A common European approach to the regulatory testing of nanomaterials

NANoREG

Grant Agreement Number 310584

Deliverable D 3.05

Development of an aquatic Mesocosms Platform allowing the evaluation of kinetics of aggregation

Due date of deliverable: *31/01/2016*

Actual submission date: *30/05/2016*

Author(s) and company:	Mélanie Auffan (CNRS), Catherine Mouneyrac (UCO), Jérôme Rose (CNRS), Carlos Fito (ITENE), Perrine Chaurand (CNRS), Armand Masion (CNRS), Hanane Perrein (UCO), Amélie Châtel (UCO) and Mélanie Bruneau (UCO)
Work package/task:	WP 3/ Task 3.2
Document status:	draft / final
Confidentiality:	confidential / restricted / public
Key words:	

DOCUMENT HISTORY

Version	*Date*	*Reason of change*
1	30/06/2016	
2	21/02/2017	Project Office harmonised lay-out
3		
4		

This project has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no 310584

Lead beneficiary for this deliverable:

Owner(s) of this document	
Owner of the content	CNRS – CEREGE, partner 7
Co-Owner 1	UCO, Partner 33
Co-Owner 2	ITENE, Partner 44

Table of Content

LIST OF FIGURES

LIST OF TABLES

1 Description of task

Task 3.2: Release of MNM

It is considered that (the potential for) exposure to a material, or product will be determined to a large extent by the combination of the activity emission potential (e.g. Marquart et al., 2011) and the substance emission potential (e.g. van Tongeren et al., 2011). The first relates to an activity or process, whereas the latter relates to material properties.

Currently, some leading institutes on Occupational Health, i.e. PEROSH , have joined efforts to benchmark (and if possible harmonize) dustiness tests for MNMs ('release to the work environment') , whereas some initiatives have been taken to design and set-up relevant rigs for release testing of MNM for different stages of the life cycle (e.g. NanoGEM, DE).

A number of subtasks were envisaged within task 3.2. The current document refers to the development / use of an Aquatic Mesocosms Platform allowing to evaluate kinetics of aggregation, settling and transformation of NMs Development of Adjustable Pocket Sized Mesocosms. This approach included continental as well as marine aquatic environments.

2 Description of work & main achievements

2.1 Summary

This deliverable "Development of an aquatic Mesocosms Platform" is part of the work in task 3.2. and involved partner n°7 (CNRS-CEREGE), 33 (UCO) and 44 (ITENE). The aim was to implement several mesocosm facilities within the Nanoreg consortium and to demonstrate the relevance of these mesocosms as environmental exposure and bio-physical-chemical testing tool.

One of the main conclusions of NANOREG deliverable 3.1 is a severe knowledge gap regarding environmental exposure to NMs. Environmental exposure remains a complex task requiring interdisciplinary efforts from Physical-chemists, (micro)biologists, and ecotoxicologists. All together they need to conduct meaningful experiments to study the environmental risk of engineered nanomaterials with access to relevant mechanistic data across several spatial and temporal scales.

The objective of the mesocosm testing is to help bridge this gap by providing a reliable methodology to obtain quantitative time- and spatially resolved data on the distribution of NMs within a simulated ecosystem. In the nanoREG project, we proposed a laboratory-scale mesocosm facility to serve as a platform for investigating NM exposure and impacts. We opted for modular, small size (3 L) and medium size (60 L) indoor mesocosms. Such experimental design allows the simultaneous monitoring of a number of parameters (*e.g.* aggregation, settling, mass balance, trophic transfer, biotransformation, oxidative stress, microbial diversity) under environmentally meaningful conditions.

The efficiency of the mesocosm platform was tested based on several pilot studies taking into account the life cycle of different nanoproducts (diesel additives, cement, plastic films) containing different pristine nanoparticles (TiO_2, CeO_2, ZnO, Ag). Results indicated that the developed experimental systems can operate with different physical and physico-chemical features (*e.g.* water quality and depth, sediment mineralogy and depth, current velocity, tidal reservoirs, *etc.*) and biota, owing to a high flexibility. Results from indoor mesocosms provide both a high degree of complexity in the system and a reduced uncertainty regarding the fate of the NMs. This platform allows for simultaneous evaluation of physico-chemical properties and their relationship to the biological systems *in situ* as the ecosystem and NMs evolve. This platform is particularly well adapted to probe physical stability of NMs within the water column. Indeed indoor mesocosm design facilitates the characterization of homo and hetero aggregation of NM. More over these platforms provide more realistic conditions for considering NM transformations that have been lacking to date in many studies of NM impacts on organisms and proven to be critical in assessing the potential hazards presented by NMs.

By focusing specifically on exposure, the experiments described in DL3.5 contribute to the answers to at least 5 of the regulatory questions NANOREG WP1.

2.2 Background of the task

Current strategies for assessing the environmental safety of engineered nanomaterials (NMs) are largely based on nanoecotoxicology approaches (**Kahru et al. 2010**) focusing on a single species or strain of model organisms. Such experiments are needed to gain knowledge on the mechanisms and thresholds of toxicity (hazard), but they separate the organism from the environment and community of organisms (*i.e*, the ecosystem), in which the environmental risks of NMs ultimately need to be understood. Moreover, while the hazard to cells and organisms is extensively investigated, relatively little attention is paid to the exposure of organisms to NMs despite the pivotal role of both exposure and hazard in together driving risk. The exposure of NMs to organisms in the environment is controlled by the interplay of parameters including aggregation state and sorption of (in)organic substances (**Auffan et al. 2009**), oxidation-reduction potential, as well as ecological factors such as abiotic parameters (e.g. salinity), interacting organisms (*e.g.* microorganisms, (in)vertebrates, plants, fungi), trophic levels present (*e.g.* primary producer, primary consumer, secondary consumer) and trophic transfer potential. The effects on organisms can therefore be evaluated under relatively controlled conditions that, like larger scale mesocosm studies, allow for evaluation of trophic transfer, maternal transfers, predator-prey interactions, competitive effects, resistance and resilience

Abundant literature exists on the effects of environmental parameters taken separately, but for a robust characterization of risk, the complex ecosystem-level interplay between the organisms, their environment, and the NMs needs to be taken into account. Such experimental studies on the risk of NMs under environmentally realistic conditions require a diverse collection of expertise—including but not limited to physical chemistry, (micro)biology, and ecotoxicology. Particularly well-suited experimental units with which to engage such multidisciplinary teams are mesocosms: medium-size replicated ecosystems. 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**).* This experimental strategy has already been used to study the behavior or impacts of NMs (**Ferry et al. 2009, Cleveland et al. 2012, Buffet et al. 2012, Lowry et al. 2012**).

A broad diversity of mesocosms design exists (e.g. **Schriner et al. 1984, Sommer et al. 2007, Mohr et al. 2005**) in term of dimension, location (indoor, outdoor), etc. A common factor of all these studies is that mesocosms are considered as a small portion of the natural environment that is brought under controlled conditions. In our study we define a mesocosm as an experimental design which is (i) self-sustaining once set-up without any input of additional nutrients or resources, and (ii) that allows controlling all (or the maximum of) input and output parameters to draw a real-life mass balance whatever its dimension or location. Such a setup has been successfully applied to trace the transfer of gold NMs from the water column to the estuarine food web (**Ferry et al. 2009**). The authors modeled the edge of a tidal marsh creek using 366 L-indoor mesocosms maintained for 12 days. Clams and biofilms were observed to accumulate most of gold on a per mass basis. The long-term (18 month) distribution and transformation of silver NMs was also studied in outdoor mesocosms mimicking freshwater emergent wetlands (**Lowry et al. 2012**). Silver sulfidation was demonstrated in the terrestrial soils and subaquatic sediments and a high body burden of Ag was measured in mosquito fish and chironomids (**Lowry et al. 2012**). Another study used indoor estuarine mesocosms to monitor the leaching of Ag from consumer products incorporating NMs over 60 days. Outdoor estuarine mesocosms were used to investigate fate, behavior and impacts of CuO NMs and Ag NMs towards two endobentic invertebrates (**Buffet et al., 2013, 2014**).

The investigations described in the literature involve rather large facilities (tank size 120 L and above), to reproduce the buffer capabilities of a natural system. However, the need for multiple replicas in biological studies limits the practicality of large mesocosms due to obvious limitations in space and cost. The challenge is thus downsizing for larger setups while avoiding the artifacts of in vitro and high throughput studies (e.g. **Bone et al. 2012, Unrine et al. 2012, Thomas et al. 2011**), which do not simulate adequately an ecosystem.

In the nanoREG project, we proposed a laboratory-scale mesocosm facility to serve as a platform for investigating NM exposure and impacts. We opted for modular, small size (3 L) and medium size (60 L) indoor mesocosms. Such experimental design allows the simultaneous monitoring of a number of parameters (*e.g.* aggregation, settling, mass balance, trophic transfer, biotransformation, oxidative stress, microbial diversity) under environmentally meaningful conditions. This experimental design can accommodate several types of ecosystems such as lotic, lentic, estuarine, or lagoon environments, without requiring expensive and/or cumbersome infrastructures. This versatile tool can then be used by a large community of physical-chemists, (micro)biologists, and ecotoxicologists to study the exposure and impacts of NMs (low doses, chronic contamination) as well as the mechanistic concepts at various temporal and spatial scales. Here we show that,

with the adequate methodology, using small sized mesocosms is an approach as robust as using large(r) sized equipment.

In this deliverable, this concept is illustrated by several pilot studies performed at CNRS-CEEGE, UCO and ITENE which took into account the life cycle of different nanoproducts (diesel additives, cement, plastic films) containing different pristine nanoparticles (TiO_2, CeO_2, ZnO, Ag).

Aim of the deliverable

This deliverable "Development of an aquatic Mesocosms Platform" is part of the work in task 3.2. and involved partner n°7 (CNRS-CEREGE), 33 (UCO) and 44 (ITENE). The aim was to implement several mesocosm facilities within the Nanoreg consortium and to demonstrate the relevance of these mesocosms as bio-physical-chemical testing tool. One of the main conclusions of deliverable 3.1 is a severe knowledge gap regarding environmental exposure to NMs. The objective of the mesocosm testing is to help bridge this gap by providing a reliable methodology to obtain quantitative time- and spatially resolved data on the distribution of NMs within a simulated ecosystem.

To do so, the activities within this task included (1) setting up the mesocosm facilities, and (2) monitoring the mesocosms using several nano-enabled products with varying properties (solubility, redox sensitivity…) and different exposure protocols (acute vs. chronic).

Among the core regulatory questions for work package 3, the work described in this present deliverable addresses questions n°12 "How should human and environmental exposure be assessed in practice" and n°13 "Exposure and life cycle analysis: Which scenarios could denote potential exposure and what information do we have on them?". Associated regulatory questions are questions n°3 " What testing should be performed to identify surface modifications that occur once a NM has been released into the environment or taken up into the body?", n°6 " Fate, persistence and long-term effects: Can effective in vitro and alternative models to understand long-term effects be developed?" and n°9 "Mode of action: What are the physical and chemical properties driving exposure and (eco)toxicity of NMs at all stages of their life cycle?"

2.3 Description of the work carried out

2.3.1 Materials

Various nanomaterials selected within the priority list of Nanoreg project, were tested (Ag, ZnO, CeO_2, TiO_2). They were obtained from NanoReg on line inventory NMs (http://nanoreg-materials.eu/). However the novelty of WP3 experiments was to take into account the different forms of NMs across the life cycle of nano-enabled products.

Then pristine, formulation, and released by-products obtained by the aging of nano-enabled products (TiO_2 based cement, CeO_2 based diesel additives, Ag and ZnO based plastic films) were also tested to fully take into account the various stages of life cycle (Figure 1).

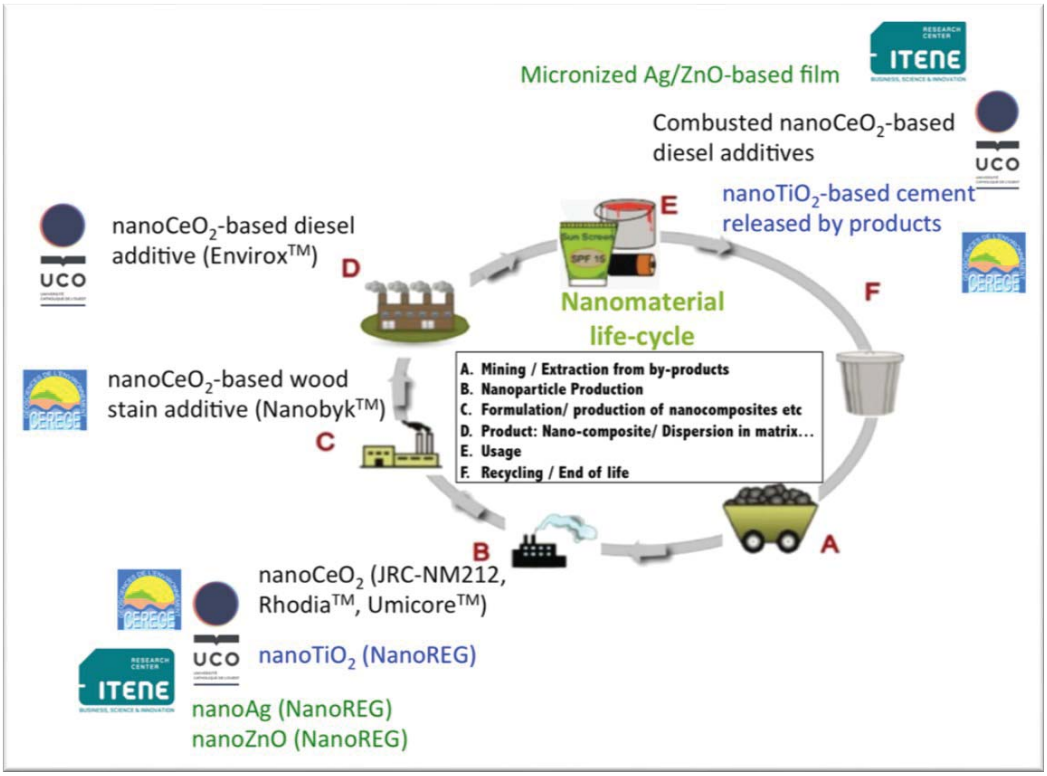


Figure 1: List of nanomaterial tested across the life-cycle.

2.3.1.1 Reference nanoparticles (stage B)

NanoCeO$_2$ NM212 JRC repository. They have a primary particle size of 30 nm ±10 (XRD and SEM). IEP was not determined, however zeta potential in DI water with 5 nM NaCl was 33,9±1,7 mV (pH was not measured in the JRC report). The specific surface area was 27.2 ± 0.9 (BET method).

NanoCeO$_2$ from Rhodia. They have a primary particle size of 3–4 nm, an average hydrodynamic diameter centered around 8 nm (in ultrapure water at pH = 3.1), and an IEP between pH 7 and 7.5. The specific surface area was estimated to be 271±177 m^2/g.

NanoCeO$_2$ from Umicore. They have a primary particle size of 31 ± 18 nm (determined using TEM), an average hydrodynamic diameter centered around 90 nm, a zeta potential of 42 ± 2 mV in the stock suspension (in ultrapure water at pH = 3.1), and an isoelectric point (IEP) of pH ~7.8 ± 2. The specific surface area was estimated to be 271±177 m^2/g.

NanoTiO$_2$ NM102 JRC repository. They have a primary particle size of 21 ±10 nm (TEM). NM-102 forms a stable suspension at pH lower than 4, with positively charged nanoparticles (exceeding 30 mV). The zeta potential varied significantly as function of pH from 40 mV at pH 2 to -45 mV around pH 12. IEP: 6 » (JRC report)

NanoAg: from the NanoReg on line inventory NMs (http://nanoreg-materials.eu/).

NanoZnO: from the NanoReg on line inventory NMs (http://nanoreg-materials.eu/).

2.3.1.2 Nano-based formulation (stage D)

NanoCeO$_2$-based wood stain additive (Nanobyk™). They are citrate-coated CeO$_2$-NPs with a primary particle size of 3.9 ± 1.8 nm (determined using TEM), an average hydrodynamic diameter centered around 8 nm, and a zeta potential of −40 ± 4 mV in ultrapure water from pH 3 to 10. The specific surface area was estimated to be 271±177 m^2/g.

NanoCeO$_2$-based diesel additive (Envirox^{TM}). A 500 mL bottle of Envirox^{TM} was supplied by Energenics Europe Ltd. It contained 21.3±0.5 g.L^{-1} of Ce. In these suspensions, the nanoparticles were spherical with an inter-reticular d_{hkl} distance of 3.1 Å close to the d_{111} of CeO_2. Using the Scherrer formula, we estimated the coherent diameters of the CeO_2 nanoparticles before combustion to 5.5±0.4 nm. These size was in agreement with the TEM size measure at 7.6 ± 1.2 nm (Figure 2).

2.3.1.3 Aged-nanoproduct (stage E)

Combusted nanoCeO$_2$-based diesel additives. Combustion was performed at 850°C to simulate the 'use' stage of the nano-CeO2 based diesel additives. Briefly, the Envirox^{TM} suspension was ultracentrifuged (at 396 750 g, 20°C for 1h). The solid phase was freeze-dried and the resulting powder was introduced in a furnace for 20 min at 850°C. After combustion, the size of the crystallites raised up to a mean size of 19 ± 10.4 nm with similar d_{hkl} distances. The polydispersity of the size of the CeO_2 nanoparticles also increased over aging as observed in the aggregate of the combusted Envirox^{TM} (Figure 2) with particles ranged between 3 and 40 nm. These results are in agreement with ref. **(Batley et al. 2013)** which have shown that before combustion CeO_2 nanoparticles were ranged from 6 to 14 nm and after combustion in a diesel engine they were transformed into ~ 43 nm particles. While the size of the CeO_2 crystallites did not changed between ambient temperature and 280°C (5.5±0.4 nm), a significant increase of the crystallites size occurred at 850°C (21 ± 2.3 nm). No change in the crystalline form (long-range order) was observed by XRD over combustion. Moreover, the short-range order was study by XAS at the Ce K-edge (Figure 3). XANES data are sensitive to the oxidation state of the Ce atoms **(Edelstein et al. 1996)**. No significant difference was observed between the XANES spectra of Ce(IV) reference compounds, of uncombusted, and of 850°C-combusted Envirox^{TM} highlighting that Ce(III) atoms were not detected in the (un)combusted CeO_2 nanoparticles. The behavior of the organic matrix surrounding the CeO_2 particles was studied by ATR-FTIR after combustion at 850°C.

