The necessity of investigating a freshwater-marine continuum using a mesocosm approach in nanosafety: the case study of TiO2 MNM-based photocatalytic cement

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Abstract

Production of Manufactured Nanomaterials (MNMs) has increased extensively due to economic interest in the current years. However, this widespread use raises concern about their impact on human and environment. Current efforts are made, both at national and international levels to help developing safer MNMs in the market. In order to assess hazards of MNMs, it is important to take into account exposome parameters in order to link fate and behavior of MNMs to their potential toxicity. In that context, the aim of this study was to compare exposure and impact of TiO_2 MNMs-based cement at different levels of its life cycle (TiO_2MNMs, cement containing TiO_2 MNMs) between two exposure mesocosm scenarios mimicking : marine conditions using the bivalve *Scrobicularia plana* and freshwater conditions using the gastropod *Planorbarius corneus,* for 28 days. These approaches allows measurements of physical-chemical parameters throughout the duration of the exposure. Similar results were observed in both exposure conditions since in the two scenarios Ti was removed from the water column and accumulated in surficial sediments. While in *P. corneus,* statistically different concentrations of Ti were measured in the digestive glands compared to controls following exposure to TiO_2 MNMs, elevated background of Ti concentrations were measured in the controls of *S. plana* that did not allow to discriminating any bioaccumulation process. In addition, both TiO_2 MNMs and TiO_2MNM-based cement exposed *S. plana* did not present any activation of the p38 mitogen-activated protein kinase (MAPKs).This study demonstrates the challenge of using freshwater-marine continuum using a mesocosm approach in nanosafety.

Keywords:Mesocosm, TiO_2MNMs, *Scrobicularia plana*, *Planorbarius corneus*

INTRODUCTION

Releases of Manufactured Nanomaterial (MNMs) can occur during any stage of the MNM life cycle i.e. production stage of nanoproduct, the use/application stage of these nanoproducts and their ultimate end-of-life management/disposal. A comprehensive understanding of the potential for such releases along the whole life cycle and their possible effects is crucial to ensure the safe and sustainable use of these new materials (Salieri et al., 2018). Among all MNMs, TiO_2 MNMs are introduced into many products such as paints, plastics, food additives, sunscreens and other care products (Yin 2012). The use of TiO_2 MNMs into cement is a recent application in building material industry. Thanks to its photocatalytic properties, it confers to the building material air decontamination, self-sterilizing, self-cleaning and anti-fogging abilities (Jimenez et al., 2016). For those reasons, photocatalytic cement appears to be an appealing market for industry that represents from 0.1 to 1% of the total European production of TiO_2 and hence about 10.2t to 102t of TiO_2 MNMs released each year (Nanotechproject.org). TiO_2MNMs incorporated in the cement matrix are usually non-coated anatase with a mass concentration ranging from 0.3 to 10 wt% (Ruot et al., 2009). Degradation of TiO_2 MNM-based cement is likely to happen at each stage of the cement life cycle (production, manufacturing, use disposal and recycling) (Bossa et al., 2017). The main causes of release of TiO_2MNMsfrom photocatalytic coatings is the stripping of the TiO_2 from the coatings because of the water flow, the dispersion of agglomerate under NaCl and UV light condition and finally its discharge from loosening caused by mechanical damage (Olabarietta et al., 2012). A recent study showed that TiO_2 MNMs were released (18.7 ± 2.1 to 33.5 ± 5.1 mg of Ti/m^2 of cement after 168 h) from photocatalytic cement pastes that were leached at a lab-scale to produce a range of degradation rates, meaning that in the worst-case scenario of weathering a negligible mass of TiO_2 (0.04w.%) was released from the photocatalytic cement (Bossa et al., 2017). TiO_2 MNMs release comes from a very thin active surface layer where both the cement surface chemistry and its pore network morphology control the TiO_2MNMs diffusion (Bossa, 2019).

Laboratory studies have extensively demonstrated the adverse effects of TiO_2 MNMs on many aquatic organisms (Ramsden et al., 2009; Binh et al., 2016; Hall et al., 2009; Kulacki et al., 2012). Nevertheless, those studies have been conducted in simple systems (microcosm) and they did not reflect the MNMs fate and behavior in the environment due to processes such as sorption and aggregation of MNMs (Auffan et al., 2014). As a consequence, for a strongly characterization of risk, the combination of the organism, its environment and MNMs needs to be taken into account and requires interdisciplinary expertise in physical-chemistry,

biology and ecotoxicology (Auffan et al., 2014). In that context, mesocosms represent relevant experimental systems for investigating the complex issue of exposure that enable to get quantitative time- and spatial data on the distribution of MNMs within a simulated ecosystem. A mesocosm refers to "an experimental system that simulates real-life conditions as closely as possible, while allowing the manipulation of environmental factors" (FAO, 2009). As stated during the H2020 European commission funded NANoREG project (Project #646221), mesocosms have been shown to provide a reliable methodology to obtain quantitative time- and spatially regarding exposure-driven environmental risk assessment.

In this context, two indoor mesocosm platforms (one marine and one freshwater) were conceived with small size tanks (60 L) for investigating MNM exposure and impacts on aquatic species that allows to recreate freshwater and estuarine conditions (sediment, tide cycles, controlled temperature, salinity and redox potential) (Auffan et al. 2019). TiO_2 MNMs and TiO_2 MNM-based cement fate, behavior, bioaccumulation and toxicity were evaluated after a 28 day exposure in two mollusks representative of the estuarine and freshwater ecosystems : the bivalve *Scrobicularia plana* and the gastropod *Planorbarius corneus*. *Scrobicularia plana* is an endobenthic bivalve involved in the functioning and the structure of estuarine ecosystems and has also been largely demonstrated to represent a relevant model for biomonitoring (Mouneyrac et al., 2014). *Planorbarius corneus* is a hermaphroditic snail that inhabits small temporary ponds. This species belongs to the *Planorbidae*, the largest family of aquatic pulmonate gastropods distributed all over the world (Jopp, 2006). *Planorbarius corneus* play an important role in trophic chains as grazer and as prey (Wojdak and Trexler, 2010) and have already been used as model organisms for exposure and toxicity studies in mesocosms (Tella et al 2014, 2015, Auffan 2018).

The originality of the project was to compare the fate and behavior of a nano-enable product (TiO_2 MNM-based cement) in these two freshwater and marine ecosystems. Two stages of the MNM lifecycle were considered : the formulation stage using bare TiO_2MNMs and end of life stage of the cement containing TiO_2MNMs using cement leachate. Freshwater and marine mesocosms were used to obtain resolved data on the distribution, transformation and impact towards these mimicked ecosystems depending on the physical-chemical properties of the environment (salinity, pH, conductivity, tide cycle or not…) and the physical-chemical properties of the contaminant at two stages of the lifecycle. One of the major drawbacks in assessing environmental impact of MNMs is that the environmental concentrations are not yet known. Herein, we used between 1and 1.2 $mg.L^{-1}$of TiO_2 MNMs which belong to the threshold values estimated in highly exposed areas (Bourgeault et al., 2015).

MATERIAL AND METHODS

TiO_2NPs

Primary size and shape of TiO_2 MNMs (NM212, JRC repository) were determined by an ultra-high resolution scanning electron microscopy SEM LEO 1530 (LEO Electron Microscopy Ltd, Cambridge, England) and Transmission Electron Microscopy (analytical TEM Field Emission Gun (FEG) 200 KV Osiris from Tecnai, FEI, Japan). The SEM and TEM images corroborate the nanometric size of the particles (see SI, figure S1). X-ray diffraction (XRD) patterns of the samples were obtained using a Bruker D8 X-ray diffraction (XRD) system in a θ-2θ mode and Cu Kα X-ray source. The TiO_2 MNMs have a body-centered tetragonal anatase crystal structure (see SI). The width of the XRD peaks were used to calculate a crystallite size about 9 nm. A specific surface area of 223 m^2/g was determined by the Brunauer-Emmett-Teller (BET) adsorption method using the N_2 adsorption isotherm measured at 77K (BELSORP-max, BEL Japan Inc.).

TiO_2 MNMs-based cement

Photocatalytic white Portland cement incorporating TiO_2 MNMs was provided by a European cement manufacturer (Calcia, France) through the French Technical Association of Industries of Hydraulic Binders (ATILH, France). This cement is mainly composed of Ca and Si (≈66wt % CaO and ≈23 wt% SiO_2) and contains 2.85 wt% of TiO_2 (anatase) (Bossa et al., 2017). Cylindersof hardened cement (4 cm diameter, 8 cm high) were obtained after 28 days of hydration at 20°C with a water/cement ratio of 0.5. Hardened cement pastes exhibit a complex mineralogy, i.e. a mixture of hydrated minerals (portlandite, calcium silicate hydrates, ettringite) and residual anhydrous minerals (di and tricalcium silicates). TiO_2MNMs are homogeneously dispersed in the hydrated paste, with the exception of few high spotsof50 µm (Bossa et al., 2017).

