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

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13 **Abstract**

14 Production of Manufactured Nanomaterials (MNMs) has increased extensively due to
15 economic interest in the current years. However, this widespread use raises concern about
16 their impact on human and environment. Current efforts are made, both at national and
17 international levels to help developing safer MNMs in the market. In order to assess hazards
18 of MNMs, it is important to take into account exposome parameters in order to link fate and
19 behavior of MNMs to their potential toxicity. In that context, the aim of this study was to
20 compare exposure and impact of TiO₂ MNMs-based cement at different levels of its life cycle
21 (TiO₂MNMs, cement containing TiO₂ MNMs) between two exposure mesocosm scenarios
22 mimicking : marine conditions using the bivalve *Scrobicularia plana* and freshwater
23 conditions using the gastropod *Planorbarius corneus*, for 28 days. These approaches allows
24 measurements of physical-chemical parameters throughout the duration of the exposure.
25 Similar results were observed in both exposure conditions since in the two scenarios Ti was
26 removed from the water column and accumulated in surficial sediments. While in *P. corneus*,
27 statistically different concentrations of Ti were measured in the digestive glands compared to
28 controls following exposure to TiO₂ MNMs, elevated background of Ti concentrations were
29 measured in the controls of *S. plana* that did not allow to discriminating any bioaccumulation
30 process. In addition, both TiO₂ MNMs and TiO₂MNM-based cement exposed *S. plana* did not
31 present any activation of the p38 mitogen-activated protein kinase (MAPKs). This study
32 demonstrates the challenge of using freshwater-marine continuum using a mesocosm
33 approach in nanosafety.

34 **Keywords:** Mesocosm, TiO₂MNMs, *Scrobicularia plana*, *Planorbarius corneus*

35 INTRODUCTION

36 Releases of Manufactured Nanomaterial (MNMs) can occur during any stage of the
37 MNM life cycle i.e. production stage of nanoproduct, the use/application stage of these
38 nanoproducts and their ultimate end-of-life management/disposal. A comprehensive
39 understanding of the potential for such releases along the whole life cycle and their possible
40 effects is crucial to ensure the safe and sustainable use of these new materials (Salieri et al.,
41 2018). Among all MNMs, TiO₂ MNMs are introduced into many products such as paints,
42 plastics, food additives, sunscreens and other care products (Yin 2012). The use of TiO₂
43 MNMs into cement is a recent application in building material industry. Thanks to its
44 photocatalytic properties, it confers to the building material air decontamination, self-
45 sterilizing, self-cleaning and anti-fogging abilities (Jimenez et al., 2016). For those reasons,
46 photocatalytic cement appears to be an appealing market for industry that represents from 0.1
47 to 1% of the total European production of TiO₂ and hence about 10.2t to 102t of TiO₂ MNMs
48 released each year (Nanotechproject.org). TiO₂MNMs incorporated in the cement matrix are
49 usually non-coated anatase with a mass concentration ranging from 0.3 to 10 wt% (Ruot et al.,
50 2009). Degradation of TiO₂ MNM-based cement is likely to happen at each stage of the
51 cement life cycle (production, manufacturing, use disposal and recycling) (Bossa et al., 2017).
52 The main causes of release of TiO₂MNMs from photocatalytic coatings is the stripping of the
53 TiO₂ from the coatings because of the water flow, the dispersion of agglomerate under NaCl
54 and UV light condition and finally its discharge from loosening caused by mechanical damage
55 (Olabarietta et al., 2012). A recent study showed that TiO₂ MNMs were released (18.7 ± 2.1
56 to 33.5 ± 5.1 mg of Ti/m² of cement after 168 h) from photocatalytic cement pastes that were
57 leached at a lab-scale to produce a range of degradation rates, meaning that in the worst-case
58 scenario of weathering a negligible mass of TiO₂ (0.04w.%) was released from the
59 photocatalytic cement (Bossa et al., 2017). TiO₂ MNMs release comes from a very thin active
60 surface layer where both the cement surface chemistry and its pore network morphology
61 control the TiO₂MNMs diffusion (Bossa, 2019).

62 Laboratory studies have extensively demonstrated the adverse effects of TiO₂ MNMs
63 on many aquatic organisms (Ramsden et al., 2009; Binh et al., 2016; Hall et al., 2009; Kulacki
64 et al., 2012). Nevertheless, those studies have been conducted in simple systems (microcosm)
65 and they did not reflect the MNMs fate and behavior in the environment due to processes such
66 as sorption and aggregation of MNMs (Auffan et al., 2014). As a consequence, for a strongly
67 characterization of risk, the combination of the organism, its environment and MNMs needs
68 to be taken into account and requires interdisciplinary expertise in physical-chemistry,

69 biology and ecotoxicology (Auffan et al., 2014). In that context, mesocosms represent
70 relevant experimental systems for investigating the complex issue of exposure that enable to
71 get quantitative time- and spatial data on the distribution of MNMs within a simulated
72 ecosystem. A mesocosm refers to “an experimental system that simulates real-life conditions
73 as closely as possible, while allowing the manipulation of environmental factors” (FAO,
74 2009). As stated during the H2020 European commission funded NANoREG project (Project
75 #646221), mesocosms have been shown to provide a reliable methodology to obtain
76 quantitative time- and spatially regarding exposure-driven environmental risk assessment.
77 In this context, two indoor mesocosm platforms (one marine and one freshwater) were
78 conceived with small size tanks (60 L) for investigating MNM exposure and impacts on
79 aquatic species that allows to recreate freshwater and estuarine conditions (sediment, tide
80 cycles, controlled temperature, salinity and redox potential) (Auffan et al. 2019). TiO₂ MNMs
81 and TiO₂ MNM-based cement fate, behavior, bioaccumulation and toxicity were evaluated
82 after a 28 day exposure in two mollusks representative of the estuarine and freshwater
83 ecosystems : the bivalve *Scrobicularia plana* and the gastropod *Planorbarius corneus*.
84 *Scrobicularia plana* is an endobenthic bivalve involved in the functioning and the structure of
85 estuarine ecosystems and has also been largely demonstrated to represent a relevant model for
86 biomonitoring (Mouneyrac et al., 2014). *Planorbarius corneus* is a hermaphroditic snail that
87 inhabits small temporary ponds. This species belongs to the *Planorbidae*, the largest family of
88 aquatic pulmonate gastropods distributed all over the world (Jopp, 2006). *Planorbarius*
89 *corneus* play an important role in trophic chains as grazer and as prey (Wojdak and Trexler,
90 2010) and have already been used as model organisms for exposure and toxicity studies in
91 mesocosms (Tella et al 2014, 2015, Auffan 2018).

92 The originality of the project was to compare the fate and behavior of a nano-enabled product
93 (TiO₂ MNM-based cement) in these two freshwater and marine ecosystems. Two stages of the
94 MNM lifecycle were considered : the formulation stage using bare TiO₂ MNMs and end of life
95 stage of the cement containing TiO₂ MNMs using cement leachate. Freshwater and marine
96 mesocosms were used to obtain resolved data on the distribution, transformation and impact
97 towards these mimicked ecosystems depending on the physical-chemical properties of the
98 environment (salinity, pH, conductivity, tide cycle or not...) and the physical-chemical
99 properties of the contaminant at two stages of the lifecycle. One of the major drawbacks in
100 assessing environmental impact of MNMs is that the environmental concentrations are not yet
101 known. Herein, we used between 1 and 1.2 mg.L⁻¹ of TiO₂ MNMs which belong to the
102 threshold values estimated in highly exposed areas (Bourgeault et al., 2015).