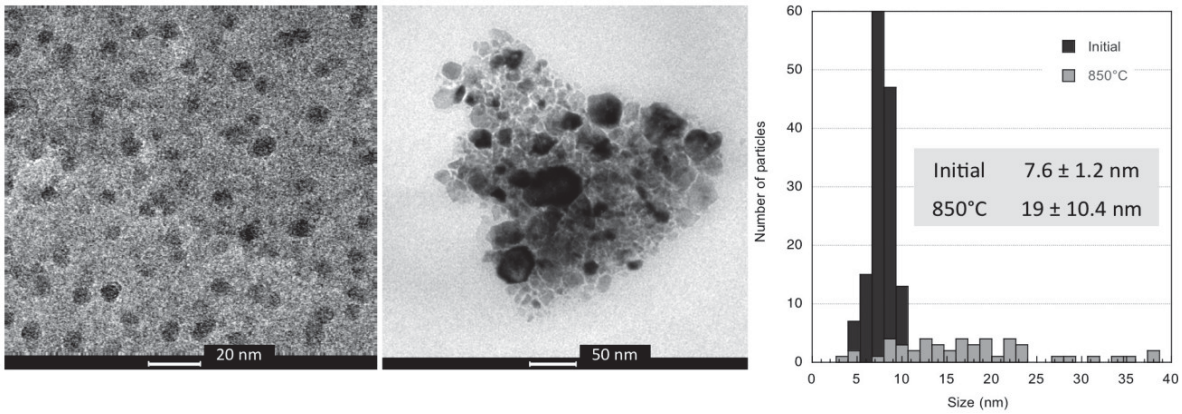


Figure 2: TEM images of the uncombusted and combusted Envirox^{TM} and the corresponding size distribution. The inset corresponds to the mean TEM size ± standard deviation.

By-products obtained from cement leaching. Photocatalytic cements were fragmented aged and the obtained nanomaterials degradation residues were used to expose mesocosms at a dose of 1 mg TiO_2/L. An industrial partner provided hardened cement pastes of photocatalytic white Portland cement incorporating TiO_2 NMs.The cement pastes were obtained by mixing anhydrous cement powder with ultra-pure water (UPW) at water to cement ratio (w/c) of 0.55 and curing in a cylindrical container.

Accelerated aging generation was simulated at lab-scale to cater for mesocosms dose injection. Finely crushed cement paste was leached in a batch test with an elevated liquid-to-solid ratio (L/S) of 100 and ultra-pure water (UPW) as leachate. After a first pH equilibrium step (12<pH<13), the cement solution was neutralized at pH 7.8 to mimic fate and transfer of aged cement residues into natural aquatic media. Nitric acid was slowly added to the cement solution. Aged cement solution with a stabilized pH of 7.8 and conductivity around 13 mS/cm was then injected into mesocosms. Its TiO_2 NMs concentration measured by ICP-MS was around 150 mg TiO_2 NMs/L.

Weathered PET and PP films. The selected samples to be assessed in the mesocosms study included: Polypropilene (PP), Polyethylene terephthalate (PET), PET+Ag, PET+ZnO, and PP+Ag. Polymer/nanofiller nanocomposites can be prepared by different routes, including *in situ* polymerization, solution processing and melt mixing. The latter is the most common, given its simplicity and high yield, the compatibility with current

industrial processes and the environmental advantage of a solvent-free procedure. Melt mixing may employ different kinds of equipment. At the industrial scale, twin screw extruders of the co-rotating type became popular. For a given incorporation level, the characteristic parameters that contribute greatly in modifying the properties of composites are the filler loading, their size and shape and their affinity towards matrix material (affinity of the inorganic fillers/polymer interface). Later is one of the critical factors.

Polymer nanocomposites were processed at ITENE facilities by extrusion processing technique (Figure 3). The extrusion equipment used is a model DSE 20/40D supplied by Brabender, with Plastograph model GmbH driver (Figure 3). The extruder comprises a double screw with a working range of 25 to 350 ° C and an output of 0.5 to 20 kg / h of material.

Figure 3: Extrusion of tested nanocomposites.

Materials were processed with a theoretic nanoparticle´s content of 4.5%, which is a typical content of nanofiller in nanocomposites processing. Nanocomposites were then subjected to accelerated aging under controlled laboratory conditions based on the protocol stablished by the standard UNE-EN ISO 4892-3 (ISO). The employed equipment was a chamber QUV-se de Q-PANEL LAB PRODUCTS equipped with a UVB-313 (emission peak at 313 nm) lamp (Figure 4). Total time of exposure was 500h and the exposure area was a test specimen of 7.5 x 15 cm and 1 cm of thickness. Such conditions emulate degradation that nanocomposites suffer once released in the environment. Aging was carried out following the UNE -EN ISO 4892-3: 2006 by exposing the material to UV light, high temperatures and water. For that, cycles of 4 hours under UV light (313 nm) at 70 ° C and 4h of condensation at 50 ° C were followed.

Figure 4: Aging chamber employed, in AIDICO facilities (Technological institute of construction).

Figure 5 shows the appearance of the samples before and after 500h of exposure to UV radiation, temperature and water.

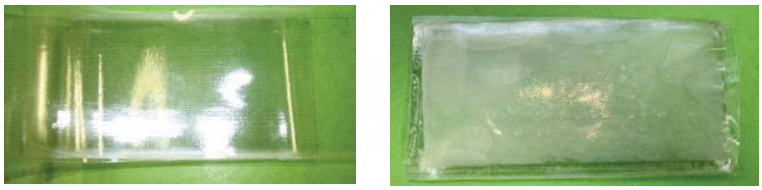


Figure 5: PET before (left) and after (right) aging

Finally, polymer nanocomposites were micronized and their final content on nanofiller determined with a thermogravimetry. Using a thermogravimetric balance model Q5000IR from TA instruments, processed films were subjected to the same cycle of thermal degradation. From the residue remaining after completion of the thermal cycle, once the polymer has been completely degraded, the inorganic content was determined. For calculating the nanoparticles content, the thermogravimetric analysis (TGA) was performed on nanoparticles, blank polymers and on nanocomposites (Figure 6). Table X shows such content.

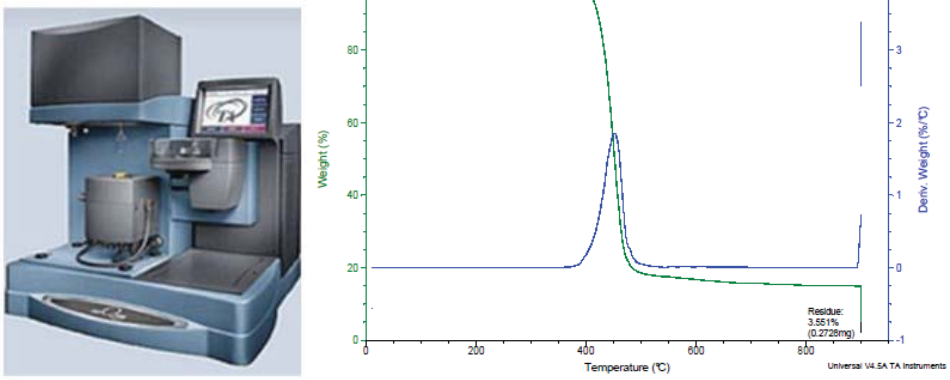


Figure 6: Thermogravimetric balance model Q5000IR, from TA instruments (left) and Thermogram of PET+silver nanoparticles obtained by TGA.

Table 1. Determined nanoparticle percentage in polymer nanocomposites.

Nanocomposites	NP %
PET-ZnO weathered	4,5
PET-Ag weathered	4,5
PP-Ag weathered	6.6

Although polymers are not considered hazardous because their high molecular weight, when decreasing their size, they generate environmental concern. In the past few years, such effect in organisms of different ecosystems is being also studied (**Wagner et al. 2014, Wright et al. 2013**). Thus, micronisation of samples was undertaken in order to obtain a homogenized powder of nanocomposite in a narrow size range able to be bio-dispersible and undertaken by organisms used in the mesocosms.

2.3.2 Methods

2.3.2.1 2 Freshwater 3L-mesocosms (ITENE)

- ***Selected concentrations to be studied***

Selection of concentrations employed in mesocosms tests are related to the lowest values of 48 h EC_{50} observed for each material in tests previously conducted of acute toxicity (19.1 ± 6.8 mg/L in *P. subcapitata* for CeO_2 NMs and 1.3 ± 0.21 mg/L in *D. magna* for TiO_2 NMs). In the case of nanocomposites, selection of the concentrations to be studied where based on previous ecotoxicity assays conducted as well as the content in nanofillers of the nanocomposite selected, and considering nanoparticles are in their major part encapsulated in the matrix of the polymer. Table 2 below shows the percentage of nanofiller in the micronized polymeric matrix selected. Taken into account these values, tested concentrations for the study of ecotoxicological effects and their distribution in the food chain were those shown in Table 2.

Table 2: Nanomaterials concentration (mg/L) in the aquariums.

Sample	Nanocomposite concentration in the aquaria (mg/L)	NP concentration (mg/L)
PETw	111.1	-
	222.2	-
PPw	15.2	-
	76.0	-
PET-ZnO w	111.1	5
	222.2	10
PET-Ag w	22.2	1
	111.1	5
PP-Ag w	15.2	1
	76.0	5
CeO_2	-	5
		10
TiO_2	-	0.5
		1
Ag	-	0.1
		0.2
		1
		5
ZnO	-	0.3
		1

In order to achieve the final concentrations proposed, a stock solution of each material was prepared in commercial water "Cortés" and sonicated for 1h. Calculated aliquots required to reach the final concentrations were transferred to each aquaria.

- ***Organisms***

For mesocosm studies, the algae, ostracods and daphnids organisms were obtained from MicroBioTests Inc., Gent, Belgium. Periphyton culture was obtained from vegetation from the Mediterranean lake, a natural spring "Riu Verd" located in the locality of Massalavés (Valencia, Spain) (39° 8′ 28″ N, 0° 31′ 16″ W).

Daphnia magna (FTM Daphtoxkit Magna, Belgium) was used as reference organisms of zooplankton species. The ephippias were washed with tap water to remove any traces of preservation medium and were transferred to a glass with daphnids standard medium. The incubation process was carried out during 4 days under illumination and 23°C.

Heterocypris incongrues (Ostracodtoxkit, Beligum) was used as a reference organism in the zoobenthos compartment, suitable to detect and quantify the toxicity in freshwater sediments. Immobilized resistance forms were transferred to a glass with standard water prepared from the kit. Incubation was carried out for 52h at 25 ° C under continuous light conditions.

Selenastrum capricornutum (Algaltoxkit F, Belgium) unicellular green alga was used as representative organism of phytoplankton compartment, usually used to estimate the phytotoxic potential of surface or underground fresh water. The starting of an inoculum of 50 mL of a $2.5x10^6$ density cells/mL was placed in a 2L flask with water Cortes with Jaworsky media giving a final 62 500 cells/mL cell density.

Periphyton is defined as a complex community of microbiota (algae, bacteria, fungi, animals, organic and inorganic debris) adhered to a substrate. The slides were left for ten days in an aquarium with river water in order to inoculate.

- ***Mesocosm experiments***

The experiment was conducted in an aquatic ecosystem model reproducing the environmental conditions of a Mediterranean shallow lake. Mesocosm compartments covered freshwater and representative organisms including daphnia (zooplankton), algae and ostracods (zoobentos), as well as periphyton. The planktonic alga was *Selenastrum capricornutum*, a small chlorophyte easily edible by zooplankton. As a filter feeder zooplankton, the cladoceran *Daphnia magna* was used. An ostracod, *Heterocypris incongruens* was introduced as grazer and detritivorous benthic organism. Periphyton culture was obtained from vegetation from the Mediterranean lake. These organisms were selected in order to include different feeding groups to study and compare how each group up-takes the nanoparticles. All the organisms were previously acclimated under experimental conditions.

To estimate the characteristics of the sediment matter of the mesocosms, a petri dish (3 cm diameter and 1 cm height) was also placed in the bottom of the aquaria. Rectangular shaped plastic (acrylic polystyrene) aquaria with a capacity of 5 L and with an enclosure of 24 cm long, 17 cm width and 13 cm deep were used as containers. Aquarias contained a final volume of 3 L.

The tests were carried out at controlled climatic conditions (light, temperature). The aquaria were placed randomly in the chamber. Daily, water of each aquarium was slightly stirred to mimic natural water movements and position was changed randomly weekly. Moreover, losses of water by evaporation were filled with Milli-Q water every three days in order to maintain constant the final volume of 3 L and salts and organisms concentration. Commercial water “Cortés” was employed in the aquaria, from spring of Penyagolosa (Spain) which composition is recompiled in Table 3.

The experiments were run for 16-20 days, in a chamber programmed at 20°C and cycles of light: 12h light/ 12h dark. Four or three repetitions were introduced for each studies sample. At least four controls were introduced in each experiment.

Table 3: “Cortés” water employed in mesocosm studies.

Type	Amount
Dry residue	270 mg/L
Bicarbonate	257 mg/L
Sulphat	17.6 mg/L
Clorures	8.7 mg/L
Calcium	80.5 mg/L
Magnesium	5.7 mg/L
Potasium	1 mg/L
Sodium	4.9 mg/L
Fluorine	< 1 mg/L

Along the duration of this task of the NanoReg project, three tests, separated on time, were performed:

- First mesocosm experiment: Ag and ZnO
- Second mesocosm experiment: Ag, ZnO, PP, PET, PPAg, PETAg, PET ZnO (weathered)
- Third mesocosm experiment: CeO_2 and TiO_2.

At starting point of the experiments and each week, water conductivity, pH, temperature, dissolved oxygen concentration and percentage of oxygen saturation were measured. Also each week growth of *S. capricornutum* was estimated. For that aim, a sample of 3 mL of water was taken each week, placed in a glass tube, and fixed with Lugol and recount of number of cells per square centimetre registered.

After three weeks, the organisms (cladocers, ostracosds, periphyton and phytoplankton) as well as water samples and sediment included in the mesocosm were collected and stored in a freezer at -20ºC for further measurement of Ag, Zn, Ti and Ce uptake. In the crustaceans (*D. magna* and *H. incongruens*), filters with alga, periphyton and sediment samples, the samples were digested with 1 mL of acid nitric and diluted with deionized water Milli-Q at a final volume. For all the organisms the concentration (µg/g) for dry weight was calculated.

In the case of water samples, boiling was not necessary because there are not tissues to be digested, and the dilution was with Mili-Q water. In addition, a recount of cladocers, ostracods as well as algae cells was undertaken. The aquaria and culture chamber are shown in Figure 7 and some of the collected samples to be analyzed are given in Figure 8.

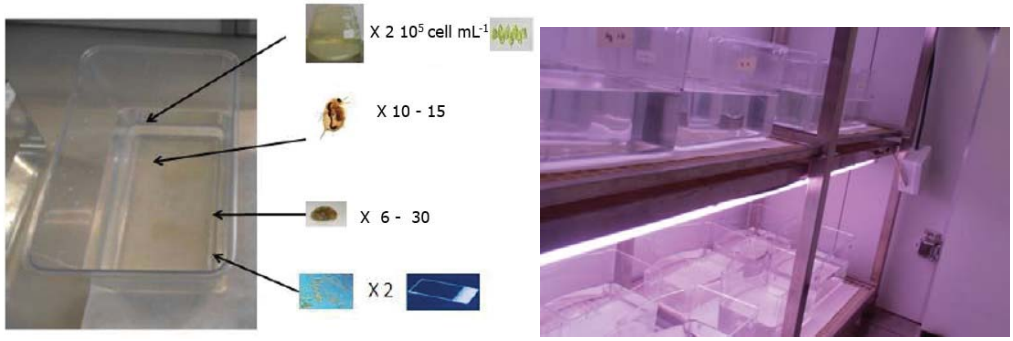


Figure 7: Aquaria (left) and Culture Chamber (right).

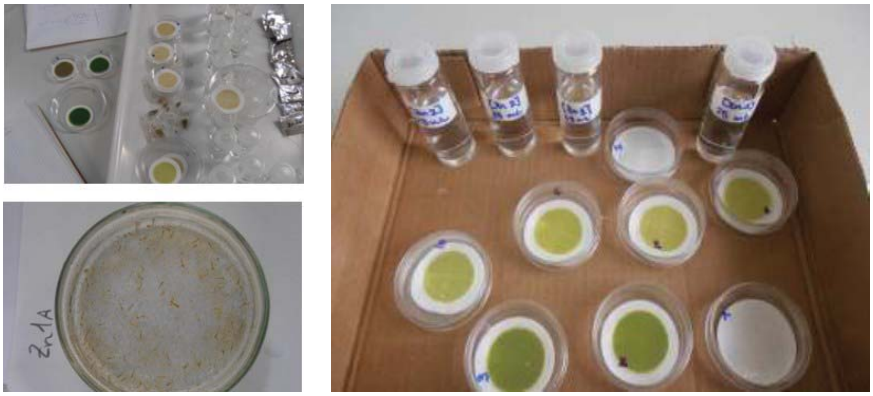


Figure 8: Sampled items of the different compartments of mesocosm to be analysed.

Each aquarium was identified with a number and the final concentration of NPs. 10-15 *D. magna* and 6-30 *H. incongruens* were introduced in each of the aquaria with water “Cortés”. Selected organisms were a difference of less than 48h old. 5 ml of *Selenastrum capricornutum* were added in order to reach a concentration of $2x10^5$ cells/mL in the aquarium (V = 3 L). Two glass slides, one inoculated with periphyton, were placed in each of the bottom of the aquarium. Finally, the sediment trap, Petri dish of 3 cm of diameter, was placed on the bottom and in the middle of the aquarium. Once all the elements were introduced, 250 mL of stock solutions of nanomaterial/nanocomposite were added, reaching the 3 L final volume in each aquarium.

All samples were tested freshly prepared in water “Cortés” medium, after 50 minutes of ultrasonication, 20-25ºC.

- ***Analytical procedures***

Phytoplankton. To characterize the phytoplankton compartment, 250 mL of water are filtered through a 0.45 µm nylon filter pre-weighed on an analytical balance with 5 decimals. The filter is placed in a petri dish which is inserted in a desiccator for 24h. After drying, the filter is weighed again to determine the filter weight of phytoplankton retained therein. For quantification of nanomaterial, the filter is introduced into a digestion tube with 3 mL of nitric acid (HNO_3 69.5%) with holed parafilm covering. Cold digestion is conducted for about 12h. After this step, it is placed in a container with boiling water for 2h. Both the cold digestion as hot digestion, were performed in a fume hood. The content of the digestion tube was transferred to a falcon tube and carried with 9 mL of Milli-Q water. After centrifuging for 5 minutes at 5000 rpm they were pipetted 0.75 mL of supernatant and placed in a new Falcon tube which was filled up to 5 mL.

Water. Water is collected in a 30 ml falcon tube when phytoplankton is filtered water. 30 mL of nitric acid were added and allowed to stand for 24 hours before analysis.

Periphyton. The slides with periphyton were rinsed slightly to remove any sediment. With a small brush, the surface was scraped and washed with Milli-Q water and put in a Falcon tube filled up to 40 mL with Milli-Q water. Once the sample collected, analytical procedure is analogous to that described for phytoplacton compartment.