Cement degradation residue generation was simulated using accelerated lab-scale approach to conduct a so-called « worst-case scenario » and to generate a quantity of cement degradation residues for mesocosms dosing. Finely crushed cement pastes were leached in batch using ultra-pure water (UPW) with an elevated liquid-to-solid ratio (L/S) of 100. After pH stabilization (pH > 12.5), the suspension of cement degradation residues was neutralized with slow addition of nitric acid (22.37 $mol.L^{-1}$) reach a pH of 7.8 and a conductivity of 13 $mS.cm^{-1}$. The solution is composed of a dissolved fraction (mainly Ca and Si, 203 and 3.96 $mg.L^{-1}$ respectively) and a particulate fraction with a TiO_2 concentration of 143.7±2.6 $mg.L^{-1}$. In

accordance with the very low TiO_2 MNM solubility, no TiO_2 MNM dissolution was observed (Table S1). The concentrations of Ca and Si in the dissolved fraction reveal the high degradability of the cement matrix in natural media (pH 7.8). Presence of free TiO_2 MNMs, i.e. not embedded in cement matrix, in the solution of cement degradation residues is then suspected. In neutralized cement degradation residues, TiO_2 MNMs were observed mainly associated with aggregates larger than 0.45 µm and with SiAl intermix chains. SiAl chains are supposed to be geopolymers stable at pH 7 and resulting from secondary precipitation/ polymerization during the pH neutralization process (Bossa et al., 2017).

Animals

Individuals of *Scrobicularia plana* (15-20 mm length) were hand-collected in November 2015 in the intertidal mudflat on the French Atlantic coast (Bay of Bourgneuf: N 47°01'50.35", W 1°59'04'80'') and transported in cool boxes to the laboratory. Then, bivalves were immediately placed into aerated artificial water (Tropic marin®) at 30 practical salinity units (psu) during 5 days in a temperature controlled room at 15°C (temperature experienced in the field at the collection time). A natural inoculum containing picoplankton as primary producer (bacteria, algae, protozoa, *etc.*) was also collected in the intertidal mudflat. Physico-chemical parameters of seawater from the collection site were : pH= 7.7; salinity 28 psu; conductivity 40 $mS.cm^{-1}$.

Individuals of *Planorbarius corneus* (L., 1758) (Great Ram's Horm snail, benthic grazer) were hand-collected in October 2015 in a non-contaminated temporary pond part of the Natura 2000 reserve network in south of France (Bonne Cougnes: N 43°20'47.04", E 6°15'34.786'', 246 m a.s.l.) and transported in pond water to the laboratory. A natural inoculum containing picobenthosas primary producer (bacteria, algae, protozoa, *etc.*) was also sampled in the pond. Physico-chemical parameters of the pond water from the collection site were: water temperature 14.9°C; conductivity 819 $mS.cm^{-1}$; concentration of dissolved oxygen 0.72 $mg.L^{-1}$ (6.6%).

Mesocosm setup

Freshwater mesocosms. The mesocosm experiments were already described by Auffan et al. (2014), Tella et al. (2014), Tella et al. (2015), Auffan et al. (2018)(figure 1). Tanks (750 x 200 x 600 mm) were filled with 6-8 cm artificial sediments containing 89% SiO_2, 10% kaolinite and 1% of $CaCO_3$ w/w adapted from OECD guideline (OECD, 2004). Primary producers were brought by ~300 g of water-saturated natural sediment (sieved at 200 µm) laid at the

surface. The mesocosms were then gently filled with 50 L of Volvic water with pH and conductivity close to the natural pond water (pH 7, 11.5 mg.L^{-1} Ca^{2+}, 13.5 mg.L^{-1} Cl^{-}, 71 mg.L^{-1} HCO_3^{2-}, 8 mg.L^{-1} Mg^{2+}, 6.3 mg.L^{-1} NO_3^{-}, 6.2 mg.L^{-1} K^{+}, 11.6 mg.L^{-1} Na^{+}).

After 2 days, the physical-chemical parameters of the mesocosms were stabilized (turbidity, pH, dissolved O2, redox). Then, 19 adult *P. corneus* were added per mesocosm and acclimatized for one week. Organisms were exposed for 28 days in a temperature controlled room (18°C) and under controlled light (photoperiod day/night 10:14). Physico-chemical parameters including temperature (°C), pH, redox potential in the water column and the sediment (mV), and dissolved oxygen (mg O2.L-1) were monitored every 2 min during the whole duration of the experiment. Among the 6 freshwater mesocosms, 2 were kept as negative control (without contamination), 2 were exposed to TiO2 MNMs (considered as positive control), and 2 were exposed to cement leachate. While the first control (negative control) condition allows to follow animal behavior (mortality) during the experiment, the second one serve as a positive control experiment to follow TiO2 bioaccumulation by the organisms in order to compare with animals treated with cement for both freshwater and marine systems.

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Marine mesocosms. Each experimental unit was composed with two tanks (mesocosm and reserve tank), two pumps (Eheim compact 300L.h^{-1}) and a mechanical timer (IDK PMTF 16A) allowing to mimicking the tidal cycle (figure 1). The mesocosm tank (750 x 200 x 600 mm) was filled with 14 kg synthetic sediment (90% SiO_2, 9% kaolinite, 1% $CaCO_3$ w/w) adapted from OECD guideline (OECD, 2004). Three hundred g of water-saturated natural inoculums was laid at the surface to bring primary producers. Then, each mesocosm tank was flooded with 35 L of the artificial seawater Tropic Marin™ (Tropicarium Buchshlag Dreieich, Germany) at 30psu. The reserve tank (700 x 500 x 37 mm) was placed under the first one (mesocosm tank) to collect removed water from the mesocosm tank during low tide. Reservoir tank was always filled with a minimum of 25 L of artificial seawater at 30 psu to constantly maintain the measuring probe immersed.

After one week, the physical-chemical parameters of the mesocosms were stabilized (turbidity, pH, dissolved O_2, redox). Then, 55 *S. plana* were added per mesocosm and acclimatized for 5 days. Organisms were exposed for 28 days in a temperature controlled room (15°C), under controlled light (photoperiod 16:8) and at a tidal cycle (6h of low tide, 6h of high tide; two tides /day). Physical-chemical parameters including temperature (°C), salinity (psu), pH, redox potential (mV), dissolved oxygen (mg O_2.L^{-1}) were monitored every

15 min into the water column from the reserve tank during the whole duration of the experiment. Among the 9 marine mesocosms, 3 were kept as negative control (without contamination), 3 were exposed to TiO_2 MNMs (positive control) and 3 were exposed to cement leachate.

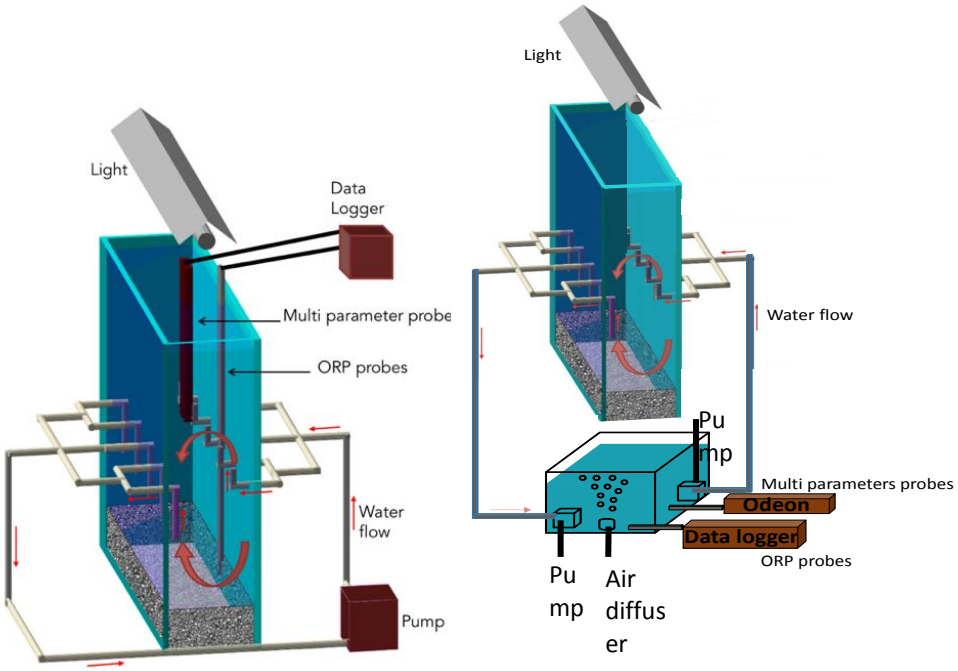


Figure1. Scheme of the freshwater (left) and marine (right) mesocosms.

Mesocosm dosing

Aqueous suspensions of TiO_2 MNMs and TiO_2 MNM-based cement degradation residues (with a stabilized pH of 7.8) were prepared prior injections in the mesocosms. Aliquots of these suspensions were digested using an Ultra WAVE microwave digestion system with 1 mL HNO_3 67% (NORMATOM) and 1 mL HF 47%-51% (PlasmaPure) and analysed by ICP-MS (Perkin Elmer© Nexion 300 ICP-MS) for their total Ti contents. A concentration of 4.86±0.13 $g.L^{-1}$ TiO_2(n=3) was found for the TiO_2 MNM suspension (2.91±0.08 $g.L^{-1}$Ti). The TiO_2 concentration measured in the solutions of cement degradation residues (n=6) was 143.7±2.6 $mg.L^{-1}$ (86±1.6 $mg.L^{-1}$Ti). Both suspensions were close to the targeted concentrations and showed excellent recovery yields after digestion (> 95%).