103 MATERIAL AND METHODS

104 *TiO₂NPs*

105 Primary size and shape of TiO₂ MNMs (NM212, JRC repository) were determined by an
106 ultra-high resolution scanning electron microscopy SEM LEO 1530 (LEO Electron
107 Microscopy Ltd, Cambridge, England) and Transmission Electron Microscopy (analytical
108 TEM Field Emission Gun (FEG) 200 KV Osiris from Tecnai, FEI, Japan). The SEM and
109 TEM images corroborate the nanometric size of the particles (see SI, figure S1). X-ray
110 diffraction (XRD) patterns of the samples were obtained using a Bruker D8 X-ray diffraction
111 (XRD) system in a θ -2 θ mode and Cu K α X-ray source. The TiO₂ MNMs have a body-
112 centered tetragonal anatase crystal structure (see SI). The width of the XRD peaks were used
113 to calculate a crystallite size about 9 nm. A specific surface area of 223 m²/g was determined
114 by the Brunauer-Emmett-Teller (BET) adsorption method using the N₂ adsorption isotherm
115 measured at 77K (BELSORP-max, BEL Japan Inc.).

116

117 *TiO₂MNMs-based cement*

118 Photocatalytic white Portland cement incorporating TiO₂ MNMs was provided by a European
119 cement manufacturer (Calcia, France) through the French Technical Association of Industries
120 of Hydraulic Binders (ATILH, France). This cement is mainly composed of Ca and Si (\approx 66wt
121 % CaO and \approx 23 wt% SiO₂) and contains 2.85 wt% of TiO₂ (anatase) (Bossa et al., 2017).
122 Cylinders of hardened cement (4 cm diameter, 8 cm high) were obtained after 28 days of
123 hydration at 20°C with a water/cement ratio of 0.5. Hardened cement pastes exhibit a complex
124 mineralogy, i.e. a mixture of hydrated minerals (portlandite, calcium silicate hydrates,
125 ettringite) and residual anhydrous minerals (di and tricalcium silicates). TiO₂MNMs are
126 homogeneously dispersed in the hydrated paste, with the exception of few high spots of 50 μ m
127 (Bossa et al., 2017).

128 Cement degradation residue generation was simulated using accelerated lab-scale approach to
129 conduct a so-called « worst-case scenario » and to generate a quantity of cement degradation
130 residues for mesocosms dosing. Finely crushed cement pastes were leached in batch using
131 ultra-pure water (UPW) with an elevated liquid-to-solid ratio (L/S) of 100. After pH
132 stabilization (pH > 12.5), the suspension of cement degradation residues was neutralized with
133 slow addition of nitric acid (22.37 mol.L⁻¹) reach a pH of 7.8 and a conductivity of 13 mS.cm⁻¹
134 ¹. The solution is composed of a dissolved fraction (mainly Ca and Si, 203 and 3.96 mg.L⁻¹
135 respectively) and a particulate fraction with a TiO₂ concentration of 143.7 \pm 2.6 mg.L⁻¹. In

136 accordance with the very low TiO₂ MNM solubility, no TiO₂ MNM dissolution was observed
137 (Table S1). The concentrations of Ca and Si in the dissolved fraction reveal the high
138 degradability of the cement matrix in natural media (pH 7.8). Presence of free TiO₂ MNMs,
139 i.e. not embedded in cement matrix, in the solution of cement degradation residues is then
140 suspected. In neutralized cement degradation residues, TiO₂ MNMs were observed mainly
141 associated with aggregates larger than 0.45 μm and with SiAl intermix chains. SiAl chains are
142 supposed to be geopolymers stable at pH 7 and resulting from secondary precipitation/
143 polymerization during the pH neutralization process (Bossa et al., 2017).

144

145 *Animals*

146 Individuals of *Scrobicularia plana* (15-20 mm length) were hand-collected in November 2015
147 in the intertidal mudflat on the French Atlantic coast (Bay of Bourgneuf: N 47°01'50.35", W
148 1°59'04'80") and transported in cool boxes to the laboratory. Then, bivalves were
149 immediately placed into aerated artificial water (Tropic marin[®]) at 30 practical salinity units
150 (psu) during 5 days in a temperature controlled room at 15°C (temperature experienced in the
151 field at the collection time). A natural inoculum containing picoplankton as primary producer
152 (bacteria, algae, protozoa, etc.) was also collected in the intertidal mudflat. Physico-chemical
153 parameters of seawater from the collection site were : pH= 7.7; salinity 28 psu; conductivity
154 40 mS.cm⁻¹.

155 Individuals of *Planorbarius corneus* (L., 1758) (Great Ram's Horn snail, benthic grazer)
156 were hand-collected in October 2015 in a non-contaminated temporary pond part of the
157 Natura 2000 reserve network in south of France (Bonne Cournes: N 43°20'47.04", E
158 6°15'34.786", 246 m a.s.l.) and transported in pond water to the laboratory. A natural
159 inoculum containing picobenthos as primary producer (bacteria, algae, protozoa, etc.) was also
160 sampled in the pond. Physico-chemical parameters of the pond water from the collection site
161 were: water temperature 14.9°C; conductivity 819 mS.cm⁻¹; concentration of dissolved
162 oxygen 0.72 mg.L⁻¹ (6.6%).

163

164 *Mesocosm setup*

165 *Freshwater mesocosms.* The mesocosm experiments were already described by Auffan et al.
166 (2014), Tella et al. (2014), Tella et al. (2015), Auffan et al. (2018)(figure 1). Tanks (750 x 200
167 x 600 mm) were filled with 6-8 cm artificial sediments containing 89% SiO₂, 10% kaolinite
168 and 1% of CaCO₃ w/w adapted from OECD guideline (OECD, 2004). Primary producers
169 were brought by ~300 g of water-saturated natural sediment (sieved at 200 μm) laid at the

170 surface. The mesocosms were then gently filled with 50 L of Volvic water with pH and
171 conductivity close to the natural pond water (pH 7, 11.5 mg.L⁻¹ Ca²⁺, 13.5 mg.L⁻¹ Cl⁻, 71
172 mg.L⁻¹ HCO₃²⁻, 8 mg.L⁻¹ Mg²⁺, 6.3 mg.L⁻¹ NO₃⁻, 6.2 mg.L⁻¹ K⁺, 11.6 mg.L⁻¹ Na⁺).

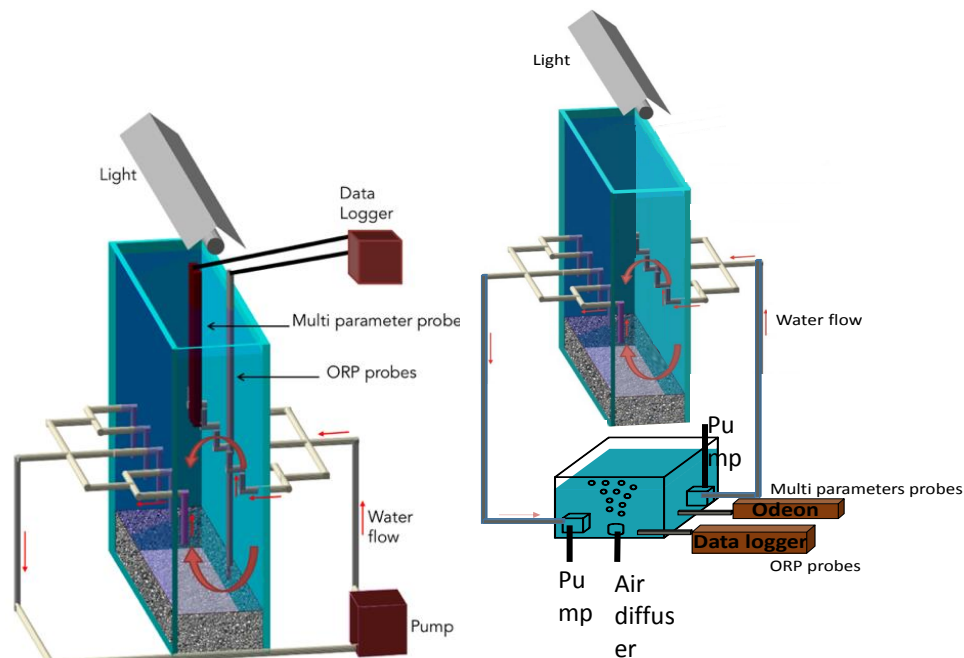
173 After 2 days, the physical-chemical parameters of the mesocosms were stabilized (turbidity,
174 pH, dissolved O₂, redox). Then, 19 adult *P. corneus* were added per mesocosm and
175 acclimatized for one week. Organisms were exposed for 28 days in a temperature controlled
176 room (18°C) and under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

186 .