Sediment. The sediment was collected by suction from the petri dish using a Pasteur pipette and stored in a falcon tube. It is trimmed to 5 mL with Milli-Q water. 3 mL is filtered with a 0.22 µm filter previously weighed on an analytical balance of 5 decimals. Analytical procedure followed later is the same as for the Phytoplankton compartment, taking 0.3 mL of supernatant.

Zoobenthos. Heterocypris Incongrues were collected from the aquarium using a Pasteur pipette entering them into a 0.2 mL eppendorf previously weighed in an analytical balance with precision of 5 decimals. Most of water was removed by an absorbent paper and it is left for 24 h in a desiccator in order to determine the dry weight of collected ostracods. The organisms were transferred to a digestion tube and with 1 mL of nitric acid (HNO_3 69.5%) for performing a cold digestion for about 12 h, and a subsequent hot digestion for 2h, always within an exhaust hood fan. The result of the digestion was carried in a falcon tube filled up to 10 mL with Milli-Q water.

Zooplankton. To capture all of Daphnia magna organisms, the aquarium water is filtered through a 100 µm pore diameter filter. They were collected with a Pasteur pipette and passed again through the filter. They were carefully transferred to an Eppendorf of 1.5 mL, pre-weighed. The process followed was similar to that described for Zoobenthos compartment, except that it was digested with 0.5 mL and filled up to a final volume of 5 mL in the falcon tube.

- ***Analysis performed***

Microscopic characterization. NMs and micronized nanocomposite were visualized and characterized with a scanning electron microscopy (S-4800, HITACHI). Also a X ray microanalysis (EDX) was undertaken in the same microscopy (20,0 kV) to determine the sample composition of nanocomposites.

Nanomaterials content in the nanocomposites. The real NMs content in nanocomposites was determined by a thermogravimetric analysis (TGA) (Q5000IR thermogravimetric balance, TA instruments).

Analytical balance. Model CPA 225D, Sartorius.

Cell count. Initial algal cell concentration were stablish using a Jasco V-630 UV-VIS Spectrophotometer, λ 630 nm. The count of algal cells from aquaria was carried out using a haemacytometer counting chamber under the microscope at 20X magnifications. And the growth rate was calculated as

$$\mu = (\ln N_n - \ln N_0) / (t_n - t_0)$$

where t_0 is the time at the beginning of the test, and t_n the time (in days) at the end of the test. N is the number of cells/mL calculated, in t_0 (at the beginning of the experiment) and in N_n at the end.

Nanomaterials concentration on mesocosm samples. Ag and Zn concentrations were measured by inductively coupled plasma mass spectrometry (ICP-MS). In this study, the mass spectrometer ICP-MS PerkinElmer SCIEX - ELAN DRC II model was employed. Ce and Ti were analysed by inductively coupled plasma optical emission spectrometry (*ICP-OES)*, with a Perkin Elmer Optima 5300 DV ICP model.

Water physicochemical properties. Conductivity (± 0.01mS/cm), pH (± 0.01), temperature (± 0.10 ºC), dissolved oxygen concentration (± 0.01 mg/L) and the percentage of oxygen saturation (± 0.1%) were measured *in situ* by a combined pH-conductimeter model HI 98130 (Hanna Instruments) and an oximeter CellOx 340i (sensor CellOx 325).

2.3.2.2 Freshwater 60L-mesocosms (CNRS-CEREGE)

The CNRS-CEREGE implemented freshwater aquatic mesocosms (60L) to study the environmental fate of nanomaterials and released materials with access to relevant mechanistic data across several spatial and temporal scales. CNRS-CEREGE developed and implemented such set-up in the framework of previous projects. For the nanoREG project, the mesocosm platform was configured to simulate a pond ecosystem.

The pond selected is part of the protected Natura 2000 Reserve Network (Figure 9) and located in South of France (43.34361 N, 6.259663 E, and 107 m above sea level). Invertebrate benthic species (*Planorbarius corneus L.*, 1758, commonly named ramshorn snail) and planktonic species (*Eudiaptomus copepods*) were used as primary consumers and a natural inoculum were used as producer. These selected organisms are involved in a real food web and have different habitats and ecological functions in the pond ecosystems.

Figure 9: non-contaminated pond in which mesocosm inoculum was collected, and pictures of the mesocosms after introduction of the *P. corneus*.

Mesocosm were 750 x 200 x 600 mm glass tanks described by **(Auffan et al. 2014)**. Each mesocosm is made of monolithic glass panels of 12 mm-thick. Five holes (ø 15 mm) drilled at mid height of the large panels are connected to a pump using silicon tubes (Figure 10). The mesocosms were filled with 5–8 cm of artificial sediment made of 84±5% (dry weight) of quartz (grain size: ~60% from 0.05–0.2 mm, and ~40% from 0.2–2 mm), 15±5% of kaolinite, and ~1% of $CaCO_3$ (adapted from **(OECD 2006)**). Three hundred g (water saturated weight) of a natural inoculum collected in the pond was sieved at 0.2 mm and laid at the surface of the artificial sediment (1 mm-thick). This natural inoculum contained $CaCO_3$, SiO_2, and clay. The mesocosms were gently filled with 50 L of Volvic© water with pH and conductivity close to the natural pond water (pH 7, 11.5 mg/L Ca^{2+}, 13.5 mg/L Cl^{2}-, 71 mg/L HCO_3^{2-}, 8 mg/L Mg^{2+}, 6.3 mg/L NO_3^{2-}, 6.2 mg/L K^{+}, 11.6 mg/L Na^{+}).

Temperature, pH, conductivity, redox potential, and dissolved O_2, were measured every 5 min at mid height of the water column using multi-parameter probes (Odeon® Open X) and at the water/sediment interface (up to 10 mm below surficial sediment) and mid height of the sediment using platinum-tipped redox probes. A

day/night cycle of 10 h/14 h was applied using full spectrum light (Viva® light T8 tubes 18 W), and room temperature was kept constant **(Auffan et al. 2014)**.

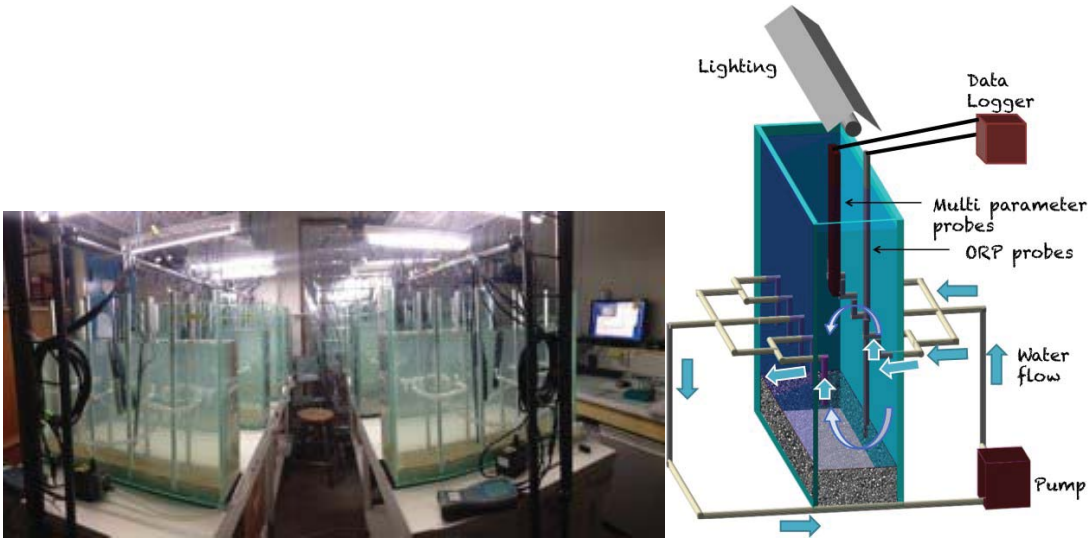


Figure 10: Picture of the freshwater mesocosm platform and schematic of the mesocosm setup.

Mesocosms experiment is divided in two phases.

- During phase I, the particles suspended by the addition of water are given time to settle, the pH, conductivity, O_2, and redox potential stabilize, and the primary producers develop. The duration of this phase depends on the target values (and variations around them) defined for each key parameter (*e.g.* ΔpH, ΔT, turbidity, ammonia). Mesocosms were run one week for stabilization. At the end of phase I, 11 mollusks (*P. corneus* (L. 1758), benthic grazers) and 200 copepods (*E. vulgaris* (Schmeil 1896), planktonic filter feeders) were introduced and the water pumps are turned on. The density of organisms is adjusted as a function of the natural environment. The duration of the acclimation depends on biological features of the species as growth rate, metabolism activity, life cycle duration. Mesocosms were run one week for organism acclimation.

- Phase II is dedicated to NMs exposure period. Two scenarios of contamination were tested with such a design : (I) a continuous point-source discharge of cerium oxide nanoparticles bare and citrate-coated and (II) a continuous point-source discharge of immersed fragmented cement (experiencing no pH gradient in its vicinity).

A multiple dosing experiment (3 times per week) was selected on a 4 weeks period (Figure 11).

- CeO_2 NMs experiment: Starting at day 0, the water columns were dosed 3 times per week (on Monday, Wednesday, and Friday) for 4 weeks with 5.2 mg of CeO_2 NMs until day 28, resulting in a final concentration of 1.1 $mg.L^{-1}$ CeO_2 NMs and a total CeO_2 mass of 52.7 mg in the mesocosms. Three mesocosms were dosed with bare CeO_2 NMs, three were dosed with citrate-coated CeO_2 NMs, and three were kept as controls.

- TiO_2 NMs experiment: Starting at day 0, the water columns were dosed 3 times per week (on Monday, Wednesday, and Friday) for 4 weeks with 4.5 mg of TiO_2 NMs/mesocosms/day until day 28 (0.09 mg TiO_2/L/mesocosms/day), resulting in a final concentration of 1.1 $mg.L^{-1}$ TiO_2 NMs and a total TiO_2 mass of 54 mg in the mesocosms.

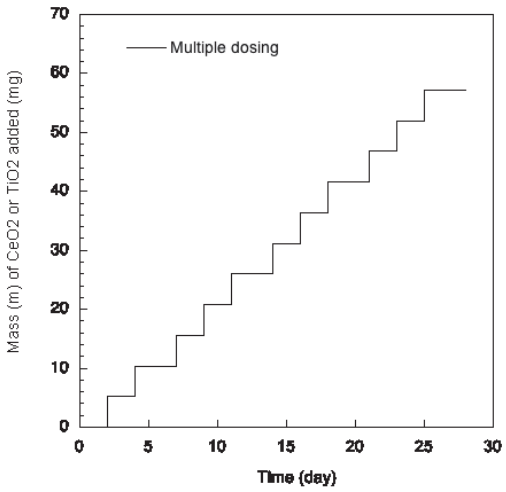


Figure 11: Experimental schedule for the 12 injections of during Phase II.

2.3.2.3 Marine 60L-mesocosms (UCO)

The same experimental design was implemented at UCO (Figure 12) to simulate estuarine (15 practical salinity unit PSU) and marine (30 PSU) ecosystems. Compared to freshwater mesocosms, a pump connected to a mechanical timer (IDK PMTF 16A) was added to mimic the tidal cycle (6h of low tide, 6h of high tide, 2 tides/day). The selected organisms for these "marine" experiments were the marine green algae (*Tetraselmis suecica)* as primary producer and the endobenthic bivalve (*Scrobicularia plana*) as a primary consumer (Figure 12). These organisms were collected from a relatively clean intertidal mudflat (Bay of Bourgneuf, (47801050.3500N, 1859004.8000W) France; French "Mussel Watch" Program, http://www.ifremer.fr/ envlit/documents/bulletins/rno) (Figure 13).

Marine mesocosms were gently filled with 60 L of artificial seawater (Tropical Marine®: Tropicarium Buchshlag Dreieich Germany) adjusted to the selected salinity (15 PSU, 30 PSU). After two days the physical-chemical parameters were stabilized (turbidity, pH, dissolved O_2). Then *S. plana* were added to each estuarine and marine mesocosms, respectively, as shown in Figure 12. Mesocosms were run one week for organism acclimation. Bivalves were fed with algae (*Tetraselmis suecica)* at 10^5 cells/mL concentration at the beginning of the experiment and then once a week.

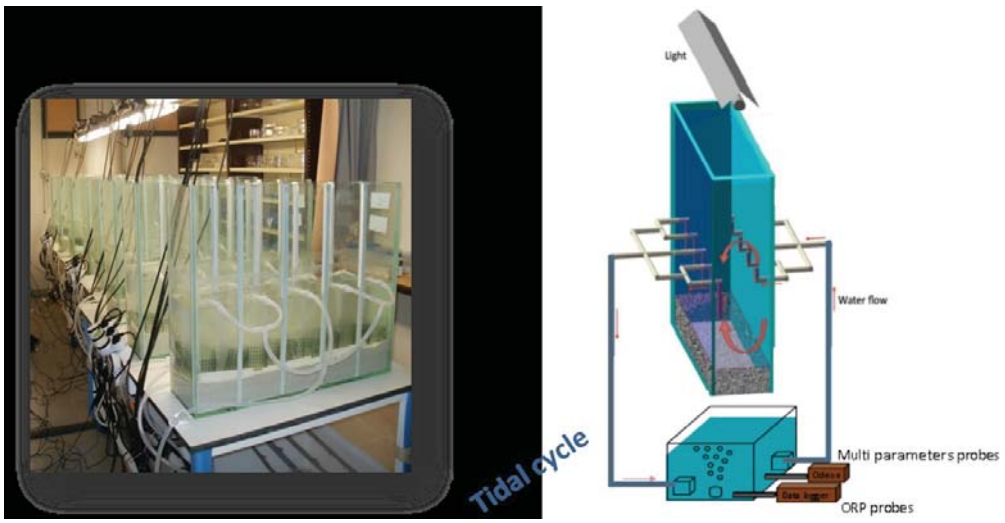


Figure 12. Picture and schematic of the marine mesocosm setup.

Figure 13 Relatively clean intertidal mudflat: the bay of Bourgneuf (France) in which organisms were collected at low tide.

Temperature, pH, conductivity, redox potential, and dissolved O_2, were measured every 5 min at mid height of the water column using multi-parameter probes (Odeon® Open X) and at the water/sediment interface (up to 10 mm below surficial sediment) and mid height of the sediment using platinum-tipped redox probes. A day/night cycle of 10 h/14 h was applied using full spectrum light (Viva® light T8 tubes 18 W), and room temperature was kept constant **(Auffan et al. 2014)**. In the case of estuarine and marine mesocosms, a pump connected to a mechanical timer (IDK PMTF 16A) allows to mimic the tidal cycle (6h of low tide, 6h of high tide, 2 tides/day).

- CeO_2 NMs experiment: The aim of this study was to estimate the fate and behaviour of Ce NMs included in a commercial fuel additive (Envirox™) at two stages of its life cycle (before and after combustion at 850°C) and of NM-212, a standardized CeO_2 NMs provided from the JRC (Joint Research Centre), in water under a salinity gradient: 15 PSU and 30 PSU. *S. plana,* were introduced (n = 12) in each mesocosm containing artificial water and standardized/artificial sediment. Four conditions were tested: a) control (without contaminants), b) NM-212, c) Envirox™, lyophilized fuel additive, and d) Envirox™ after combustion at 850°C. For all conditions and during the whole duration of the experiment, *S. plana* were fed with *Tetraselmis*. Organisms were exposed for 28 days in oxygenated water, under controlled light (16 h/8 h) and temperature (15°C). The contaminants were added every 3 days (90 µg Ce /L) during the whole duration of experiment. This concentration of exposure corresponds to 100 times predictive environmental concentration (PEC) values allowing their measure by available technics. Total Ce in the water column, labile forms of Ce accumulated in Diffusive Gradient Thin film (DGT) tools and Ce concentrations in the digestive glands of bivalves were estimated by ICP-MS. After 7, 14, 21 and 28 days of exposure, individuals were submitted to burrowing tests.

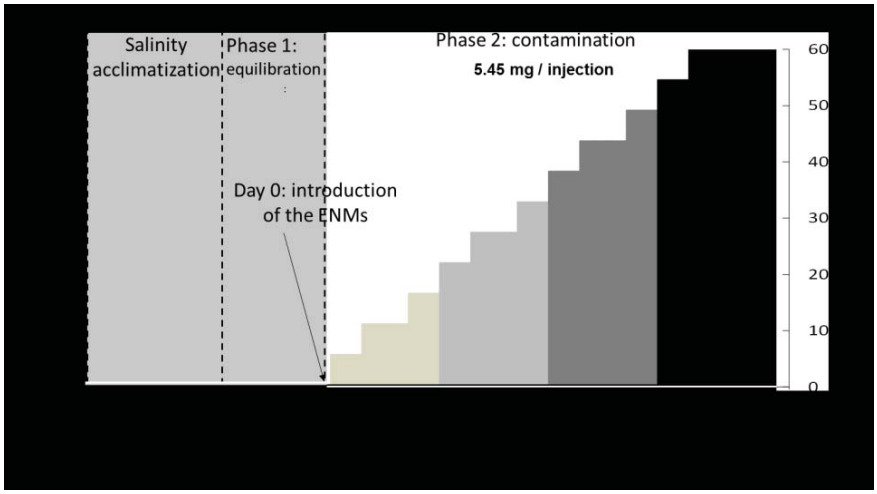


Figure 14. Experimental schedule for the 12 injections of CeO_2 based NMs.

- TiO_2 NMs experiment: The experiment was conducted in nine marine mesocosms (75x20x60 cm) containing 10 cm depth artificial sediment (89% sand / 10% kaolin / 1% $CaCO_3$) and filled with artificial seawater (30 psu). A layer of natural sediment collected on the sampling site was spread onto the artificial sediment surface (0.5 cm in depth) and was used as a food source for animals. Aquatic systems were run in triplicate for each experimental condition (control, leachates of cements containing TiO_2 NMs and TiO_2 NMs). Fifty-five organisms per mesocosm were introduced sequentially and TiO_2 NMs or leachates of cement containing TiO_2 were added regularly in 12 injections (3 injections per week during 4 weeks; 0.09 mg/L per injection) to reach a final nominal concentration of 1.1mg/L TiO_2 per mesocosm. Three groups of animals exposed in mesocosms without any contaminants were used as controls. Organisms were exposed to the 3 conditions described above in oxygenated seawater (30 psu) in a temperature-controlled room (17°C), under controlled light (16h/8h) and at a tidal cycle (6h of low tide, 6h of high tide; two tides /day). Salinity, temperature, conductivity, red-ox potential and oxygen levels were monitored continuously with probes and a Redox sensor and a Data logger. Samples (water, sediment, biota) were collected after 7, 14, 21 or 28 days for subsequent analysis (TiO_2 quantification in the water column, sediment and bivalves using ICP-MS).