A multiple dosing experiment was performed on a 4 week-period. A total of 12 dosings were achieved (3 times per week), corresponding to 0.09 $mg.L^{-1}$ of TiO_2 per injection in order to reach a final nominal concentration of 1$mg.L^{-1}$ TiO_2 per mesocosm. As the suspension of cement degradation residues is not stable over time, a new neutralized batch was prepared every two injections and stored at 4°C in dark between the two injections.

The real concentration injected over time was determined by ICP-MS and are plotted in Figure 2. For each injection, a low and realistic concentration of 0.1 mg.L^{-1} of injected TiO_2 was targeted in the mesocosm (0.06 mg.L^{-1}Ti). After 30 days (12 injections), the cumulated concentration of TiO_2 injected reached 1-1.2 mg.L^{-1} in the mesocosms.

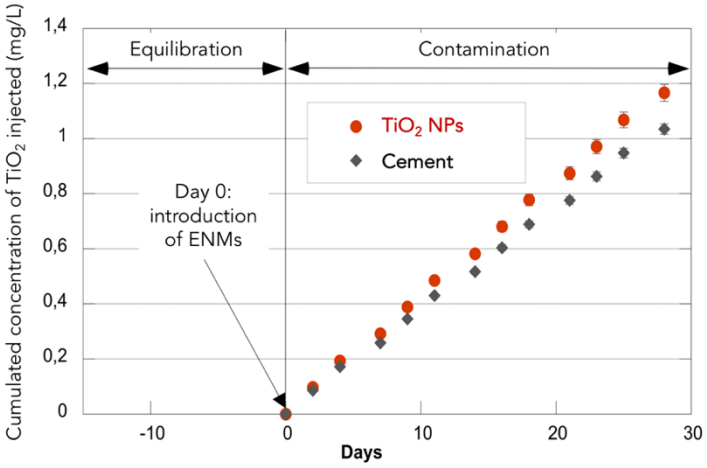


Figure 2. TiO_2 injection sequence for TiO_2MNMs and TiO_2MNM-based cement degradation residues in both freshwater and marine mesocosms over the 28 days-contamination period.

Ti quantification in the different compartment of the mesocosms

The concentration of Ti in the mesocosms following TiO_2 MNMs or cement leachate contamination was measured in the water column, in the surficial sediments and in the organisms sampled at 0, 7, 14, 21 and 28 days. Water was sampled at ~10cm from the air/water interface. Surficial sediments (between 500 µm to 1000 µm depth) were sampled at three different locations and then pooled before being dried. Samples were digested using microwaves at 180°C before analysis by ICP-MS.

For the marine mesocosms, Ti quantifications were performed by Micropolluants Technologie S.A. (Metz, France) using an Agilent 7700 ICP-MS (Agilent Technologies France). 10 mL of water or 2 mL of surficial sediments were mixed with 1mL 32-35% HCl, 1mL 67-69% HNO_3, 100 µL 94-98% H_2SO_4, and 1 mL 47-51% HF prior ICP-MS measurements. For *S. plana*, whole soft tissues were mixed with 1 mL 67-69% HNO_3, 100 µL 94-98% H_2SO_4, and 1 mL 47-51% HF. For the freshwater mesocosms, water samples (2 mL) were digested with 1 mL HNO_3 67-69% and 0.5 mL HF 47%-51%. For the surficial sediments (50 mg), a mixture of 3 acids (1 mL HCl 34%, 2 mL HNO_3 67%, 0.5 mL HF 47%-51%) was used. For *P. corneus*, the

digestive glands were dissected, before being digesting using 1 mL HNO_3 67%, 0.5 mL H_2O_2 30%-32% and 1 mL HF 47%-51%. Samples were digested using the microwave Ultra WAVE and the analysis were performed using the Perkin Elmer Nexion 300 ICP-MS. Using sediments and freshwater organisms spiked with TiO_2 nanoparticles, a recovery yield of >95% after microwave digestion and ICP-MS analysis was obtained.

Evaluation of immunotoxic effects in S. plana: pp38 level measurement by Western blot

Total whole soft tissues of *S. plana* exposed for 28 days (n=7 for each condition) were homogenized with lysis-buffer (NaCl 0.2M, DTT 1mM, protease inhibitor cocktail 0.1%) in a mortar at 4°C. The clear supernatant was then obtained by centrifugation (13,000xg, 10 min, 4°C) and stored at -20°C until analysis. Protein concentrations were determined according to Bradford (1976) using bovine serum albumin as a standard. Protein extracts were mixed with sample buffer (0.5 M Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 4% 2-mercaptoethanol, 0.05% bromophenol blue) 1:3 and boiled for 5 min at 95°C. Proteins (40 µg per lane) were separated using a 12% polyacrylamide gel. Western blot analysis was performed as previously described (Châtel et al., 2011b). After transfer, membranes were incubated with a mouse monoclonal antibody anti-Phospho-p38 MAPK (Thr_{180}/Tyr_{182}) (9216-Cell signaling Biotechnology) diluted in 1/1000 in TBS/BSA 5% or with a mouse monoclonal antibody anti-β-Actin (A5441 -SIGMA) diluted in 1/2000 in TBS/BSA 5%. After washing (3 times for 5 min), membranes were incubated with a goat anti-Mouse IgG antibody coupled with alkaline phosphatase (A3562, Sigma Aldrich) diluted 1:2000 in TBS/BSA 5%. Detection of immune complexes was carried out by colorimetric reaction using a solution of 5-bromo-4-chloro-3-indolyl phosphatase (BCIP) and nitroblue tetrazolium (NBT). After visualization, membranes were scanned and band intensity, which is the relative amount of protein expression, was quantified as Relative Optical Densities (ROD; pixels/mm^2). Results of pp38 were normalized according to actin expression.

Statistical analysis

The measured values were compared among different groups using the non-parametric test Mann–Whitney (XL-Stat software) and the Student's t-test. Statistical significance was accepted at $p<0.05$.

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RESULTS and DISCUSSION

Favorable physical-chemical conditions in freshwater and estuarine mesocosms

During the 28 days of contamination several physical-chemical parameters were monitored to assess the global response of the mesocosms to the presence of TiO_2 MNMs and cement leachate. In marine as well as in freshwater mesocosms, the average temperature, dissolved O_2, conductivity, and pH were not significantly different between negative controls and contaminated mesocosms(see SI, Figures S2 and S3).

Oxido-reductive probes indicated that freshwater was oxidative (310±20 mV), while reductive conditions prevailed in the sediments (*ca* -370 mV). Regarding the conductivity, a step by step increase from day 0 to day 28 (200 ± 1 µS.cm^{-1} to 288 ± 0.4 µS.cm^{-1}respectively) was observed. Conductivity drops were recorded during the weekly refills with ultrapure water to compensate the evaporation. On the whole, no significant differences were observed between controls and contaminated mesocosms.

Consequently, during the exposure to TiO_2 MNMs and cement leachate, the physico-chemical conditions of the 15 mesocosms remained favorable with the oxygenation, pH, temperature, redox potential in the range of natural conditions.

Homo-aggregationof TiO_2MNMs and accumulation in the surficial sediments

Bossa et al. (2017) estimated using an accelerated aging protocol that a negligible mass of TiO_2 (0.04w.%) was released from the photocatalytic cement (Bossa et al., 2017). Herein, we used an accelerated lab-scale approach to conduct a so-called « worst-case scenario » assuming that 100% of the Ti contained in the cement would have been released. Figure 3 and 4 show the Ti concentrations measured in the water column and sediments of both freshwater and marine mesocosms as a function of time. Ti was detected in both waters and sediments sampled in control mesocosms proving that a high geochemical background of this element existed in the mimicked ecosystems. Average background Ti concentrations in freshwater mesocosms were 19.2±17.2 µg.L^{-1}in the water column and 2230±928 mg.kg^{-1} in surficial sediments. Average background Ti concentrations in marine control mesocosms were 567±273mg.kg^{-1} in surficial sediments and below the quantification limit (<2.5 µg.L^{-1}) in the water column. Ti is a naturally occurring element in mineral and amorphous phases occurring in freshwater and marine environments. Ti can be found in titanium-iron oxide minerals (as ilmenite), TiO_2 minerals as rutile, anatase, and brookite. Herein, the TiO_2 MNMs used were engineered nanosized anatase minerals. Consequently even if the Ti background concentration

was elevated, it is noteworthy that the speciation and reactivity of the naturally occurring Ti-bearing phases might be different from the MNM injected.

In the surficial sediments of marine mesocosms, high concentrations of Ti were detected after 7 days (control, TiO_2 MNMs, and cement leachate) and progressively decreased until 28 days (Figure 4, right). However, because of the elevated geochemical background of Ti, no statistical difference was observed between controls and contaminated mesocosms. Similarly, no increase in Ti concentration could be evidenced in the surficial sediments of contaminated freshwater mesocosms compared to controls. Besides, the fluctuations of Ti concentration measured over time were likely related to the heterogeneity of the Ti distribution and the difficulty of sampling the surficial sediments(Figure 4). These results highlighted the challenge of a reliable quantification of Ti in that compartment.