187 **Marine mesocosms.** Each experimental unit was composed with two tanks (mesocosm and
188 reserve tank), two pumps (Eheim compact 300L.h⁻¹) and a mechanical timer (IDK PMTF
189 16A) allowing to mimicking the tidal cycle (figure 1). The mesocosm tank (750 x 200 x 600
190 mm) was filled with 14 kg synthetic sediment (90% SiO₂, 9% kaolinite, 1% CaCO₃ w/w)
191 adapted from OECD guideline (OECD, 2004). Three hundred g of water-saturated natural
192 inoculums was laid at the surface to bring primary producers. Then, each mesocosm tank was
193 flooded with 35 L of the artificial seawater Tropic Marin™ (Tropicarium Buchshlag Dreieich,
194 Germany) at 30psu. The reserve tank (700 x 500 x 37 mm) was placed under the first one
195 (mesocosm tank) to collect removed water from the mesocosm tank during low tide.
196 Reservoir tank was always filled with a minimum of 25 L of artificial seawater at 30 psu to
197 constantly maintain the measuring probe immersed.

198 After one week, the physical-chemical parameters of the mesocosms were stabilized
199 (turbidity, pH, dissolved O₂, redox). Then, 55 *S. plana* were added per mesocosm and
200 acclimatized for 5 days. Organisms were exposed for 28 days in a temperature controlled
201 room (15°C), under controlled light (photoperiod 16:8) and at a tidal cycle (6h of low tide, 6h
202 of high tide; two tides /day). Physical-chemical parameters including temperature (°C),
203 salinity (psu), pH, redox potential (mV), dissolved oxygen (mg O₂.L⁻¹) were monitored every

204 15 min into the water column from the reserve tank during the whole duration of the
205 experiment. Among the 9 marine mesocosms, 3 were kept as negative control (without
206 contamination), 3 were exposed to TiO₂ MNMs (positive control) and 3 were exposed to
207 cement leachate.



208
209 **Figure 1.** Scheme of the freshwater (left) and marine (right) mesocosms.

210
211 ***Mesocosm dosing***

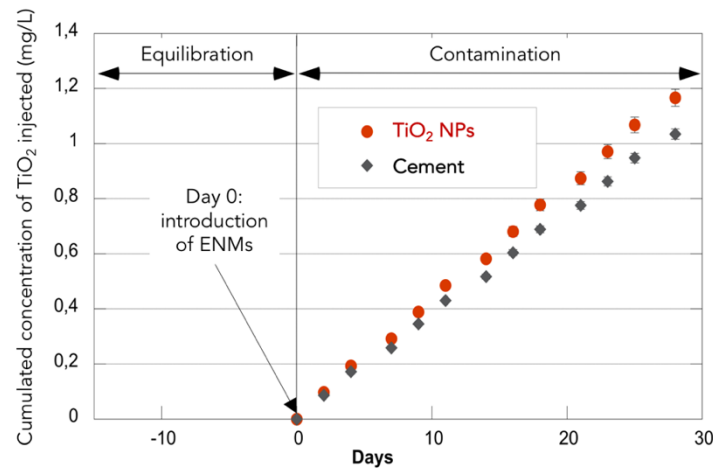
212 Aqueous suspensions of TiO₂ MNMs and TiO₂ MNM-based cement degradation residues
213 (with a stabilized pH of 7.8) were prepared prior injections in the mesocosms. Aliquots of
214 these suspensions were digested using an Ultra WAVE microwave digestion system with 1
215 mL HNO₃ 67% (NORMATOM) and 1 mL HF 47%-51% (PlasmaPure) and analysed by ICP-
216 MS (Perkin Elmer[®] Nexion 300 ICP-MS) for their total Ti contents. A concentration of
217 $4.86 \pm 0.13 \text{ g.L}^{-1} \text{ TiO}_2$ (n=3) was found for the TiO₂ MNM suspension ($2.91 \pm 0.08 \text{ g.L}^{-1} \text{ Ti}$). The
218 TiO₂ concentration measured in the solutions of cement degradation residues (n=6) was
219 $143.7 \pm 2.6 \text{ mg.L}^{-1}$ ($86 \pm 1.6 \text{ mg.L}^{-1} \text{ Ti}$). Both suspensions were close to the targeted
220 concentrations and showed excellent recovery yields after digestion (> 95%).

221 A multiple dosing experiment was performed on a 4 week-period. A total of 12 dosings were
222 achieved (3 times per week), corresponding to 0.09 mg.L^{-1} of TiO₂ per injection in order to
223 reach a final nominal concentration of $1 \text{ mg.L}^{-1} \text{ TiO}_2$ per mesocosm. As the suspension of
224 cement degradation residues is not stable over time, a new neutralized batch was prepared
225 every two injections and stored at 4°C in dark between the two injections.

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

230

231



232

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

235

236 *Ti quantification in the different compartment of the mesocosms*

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

243 For the marine mesocosms, Ti quantifications were performed by Micropolluants Technologie
244 S.A. (Metz, France) using an Agilent 7700 ICP-MS (Agilent Technologies France). 10 mL of
245 water or 2 mL of surficial sediments were mixed with 1mL 32-35% HCl, 1mL 67-69% HNO_3 ,
246 $100 \mu\text{L}$ 94-98% H_2SO_4 , and 1 mL 47-51% HF prior ICP-MS measurements. For *S. plana*,
247 whole soft tissues were mixed with 1 mL 67-69% HNO_3 , $100 \mu\text{L}$ 94-98% H_2SO_4 , and 1 mL
248 47-51% HF. For the freshwater mesocosms, water samples (2 mL) were digested with 1 mL
249 HNO_3 67-69% and 0.5 mL HF 47%-51%. For the surficial sediments (50 mg), a mixture of 3
250 acids (1 mL HCl 34%, 2 mL HNO_3 67%, 0.5 mL HF 47%-51%) was used. For *P. corneus*, the

251 digestive glands were dissected, before being digesting using 1 mL HNO₃ 67%, 0.5 mL H₂O₂
252 30%-32% and 1 mL HF 47%-51%. Samples were digested using the microwave Ultra WAVE
253 and the analysis were performed using the Perkin Elmer Nexion 300 ICP-MS. Using
254 sediments and freshwater organisms spiked with TiO₂ nanoparticles, a recovery yield of
255 >95% after microwave digestion and ICP-MS analysis was obtained.

256

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

258 Total whole soft tissues of *S. plana* exposed for 28 days (n=7 for each condition) were
259 homogenized with lysis-buffer (NaCl 0.2M, DTT 1mM, protease inhibitor cocktail 0.1%) in a
260 mortar at 4°C. The clear supernatant was then obtained by centrifugation (13,000xg, 10 min,
261 4°C) and stored at -20°C until analysis. Protein concentrations were determined according to
262 Bradford (1976) using bovine serum albumin as a standard. Protein extracts were mixed with
263 sample buffer (0.5 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 4% 2-mercaptoethanol,
264 0.05% bromophenol blue) 1:3 and boiled for 5 min at 95°C. Proteins (40 µg per lane) were
265 separated using a 12% polyacrylamide gel. Western blot analysis was performed as previously
266 described (Châtel et al., 2011b). After transfer, membranes were incubated with a mouse
267 monoclonal antibody anti-Phospho-p38 MAPK (Thr₁₈₀/Tyr₁₈₂) (9216-Cell signaling
268 Biotechnology) diluted in 1/1000 in TBS/BSA 5% or with a mouse monoclonal antibody anti-
269 β-Actin (A5441 -SIGMA) diluted in 1/2000 in TBS/BSA 5%. After washing (3 times for 5
270 min), membranes were incubated with a goat anti-Mouse IgG antibody coupled with alkaline
271 phosphatase (A3562, Sigma Aldrich) diluted 1:2000 in TBS/BSA 5%. Detection of immune
272 complexes was carried out by colorimetric reaction using a solution of 5-bromo-4-chloro-3-
273 indolyl phosphatase (BCIP) and nitroblue tetrazolium (NBT). After visualization, membranes
274 were scanned and band intensity, which is the relative amount of protein expression, was
275 quantified as Relative Optical Densities (ROD; pixels/mm²). Results of pp38 were normalized
276 according to actin expression.