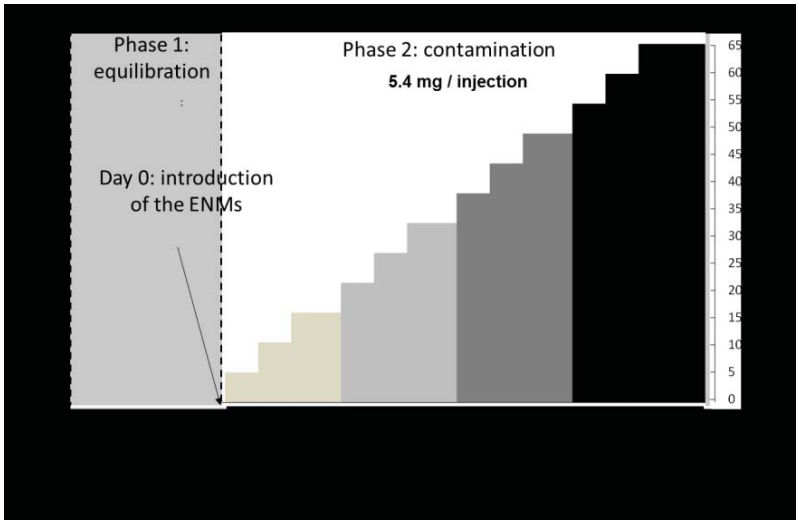


Figure 15. Experimental schedule for the 12 injections of TiO_2 based NMs.

2.3.2.4 Characterization techniques used in the 60L-mesocosms (CNRS-CEREGE, UCO)

Several physico-chemical, microbial, and biological analyses can be performed to assess both the exposure and impacts of ENMs on such a designated trophic link. A number of parameters can be monitored continuously with the appropriate probe (*e.g.* pH, temperature, Eh). Other parameters (*e.g.* metal concentration, number of colloids, picoplankton and algae concentrations) require sampling. Water, superficial sediments, cores of sediment, picoplankton, algae, and macro-invertebrates can be sampled with any desired periodicity. During sampling, special attention must be given (i) to avoid disturbing the sediment and water column properties, and (ii) to keep organism densities and NMs concentrations constant.

Chemical analysis. The distribution of the NMs or their degradation by-products is assessed by measuring their concentration in the water, sediment, biota, *etc.* using conventional analytical methods (e.g. ICP-MS or ICP-AES).

Particle Size distribution. Dynamic light scattering (DLS) using the NanoZS (Malvern, UK) was used for the sub-micrometric fractions. For particles with size larger than few microns, laser diffraction particle size analyser was used (Mastersizer 3000 Malvern, UK).

Particle counting. The number of colloidal particles suspended in mesocosms water column was monitored at 10 cm below the water surface using an optical particle counter (HIAC 8011+).

Speciation and biodistribution. A thorough characterization of the speciation, (bio)transformation, bio(distribution) of the ENMs in the water, sediment, or biota will be performed using X-ray, IR, Raman spectroscopies, Nuclear magnetic resonance, as well as electron- or X ray-based microscopy and tomography.

Dissolution. To estimate the dissolution of metal-based NMs in the experimental medium the use of DGT (Diffusive Gradients in Thin films) tools is suitable (Davison and Zhang, 1994). This technique, based on mass transport control of the chemical species of interest from water or sediment pores water, uses two hydrogel layers. A polyacrylamide gel is used as the diffusive layer, and is backed up with a second thin gel layer containing a Chelex cation-exchange resin selective for trace metals. The diffusive layer of known thickness is placed in the DGT probe on top of the binding phase and covered with a filter used to avoid biofouling. Ions diffuse through the filter and diffusive layer to reach the Chelex resin. The mass of the diffused ion, M, can be obtained by direct measurement of the ion concentration (Cr) in the resin layer with total volume of resin Vr : M = CrVr. The DGT disc units (2.5 cm diameter corresponding to a 3.14 cm2 diffusive area) are purchased from DGT Research Ltd. A Chelex-100 resin beads and a diffusive gel with a pore size of about 5 nm are used (open pore diffusive gel) (Zhang and Davison, 1999). The thickness of gel is 0.82 mm. A filter of 0.14 mm thickness and 0.45 µm pores size covers the gel. These DGT units were used in the water compartment of marine aquatic mesocosms.

When necessary, the dissolution of NMs can be also measured by placing sealed dialysis bags (3 or 10 KDa) in the mesocosms.

2.4 Results & main achievement

2.4.1 Freshwater 3L-mesocosms contaminated with pristine Ag and ZnO NMs (ITENE)

In that first mesocosm experiment, Ag and ZnO nanomaterials were tested, together with the control treatment (without added nanomaterial), thus, a total of 5 treatments. Selected concentrations were based on the no observed adverse effect level (NOAEL) based on previous scientific literature review and undertaken ecotoxicity tests:

- ✓ Silver (Ag): 0.16 mg/L and 0.08 mg/L
- ✓ Zinc oxide (ZnO): 0.33 mg/L and 1 mg/L

The experiments were run for 16 days (Figure 16).

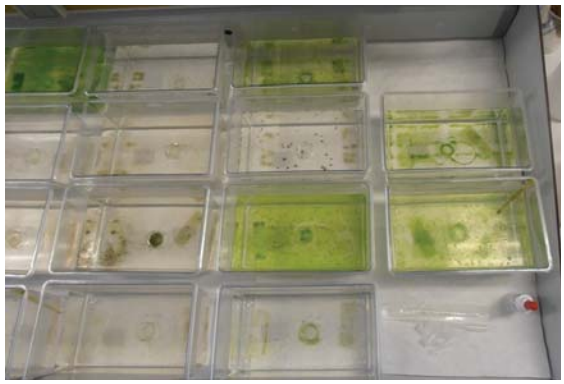


Figure 16: Aquaria after 16 days of exposure.

- ***Physical and chemical variables***

After adding the nanoparticles in the mesocosms, no significant differences in the physical and chemical variables were observed ($p > 0.05$) between the control and the treatments with NMs, therefore they did not affect these water parameters. After one week of exposure, the dissolved oxygen concentration and saturated percentage of oxygen mean values increased for all the treatments except for Zn1. Additionally, temperature increased and conductivity decreased in comparison to the values measured in the previous week. 16 days after exposure to the nanoparticles a decrease in all the parameters was observed. Changes in the oxygen concentration are usually related to the primary producers activity, therefore, this agrees with an increase on algae population the first week of the experiment for almost all the treatments and a decrease of this population after 16 days (Table 4).

Table 4. Mean and standard deviation of the physical and chemical variables for each treatment at three different times: the day of starting the experiment, a middle time and the last day of the experiment.

First day					
Treatment	**Conductivity (mS/cm^2)**	**pH**	**Temperature (°C)**	**Oxygen (%)**	**Oxygen (mg/L)**
Control	0.66±0	8.33±0.02	16.42±0.61	107.52±4.29	10.52±0.56
Ag1	0.66±0	8.32±0.02	16.65±0.42	105.7±2.95	10.29±0.37
Ag2	0.66±0	8.33±0.03	16.40±0.31	107.15±2.71	10.48±0.31
Zn1	0.66±0	8.30±0.01	16.91±0.34	103.62±2.29	10.03±0.30
Zn2	0.66±0	8.31±0.01	16.77±0.24	105.25±1.45	10.25±0.19
7 days after exposure					
Treatment	**Conductivity (mS/cm^2)**	**pH**	**Temperature (°C)**	**Oxygen (%)**	**Oxygen (mg/L)**
Control	0.61±0.01	8.72±0.52	20.87±0.45	136.70±49.90	12.10±4.54
Ag1	0.63±0.01	8.55±0.33	21.00±0.08	116.55±26.29	10.20±2.38
Ag2	0.62±0.01	8.90±0.35	20.95±0.06	126.72±33.21	11.03±2.90
Zn1	0.63±0.01	8.35±0.11	20.98±0.05	101.67±8.69	8.86±0.77
Zn2	0.62±0.01	8.63±0.14	20.92±0.05	118.44±15.62	10.85±1.37
16 days after exposure					
Treatment	**Conductivity (mS/cm^2)**	**pH**	**Temperature (°C)**	**Oxygen (%)**	**Oxygen (mg/L)**
Control	0.66 ± 0.11	8.32 ± 0.20	18.05 ± 0.26	129.10± 47.42	9.82±1.20
Ag1	0.66 ± 0.02	8.29 ± 0.07	16.97 ± 0.32	100.30 ± 4.28	9.59±0.53
Ag2	0.66 ± 0.01	8.27 ± 0.7	16.80 ± 0.47	98.57 ± 5.41	9.45±0.44
Zn1	0.67 ± 0.01	8.35 ± 0.2	16.62 ± 0.32	100.75 ± 2.75	9.68±0.23
Zn2	0.66 ± 0.02	8.53 ± 0.37	16.42 ± 0.27	112.07 ± 16.64	10.07±0.20

- ***Population changes in the mesocosms***

Planktonic primary producer, the green algae, was initially at a concentration of $2x10^5$ cells/mL. After one week of exposure to the nanoparticles, the concentration of green algae in each aquarium was estimated, as well as the population growth rate (μ) (Table 5). Growth was affected by the feeding of *D. magna* but there were not significant differences between treatments ($p > 0.05$).

Table 5. Mean of growth rate (μ) for each treatment.

Treatment	μ
Control	0,18
Ag1	0,01
Ag2	0,04
Zn1	-0,04
Zn2	0,10

Additionally, we did not find a clear relationship between the number of *D. magna* and the depletion of algae population (Figure 17). At the end of the experiment, the chlorophyll *a* for each aquarium was measured. The values ranged from 0.87 µg/L in one of the replicates of Zn2, to 114.4 µg/L in one of the controls and there were not significant differences between treatments (p >0.05).

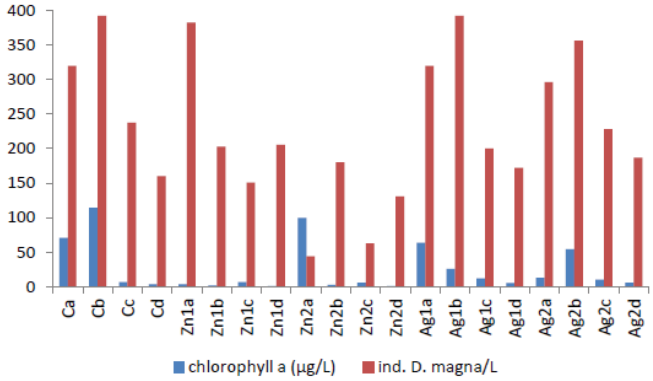


Figure 17: Chlorophyll a concentration and individuals of D.magna/L estimated in each aquaria at the end of the experiment, after 16 days of exposure.

- ***Planktonic consumer, the cladoceran D. magna***

Throughout the study period, a rapid growth of the population of *D. magna* in all the treatments was observed (much higher growing that the ostracods). At the end of the experiment, the total number of organisms found in each aquarium presented great variations, ranging from 172 (in one treatment with the high concentration of ZnO NP) to 1180 individuals (in one control) (Figure 18). Although there were no significant differences (Kruskal Wallis, $p < 0.05$) among the values for the different treatments and controls, there is a reduction in the averaged number of individuals in the Zn treatments, which was more evident for the higher Zn (Zn2) concentration. The population growth rate (μ) was also calculated: there was a great variability within treatments and thus, not significant differences were found among them.

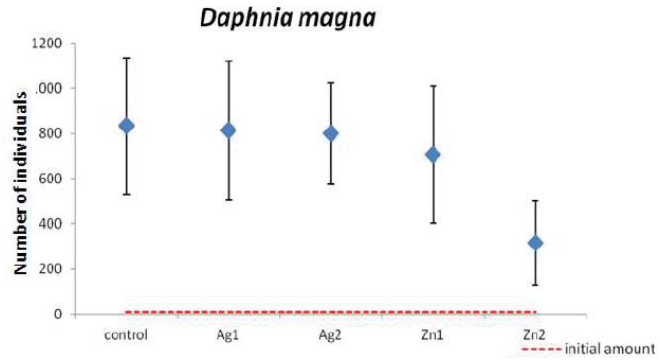


Figure 18: Mean and standard deviation of D. magna for each treatment, initial amount was ten individuals

- ***Benthic primary producers, periphyton (Pennated diatoms)***

A growth all over the bottom and walls of the aquaria was observed, as can be seen in Figure 19.

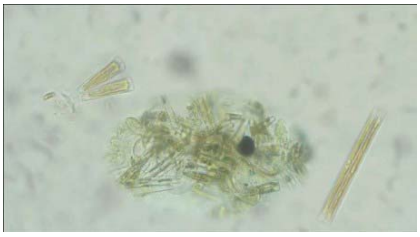


Figure 19: Growth of periphyton in the aquaria walls.

- ***Benthic consumer, ostracod H. incongruens***

A high variability in the total abundance was observed. Any living adult of *H. incongruens* in the treatments with the highest concentration of ZnO NP (Zn2), and the decrease of the population in all the replicates of Zn1 and in two of the replicates of the control treatment. In general terms the ostracod did not show a great growing in the mesocoms (Figure 20).

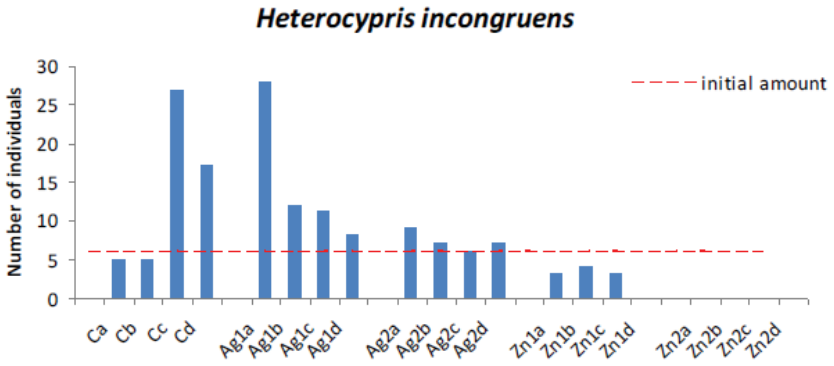


Figure 20: Growth of ostracods.

- ***Amount of Ag found in the different compartments of the mesocosms (planktonic producers, planktonic consumers, benthic producers and benthic consumers and in the water)***

At both treatments, the highest proportion of Ag was found in the periphyton, and the lowest in the ostracods. The proportion of the total amount of Ag recovered compared to the concentration introduced in the aquaria was similar to that found for the treatment with the low concentration of Ag (aprox. 12%) (Table 6).

Table 6: Mean concentrations and standard deviation of Ag estimated in each compartment, and balance of the total Ag recovered and percentages for treatment Ag1 (left) and Ag2 (right).

Treatment Ag 1	µg	%
Ag in *S. acutus*	1.6±0.4	0.68±0.2
Ag in *D. magna*	3.4±0.7	1.38±0.3
Ag in *H. incongruens*	0.8±0.3	0.33±0.1
Ag in periphyton	9.1±2.	3.64±0.8
Ag in water	16.4±2.2	6.57±0.9
Ag recovered	31.4	12.6

Treatment Ag 2	µg	%
Ag in S. acutus	3.9±1.1	0.79±0.2
Ag in D. magna	11.4±2.1	2.28±0.4
Ag in H. incongruens	1.8±0.4	0.36±0.1
Ag in periphyton	17.6±4.5	3.52±0.9
Ag in water	29.1±5.0	5.82±1.1
Ag recovered	63.8	12.8

- ***Amount of ZnO found in the different compartments of the mesocosms (planktonic producers, planktonic consumers, benthic producers and benthic consumers and in the water)***

In both treatments, the highest proportion of Zn was found in the water, followed by the periphyton, and a low percentage of the Zn was found in *D. magna* organisms, as can be observed in Table 7.

Table 7: Mean concentrations and standard deviation of Ag estimated in each compartment, and balance of the total Ag recovered and percentages for treatment ZnO1 (left) and ZnO2 (right).

Treatment Zn1	µg	%
Zn in *S. acutus*	n.d	n.d
Zn in *D. magna*	7.7±4.4	0.9±0.5
Zn in *H. incongruens*	2.0	0.2
Zn in periphyton	118.5±21.1	14.8±2.6
Zn in water	194.2±9.7	24.3±1.2
Zn recovered	**322.4**	**40.2**

Treatment Zn2	µg	%
Zn in *S. acutus*	n.d	n.d
Zn in *D. magna*	59.1±8.5	3.7±0.5
Zn in *H. incongruens*	n.d	n.d
Zn in periphyton	431.9±111.3	26.8±6.9
Zn in water	464.3±73.0	28.8±4.5
Zn recovered	**955.3**	**59.3**

- ***Discussion and conclusions***

In all treatments there is not known the environmental fate of 30-50% from the initial amount of nanoparticle introduced, which are supposed to be in the sediments. An important element of many natural freshwaters, the sediment, was not included in the system. To get a closer approach to the realistic environment we suggest the introduction of this element in future studies, because the material present in the sediment can react with nanoparticles and change bioavailability to organisms. Information about how sediment influences the behavior and fate of NM is an important point.

After 16 days of exposure, all the studied organisms have accumulated these nanomaterials, and we have proved that bioaccumulation depends on dosing in almost all the organisms, since in treatments Ag1 and Zn1 lower concentrations of the nanoparticles were found compared with the treatments with higher concentration of nanoparticles (Ag2 and Zn2).

The measurement by ICP-MS of Ag and ZnO NMs concentration in the organisms has been a challenge due to the lack of detailed protocols. Regarding to Zinc measurements, in order to be able to distinguish between Zinc associated with NMs and naturally present metal, the use of stable isotope labelled Zinc nanoparticles can be suggested. The small size and the not well known physical and chemical properties in the aquatic medium of the NMs make difficult the analysis, and generate the need of technical developments in instrumentation in order to reach lower detection limits and more reliable data.

Therefore, after 16 days of exposure, it has been demonstrated that both nanoparticles are accumulated in all the types of organisms: planktonic and benthic, consumers and producers. The highest concentration was measured in periphyton in all the treated mesocosms, and the lowest in green algae in treatments with Ag NM. Although there is not known the toxicological effects of these nanoparticles in lower concentrations in the studied organisms, the presence of the contaminant in primary and secondary producers can have long-term effects on health, reproduction and populations not only for these organisms but also organisms in higher steps of the food web.

Through this study, some preliminary results and conclusions have been obtained, but further studies are required in order to earn more knowledge regarding the environmental effects on freshwater ecosystems of these nanoparticles.

2.4.2 Freshwater 3L-mesocosms contaminated with weathered PP and PET films (ITENE)

In that second experiment of mesocosms, Ag, PP, PET, PPAg, PETAg and PETZnO were tested and compared with the controls without NMs (control treatment) after two weeks of exposure. Three replicates of each sample were studied.

- ***Environmental parameters***

Determinations of physicochemical parameters measured in the aquaria were uniform during the tests. Oxygen concentrations were 124.43±4.29 % and 11.26±0.41 mg/L, temperatures 20.63±0.19 ºC, pH 8.36±0.07 and electric conductivities of 0.51±0.06 mS/cm.