Total Ti concentrations in the water column of marine mesocosms (control, TiO_2 MNMs and cement leachate) were always below the quantification limit estimated at 2.5 $\mu g.L^{-1}$ whatever the duration of exposure. On the opposite, total Ti concentrations in the water column were always above 10 $\mu g.L^{-1}$ in freshwater mesocosms. The only condition for which a statistically significant difference was observed between the water column of controls and contaminated freshwater mesocosms was after 7 days of exposure to TiO_2 MNMs. At this time point, the difference between TiO_2 MNMs contaminated mesocosms and geochemical Ti background were 2.2±1.2 $\mu g.L^{-1}$Ti for a total Ti concentration injected of 180 $\mu g.L^{-1}$Ti (300 $\mu g.L^{-1}$ of TiO_2). Based on these values, we estimated that 98-99 % of the Ti injected was removed from the water column after 7 days. Although no increase in Ti concentration could be evidenced in the surficial sediment of freshwater mesocosms, we hypothesize that both cement leachate and TiO_2 MNMs settled at more than 98% at the surface of sediments. Based on the 31.0±0.6 mg and 35.0±0.9 mg Ti introduced respectively after 28 days in mesocosms contaminated with cement leachate and TiO_2 MNMs, the remaining of 2% of Ti particles in the water column should entail a theoretical increase in the surficial sediment of176 $mg.kg^{-1}$, which is far below the standard deviation determined for Ti background in that compartment. This explains why Ti accumulation at sediment surface remained undetected by chemical analysis. In addition, in marine mesocosms, due to the tidal system, part of the sediment were removed along with the water in the lower tank and sedimentation of TiO_2 occurred as well in this compartment favoring decrease in its concentration as compared to freshwater mesocoms.

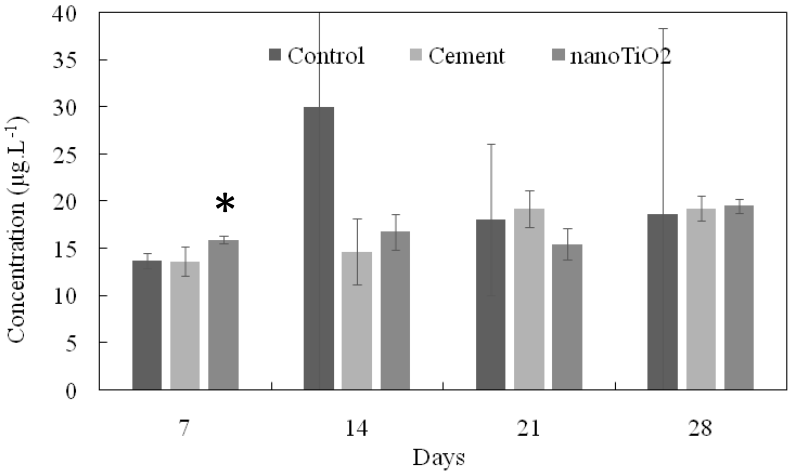


Figure 3. Total Ti concentration in the water column after 7, 14, 21 and 28 days of exposure in freshwater mesocosms. Ti concentration in the water column in marine mesocosms is not represented as values were always below the quantification limit estimatedat 2.5 µg.L^{-1}. Statistical significance was determined by the Student's t-test (*: p<0.05).

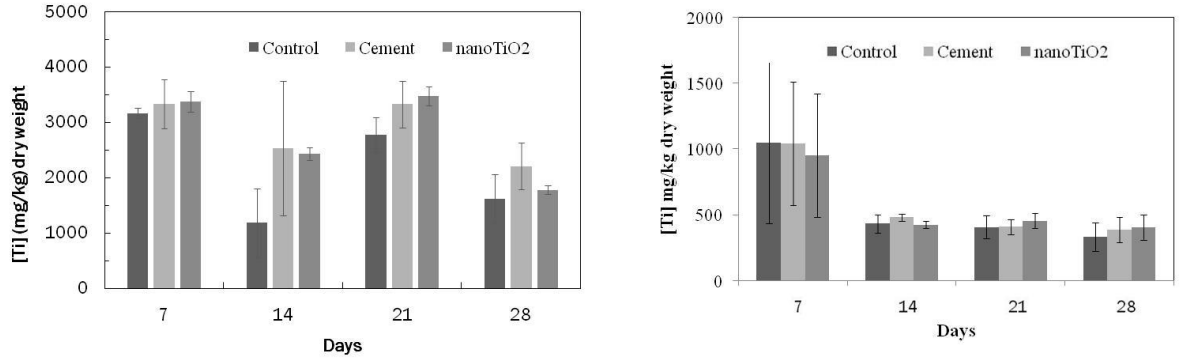


Figure 4. Total Ti concentration in surficial sediment after 7, 14, 21 and 28 days of exposure in freshwater (left) and marine (right) mesocosms.

In freshwater mesocosms, the size distribution of the suspended matter in the 0.1 µm – 1000 µm size range and the total number of suspended particles in the 0.4 µm – 0.9 µm size range were not different between control and contaminated mesocosms (Figure 5). This highlighted that the main mechanism of aggregation and settling down expected in the water column was the homo-aggregation of the TiO_2 MNMs either pristine or brought *via* cement leachate. In marine mesocosms, MNM homo-aggregation was expected to be even faster due to higher alkalinity and ionic strength as compared to freshwater environments (Peralta-Videa et al., 2011; Rocha et al., 2015;Vale et al., 2016). It is noteworthy that the aggregation of MNMs allows their deposition onto the surficial sediment (Peralta-Videa et al., 2011), resulting in high exposure of benthic organisms compared to planktonic organisms (Mohd Omar et al., 2014; Mouneyrac et al., 2014).

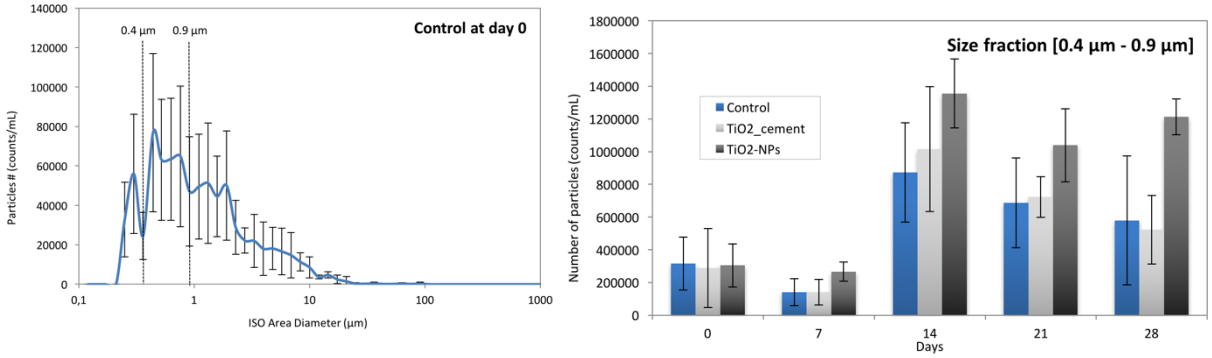


Figure 5. (left) Size distribution of colloidal particles in the water column of freshwater control mesocosms at day 0. The dotted lines mark out the 0.4 µm – 0.9 µm fraction. (right) Evolution of the number of colloidal particles in the 0.4µm – 0.9 µm range from day 0 to 28, under the three conditions tested (control, cement leachate and TiO_2 MNMs) in freshwater mesocosms.

Bioaccumulation and effects on benthic organisms

P. corneus and *S. plana* are two benthic grazer species. While *P. corneus* eat algae and biofilms at the sediment/water interface(Jones 1961), *S. plana* can either feed suspended particles from the water column but also ingest sedimentary particles (Hughes, 1969). For *S. plana* exposed in marine mesocosms, elevated background of Ti concentrations were measured in the control group (84.8±42.3 $mg.kg^{-1}$ dry weight) which did not allow to discriminating any bioaccumulation process in organisms exposed to TiO_2 MNMs and cement leachate (97.1±43.9 and 107.5±48.2 $mg.kg^{-1}$ dry weight, respectively) (figure 6).

Background concentrations of Ti measured in the digestive glands of unexposed *P. corneus* were one order of magnitude lower. Following exposure to cement leachate, no difference in Ti concentration in the digestive glands were measured with respect to control *P. corneus*. However, statistically different concentrations of Ti were measured in the digestive glands compared to controls following exposure to TiO_2 MNMs *i.e.*1.5 ± 0.2 mg $Ti.kg^{-1}$ (dry weight) and 2.5 ± 0.1 mg $Ti.kg^{-1}$ (dry weight) after 21 and 28 days of exposure respectively (figure 6). This suggests that an ingestion of Ti by *P. corneus* occurred likely resulting from the accumulation of TiO_2 MNMs on the surficial sediments.