277

278 ***Statistical analysis***

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

282

283

284

285 **RESULTS and DISCUSSION**

286

287 *Favorable physical-chemical conditions in freshwater and estuarine mesocosms*

288 During the 28 days of contamination several physical-chemical parameters were monitored to
289 assess the global response of the mesocosms to the presence of TiO₂ MNMs and cement
290 leachate. In marine as well as in freshwater mesocosms, the average temperature, dissolved
291 O₂, conductivity, and pH were not significantly different between negative controls and
292 contaminated mesocosms(see SI, Figures S2 and S3).

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

299 Consequently, during the exposure to TiO₂ MNMs and cement leachate, the physico-chemical
300 conditions of the 15 mesocosms remained favorable with the oxygenation, pH, temperature,
301 redox potential in the range of natural conditions.

302

303 *Homo-aggregation of TiO₂ MNMs and accumulation in the surficial sediments*

304 Bossa et al. (2017) estimated using an accelerated aging protocol that a negligible mass of
305 TiO₂ (0.04w.%) was released from the photocatalytic cement (Bossa et al., 2017). Herein, we
306 used an accelerated lab-scale approach to conduct a so-called « worst-case scenario »
307 assuming that 100% of the Ti contained in the cement would have been released. Figure 3 and
308 4 show the Ti concentrations measured in the water column and sediments of both freshwater
309 and marine mesocosms as a function of time. Ti was detected in both waters and sediments
310 sampled in control mesocosms proving that a high geochemical background of this element
311 existed in the mimicked ecosystems. Average background Ti concentrations in freshwater
312 mesocosms were 19.2±17.2 μg.L⁻¹ in the water column and 2230±928 mg.kg⁻¹ in surficial
313 sediments. Average background Ti concentrations in marine control mesocosms were
314 567±273mg.kg⁻¹ in surficial sediments and below the quantification limit (<2.5 μg.L⁻¹) in the
315 water column. Ti is a naturally occurring element in mineral and amorphous phases occurring
316 in freshwater and marine environments. Ti can be found in titanium-iron oxide minerals (as
317 ilmenite), TiO₂ minerals as rutile, anatase, and brookite. Herein, the TiO₂ MNMs used were
318 engineered nanosized anatase minerals. Consequently even if the Ti background concentration

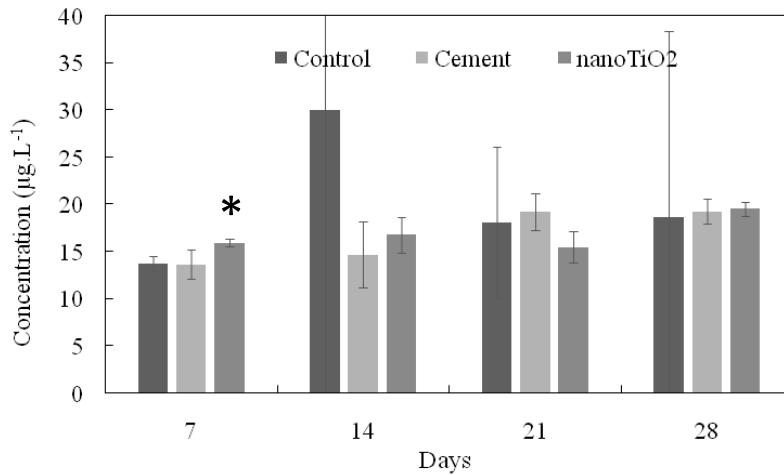
319 was elevated, it is noteworthy that the speciation and reactivity of the naturally occurring Ti-
320 bearing phases might be different from the MNM injected.

321 In the surficial sediments of marine mesocosms, high concentrations of Ti were detected after
322 7 days (control, TiO₂ MNMs, and cement leachate) and progressively decreased until 28 days
323 (Figure 4, right). However, because of the elevated geochemical background of Ti, no
324 statistical difference was observed between controls and contaminated mesocosms. Similarly,
325 no increase in Ti concentration could be evidenced in the surficial sediments of contaminated
326 freshwater mesocosms compared to controls. Besides, the fluctuations of Ti concentration
327 measured over time were likely related to the heterogeneity of the Ti distribution and the
328 difficulty of sampling the surficial sediments(Figure 4). These results highlighted the
329 challenge of a reliable quantification of Ti in that compartment.

330

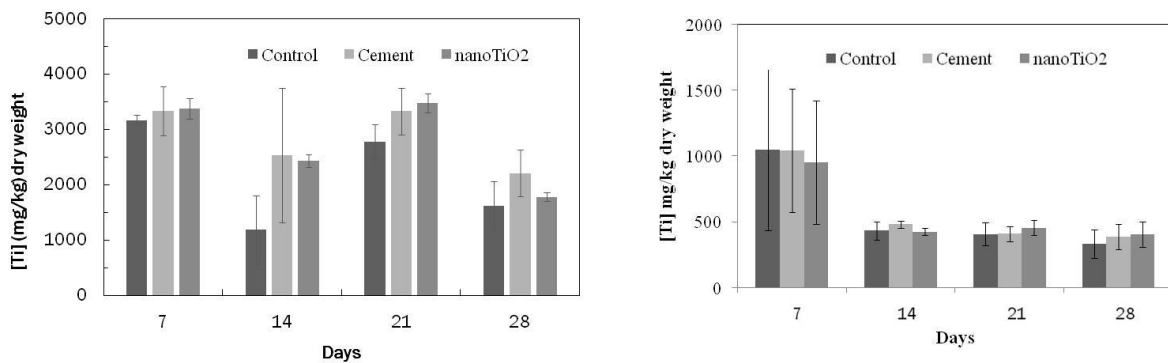
331 Total Ti concentrations in the water column of marine mesocosms (control, TiO₂ MNMs and
332 cement leachate) were always below the quantification limit estimated at 2.5 µg.L⁻¹ whatever
333 the duration of exposure. On the opposite, total Ti concentrations in the water column were
334 always above 10 µg.L⁻¹ in freshwater mesocosms. The only condition for which a statistically
335 significant difference was observed between the water column of controls and contaminated
336 freshwater mesocosms was after 7 days of exposure to TiO₂ MNMs. At this time point, the
337 difference between TiO₂ MNMs contaminated mesocosms and geochemical Ti background
338 were 2.2±1.2 µg.L⁻¹Ti for a total Ti concentration injected of 180 µg.L⁻¹Ti (300 µg.L⁻¹ of
339 TiO₂). Based on these values, we estimated that 98-99 % of the Ti injected was removed from
340 the water column after 7 days. Although no increase in Ti concentration could be evidenced in
341 the surficial sediment of freshwater mesocosms, we hypothesize that both cement leachate
342 and TiO₂ MNMs settled at more than 98% at the surface of sediments. Based on the 31.0±0.6
343 mg and 35.0±0.9 mg Ti introduced respectively after 28 days in mesocosms contaminated
344 with cement leachate and TiO₂ MNMs, the remaining of 2% of Ti particles in the water
345 column should entail a theoretical increase in the surficial sediment of 176 mg.kg⁻¹, which is
346 far below the standard deviation determined for Ti background in that compartment. This
347 explains why Ti accumulation at sediment surface remained undetected by chemical analysis.
348 In addition, in marine mesocosms, due to the tidal system, part of the sediment were removed
349 along with the water in the lower tank and sedimentation of TiO₂ occurred as well in this
350 compartment favoring decrease in its concentration as compared to freshwater mesocosms.