- ***Amount of Zn and Ag found in the different compartments of the mesocosms***

Figure 21 and Figure 22 recompile the amount of Zn and Ag found in the different compartments of the mesocosms: planktonic producers, planktonic consumers, benthic producers and benthic consumers, water and sediments.

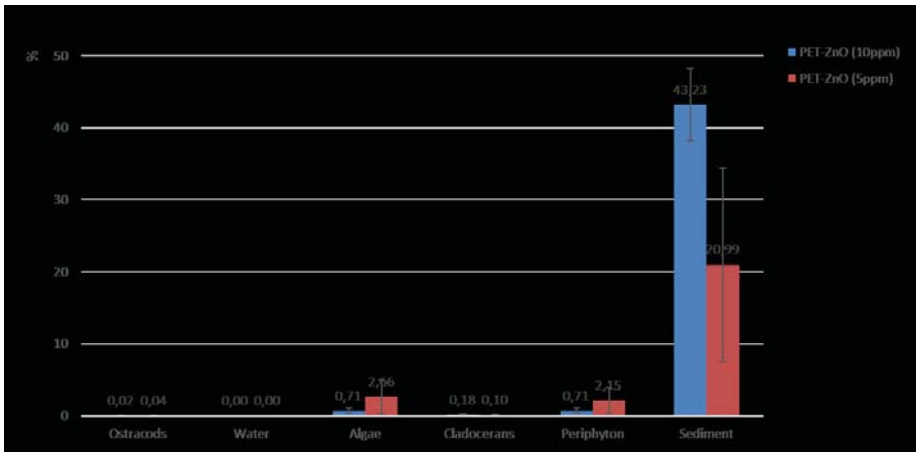


Figure 21: Results for PET ZnO nanocomposites at 1 and 5 mg/L in the mesocosms.

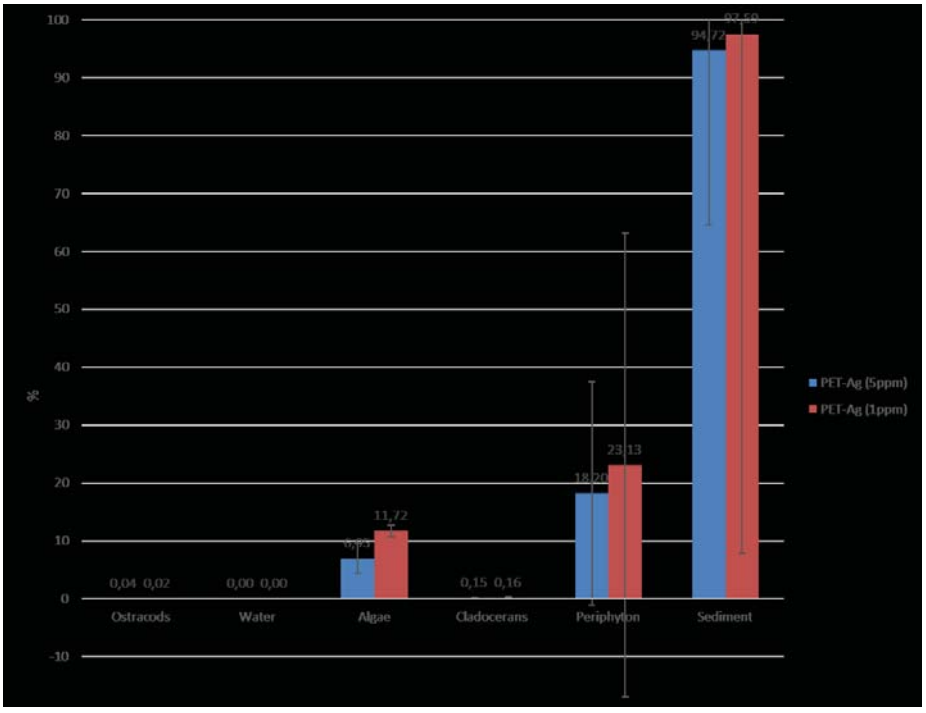


Figure 22: Results for PET Ag nanocomposites at 1 and 5 mg/L in the mesocosms.

Figure 23 and Figure 24 show the percentage of silver and zinc, respectively, contained in the different compartments for each treatment microcosm experiment microcosm. In the case of zinc, major part was quantified in the sediment compartment.

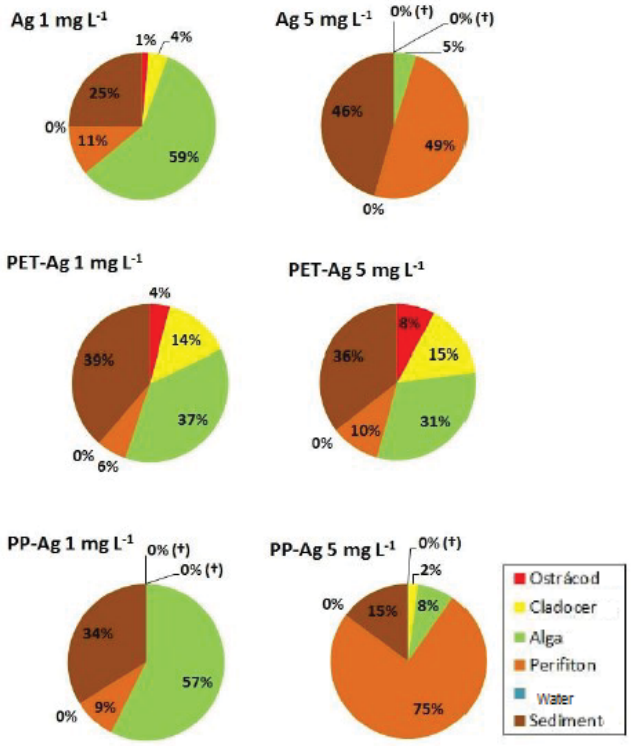


Figure 23: Percentage of Ag in the different compartments for each treatment of the experiment of microcosms († = the day of sampling no living organisms to collect were found).

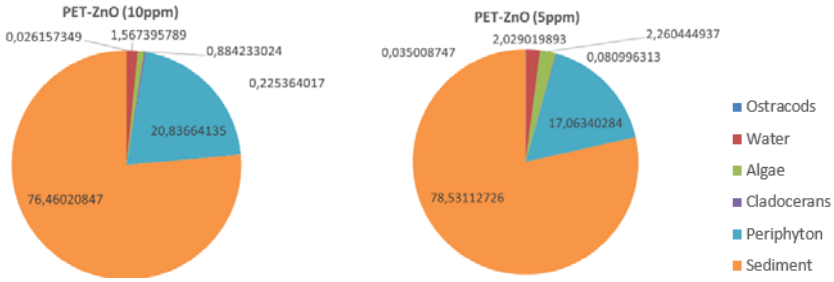


Figure 24: Percentage of Zn contained in the different compartments for each treatment of the experiment of microcosms.

Recovery of total Zn from the samples was not complete. That can be explained as PET-ZnO was less degraded during the weathering process, so part of the ZnO would have remained into the polymeric matrix and thus not 100% ZnO was disposable to be taken by organisms. In addition, part of the nanocomposite remained adhered to the aquarium walls.

In the case of PET Ag nanocoposites, also part of the nanocomposite remained adhered to the aquarium walls. Nevertheless, PET-Ag was more degraded during the weathering process, so a greater percentage of Ag was liberated to the medium. That explain why almost 100% of Ag was quantified among the different studied compartments.

- ***Discussion and conclusions***

After two weeks of exposure, all studied organisms incorporated Ag and ZnO nanoparticles released from nanocomposites. The density of nanocomposites and polymers affects to their biodisponibility and makes they affect in a different way to organisms.

Major part of initial silver content in each aquarium was recovered but in the case of Zn, a high amount of initial Zn concentration was not quantified in any of studied compartments. That can be explained as PET-ZnO was less degraded during the weathering process, so part of the ZnO would have remained into the polymeric matrix and thus not 100% ZnO was disposable to be taken by organisms. In addition, part of the nanocomposite remained adhered to the aquarium walls.

Nanocomposites with Ag supposed a greater threat because they were more degraded during the weathering process and a major % of NP was liberated.

The accumulation of ZnO in the sediment depended on dosing. The sediment was the most affected compartment, followed by periphyton and algae. That showed that this type of pollution would be especially dangerous for the organisms living in the depths. In addition, as periphyton and algae are primary consumers, the whole food chain could be affected by this problem in the long term.

Through this study, some preliminary results and conclusions have been obtained, but further studies are required in order to earn more knowledge regarding the environmental effects on freshwater ecosystems of these nanoparticles.

2.4.3 Freshwater 3L-mesocosms contaminated with pristine CeO_2 and TiO_2 NMs (ITENE)

Last mesocosm experiments were run for 21 days. In that case, CeO_2 and TiO_2 nanoparticles where assayed, at tested concentrations of 5 mg CeO_2/L, 10 mg CeO_2/L, and 1 mg TiO_2/L and 0.5 mg TiO_2/L, compared with a control without nanoparticle content. Four replicates of each sample were assayed. During the tests all mesocosms were under the same conditions or operating parameters. No significant incidents were observed presenting all mesocosms stable and living populations.

After 21 days of exposure, concentration of CeO_2 NPs and TiO_2 NPs was determined in the sediment compartments, periphyton, zoobenthos, water, algae and zooplankton. Figure 25 and Figure 26 represent the mean concentration for each of the tested concentrations of 5mg CeO_2/L, 10mg CeO_2/L, 0.5mg TiO_2/L, and 1mg TiO_2/L respectively.

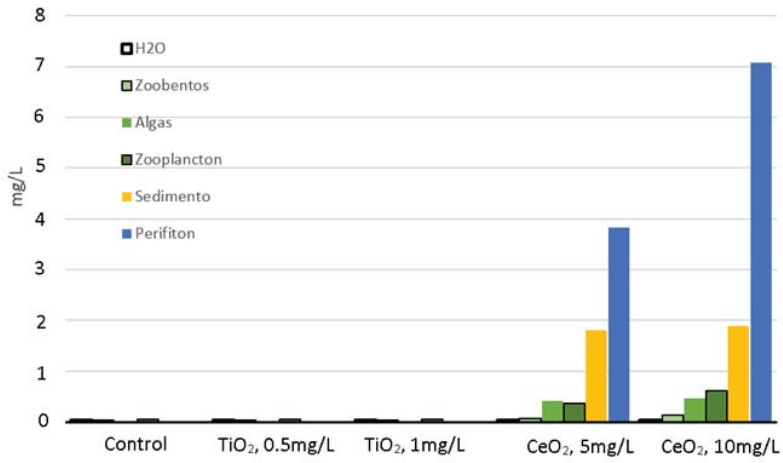


Figure 25: Concentration of TiO_2 NPs in different compartments of the mesocosms.

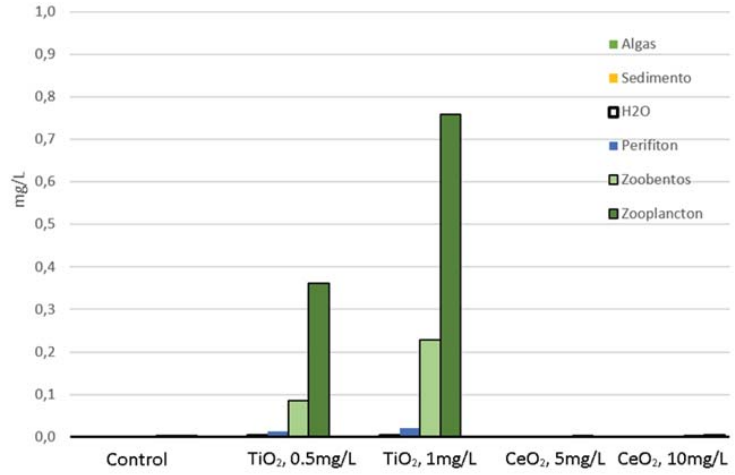


Figure 26: Concentration of CeO_2 NPs in different compartments of the mesocosms.

- ***Discussion and conclusions***

The highest concentrations of CeO_2 NPs are determined in periphyton and sediment while the values obtained in the water column are about two orders of magnitude lower. These results are consistent with those previously obtained by **Miller et al. 2010** and **Navarro et al. 2008**. The CeO_2 aggregated quickly after its introduction in the system. CeO_2 can then accumulate in the environment in soil and sediment, thus providing a way to be incorporated by plants and animals. In the long term, these deposits can lead to increased exposure of CeO_2 NPs (**Gottschalk et al. 2009**).

For TiO_2 NPs, the compartments having a higher concentration are zooplankton and zoobenthos. Although they also have a low solubility in water, TiO_2 NPs have higher capacity of resuspension when the system is agitated. This is because the TiO_2 NPs have not been incorporated or retained by the periphyton, increasing exposure and bioavailability in the water column. These results agree with the data obtained by Cañedo where the zooplankton and zoobenthos, as top level representatives of the trophic chain of artificial mesocosms, had a greater amount of NMs due to bioaccumulation (**Canedo et al. 2012, Brooks et al. 2004**).

It is important to mention that, at the end of the test, most of the introduced NMs have been recovered during the analytical procedure, indicating that the mesocosms has behaved as a closed material flow system.

In conclusion, if we compare both NMs, results show that have a very different behavior. While CeO_2 NMs have an effect on the benthos, the TiO_2 NMs produce it in the pelagic zone. This information is very useful when predictions about release into the environment and their accumulation are made, and also when control measurements or actions to recover affected area want to be carried out (**Mueller et al. 2008, 2013**).

2.4.4 Freshwater 60L-mesocosms contaminated with pristine and formulated CeO_2 NMs (CNRS-CEREGE)

Starting at day 0, the water columns were dosed 3 times per week (on Monday, Wednesday, and Friday) for 4 weeks with 5.2 mg of CeO_2 NMs until day 28, resulting in a final concentration of 1.1 mg/L CeO_2 NMs and a total CeO_2 mass of 52.7 mg in the mesocosms. Three mesocosms were dosed with bare CeO_2 NMs, three were dosed with citrate-coated CeO_2 NMs, and three were kept as controls.

2.4.4.1 Evolution of the physico-chemical parameters

Details on the parameters monitored during the experiment for 7 weeks are provided in Figure 27. Briefly, temperature (25.3 ± 0.6 °C), dissolved O_2 (7.4 ± 0.5 mg L^{-1}), pH (7.9 ± 0.1), and total organic carbon (2.0 ± 0.1 mg L^{-1}) were constant over time. ORP probes indicated that the water column was oxidative (241 ± 10 mV), while reductive conditions prevailed in sediments (−267 ± 15 mV). Conductivity increased during the experiment from 293 ± 8 µS cm^{-1} to 318 ± 15 µS cm^{-1} due to weekly refills with Volvic® water to compensate for evaporation. The concentrations of phosphates and carbonates were 3.8 × 10^{-6} mol L^{-1} and 0.2 × 10^{-3} mol

L^{-1}, respectively, in the control mesocosms after 4 weeks. The primary producers were counted weekly in both the water column and sediments. The concentrations of picoplankton and algae were between 10^4–10^5 cells mL^{-1} and $<10^3$ cells mL^{-1} in the water column and between 10^6–10^7 cells mL^{-1} and 10^5–10^6 cells mL^{-1} at the surface of the sediments, respectively. The concentration of particles with sizes ranging from 0.5 to 2.5 µm in the water column was 10^6 particles mL^{-1}, as measured using an optical counter (HIAC).

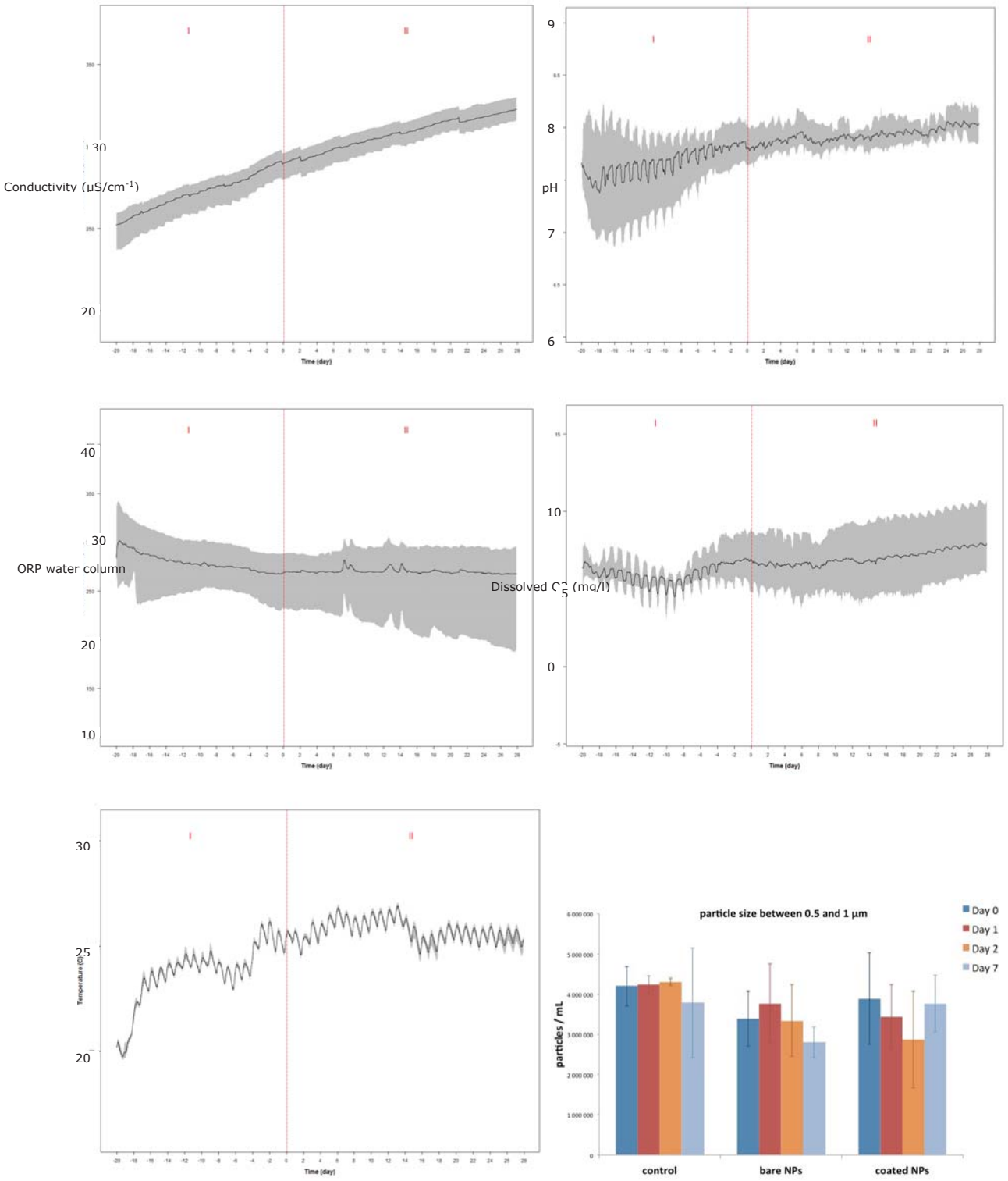


Figure 27: Evolution of the physico-chemical parameters in the water column of the mesocosms. Redox potential, dissolved oxygen, pH, and conductivity were measured during phases I (stabilization) and II (contamination). Day 0 corresponds to the first dosing of NPs. The grey surface is defined by the maximum and minimum values of each parameter, and the dark line corresponds to the average values of the 9 mesocosms. One measurement was performed every 5 min. (bottom right) Number of suspended materials (size ranged between 0.5 and 1 µm) in the mesocosm water column as a function of the NPs injected (bare versus coated) during the first 7 days.