Some authors have shown abilities to discriminate natural Ti from TiO_2 NMs. For example, Bourgeault et al. (2015) resolved this lack of sensitivity using isotopically modified TiO_2 nanoparticles (^{47}Ti) to characterize the processes governing Ti bioaccumulation in a freshwater environment. Thanks to the ^{47}Ti labeling, they detected bioaccumulation of NPs in *Dreissena polymorpha* exposed for 1 h at environmental concentrations *via* water (7–120 µg/L of $^{47}TiO_2$ NPs) and *via* their food (4–830 µg/L of $^{47}TiO_2$ NPs mixed with 1×10^6

cells/mL of cyanobacteria) despite the high natural Ti background, which varied in individual mussels. Such a methodology would be particularly relevant in mesocosms experiment mimicking real environments with high Ti background.

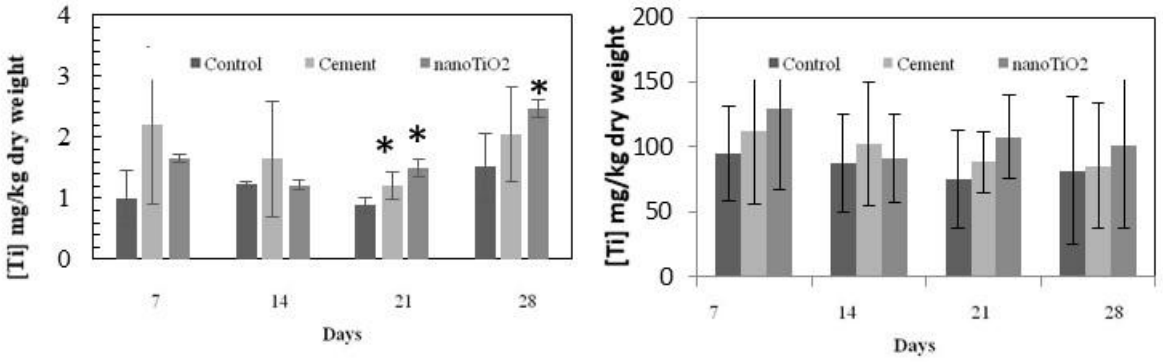


Figure 6. Total Ti concentration in the digestive gland of *P. corneus* (left) and total soft tissues of *S. plana* (right) after 7, 14, 21 and 28 days of exposure in mesocosms. The statistical significant was determined by the Student's t-test (*: $p<0.05$; ** : $p<0.01$).

During the 28 days of exposure in mesocosms, the survival rates of *P. corneus* and *S. plana* (<2% of mortality, data not shown), picoplankton and picobenthos (see SI, Figure S4) were not affected by the presence of TiO_2 MNMs and cement leachate. Hence, no acute toxicity was observed towards these two trophic levels. Nevertheless, a thorough characterization of the biological responses (at the individual, sub-individual, and community levels) is still needed to better understand the biological mechanisms of interactions.

One of the main effect associated with TiO_2 toxicity was the induction of immune responses. The MAPKs represent a superfamily of protein Ser/Thr-kinases, highly conserved through evolution (Kyriakis and Avruch, 2001; Roux and Blenis, 2004), that can transduce stress signals into cellular responses (Kultz and Avila, 2001; Kyriakis and Avruch, 2001). Three subfamilies of the MAPKs have been well described in mammals: the extracellular regulated protein kinase (ERK), the c-Jun NH_2-terminal kinases (JNK) and the p38-MAPK. ERKs have been more associated to cell division, growth and differentiation whereas JNKs and p38-MAPKs are activated in various types of stress going from osmotic stress to chemical stress and can trigger cell survival or death (apoptosis), depending on their isoform and/or cell type (Canesi et al.,2001, 2002, 2006; Châtel et al., 2009, 2011a,b). They have been demonstrated to play a key role in immune responses in mammals (Caffrey et al., 1999) but also in invertebrates and more particular in bivalves (Canesi et al., 2001; 2002; 2006). Moreover p38

MAPK have been shown to be induced by TiO2 exposure in bivalves (Couleau et al., 2012). P38 MAPK in *S. plana* have been studied in the laboratory and antibody matching has already been verified on this specie as opposed to *P.corneus*. Since no ingestion of Ti by *S. plana* was identified by ICP-MS, western blot analysis of *S. plana* using specific antibodies directed against the phosphorylated form of p38 MAPK were used (Figure 7).

In the present study, membrane revelation showed that the molecular weight of pp38 in *S. plana* is around 38 KDa. After normalization of the different bands representing pp38 expression with actin, results revealed that there was no significant modification in pp38 expression level for any of the conditions tested (TiO_2 MNMs or cement leachate), as compared to control group (figure 7). This suggested no stimulation of immune response of *S. plana* following 28 days of exposure. Couleau et al. (2012) observed in the freshwater mussel *D. polymorpha* exposed to TiO_2 (0.1 to 25 $mg.L^{-1}$ during 24 h), that ERK ½ was activated for all concentrations tested in this study whereas p38 activation was only observed when bivalves were exposed to 5 and 25 $mg.L^{-1}$) indicating that p38 phosphorylation was less sensitive than ERK ½ to this contaminant. This could be the case in the present study. Moreover, an *in vitro M. galloprovincialis* hemocyte exposure to TiO_2 (10 $\mu g.ml^{-1}$) for 5 to 60 min showed that pp38 activation was transient, with an increase in phosphorylated form of p38 after 5 and 15 min, followed by a dephosphorylation at 30 and 60 min (Canesi et al., 2010). Even though p38 is implicated in immune responses, absence of p38 activation does not mean that immune response is not present. However, in the present study, the long duration of exposure (28 days) could explain the absence of pp38 response depicted in *S. plana* and investigation of its response at shorter time points would have been interesting to perfom.

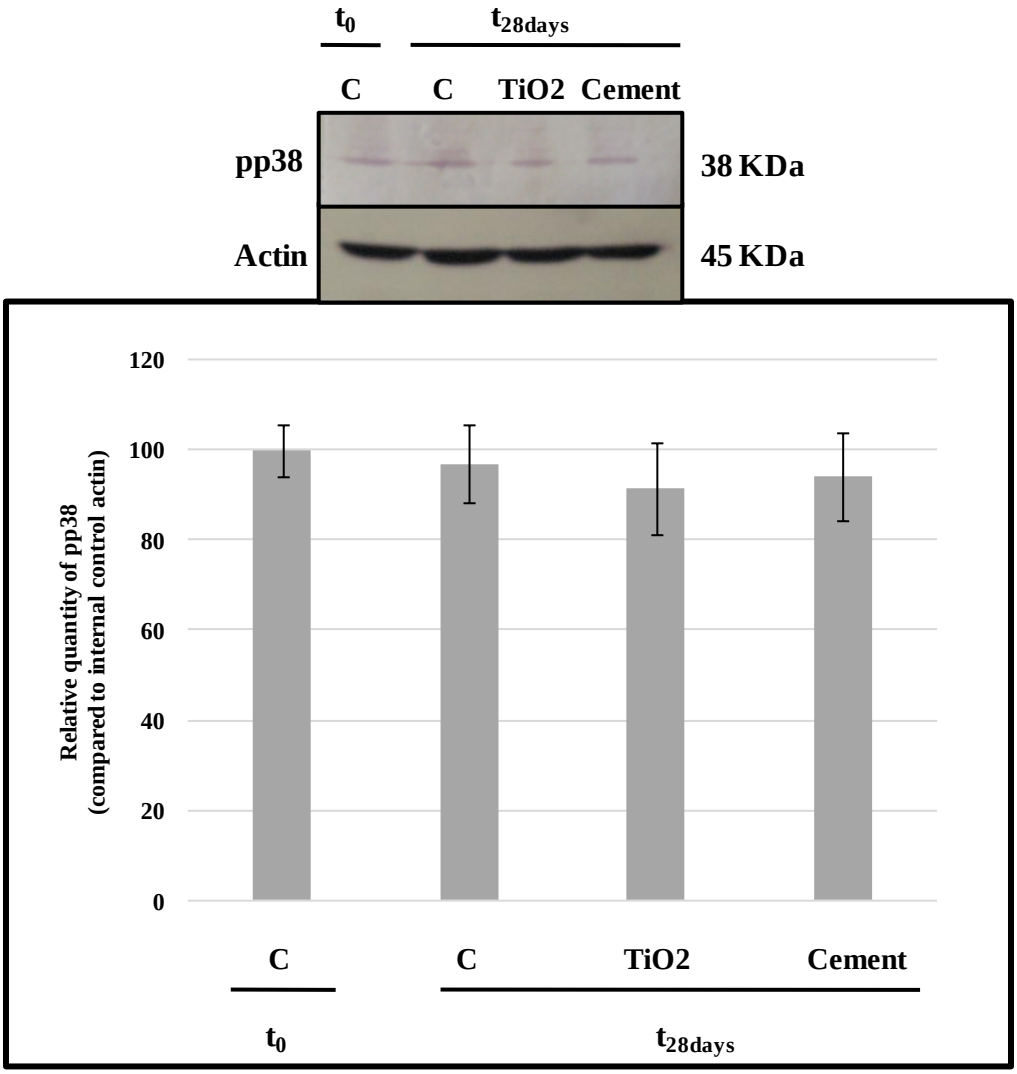


Figure 7. Expression of pp38 in *S. plana* control, TiO_2 MNMs or cement leachate, analyzed by Western blot. Results have been normalized to actin expression levels.