351



352

353 **Figure 3.** Total Ti concentration in the water column after 7, 14, 21 and 28 days of exposure
 354 in freshwater mesocosms. Ti concentration in the water column in marine mesocosms is not
 355 represented as values were always below the quantification limit estimated at $2.5 \mu\text{g.L}^{-1}$.
 356 Statistical significance was determined by the Student's t-test (*: $p < 0.05$).
 357

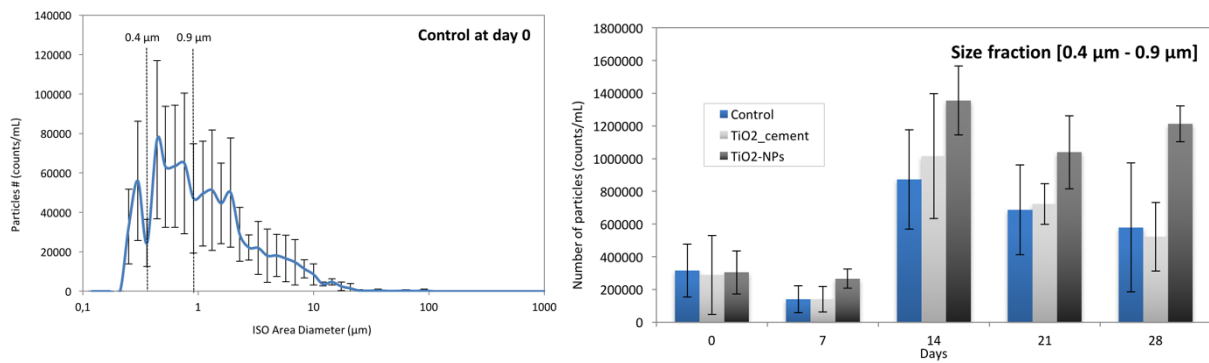


358

359 **Figure 4.** Total Ti concentration in surficial sediment after 7, 14, 21 and 28 days of exposure
 360 in freshwater (left) and marine (right) mesocosms.
 361

362

363 In freshwater mesocosms, the size distribution of the suspended matter in the $0.1 \mu\text{m} - 1000$
 364 μm size range and the total number of suspended particles in the $0.4 \mu\text{m} - 0.9 \mu\text{m}$ size range
 365 were not different between control and contaminated mesocosms (Figure 5). This highlighted
 366 that the main mechanism of aggregation and settling down expected in the water column was
 367 the homo-aggregation of the TiO₂ MNMs either pristine or brought *via* cement leachate. In
 368 marine mesocosms, MNM homo-aggregation was expected to be even faster due to higher
 369 alkalinity and ionic strength as compared to freshwater environments (Peralta-Videa et al.,
 370 2011; Rocha et al., 2015; Vale et al., 2016). It is noteworthy that the aggregation of MNMs
 371 allows their deposition onto the surficial sediment (Peralta-Videa et al., 2011), resulting in
 372 high exposure of benthic organisms compared to planktonic organisms (Mohd Omar et al.,
 2014; Mouneyrac et al., 2014).



373

374 **Figure 5.** (left) Size distribution of colloidal particles in the water column of freshwater
 375 control mesocosms at day 0. The dotted lines mark out the 0.4 µm – 0.9 µm fraction. (right)
 376 Evolution of the number of colloidal particles in the 0.4µm – 0.9 µm range from day 0 to 28,
 377 under the three conditions tested (control, cement leachate and TiO₂ MNMs) in freshwater
 378 mesocosms.

379

380 *Bioaccumulation and effects on benthic organisms*

381 *P. corneus* and *S. plana* are two benthic grazer species. While *P. corneus* eat algae and
 382 biofilms at the sediment/water interface (Jones 1961), *S. plana* can either feed suspended
 383 particles from the water column but also ingest sedimentary particles (Hughes, 1969). For *S.*
 384 *plana* exposed in marine mesocosms, elevated background of Ti concentrations were
 385 measured in the control group (84.8±42.3 mg.kg⁻¹ dry weight) which did not allow to
 386 discriminating any bioaccumulation process in organisms exposed to TiO₂ MNMs and cement
 387 leachate (97.1±43.9 and 107.5±48.2 mg.kg⁻¹ dry weight, respectively) (figure 6).

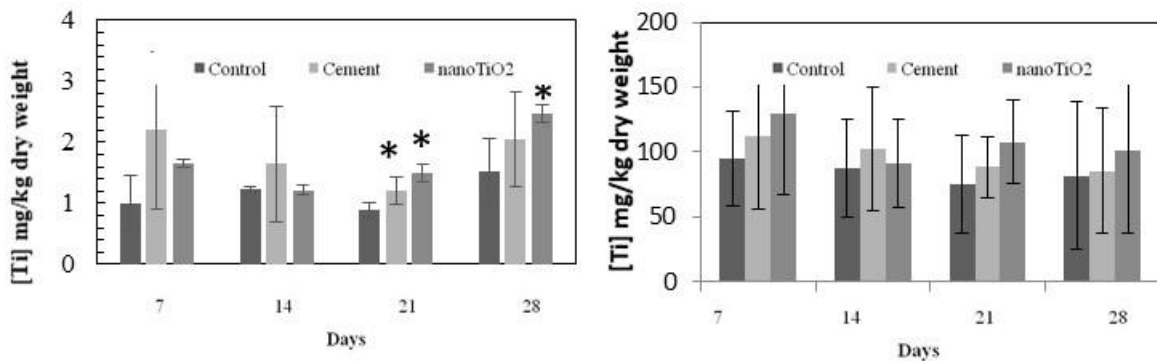
388 Background concentrations of Ti measured in the digestive glands of unexposed *P. corneus*
 389 were one order of magnitude lower. Following exposure to cement leachate, no difference in
 390 Ti concentration in the digestive glands were measured with respect to control *P. corneus*.
 391 However, statistically different concentrations of Ti were measured in the digestive glands
 392 compared to controls following exposure to TiO₂ MNMs *i.e.* 1.5 ± 0.2 mg Ti.kg⁻¹ (dry weight)
 393 and 2.5 ± 0.1 mg Ti.kg⁻¹ (dry weight) after 21 and 28 days of exposure respectively (figure 6).
 394 This suggests that an ingestion of Ti by *P. corneus* occurred likely resulting from the
 395 accumulation of TiO₂ MNMs on the surficial sediments.

396 Some authors have shown abilities to discriminate natural Ti from TiO₂ NMs. For example,
 397 Bourgeault et al. (2015) resolved this lack of sensitivity using isotopically modified TiO₂
 398 nanoparticles (⁴⁷Ti) to characterize the processes governing Ti bioaccumulation in a
 399 freshwater environment. Thanks to the ⁴⁷Ti labeling, they detected bioaccumulation of NPs in
 400 *Dreissena polymorpha* exposed for 1 h at environmental concentrations *via* water (7–120
 401 µg/L of ⁴⁷TiO₂ NPs) and *via* their food (4–830 µg/L of ⁴⁷TiO₂ NPs mixed with 1 × 10⁶