2.4.4.2 Ce distribution between the water column, the sediment and the organisms

- ***Distribution between the water column and the surficial sediments***

Surface and deep waters were sampled 1.5 h, 3 h, 6 h, 24 h, and 48 h after the first injection of 5.2 mg of CeO_2 (*i.e.* a Ce concentration of 100 µg L^{-1}) in each mesocosm.

For bare CeO_2, a concentration gradient between the top (9.9 ± 0.9 µg L^{-1} at ~10 cm from the air/water interface) and the bottom of the water column (3 ± 1 µg L^{-1} at ~5 cm from the water/sediment interface) was observed after 1.5 h, after which the Ce concentration between the top and the bottom of the water column did not significantly evolve. 93 ± 3% of the Ce initially introduced at the top of the water column settled out of the water column after 48 h. For the coated CeO_2, a homogeneous Ce distribution (71 ± 12 µg L^{-1}) between the top and the bottom of the water column was reached after 6 hours. Only 29 ± 12% of the Ce initially introduced had settled out after 48 h. These results highlight that waiting for 2 to 3 days between each dosing of NPs was enough to reach a steady state in terms of distribution in the water column. After 4 weeks, the total Ce concentration in the water column remained stable at 5 ± 2 µg L^{-1} and 49 ± 7 µg L^{-1} for bare and citrate-coated CeO_2 NMs, respectively.

While the Ce concentration in the water column was ten times higher for the coated NMs than that for the bare NMs, similar Ce concentrations were measured in the surficial sediment for both types of CeO_2 NMs (Figure 28). The concentration in the sediment reached 99 ± 42 mg kg^{-1} after 4 weeks with a sedimentation rate of 1.0 ± 0.1 mg per day, which corresponds to ~99% (bare NPs) and ~75% (coated NPs) settling of Ce injected after 28 days. These percentages are in agreement with previous studies simulated using a single-pulse input of CeO_2 or Ag NMs in aquatic mesocosms **(Lowry et al. 2012, Zhang et al. 2012, Tella et al. 2014)**.

Using batch experiments, we highlighted that two aggregation mechanisms co-occurred in the water column of the mesocosms. For bare CeO_2 NMs, homo-aggregation appeared to be the most favorable mechanism; these particles settled out very quickly due to the relative number of particles in suspension and particle surface charges. For coated CeO_2 NMs, both homo-aggregation and hetero-aggregation were favorable, but a few days under artificial daylight were required to degrade the citrate coating in the water column.

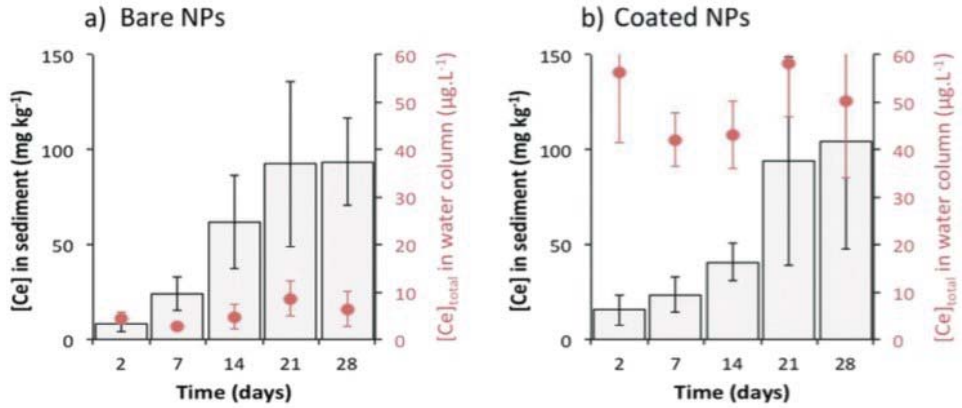


Figure 28. **Long-term distribution of Ce between the water column and the surficial sediments after 28 days of CeO_2 NMs contamination for (a) bare CeO_2 NMs and (b) coated CeO_2 NMs. Water was sampled at ~10 cm from the air/water interface. Data represent the average of three replicates ± standard deviation and are corrected from background concentration determined in control mesocosms.**

- ***Interaction with biota***

Table 1 presents the concentrations of Ce taken up or adsorbed onto the surface of *P. corneus* and *E. vulgaris* after exposure to bare and citrate-coated CeO_2 NMs.

Eudiaptomus are planktonic filter feeders that pass water currents through sieve- or comb-like structures to feed. The Ce concentrations in the copepods (whole organism) after 2 weeks were 288 ± 54 mg kg^{-1} (dry weight) and 3356 ± 625 mg kg^{-1} for bare and coated CeO_2 NMs, respectively. A similarly large amount was

previously observed in daphnia after exposure to 0.1 mg L^{-1} TiO_2 NMs **(Zhu et al. 2010)**. These planktonic filter feeders live and feed in the water column and can ingest **(Auffan et al. 2013)** and adsorb Ce on the cuticle **(Artells et al. 2013)**. The longer persistence of Ce in the water column after dosing with citrate-coated NMs is in agreement with the high amount of Ce measured in and/or on the surface of the copepods exposed to coated NMs.

Planorbarius are benthic grazers that ate algae and biofilms at the sediment/water interface. Consequently, the Ce concentration measured in their digestive glands depends on the Ce concentration in the surficial sediments and the settling kinetics of the homo- and hetero-aggregates. The Ce concentration measured in whole *P. corneus* was not statistically different between bare (2.1 ± 1.6 to 6.3 ± 6.0 mg kg^{-1}) and coated NMs (1.3 ± 0.9 to 5.1 ± 3.7 mg kg^{-1}) over time (Table 1), which is in agreement with the lack of statistically significant difference in Ce concentrations measured in surficial sediments.

In addition to ecological strategies, one important difference between *E. vulgaris* and *P. corneus* is the specific surface area (SSA) available to adsorb Ce. The small size and large SSA of *E. vulgaris* could explain why the concentration in the whole copepods is several orders of magnitude higher than that in the whole *P. corneus* mollusks.

Table 1. Concentration of cerium (Ce) (mg kg^{-1} dry weight) measured in whole planktonic *Eudiaptomus copepods* as well as in whole benthic *Planorbarius* mollusks and their digestive gland exposed to cerium oxide nanoparticles (CeO2-NPs) over 28 days in mesocosms. For copepods, masses were estimated from biovolumes considering 20% of dry matter in organisms. DG: Digestive gland. N/A: Not analyzed

	Bare CeO_2-NPs			Coated CeO_2-NPs		
	Planktonic	Benthic		Planktonic	Benthic	
	Whole	Whole	DG	Whole	Whole	DG
7 d	288 ± 54	2.1 ± 1.6	31 ± 25	97 ± 18	5.1 ± 3.7	82 ± 62
14 d	111 ± 21	6.3 ± 6.0	170 ± 170	3356 ± 625	1.6 ± 1.1	16 ± 18
21 d	N/A	4.4 ± 4.1	140 ± 130	N/A	1.3 ± 0.9	9 ± 8
28 d	N/A	2.8 ± N/A	85 ± N/A	N/A	3.4 ± 2.4	60 ± 42

2.4.4.3 Ce speciation and biotransformation

- ***CeO_2 dissolution in the water column***

The concentration of dissolved Ce (<10 kDa) was measured in the water column of the mesocosms weekly over 28 days. The dissolved Ce concentration stabilized after 2 days at 1.1 ± 0.2 µg L^{-1} for bare and 18 ± 2 µg L^{-1} for citrate-coated CeO_2 NMs, which correspond to 22 ± 4% and 36 ± 5% of the total Ce introduced, respectively. The highest chemical stability of bare CeO_2 NMs in the water column was also confirmed by XANES at the Ce L_3-edge at which Ce^{III} was not detected. For both types of CeO_2 NMs, the concentrations of dissolved Ce measured in the mesocosms were not in agreement with the low solubility expected for Ce oxyhydroxide ($K_{sp}Ce(OH)_3 = 6.3 \times 10^{-24}$ at 25 °C), which suggests the presence of soluble inorganic or organic complexes of Ce in the mesocosms.

The Ce speciation in the water column was geochemically modeled by considering the amount of cations and anions present in Volvic® water and the concentrations of phosphates and carbonates measured in the water column of the mesocosms. For bare CeO_2 NMs, the results of modeling are quantitatively in agreement with the Ce concentrations measured in the water column: Ce^{III}–CO_3^+ aqueous complexes represent 20% of the total Ce (*i.e.* 0.9 µg L^{-1}) in equilibrium with $CeO_{2(s)}$ at pH = 7.9. However, these Ce^{III}– CO_3^+ complexes were not sufficient to account for the dissolved Ce measured in the mesocosms dosed with coated CeO_2 NMs.

Another parameter that needed to be taken into account was the concentration of citrate present in the stock suspension of the coated CeO_2 NMs. An estimated concentration of 1.3×10^{-4} mol L^{-1} of citrate was introduced into the mesocosms during the injection of the coated CeO_2 NMs. The Ce speciation was then modeled by considering the Ce^{III}–Cit^0 complexes, and the results obtained were quantitatively in agreement with the Ce concentrations measured in the water column: Ce^{III}–Cit^0 aqueous complexes represent 70% of the total Ce (34 µg L^{-1}) in equilibrium with $CeO_{2(s)}$ at pH = 7.9.

- ***CeO_2 biotransformation***

The speciation (local atomic order and valence) of the Ce accumulated in the surficial sediments was studied by X-ray Absorption Spectroscopy (XAS) at the Ce L_3-edge. However, standard transmission or total fluorescence XAS spectra do not have the required energy resolution to unambiguously identify Ce^{III} in the structure of CeO_2-NPs. HERFD-XAS (High-Energy Resolution Fluorescence-Detected XAS) could address this deficiency by offering more defined edge and pre-edge features for analysis. One of the most interesting features of HERFD-XAS is the ~2 eV difference between the pre-edges of Ce^{III} and Ce^{IV} reference compounds: 5719 eV for Ce^{III} and 5721 eV for Ce^{IV}. As shown in Figure 29, the pre-edges of Ce in both sediments exposed to bare and coated NMs were centered at 5721 eV, leading to the conclusion that no reduction of Ce^{IV} to Ce^{III} occurred in the surficial sediments after 28 days in the mesocosms.

The speciation of Ce in the digestive glands of *P. corneus* after 28 days was also investigated using XANES at the Ce L_3-edge (Figure 29). Significant reduction of Ce to Ce^{III} was observed for both bare and coated CeO_2 NMs in the digestive glands. A peak appeared on the XANES spectra of CeO_2 NMs at the energy of the edge observed for the Ce^{III}–cysteine reference compound. The intensity of the peak is higher for bare CeO_2 NMs than that for coated CeO_2 NMs, highlighting the more important bioreduction of Ce following bare CeO_2 NM ingestion.

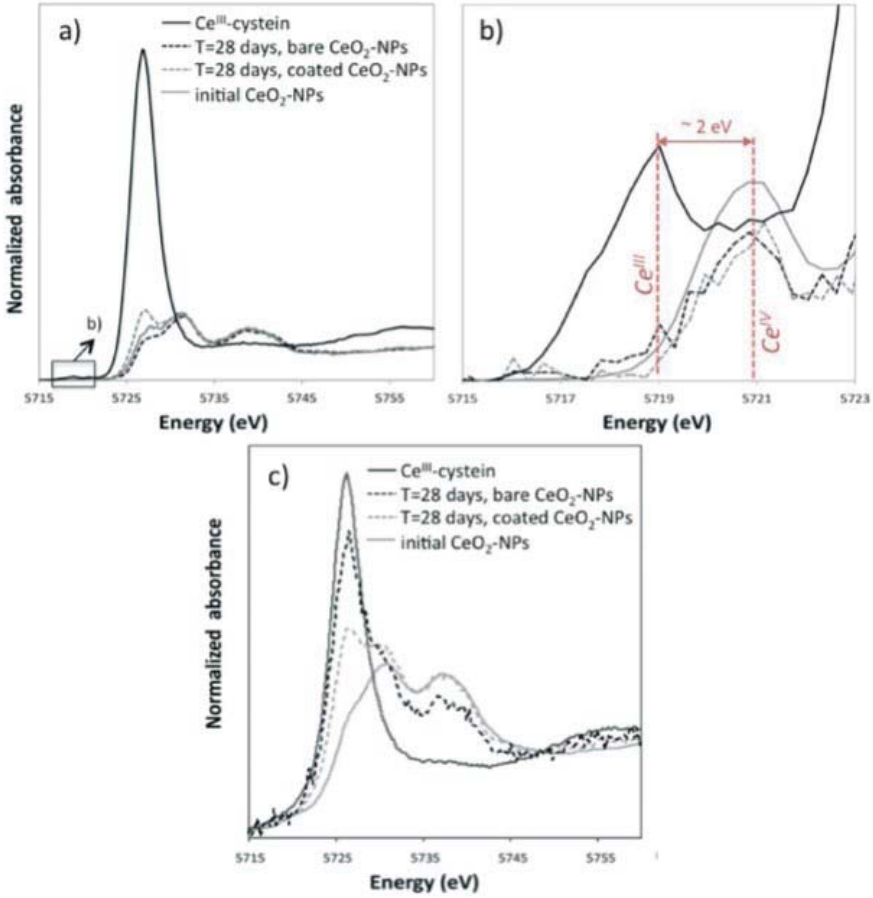


Figure 29: Cerium (Ce) L_3-edge X-ray absorption near-edge structure (XANES) spectra of Ce accumulated on the surficial sediments (using high- energy resolution fluorescence-detected XAS [HERFD-XAS]) (a, b) and in the digestive gland of *Planorbarius corneus* at day 28 (total fluorescence XAS). (c). Details of the pre-edge area are shown in (b). Experimental spectra are compared to Ce^{III}–cysteine and initial nanoparticle reference spectra.

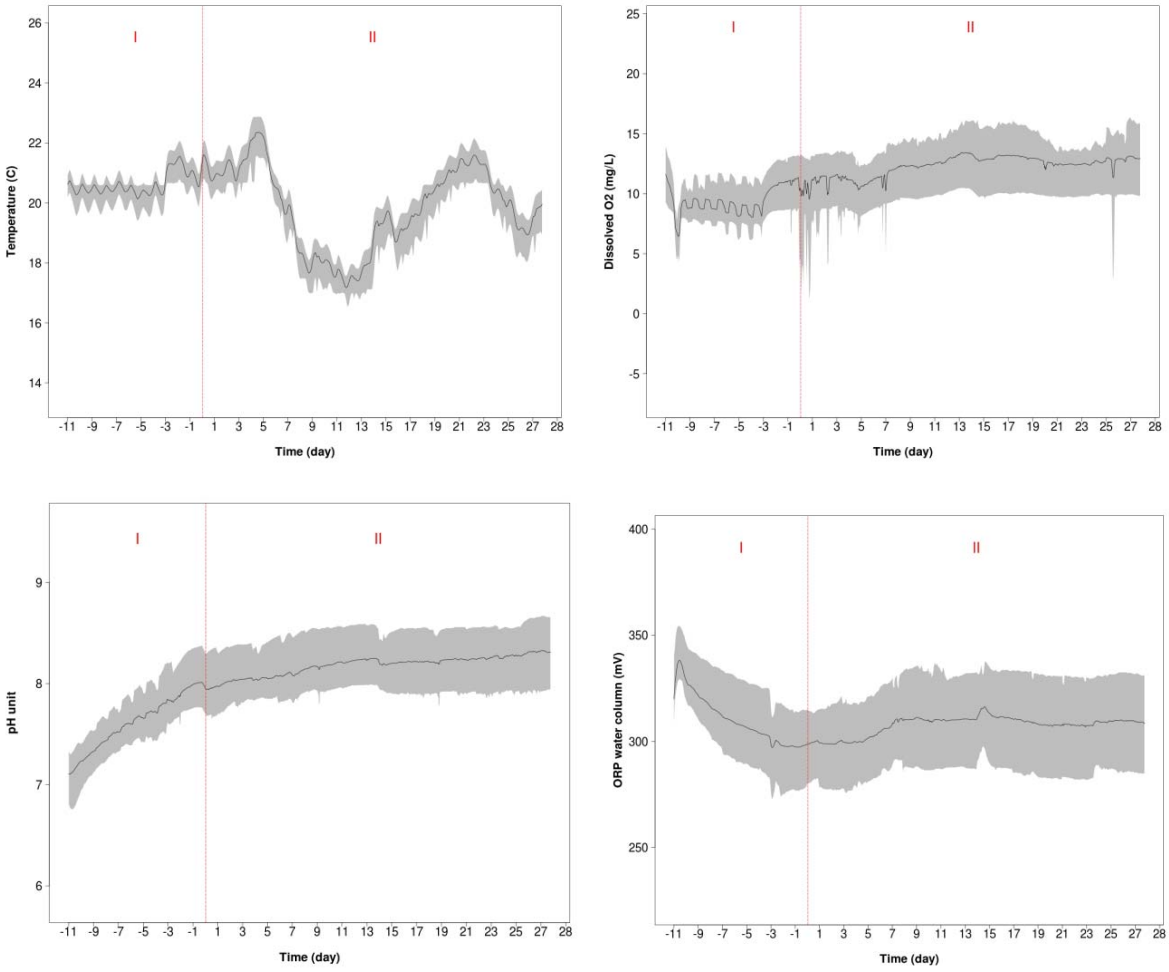
2.4.5 Freshwater 60L-mesocosm contaminated with pristine TiO_2 NMs and fragmented cement (CNRS-CEREGE)

Starting at day 0, the water columns were dosed 3 times per week (on Monday, Wednesday, and Friday) for 4 weeks with 4.5 mg of TiO_2 NMs/mesocosms/day until day 28 (0.09 mg TiO_2/L/mesocosms/day), resulting in a

final concentration of 1.1 mg.L^{-1} TiO_2 NMs and a total TiO_2 mass of 54 mg in the mesocosms.