Conclusion

In the present study, impact of TiO_2 MNMs and TiO_2 MNM-based cement leachate was investigated in marine and freshwater mesocosms, that allows to reproduce realistic environmental exposure (mid-term duration, low doses, chronic contamination,…) and in particular, a tidal system in the marine mesocosm. Such tools have been demonstrated as being relevant since they take into account both hazard and exposure and hence the synergetic and/or antagonistic effects of different physico-chemical parameters (temperature, pH, redox potential, conductivity), that is of great interest for the regulation of the use of NMs (Auffan et al., 2019).The originality of the project was also to compare the exposure and potential impact of TiO_2 and cement containing TiO_2 MNMs on two species, representative of the estuarine/freshwater continuum(with a salinity gradient),that are particular targets towards

nanomaterials at the water/sediment interface. The use of similar design for both freshwater and marine systems showed challenging points and limitations, that enable consistent comparison of fate and behavior of NMs in different species. However, even with same procedures, characterization of TiO_2 in different abiotic compartments was difficult, as stated in this study, and made challenging investigations of differences in bioaccumulation and effects in these benthic species. The standardization of stable test suspensions for nanomaterials is an ongoing challenge as it is well documented that the preparation method influences the interpretation of toxicity according to the exposure. In the context of developing a regulatory framework this is a challenge as it necessary that all nanomaterials have equivalent preparation techniques in order to have a better standardization of experiments and hence a more comparable set of data generated for nanomaterial-related data categorization.

Finally, studying the impact of TiO_2 at different stages of the TiO2 MNMs life cycle allows identifying critical stages at which Safe by design (SbD) concepts can be implemented to reduce adverse impacts or decrease exposure to MNMs.

REFERENCES

Auffan, M., Tella, M., Santaella, C., Brousset, L., Paillès, C., Barakat, M., Espinasse, B., Artells, E., Issartel, J., Masion, A., Rose, J., Wiesner, M.R., Achouak, W., Thiéry, A. and Bottero, J.Y., 2014. An adaptable mesocosm platform for performing integrated assessments of nanomaterial risk in complex environmental systems. Sci. Rep. 4, 5608. https://doi.org/10.1038/srep05608

Auffan, M., Masion, A., Mouneyrac, C., de Garidel-Thoron, C., Hendren, C., Thiery, A., Santaella, C., Giamberini, L., Bottero, J.Y., Wiesner, M. and Rose, J., 2019. Contribution of mesocosm testing to a single-step and exposure-driven environmental risk assessment of engineered nanomaterials. NanoImpact. 13, 66-69. https://doi.org/10.1016/j.impact.2018.12.005

Auffan, M., Liu, W., Brousset, L., Scifo, L., Pariat, A., Sanles, M., Chaurand, P., Angeletti, B., Thiery, A., Masion, A. and Rose, J., 2018. Environmental Exposure of a Simulated Pond Ecosystem to Cuo Nanoparticle Based-Wood Stain Throughout Its Life Cycle. Environ. Sc.: Nano. 5, 2579-2589. https://doi.org/10.1039/C8EN00712H

Bour, A., Mouchet, F., Cadarsi, S., Silvestre, J., Verneuil, L., Baqué, D., Chauvet, E., Bonzom, J. M., Pagnout, C., Clivot, H., Fourquaux, I., Tella, M., Auffan, M., Gauthier, L. and Pinelli, E., 2016. Nanoparticles on a freshwater experimental trophic chain: A study in environmentally relevant conditions through the use of mesocosms. Nanotoxicology. 10 (2), 245-55. https://doi.org/10.3109/17435390.2015.1053422

Bourgeault, A., Cousin, C., Geertsen, V. , Cassier-Chauvat, Chauvat, C., F., Duruphy, O., Chanéac, C. and Spalla, O., 2015. The challenge of studying TiO2 nanoparticle bioaccumulation at environmental concentrations:crucial use of a stable isotope tracer. Environ. Sci. Technol. 49(4), 2451-9. https://doi.org/10.1021/es504638f

Caffrey, D.R., O'Neill, L.A.J. and Shields, D.C., 1999. The evolution of the MAP kinase pathways: coduplication of interacting proteins leads to new signaling cascades. J. Mol. Evol. 49, 576- 582. http://dx.doi.org/10.1007/PL00006578

Canesi, L., Betti, B., Ciacci, M. and Gallo, G., 2001. Insulin-like effect of zinc in *Mytilus* digestive gland cells: modulation of tyrosine kinase-mediated cell signaling. Gen. Comp. Endocrinol. 122, 60-66. https://doi.org/10.1006/gcen.2001.7612

Canesi, L., Betti, M., Ciacci, C., Scarpato, A., Citterio, B., Pruzzo, C. and Gallo., G., 2002. Signaling pathways involved in the physiological response of mussel hemocytes to bacterial challenge: the role of stress-activated p38 MAP kinases. Dev. Comp. Immunol. 26, 325-334. https://doi.org/10.1016/S0145-305X(01)00078-7

Canesi, L., Ciacci, C., Lorusso, L.C., Betti, M., Guarnieri, T., Tavolari, S. and Gallo, G., 2006. Immunomodulation by 17 beta-estradiol in bivalve hemocytes. Am. J. Physiol. Regul. Integr. Comp. Physiol. 291, R664-673. https://doi.org/10.1152/ajpregu.00139.2006

Canesi, L., Ciacci, C., Vallotto, D., Gallo, G., Marcomini, A. and Pojana, G., 2010. *In vitro* effects of suspensions of selected nanoparticles (C60 fullerene, TiO2, SiO2) on *Mytilus* hemocytes. Aquat. Toxicol. 96 (2), 151-8. https://doi.org/10.1016/j.aquatox.2009.10.017

Châtel, A., Hamer, B., Talarmin, H., Dorange, G., Schroder, H.C. and Muller, W.E., 2009. Activation of MAP kinase signaling pathway in the mussel *Mytilus galloprovincialis* as biomarker of environmental pollution. Aquat. Toxicol. 96, 247-255. https://doi.org/10.1016/j.aquatox.2009.11.002

Châtel, A., Talarmin, H., Hamer, B., Schroder, H.C., Muller, W.E. and Dorange, G., 2011a. MAP kinase cell signaling pathway as biomarker of environmental pollution in the sponge *Suberites domuncula*. Ecotoxicology. 20, 1727-1740. http://dx.doi.org/10.1007/s10646-011-0706-1

Châtel, A., Hamer, B., Jaksic, Z., Vucelic, V., Talarmin, H., Dorange, G., Schroder, H.C. and Muller, W.E. 2011b. Induction of apoptosis in mussel *Mytilus galloprovincialis* gills by model cytotoxic agents. Ecotoxicology. 20, 2030-2041. https://doi.org/10.1007/s10646-011-0746-6

Couleau, N., Techer, D., Pagnout, C., Jomini, S. , Foucaud, L. , Laval-Gilly, P. , Falla, J. and Bennasroune, A., 2012. Hemocyte responses of *Dreissena polymorpha* following a short-term in vivo exposure to titanium dioxide nanoparticles: preliminary investigations. Sci. Total Environ. 438, 490-7. https://doi.org/10.1016/j.scitotenv.2012.08.095

Farré, M., Gajda-Schrantz, K., Kantiani, L. and Barceló, D., 2009. Ecotoxicity and analysis of nanomaterials in the aquatic environment. Anal. Bioanal. Chem. 393,81-95. https://doi.org/10.1007/s00216-008-2458-1

Hughes, R.N., 1969. A study of feeding in *Scrobicularia plana*. J. mar. biol. Ass. U.K. 49, 805-823. https://doi.org/10.1017/S0025315400037309

Jones, J. D., 1961. Aspects of Respiration in Planorbis Corneus L. And Lymnaea Stagnalis L. (Gastropoda: Pulmonata). Comp. Biochem. Physiol. 4,1-29. https://doi.org/10.1016/0010-406X(61)90042-1

Kyriakis, J.M. and Avruch, J., 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol. Rev. 81, 807-869. https://doi.org/10.1152/physrev.2001.81.2.807

Kultz, D. and Avila, K., 2001. Mitogen-activated protein kinases are *in vivo* transducers of osmosensory signals in fish gill cells. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 129,821-829. https://doi.org/10.1016/S1096-4959(01)00395-5

Mohd Omar, F., Abdul Aziz, H. and Stoll, S., 2014. Aggregation and disaggregation of ZnO nanoparticles: Influence of pH and adsorption of Suwannee River humic acid. Sci. Total Environ. 468–469,195–201. https://doi.org/10.1016/j.scitotenv.2013.08.044

Mouneyrac, C., Linot, S., Amiard, J.C., Amiard-Triquet, C., Métais, I., Durou, C., Minier, C. and Pellerin, J., 2008. Biological indices, energy reserves, steroid hormones and sexual maturity in the infaunal bivalve *Scrobicularia plana* from three sites differing by their level of contamination. Gen. Comp. Endocrinol. 157(2), 133-141. https://doi.org/10.1016/j.ygcen.2008.04.010

Peralta-Videa, J.R., Zhao, L., Lopez-Moreno, M.L., de la Rosa, G., Hong, J. and Gardea-Torresdey, J.L., 2011. Nanomaterials and the environment: a review for the biennium 2008–2010. J. Hazard. Mater. 186, 1-15. https://doi.org/10.1016/j.jhazmat.2010.11.020