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

405

406



407

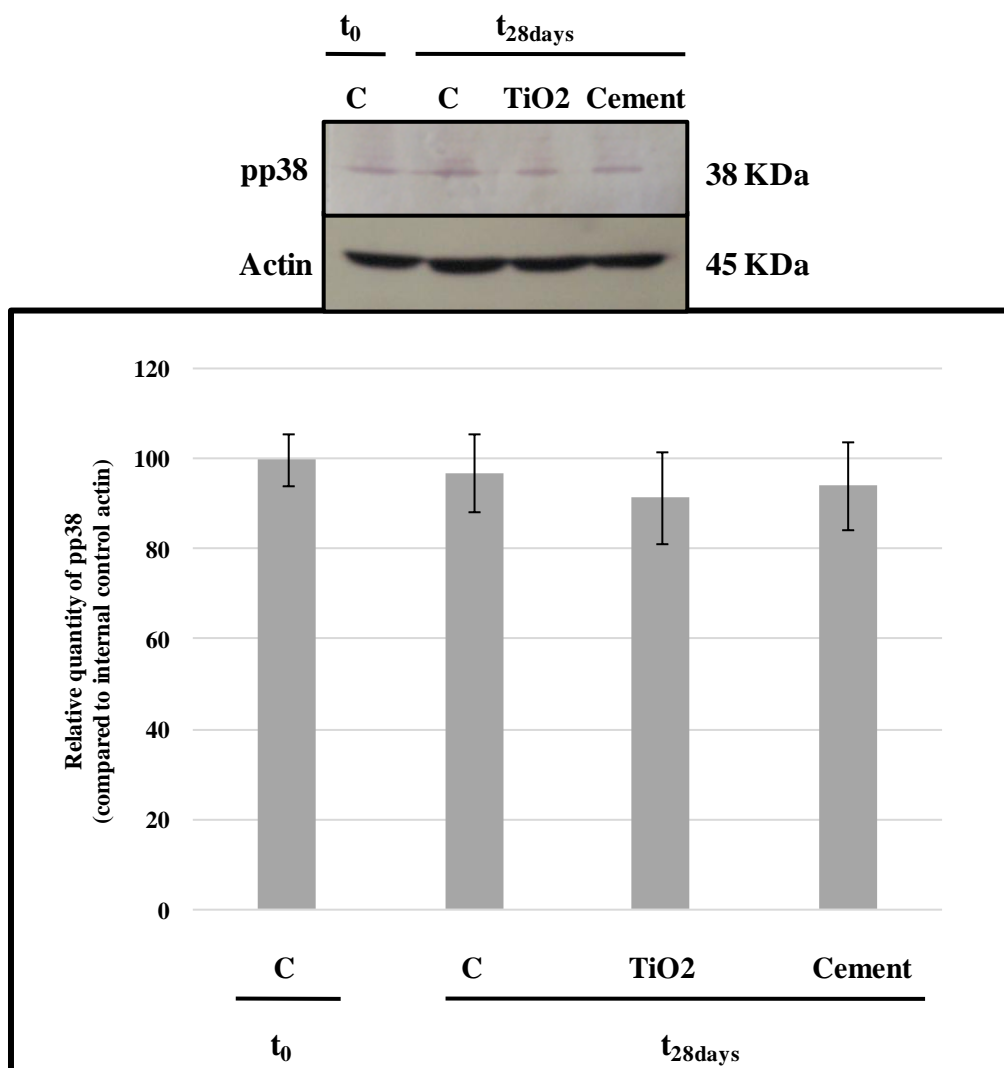
408 **Figure 6.** Total Ti concentration in the digestive gland of *P. corneus* (left) and total soft
409 tissues of *S. plana* (right) after 7, 14, 21 and 28 days of exposure in mesocosms. The
410 statistical significant was determined by the Student's t-test (*: $p < 0.05$; **: $p < 0.01$).

411

412 During the 28 days of exposure in mesocosms, the survival rates of *P. corneus* and *S. plana*
413 (<2% of mortality, data not shown), picoplankton and picobenthos (see SI, Figure S4) were
414 not affected by the presence of TiO₂ MNMs and cement leachate. Hence, no acute toxicity
415 was observed towards these two trophic levels. Nevertheless, a thorough characterization of
416 the biological responses (at the individual, sub-individual, and community levels) is still
417 needed to better understand the biological mechanisms of interactions.

418 One of the main effect associated with TiO₂ toxicity was the induction of immune responses.
419 The MAPKs represent a superfamily of protein Ser/Thr-kinases, highly conserved through
420 evolution (Kyriakis and Avruch, 2001; Roux and Blenis, 2004), that can transduce stress
421 signals into cellular responses (Kultz and Avila, 2001; Kyriakis and Avruch, 2001). Three
422 subfamilies of the MAPKs have been well described in mammals: the extracellular regulated
423 protein kinase (ERK), the c-Jun NH₂-terminal kinases (JNK) and the p38-MAPK. ERKs have
424 been more associated to cell division, growth and differentiation whereas JNKs and p38-
425 MAPKs are activated in various types of stress going from osmotic stress to chemical stress
426 and can trigger cell survival or death (apoptosis), depending on their isoform and/or cell type
427 (Canesi et al., 2001, 2002, 2006; Châtel et al., 2009, 2011a,b). They have been demonstrated
428 to play a key role in immune responses in mammals (Caffrey et al., 1999) but also in
429 invertebrates and more particular in bivalves (Canesi et al., 2001; 2002; 2006). Moreover p38

430 MAPK have been shown to be induced by TiO₂ exposure in bivalves (Couleau et al., 2012).
431 P38 MAPK in *S. plana* have been studied in the laboratory and antibody matching has already
432 been verified on this specie as opposed to *P. corneus*. Since no ingestion of Ti by *S. plana* was
433 identified by ICP-MS, western blot analysis of *S. plana* using specific antibodies directed
434 against the phosphorylated form of p38 MAPK were used (Figure 7).
435 In the present study, membrane revelation showed that the molecular weight of pp38 in *S.*
436 *plana* is around 38 KDa. After normalization of the different bands representing pp38
437 expression with actin, results revealed that there was no significant modification in pp38
438 expression level for any of the conditions tested (TiO₂ MNMs or cement leachate), as
439 compared to control group (figure 7). This suggested no stimulation of immune response of *S.*
440 *plana* following 28 days of exposure. Couleau et al. (2012) observed in the freshwater mussel
441 *D. polymorpha* exposed to TiO₂ (0.1 to 25 mg.L⁻¹ during 24 h), that ERK ½ was activated for
442 all concentrations tested in this study whereas p38 activation was only observed when
443 bivalves were exposed to 5 and 25 mg.L⁻¹) indicating that p38 phosphorylation was less
444 sensitive than ERK ½ to this contaminant. This could be the case in the present study.
445 Moreover, an *in vitro* *M. galloprovincialis* hemocyte exposure to TiO₂ (10 µg.ml⁻¹) for 5 to 60
446 min showed that pp38 activation was transient, with an increase in phosphorylated form of
447 p38 after 5 and 15 min, followed by a dephosphorylation at 30 and 60 min (Canesi et al.,
448 2010). Even though p38 is implicated in immune responses, absence of p38 activation does
449 not mean that immune response is not present. However, in the present study, the long
450 duration of exposure (28 days) could explain the absence of pp38 response depicted in *S.*
451 *plana* and investigation of its response at shorter time points would have been interesting to
452 perform.



453
 454 **Figure 7.** Expression of pp38 in *S. plana* control, TiO₂ MNMs or cement leachate, analyzed
 455 by Western blot. Results have been normalized to actin expression levels.
 456

457 **Conclusion**

458 In the present study, impact of TiO₂ MNMs and TiO₂ MNM-based cement leachate was
 459 investigated in marine and freshwater mesocosms, that allows to reproduce realistic
 460 environmental exposure (mid-term duration, low doses, chronic contamination,...) and in
 461 particular, a tidal system in the marine mesocosm. Such tools have been demonstrated as
 462 being relevant since they take into account both hazard and exposure and hence the synergetic
 463 and/or antagonistic effects of different physico-chemical parameters (temperature, pH, redox
 464 potential, conductivity), that is of great interest for the regulation of the use of NMs (Auffan
 465 et al., 2019).The originality of the project was also to compare the exposure and potential
 466 impact of TiO₂ and cement containing TiO₂ MNMs on two species, representative of the
 467 estuarine/freshwater continuum(with a salinity gradient),that are particular targets towards

468 nanomaterials at the water/sediment interface. The use of similar design for both freshwater
469 and marine systems showed challenging points and limitations, that enable consistent
470 comparison of fate and behavior of NMs in different species. However, even with same
471 procedures, characterization of TiO₂ in different abiotic compartments was difficult, as stated
472 in this study, and made challenging investigations of differences in bioaccumulation and
473 effects in these benthic species. The standardization of stable test suspensions for
474 nanomaterials is an ongoing challenge as it is well documented that the preparation method
475 influences the interpretation of toxicity according to the exposure. In the context of
476 developing a regulatory framework this is a challenge as it necessary that all nanomaterials
477 have equivalent preparation techniques in order to have a better standardization of
478 experiments and hence a more comparable set of data generated for nanomaterial-related data
479 categorization.