2.4.5.1 Evolution of the physico-chemical parameters

Details of the parameters monitored during the experiments for 6 weeks are provided in Figure 30. Briefly, after period 1, dissolved O_2 (12 ±3 mg/l), pH (8.2 ±0.15) and ORP (310 mv ±20) were almost constant during the inoculation period. It is worth noting that the conductivity for the mesocosms incubated with by-products obtained from cement leaching increased faster that for the control systems and the mesocosms inoculated with pristine nano-TiO_2. This main difference can be related to the partial dissolution of the cementitious matrix.



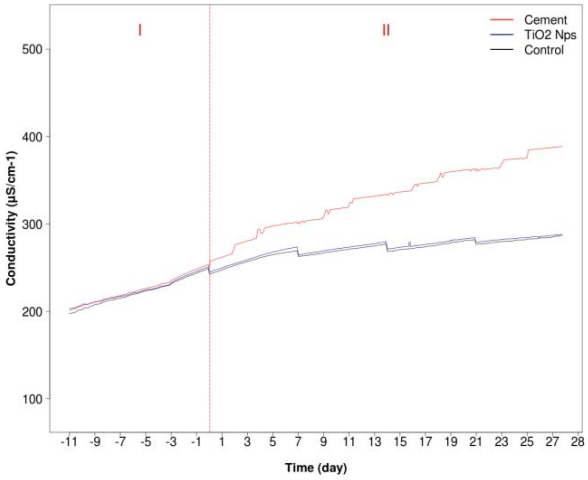
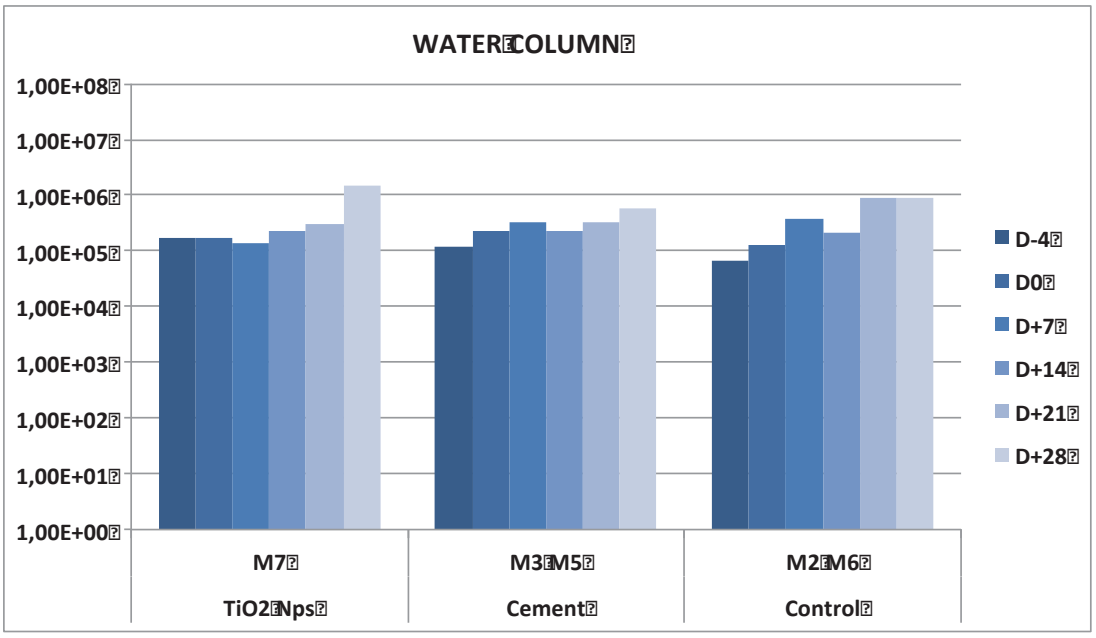


Figure 30. Evolution of the physico-chemical parameters in the water column of the mesocosms. Redox potential, dissolved oxygen, pH, and conductivity were measured during phases I (stabilization) and II (contamination). Day 0 corresponds to the first dosing of NPs. The grey surface is defined by the maximum and minimum values of each parameter, and the dark line corresponds to the average values of the 9 mesocosms. One measurement was performed every 5 min.

2.4.5.2 Number of picoplankton in the water column and surficial sediments

The total number of picoplankton for all mesocosms, before and after inoculation is details in Figure 31 for the water column and the sediments. The comparison between the three different conditions revealed that no statistical difference exists.



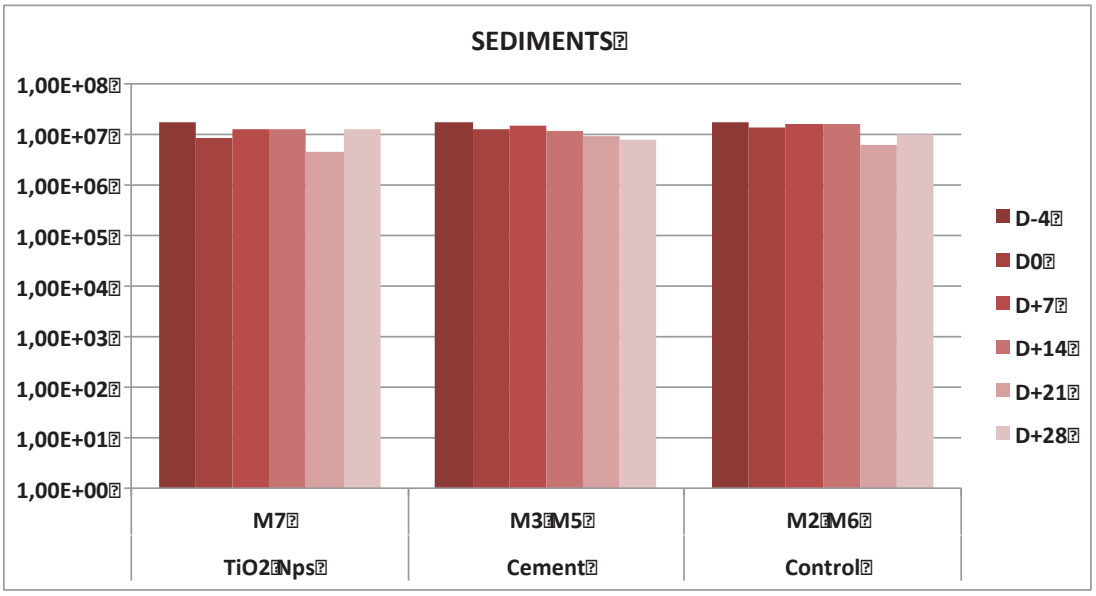


Figure 31. Evolution of the number of picoplankton in the water column and in the surficial sediment. Water was sampled at ~10 cm from the air/water interface and sediment at less than 1 mm of depth.

The chemical analysis of the water column, the surficial sediments, cores of sediment, and organisms are in progress to determine the distribution of Ti between all these compartments.

2.4.6 Marine 60L-mesocosms contaminated with pristine CeO_2 NMs and combusted diesel additives (UCO)

2.4.6.1 Evolution of the physico-chemical parameters

Physico-chemical (pH, Redox potential, dissolved O_2 and temperature) parameters at 15 PSU (Figure 32) or 30 PSU (Figure 33) remained stable during 28 days and were not statistically different between controls and contaminated mesocosms.

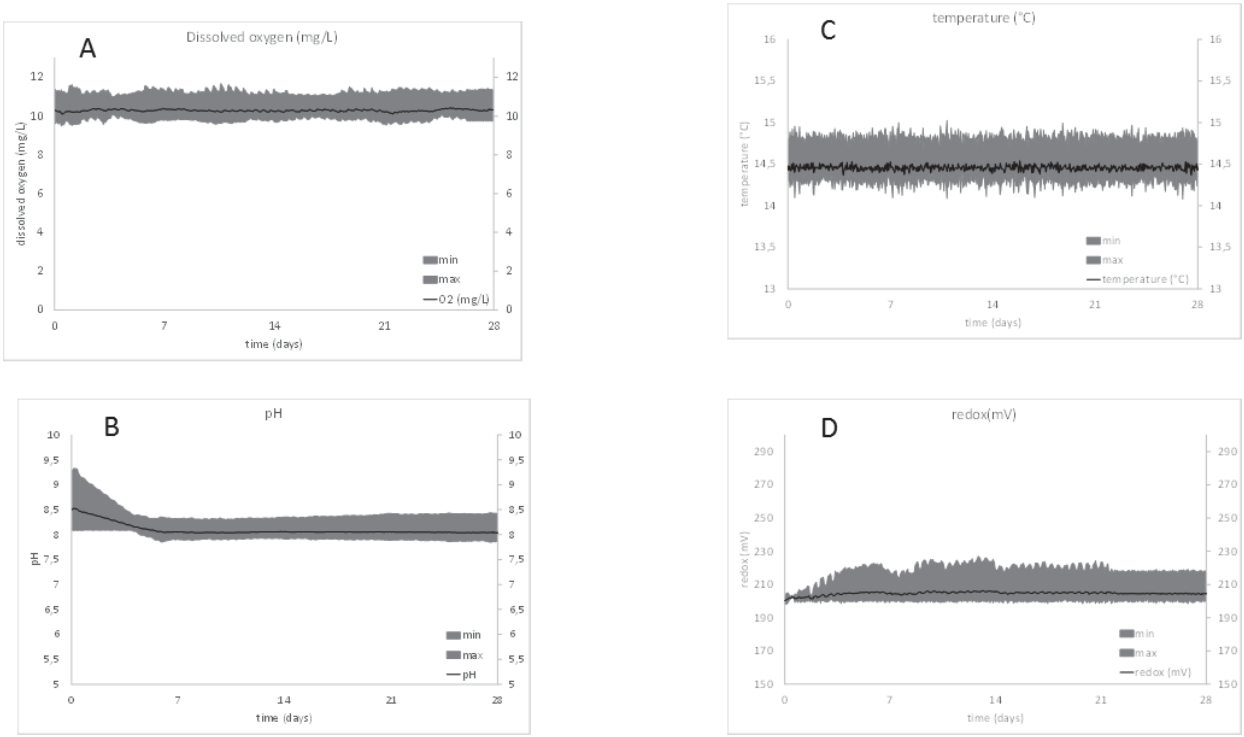


Figure 32 Evolution of the physico-chemical parameters. Dissolved oxygen (A), pH (B), temperature (C) and redox potential (D) in the water column at 15 PSU during phase II. The grey surface is defined by the maximum and minimum values of each parameter, and the dark line corresponds to the average values of the 9 mesocosms. One measurement was performed every 5 min.

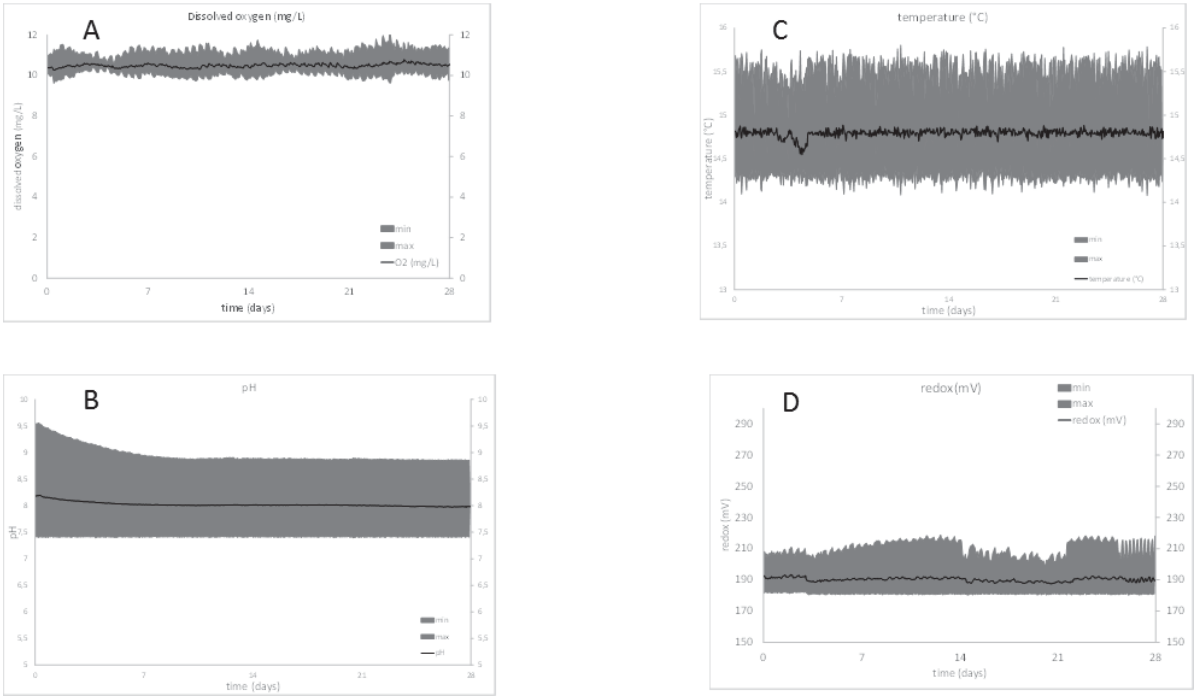


Figure 33: Evolution of the physico-chemical parameters. Dissolved oxygen (A), pH (B), temperature (C) and redox potential (D) in the water column at 30 PSU during phase II. The grey surface is defined by the maximum and minimum values of each parameter, and the dark line corresponds to the average values of the 9 mesocosms. One measurement was performed every 5 min.

2.4.6.2 Total Ce quantification in the water column

Results shown that Ce concentrations were usually greater in marine mesocosms spiked with NM-212 and Envirox^{TM} compared with controls and the combusted Envirox^{TM}. Ce concentrations increased between 7 and 21 days followed by a decrease at 28 days corresponding to the end of the experiment. A significant effect of salinity was depicted for Envirox^{TM} with greater concentrations at 30 PSU vs 15 PSU at 14 and 21 days of exposure. The quantification of Ce has been realized in the water column of marine mesocosms used for *S. plana* exposure. A mineralization procedure involving HNO_3 and H_2O_2 (block heater at 113°C during 1h) was followed by an ICP-Ms analysis. The Figure 34 illustrates the total Ce concentrations measured in the water column according to the different conditions of exposure (seawater only: controls; NM-212; Envirox^{TM} before combustion: Envirox^{TM} and after combustion: Comb Envirox^{TM}), the day of sampling (7, 14, 21 and 28 days) and the salinity tested (15 and 30 PSU).

- ***Influence of exposure conditions on total Ce concentrations***

At 15 PSU and at 7 days of exposure, Ce concentrations were significantly ($p<0.005$) higher in water from mesocosms spiked by Envirox^{TM} compared with the three other exposure conditions (controls, NM-212, Comb Envirox^{TM}). At 30 psu, Ce water concentrations were also higher for Envirox^{TM} but compared with controls only. At 14 and 21 days, greater Ce concentrations were observed in mesocosms spiked with NM-212 and Envirox^{TM} *vs* controls and Comb Envirox^{TM} at 15 PSU, whereas at 30 PSU, significant greater concentrations were found only for Envirox^{TM} compared with the three other conditions. At the end of the experiment (28 days) and at both salinities tested, Ce concentrations were significantly higher in mesocosms contaminated by Envirox^{TM} and NM-212 compared with controls and Comb Envirox^{TM} (except at 15 PSU where Ce concentrations were not significantly different between Envirox^{TM} and Comb Envirox^{TM}).

- ***Influence of exposure time***

At both salinities (15 and 30 PSU) tested and between 7 and 21 days, a significant increase of Ce water concentrations with the time of exposure was observed in mesocosms contaminated by NM-212. After 21 days, Ce concentrations decreased until the end of the experiment (28 days) (only significant at 15 PSU). In mesocosms spiked with Envirox^{TM} at 15 PSU, significantly higher Ce concentrations were found at 7 and 21 days compared with 14 and 28 days of exposure. At 30 PSU and for the same exposure condition (Envirox^{TM}),

increasing concentrations were observed from 7 to 21 days followed by a significant decrease at 28 days. Concerning Comb Envirox™, at 7 days of exposure and at 15 PSU, Ce concentrations were significantly higher compared with 28 days, whereas at 30 PSU, greater concentrations were found at 14 days compared with the other times of exposure (7, 21 and 28 days).

- ***Influence of salinity***

Ce concentrations were significantly greater at 30 psu compared to 15 psu for NM212, Envirox™ and Comb Envirox™ at respectively 14, 21 and 28 days of exposure. Ce concentrations significantly higher at 15 psu *vs* 30 psu were observed only for NM212 at 21 days of exposure.

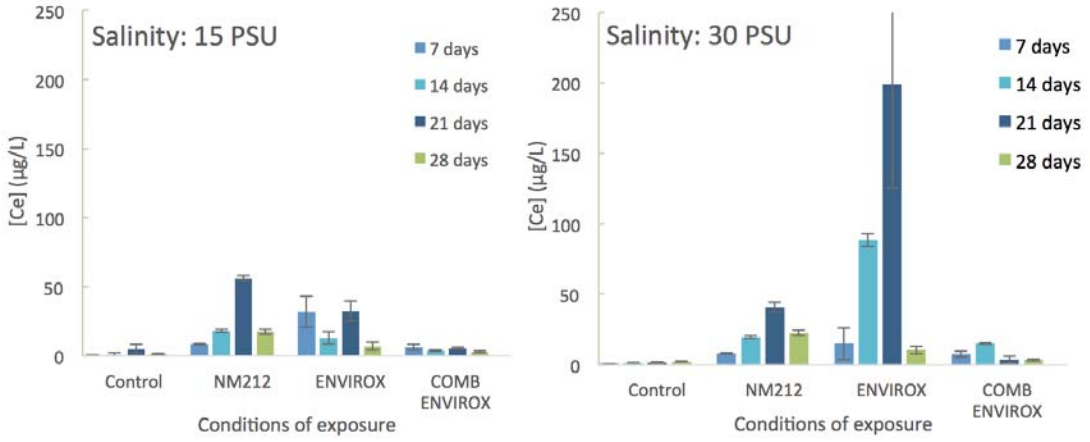
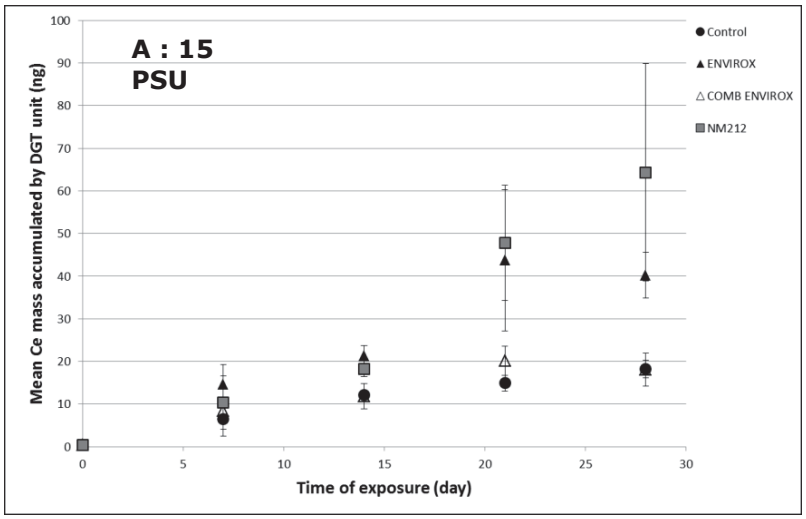


Figure 34 Means of total Ce concentrations (µg.L^{-1}) and standard deviations in the water column of marine mesocosms after 7, 14, 21 and 28 days of exposure according to the four experimental conditions (control, NM-212 reference NM (NM212), Envirox™ and Comb Envirox™) at the two salinities (left: 15 PSU and right: 30 PSU) tested.