Rocha, M., Fernandes, C., Pereira, C., Rebelo, S.L.H., Pereira, M.F.R. and Freire, C., 2015. Gold-supported magnetically recyclable nanocatalysts: a sustainable solution for the reduction of 4-nitrophenol in water. RSC Adv. 5, 5131-5141. https://doi.org/10.1039/c4ra15865b

Roux, P.P. and Blenis, J., 2004. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol. Mol. Biol. Rev. 68, 320-344. https://doi.org/10.1128/MMBR.68.2.320-344.2004

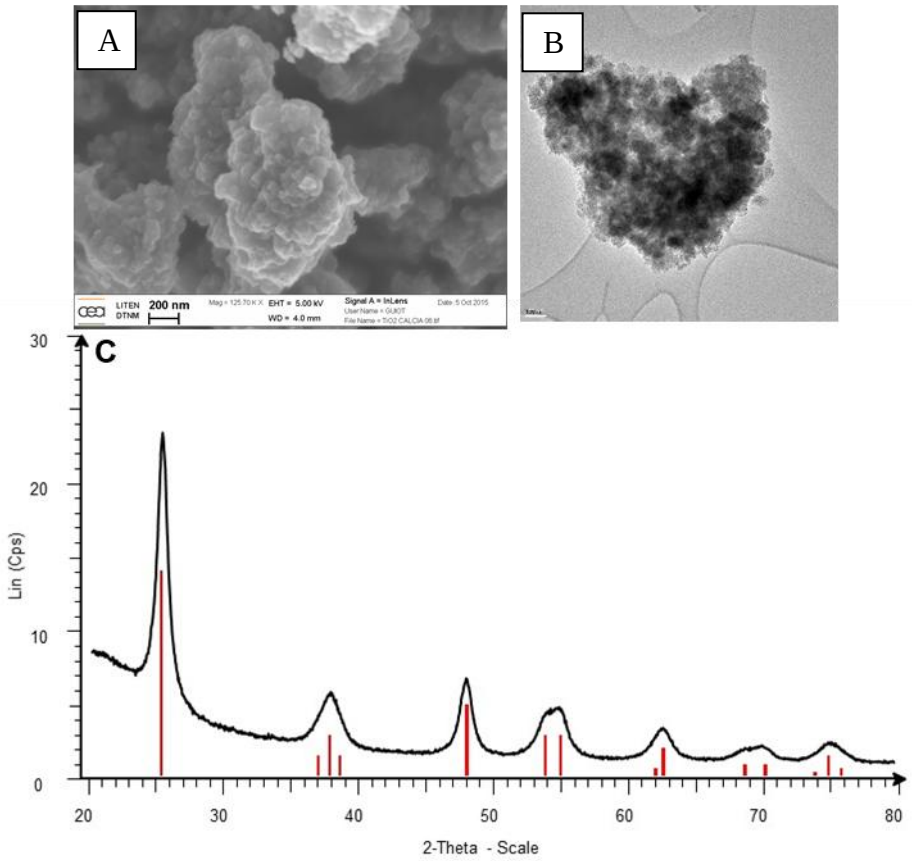
Tella, M., Auffan, M., Brousset, L., Issartel, J., Kieffer, I., Pailles, C., Morel, E., Santaella, C., Angeletti, B., Artells, E., Rose, J., Thiery, A., Bottero, J.Y., 2014. Transfer, Transformation and Impacts of Ceria Nanomaterials in Aquatic Mesocosms Simulating a Pond Ecosystem. Environmental Science & Technology. 48, 9004–9013. https://doi.org/10.1021/es501641b

Tella, M., Auffan, M., Brousset, L., Morel, E., Proux, O., Chaneac, C., Angeletti, B., Pailles, C., Artells, E., Santaella, C., Rose, J., Thiery, A., Bottero, J.Y., 2015. Chronic Dosing of a Simulated Pond Ecosystem in Indoor Aquatic Mesocosms: Fate and Transport of Ceo_2 Nanoparticles. Environmental Science-Nano. 2, 653-663. http://dx.doi.org/10.1039/C5EN00092K

Vale, G., Mehennaoui, K., Cambier, S., Libralato, G., Jomini, S. and Domingos, R.F., 2016. Manufactured nanoparticles in the aquatic environment-biochemical responses on freshwater organisms: A critical overview. Aquat. Toxicol. 170,162-174. https://doi.org/10.1016/j.aquatox.2015.11.019

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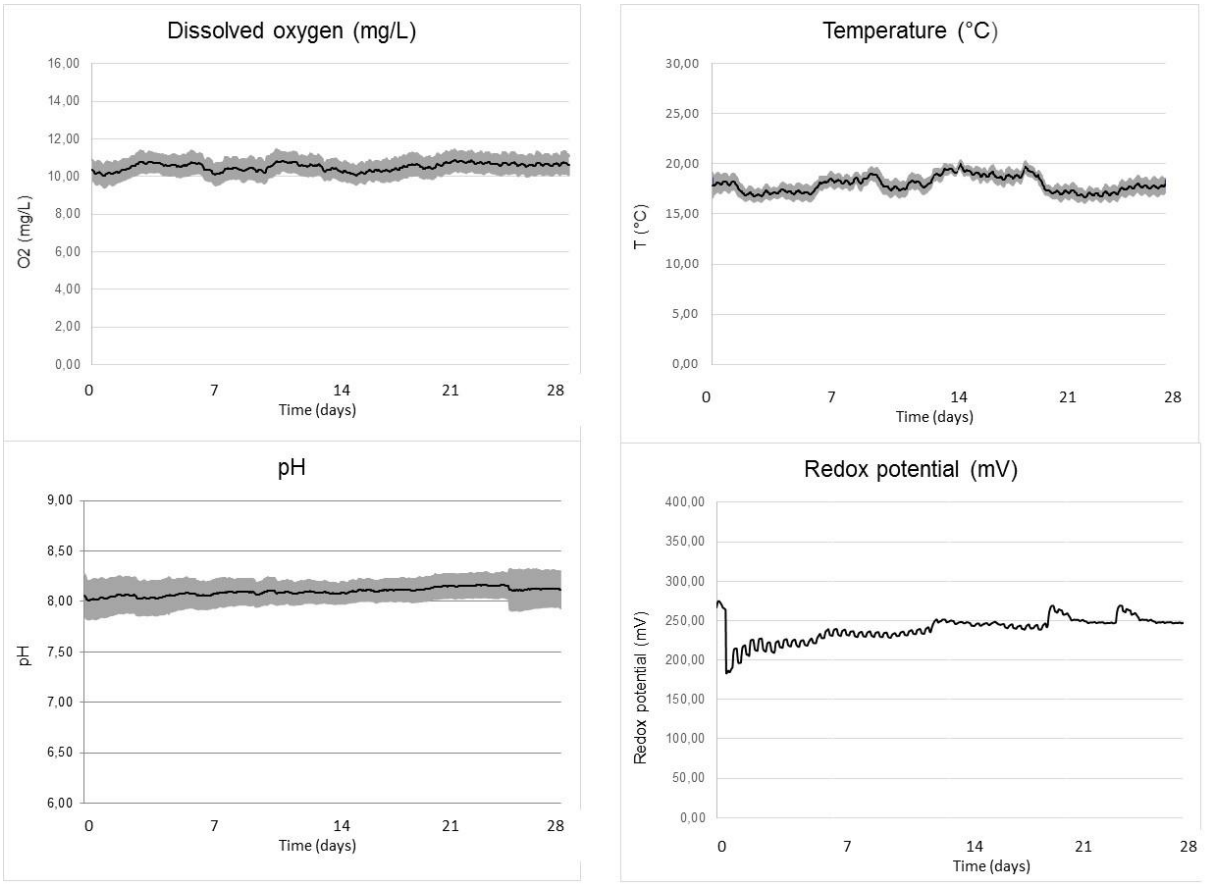
624
625 **Figure S1.** Primary size and shape of TiO_2MNMs determined by an ultra-high resolution
626 scanning electron microscopy (SEM) (A) and Transmission Electron Microscopy (TEM) (B).
627 Body-centered tetragonal anatase crystal structure of TiO_2MNM observed by X-ray
628 diffraction (XRD) (C).
629

630 **Table 1.** Concentrations of Ti, Ca, Al, Fe, Ca and Si after cement degradation in the total
631 (dissolved + particulate fractions) and soluble fraction solution
632

	Ti	Na	Al	Fe	Ca	Si
Total ($mg.L^{-1}$)	**89.91**	1.89	54.06	12.10	2216.00	2407.80
Soluble fraction (<3 kDa) (µg/l)	3.22	182.00	1.00	1.00	2.03E+05	3960.00

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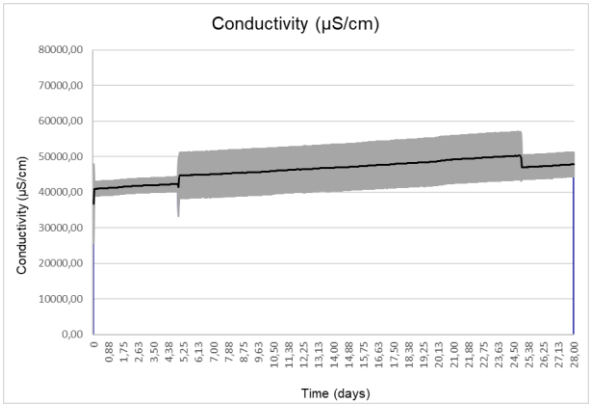


Figure S2. Evolution of the physical-chemical parameters in the water column of the marine mesocosms. Temperature, Redox potential, dissolved oxygen, pH were measured during phases I (stabilization) and II (contamination). Day 0 corresponds to the first dosing of NPs. The grey surface is defined by the maximum and minimum values of each parameter, and the dark line corresponds to the average values of the 9 mesocosms. One measurement was performed every 5 min.

