TiO$_2$ on algae growth inhibition effect in standard conditions (UNI EN ISO 10253, 2006) (A) and in presence of alginate (B); data with different letters (a and b) are significantly different ($p < 0.05$).
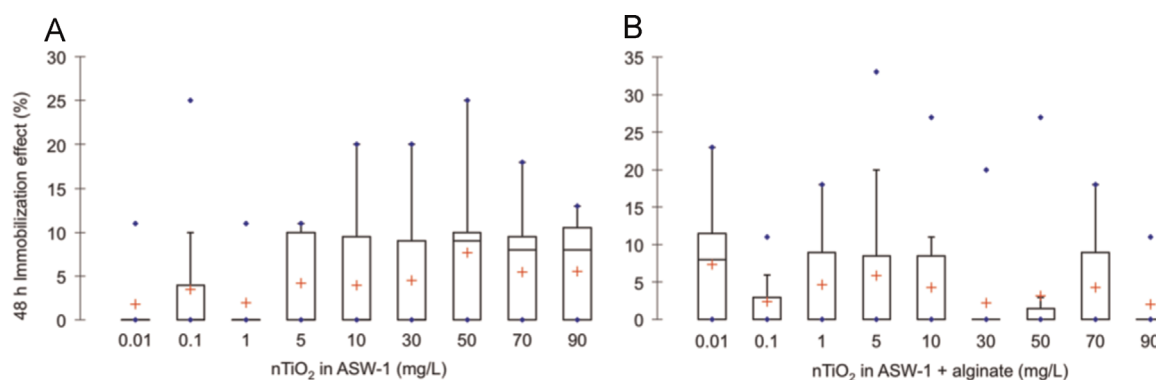
**Fig. 5.** Cumulative immobilisation after 48 h exposure to nano-TiO$_2$ of *A. franciscana* without (A) and with (B) alginate with any statistically significant effect.

did not allow the calculation of the relative EC50 value. As illustrated in Fig. S4, after 48 h of exposure to the reference toxicant diluted in ASW-1 the EC50 and its relative 95% confidence limits were 5.52 (4.90–6.23) (TSK) mg/L, whereas the CuSO$_4$ 5H$_2$O diluted in the ASW-1 alginate-enriched solution did not show effects greater than 40%.

Alginate may act as a confounding factor reducing the toxicity effects of CuSO$_4$ 5H$_2$O, but not of K$_2$Cr$_2$O$_7$. This can be explained considering its binding properties with divalent cations as studied by Fatin-Rouge et al. (2006) and Liu et al. (2010). Because of alginate relative affinity to bind to divalent ions (i.e. Pb$^{2+}$ ≥ Cu$^{2+}$ > Zn$^{2+}$ > Ni$^{2+}$), the complex of Cu$^{2+}$ originated from CuSO$_4$ 5H$_2$O was probably less bioavailable to *A. franciscana* than in standard conditions (without alginate). This kind of interactions must be seriously taken into consideration when using alginate as an agent to stabilise the nano-dispersions in order to avoid any underestimation of its potential adverse effects. Alginate should not present similar effects with nano-TiO$_2$ being the +3 and +4 the most common oxidation states.

### 3.3.2. Nano-TiO$_2$ effects to P. tricornutum

Ecotoxicological data from algae exposed to uncoated nano-TiO$_2$ are shown in Fig. 4A and B as box-plots referred to the absence and the presence of alginate in the ASW-2 testing exposure medium, respectively. From Fig. 4A, the maximum effect (18%) was observed at 45 mg/L of nano-TiO$_2$, whereas not significantly different results ($p < 0.05$) (5% and 7%) were detected at 0.9 and 9 mg/L of nano-TiO$_2$, in that order. Conversely, from very low to background effects were observed when nano-TiO$_2$ was diluted with the alginate-rich ASW-2 as shown in Fig. 4B. ANOVA tests ($p < 0.05$) outlined that the results from the two exposure scenarios were significantly different. The alginate in ASW-2, used to stabilise nano-TiO$_2$ concentration during ecotoxicological testing, also affected the nano-TiO$_2$ bioavailability. A capping or coating effect is likely to occur, thus false negatives may be possible. Moreover, *P. tricornutum* is a benthonic species, which, once inoculated into the exposure solution/dispersion, tends to rapidly settle to the bottom of the testing container. Actually, the association between the benthonic attitude of the algae and the relatively fast nano-TiO$_2$ deposition (i.e. almost fully settled after 72 h in ASW-2) (Keller et al., 2010) may lead, under standard conditions, to an overexposure scenario and thus positive toxicity effects. Therefore the alginate is able to stabilise dispersed nano-TiO$_2$ in reconstructed seawater keeping constant the exposure conditions in toxicity testing, but lowering the bioavailability of nTiO$_2$ compared to the standardised protocol.

### 3.3.3. Nano-TiO$_2$ effects to A. franciscana

Ecotoxicological data after 48 h exposure to the tested nano-TiO$_2$ are shown in Fig. 5 as box-plots from testing under

standard conditions (Fig. 5A) and after the addition of alginate (Fig. 5B) in ASW-1. Toxicity data after 24 h contact time to nano-TiO$_2$ were not presented due to the fact that no significant effects were detected between the negative controls and the treatments including the presence of alginate as well. After 48 h of cumulative exposure, no significant immobilisation effects were observed either with or without alginate with a maximum average effect in both cases of about 10%, which represents the acceptability threshold for the negative control. The presence of alginate in the nano-TiO$_2$ dispersion did not significantly influence ($p < 0.05$) the ecotoxicological results regardless the tested dispersions from 0.01 to 90 mg/L. However, due to the lack of toxicity data of nano-TiO$_2$ on other marine crustaceans and the relative sensitivity of *Artemia* spp. as reference biological model (Libralato, 2014) the presented results can be considered as preliminary to in-depth investigation followed by literature comparison.

## 4. Conclusions

Alginate, as an environmentally occurring biopolymer, acted as an effective stabiliser of uncoated nano-TiO$_2$ dispersions in artificial seawater. A concentration of 0.45 g alginate/L stabilised the nano-TiO$_2$ suspensions maintaining constant exposure conditions over the whole toxicity-testing period. This was also confirmed by the good reproducibility of the size distribution of various nano-TiO$_2$ dispersions.

The ecotoxicity tests carried on with *P. tricornutum* and *A. franciscana* showed that the considered alginate concentrations did not generate *per se* any adverse effect significantly different from the negative controls, but it can limit the bioavailability of ENMs during the exposure phase. Thus caution should be paid to its use in seawater ecotoxicity tests and a case-by-case approach is always strongly recommended.

## Appendix A. Suplementary Information

Supplementary data associated with this article can be found in

the online version at http://dx.doi.org/10.1016/j.ecoenv.2015.03.030.

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