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

483

484

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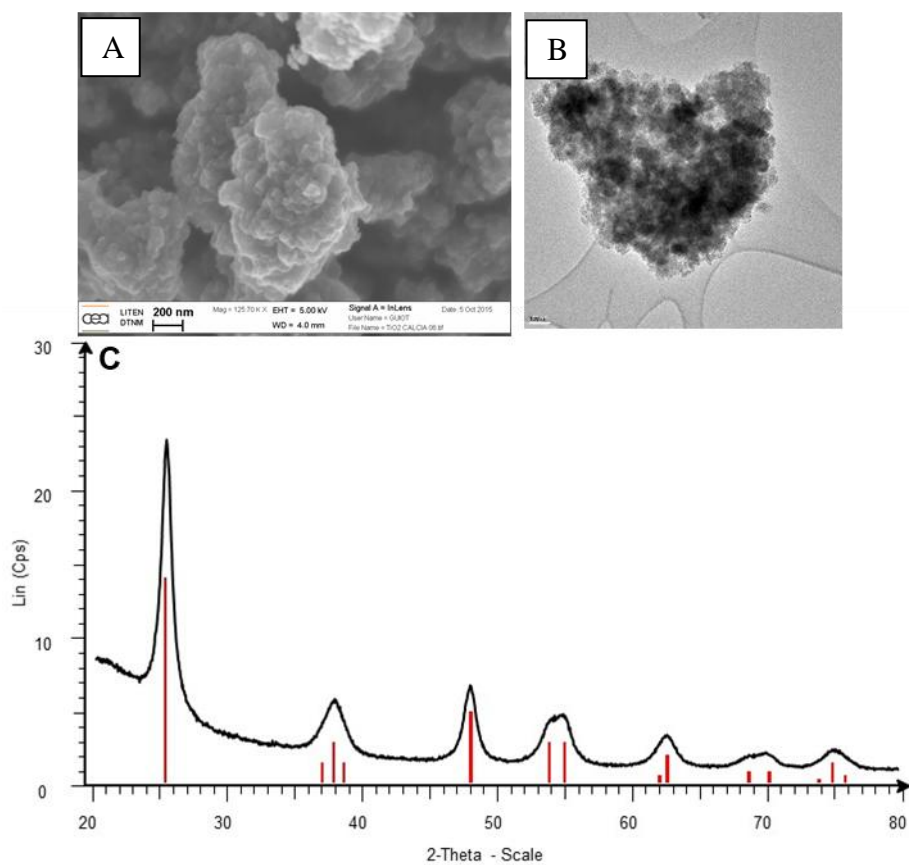
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620

621

622



624
625 **Figure S1.** Primary size and shape of TiO₂MNMs 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 TiO₂MNM 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

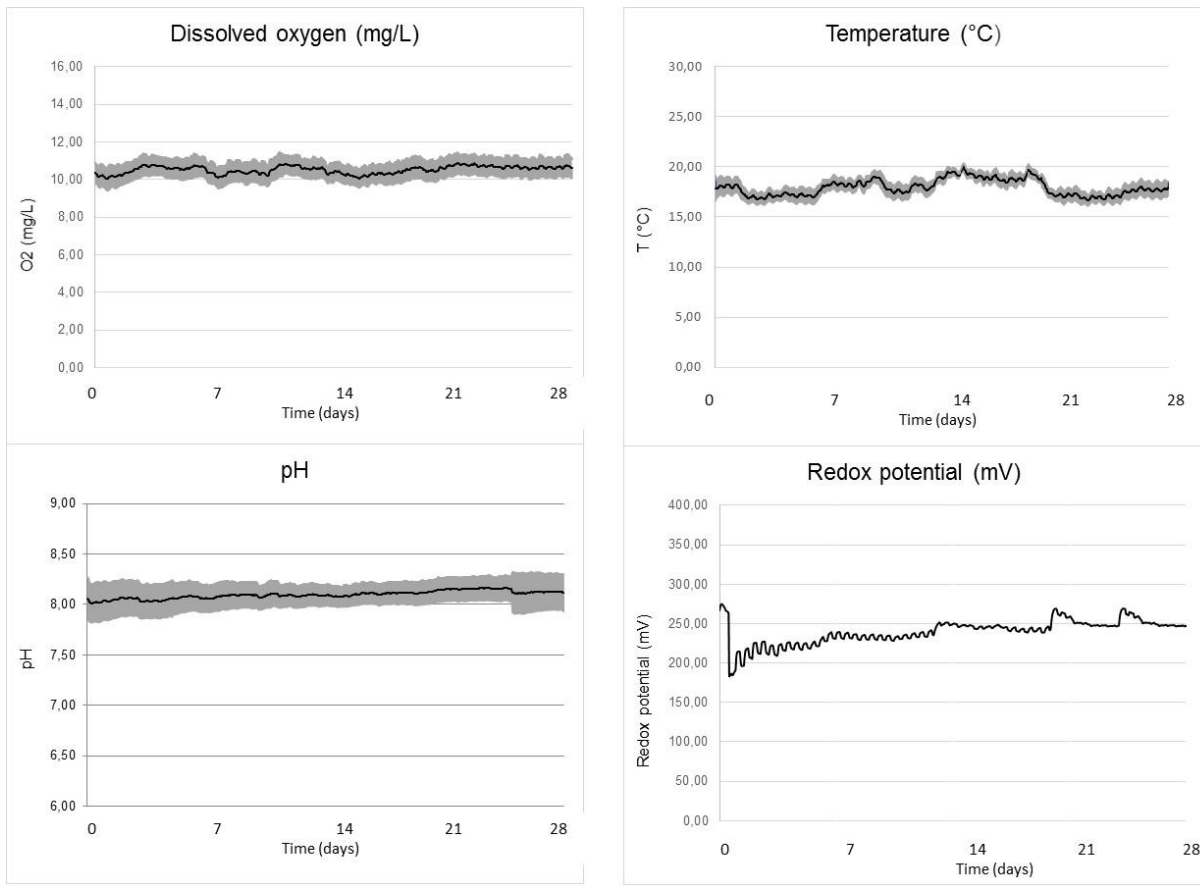
	Ti	Na	Al	Fe	Ca	Si
Total (mg.L ⁻¹)	89.91	1.89	54.06	12.10	2216.00	2407.80
Soluble fraction (<3 kDa) (µg/l)	3.22	182.00	1.00	1.00	2.03E+05	3960.00