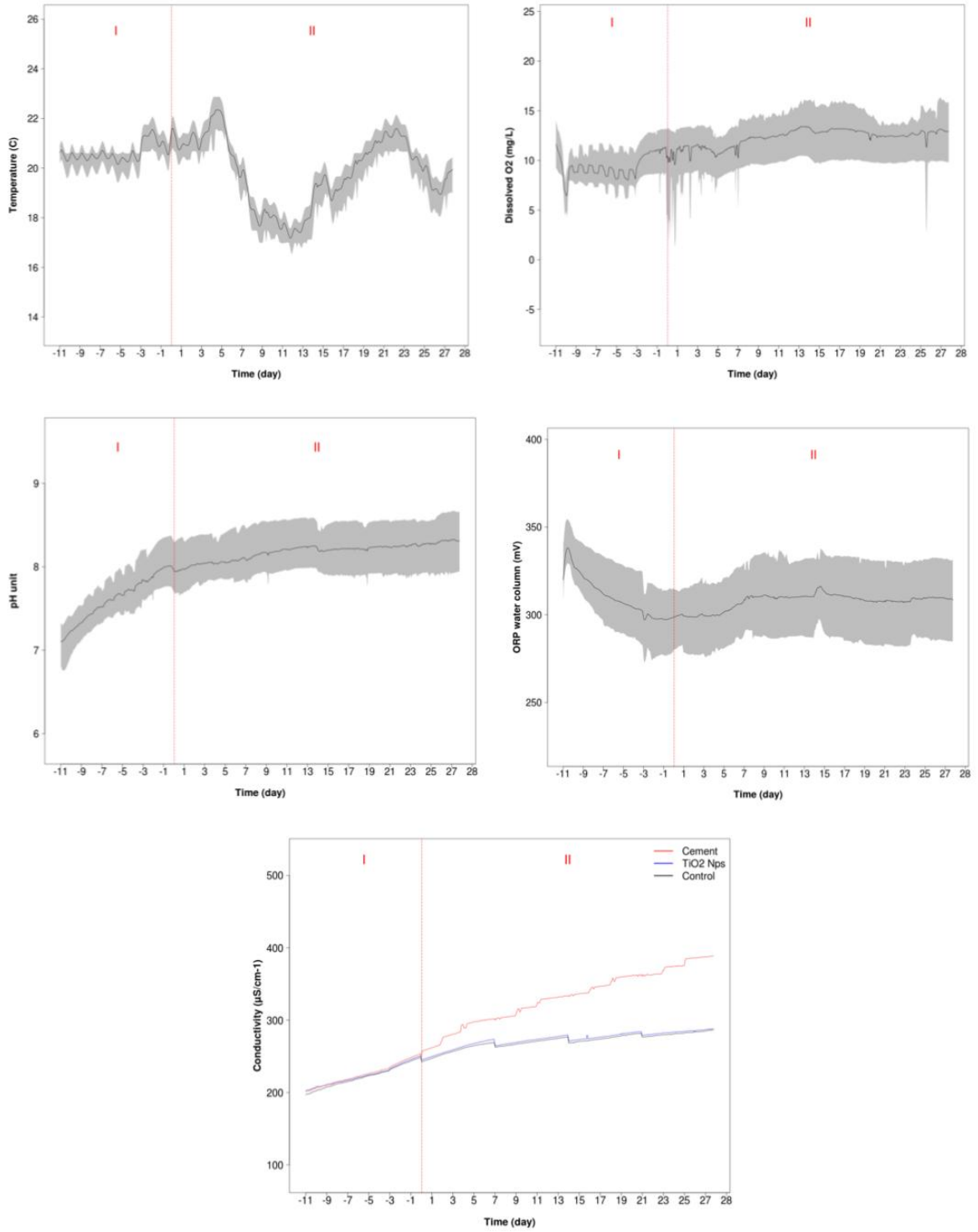


Figure S3. Evolution of the physical-chemical parameters in the water column of the freshwater mesocosms. Oxidation-Reduction potential (ORP), dissolved oxygen, pH, and conductivity were measured during phases I (stabilization) and II (contamination). Day 0 corresponds to the first dosing of NPs. The grey surface is defined by the maximum and minimum values of each parameter, and the dark line corresponds to the average values of the 6 mesocosms. The conductivity were not merged since a different trend was observed in the mesocosms exposed to cement leachate. One measurement was performed every 5 min.

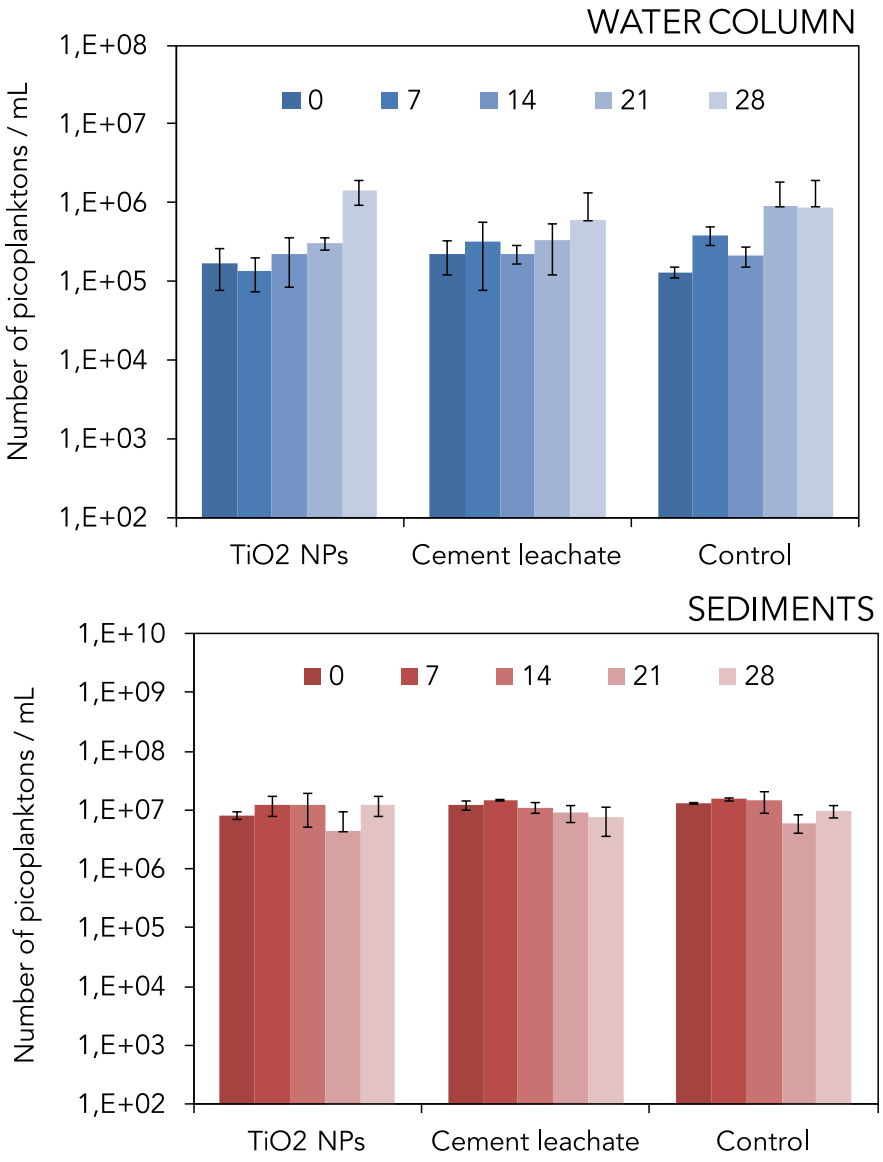


Figure S4. Concentration of picoplankton in water column at 10 cm below water surface (Top) and in surficial sediments(depth: 0.5 to 1 mm) (B)on a weekly basis.

Five mL of water and 15 mL of sediment were sampled, treated with formaldehyde (3.7%), and stored at 4°C before counting. Before picoplankton counting, 1 mL of each water column sample was centrifuged (5.9 × g at 4°C for 15 min), and 200 µL of each sediment sample was treated with 800 µL of 0.1 mM sterile tetrasodium pyrophosphate and vortexed with a steel ball for 30 seconds. For the counting, 10 µL of each sample was mixed with 5µL of 3µM SYTO® 9 Green Fluorescent Nucleic Acid Stain and dropped on a glass slide. Concentration of picoplankton was the mean ± standard deviation of five counts.