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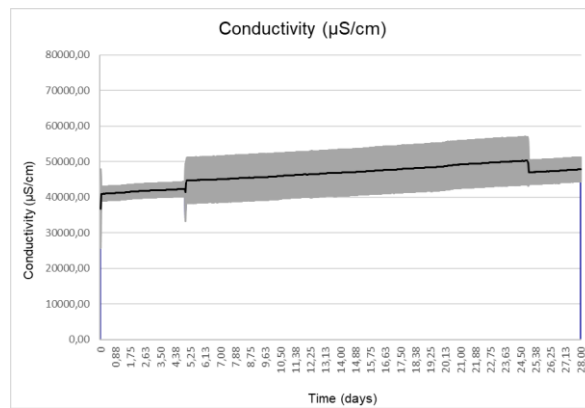
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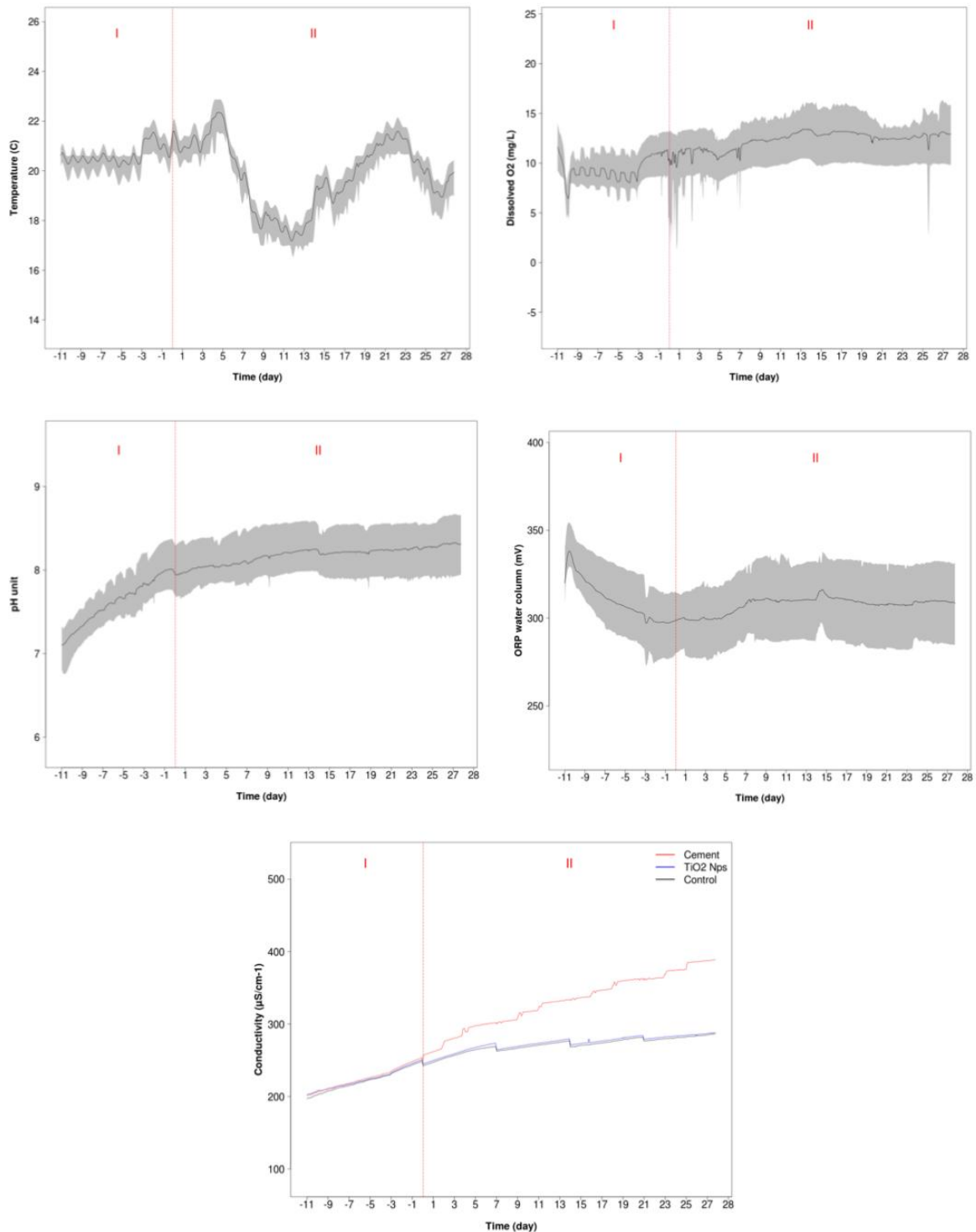
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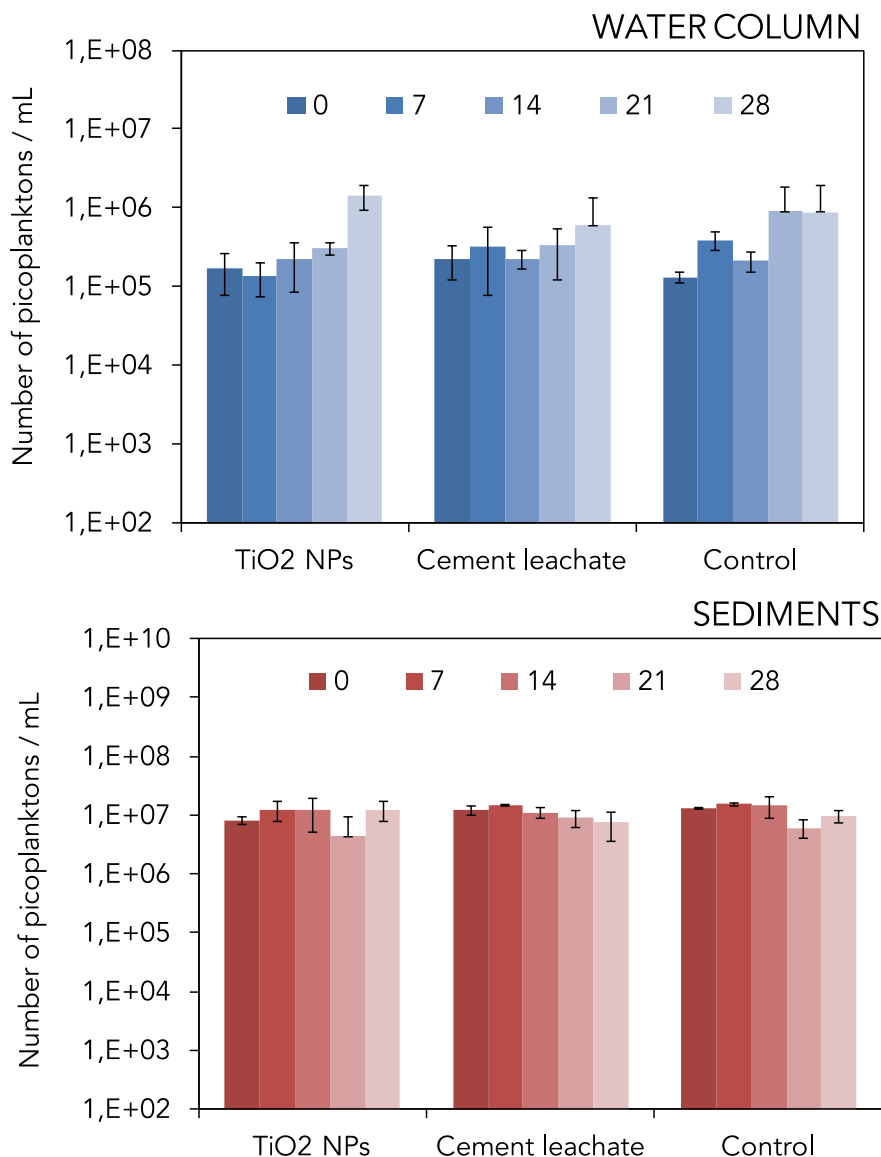
639 **Figure S2.** Evolution of the physical-chemical parameters in the water column of the marine
640 mesocosms. Temperature, Redox potential, dissolved oxygen, pH were measured during
641 phases I (stabilization) and II (contamination). Day 0 corresponds to the first dosing of NPs.
642 The grey surface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

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648 **Figure S3.** Evolution of the physical-chemical parameters in the water column of the
 649 freshwater mesocosms. Oxidation-Reduction potential (ORP), dissolved oxygen, pH, and
 650 conductivity were measured during phases I (stabilization) and II (contamination). Day 0
 651 corresponds to the first dosing of NPs. The grey surface is defined by the maximum and
 652 minimum values of each parameter, and the dark line corresponds to the average values of the
 653 6 mesocosms. The conductivity were not merged since a different trend was observed in the
 654 mesocosms exposed to cement leachate. One measurement was performed every 5 min.



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

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

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