2.4.6.3 Labile Ce fraction released from NMs in the water column, and the sediments

At both salinities tested (15 PSU, 30 PSU) and during the whole duration of the exposure, DGT in Comb Envirox™ mesocosms did not accumulate more Ce than DGT in controls mesocosms. These results indicate that no release of labile Ce from altered Envirox™ (Comb Envirox™) NMs occurred. In contrast, concerning mesocosms contaminated by not altered Envirox™ NMs and NM-212, a significant accumulation of Ce by DGT was observed at both salinities tested from day 14 to day 28, compared to DGT in control mesocosms.



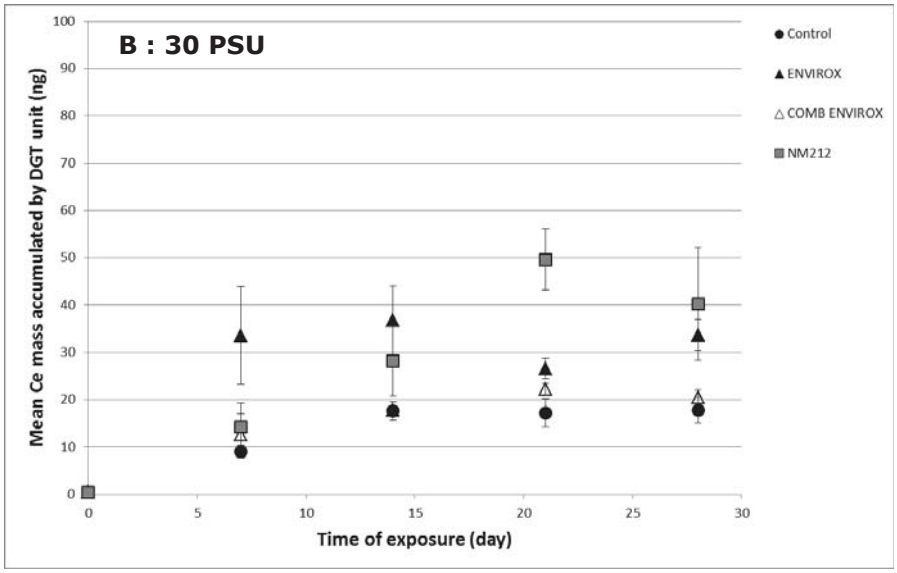


Figure 35. Mean masses of cerium (Ce) accumulated by diffusive gradients in thin films (DGT) units (ng) at successive times of exposure deployed in marine mesocosms at 15 PSU (A) and 30 PSU (B) with *Scrobicularia plana* (n=3 per sampling day and treatment).

2.4.7 Marine 60L-mesocosms contaminated with pristine TiO_2 NMs and fragmented cement (UCO)

pH (8.10 ± 0.14), Redox potential (239 ± 0.9 mV), dissolved O_2 (10.52 ± 0.54 mg/L), temperature (17.95 ± 0.63 °C) and conductivity (46318 ± 5408 µS/cm) parameters at 30 PSU remained stable during 28 days and were not different between controls and contaminated mesocosms (Figure 36).

Table 2. Percentage of Ti measured in different compartments of the marine mesocosms at the end of the experiment (day 28).

	Mass balance	% of Ti in the surficial sediment	% of Ti in the water column	% of Ti in *S. plana*
TiO_2 ref	67 ± 2%	99.92 ± 0.0005%	< 0.01%	0.08 ± 0.0005%
TiO_2 cement	34 ± 6%	99.91 ± 0.0002%	< 0.01%	0.09 ± 0.0002%

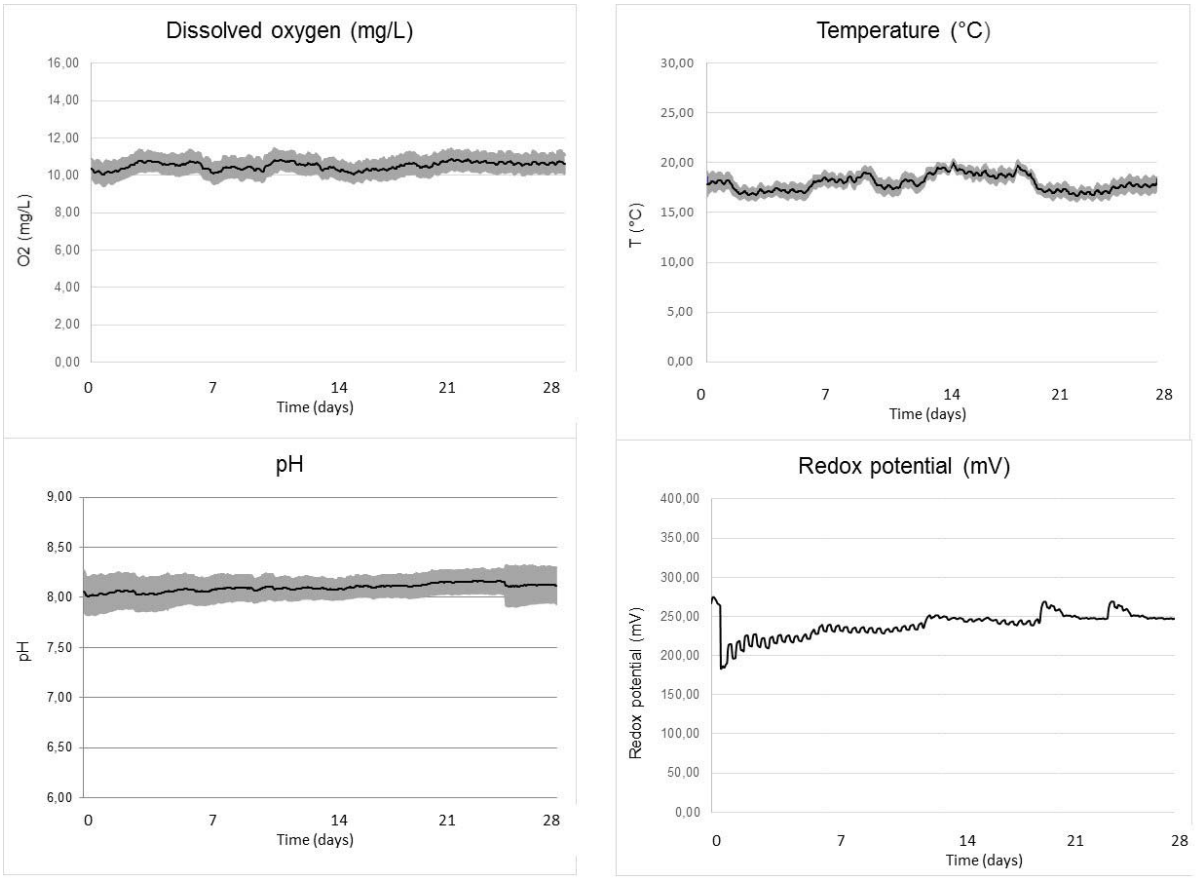


Figure 36 Evolution of the physico-chemical parameters. Dissolved oxygen, pH, temperature and redox potential in the water column at 30 PSU during phase II. The grey surface is defined by the maximum and minimum values of each parameter, and the dark line corresponds to the average values of the 9 mesocosms. One measurement was performed every 15 min.

After 28 days of reference TiO_2 exposure, 67 ± 2 % of Ti was recovered while 34 ± 6% of Ti was found for cement TiO_2 exposure. Results showed that the majority of Ti was mainly found in surficial sediments (~ 99%) for both conditions, probably due to the aggregation and hence to sedimentation of these NMs (Table 2).

2.5 Evaluation and conclusions

Indoor aquatic mesocosms: an adaptable tool for performing integrated environmental risk assessment of nanomaterials (CNRS-CEREGE, UCO, ITENE)

Physical-chemists, (micro)biologists, and ecotoxicologists need to conduct meaningful experiments to study the environmental risk of engineered nanomaterials with access to relevant mechanistic data across several spatial and temporal scales. Indoor aquatic mesocosms that can be tailored to virtually mimic any ecosystem appear as a particularly well-suited device. In this NANOREG deliverable, this concept was illustrated by several pilot studies taking into account the life cycle of different nanoproducts (diesel additives, cement, plastic films) containing different pristine nanoparticles (TiO_2, CeO_2, ZnO, Ag).

These pilot studies highlight that the exposure of the organisms (benthic *vs.* planktonic) will strongly depend on the contamination scenario. According to the NMs and the contamination scenarios, these experimental systems can operate with different physical and physico-chemical features (*e.g.* water quality and depth, sediment mineralogy and depth, current velocity, tidal reservoirs, *etc.*) and biota, owing to a high flexibility. This approach can be adapted in response to ecotoxicological benchmarks. This allows for testing of the Contaminant of Potential Ecological Concern (COPEC) that may be causing risk or adverse effects to biota at

a site. Of course, outdoor mesocosms may provide a more representative simulation of natural ecosystems [31,32] and results obtained with our experimental design should be extrapolated with caution. Variations representative of natural aquatic environment induced by the wind, direct sunlight, drought, rain, spatial migration, flora *etc.* are not taken into consideration. However, these indoor mesocosms provide both a high degree of complexity in the system and a reduced uncertainty regarding the fate of the NMs. This platform allows for simultaneous evaluation of physico-chemical properties and their relationship to the biological systems *in situ* as the ecosystem and NMs evolve. This platform is particularly well adapted to probe physical stability of NMs within the water column. Indeed indoor mesocosm design facilitates the characterization of homo and hetero aggregation of NM. More over these platforms provide more realistic conditions for considering NM transformations that have been lacking to date in many studies of NM impacts on organisms and proven to be critical in assessing the potential hazards presented by NMs.

The effects on organisms can therefore be evaluated under relatively controlled conditions that, like larger scale mesocosm studies, allow for evaluation of trophic transfer, maternal transfers, predator-prey interactions, competitive effects, resistance and resilience. However, the complexity of food web and the species diversity will be reduced. This will exacerbate the exposure and impacts compared to real natural environments. It will provide the upper limit of the effects expected in the mimicked ecosystems.

This experimental approach requires a strong integration of disciplinary researches. It offers physico-chemists, (micro)biologists, ecotoxicologists and ecologists the possibility of conceiving robust experiments to study the exposure and impacts of NMs (low doses, chronic inputs) in a fashion that accommodates the control required to elucidate underlying mechanisms at various time and spatial scales. By simultaneously creating representative conditions for environmental transformation and ecosystem exposure, the platform facilitates the integration of the complementary approaches into an environmental risk assessment model related to nanotechnologies based on reliable exposure and impact data.

By focusing specifically on exposure, the experiments described above contribute to the answers to the regulatory questions mentioned earlier. The complexity of environmental exposure is the result of the interaction of a number of biological- and physico-chemical processes. These processes may have synergetic or antagonist effects in terms aggregation/dispersion, uptake by biota, etc. Question 12 deals with how environmental exposure should be assessed in practice. Using mesocosm testing does account for the complexity in a reliable manner in a single experiment, which is far more difficult to achieve with a combination of tests addressing each process.

Accelerated mechanical (e.g. abrasion) and chemical (e.g. leaching) aging tests yield valuable data to determine the transfer factor, i.e. the ability to release NMs from a product, which was found to be a major knowledge gap in D3.1. Their usefulness is greatest in the use phase of the life cycle. At the end of life stage however, the environmental distribution is best characterized when considering both physical-chemical and biological interactions in a single experiment, which underlines the attractiveness and relevance of mesocosm testing. Although not to the point of standardization, general guidelines on how to conduct such experiments can be derived, for different exposure scenarios (questions n°13 and 9).

The results presented above demonstrate the capability of the present testing strategy to characterize/distinguish acute vs. chronic exposure, account for varying surface chemistry (coated vs. uncoated) by quantifying the distribution of NMs and their alteration residues within the different compartments of an ecosystem. The time resolved monitoring allows to distinguish between transient reservoirs and NMs accumulation sinks (questions n°3 and 6).

Mesocosms are one of the rare bio-physico-chemical exposure characterization tools, but their application of course extends beyond the strict perimeter of work package 3 by addressing ecotoxicity in a realistic setting. This provides obvious links with the other Nanoreg work packages and increases the number of regulatory questions to which this testing strategy provides answers

2.6 Data management

Data generated during mesocosm experiments are temporarily stored at the CNRS-CEREGE (SERENADE intranet) and can be available upon request. Datasets were entered by organizing the necessary information within Excel templates.

It is worth remembering that it was decided by the NANOREG MC that exposure dataset will not be included within the NANOREG database. However the PROSAFE momentum concerning database allows to broaden the possibility to integrate mesocosms datasets within structured databases. Data from mesocosm were cured by consideration of existing ontology, specifically developed by e-nanomaper and the US CEINT NanoInformatics Knowledge Commons (NIKC) (CEINT, Duke University, NC, USA). Part of these data, are already included in the NIKC database.

3 Deviations from the work plan

The deliverable 3.5 was initially due by month 34 (January 2016). The set-up of mesocosms was a little bit longer than expected, mainly due to the generation of released nano by-products that can simulate various stages of life cycle. In agreement with the MC and WP 3 leaders, the deliverable was postponed by 4 months. However the delay will not affect the review of Nanoreg deliverables by the PROSAFE Task force in preparation of the meeting in November 2016.

4 References / Selected sources of information (optional)

Artells E et al. (2013) Exposure to Cerium Dioxide Nanoparticles Differently Affect Swimming Performance and Survival in Two Daphnid Species. PlosOne 8: e71260.

Auffan M et al. (2013) Role of Molting on the Biodistribution of Ceo2 Nanoparticles within Daphnia Pulex. Water Research 47: 3921-3930.

Auffan M et al. (2014) An Adaptable Mesocosm Platform for Performing Integrated Assessments of Nanomaterial Risk in Complex Environmental Systems. Scientific reports 4: 5608.

Auffan M et al. Towards a definition of inorganic nanoparticles from an environmental, health, and safety perspective. Nat Nanotechnol 4, 634-641 (2009).

Bone A. J. et al. Biotic and abiotic interactions in aquatic microcosms determine fate and toxicity of Ag nanoparticles: Part 2. Toxicity and Ag speciation. Environ Sci Technol 46, 6925-6933 (2012).

Brooks BW et al. Laboratory and field responses to cadmium: an experimental study in effluent-dominated stream mesocosms. Environmental Toxicology, Volume 23, Issue 4, 2004, 1057–1064

Buffet PE et al. 2013. A mesocosm study of fate and effects of CuO nanoparticles on endobenthic species (Scrobicularia plana, Hediste diversicolor). Environmental Science and Technology. 47(3), 1620-1628.

Buffet PE et al. 2014. A marine mesocosm study on the environmental fate of silver nanoparticles and toxicity effects on two endobenthic species: The ragworm Hediste diversicolor and the bivalve mollusc Scrobicularia plana. Science of The Total Environment, 470–471, 1151-1159.

Cañedo A et al. El mesocosmos Sostaqua: recreación de un ecosistema fluvial bajo condiciones experimentales controladas. Tecnología del agua. 2012

Cleveland D et al. Pilot estuarine mesocosm study on the environmental fate of silver nanomaterials leached from consumer products. Sci Total Environ 421–422, 267-272 (2012).

Crossland NO et al. The design of mesocosm experiments. Environ Toxicol Chem 11, 1-4 (1992).

Davison W et al. (1994) In-Situ Speciation Measurements of Trace Components in Natural-Waters Using Thin-Film Gels. Nature 367, 546–548.

FAO in Biosafety of genetically modified organisms: basic concepts, methods and issues (FAO, Rome, 2009).

Ferry JL et al. Transfer of gold nanoparticles from the water column to the estuarine food web. Nat Nanotech 4, 441-444 (2009).

Gottschalk et al. Modelled environmental concentrations of engineered nanomaterials (TiO2, ZnO, Ag, CNT, Fullerenes) for different regions. Environmental Science & Technology 2009; 43(24): 9216-9222.

ISO (2006). ISO 4892-3:2006 Plastics -- Methods of exposure to laboratory light sources. Part 3: Fluorescent UV lamps. Geneva, International Organization for Standardization (ISO).

Lowry GV et al. (2012) Long-Term Transformation and Fate of Manufactured Ag Nanoparticles in a Simulated Large Scale Freshwater Emergent Wetland. Environmental Science & Technology 46: 7027-7036.

Marquart H et al. (2011) Classification of Occupational Activities for Assessment of Inhalation Exposure. Ann. Occup. Hyg. 55, 989–1005 17p.

Miller L et al. Impacts of metal oxide nanoparticles on marine phytoplankton, Environmental Science & Technology , vol. 44, no. 19, pp. 7329-7334, 2010.

Mohr S et al. Enhanced experimental flexibility and control in ecotoxicological mesocosm experiments: a new outdoor and indoor pond and stream system. Environ Sci Pollut R 12, 5-7 (2005).

Mueller and Nowack. Exposure Modeling of Engineered Nanoparticles in the Environment. Environ. Sci. Technol. 2008, 42, 4447–4453.

Mueller et al. Modeling the flows of engineered nanomaterials during waste handling. Environ. Sci.: Processes Impacts, 2013, 15, 251

Navarro et al. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi, Ecotoxicology, vol. 17, pp. 372-386, 2008.

OECD (2006) Oecd Guidelines for the Testing of Chemicals. Sediment-water Lumbricus toxicity test using spiked sediment. Paris, France: Organization for Economic Cooperation and Development.

Shriner C et al. Use of artificial streams for toxicological research. Crit Rev Toxicol 13, 253-281 (1984).

Sommer U et al. An indoor mesocosm system to study the effect of climate change on the late winter and spring succession of Baltic Sea phyto- and zooplankton. Oecologia 150, 655-667 (2007).

Tella M. et al. (2014) Transfer, Transformation and Impacts of Ceria Nanomaterials in Aquatic Mesocosms Simulating a Pond Ecosystem. Environmental Science & Technology 48: 9004–9013.

Thomas CR et al. Nanomaterials in the environment: from materials to high-throughput screening to organisms. ACS Nano 5, 13-20 (2011).

Unrine JM et al. Biotic and abiotic interactions in aquatic microcosms determine fate and toxicity of Ag nanoparticles. Part 1. Aggregation and dissolution. Environ Sci Technol 46, 6915-6924 (2012).

Van Tongeren M et al. (2011) Advanced REACH Tool: Development and Application of the Substance Emission Potential Modifying Factor. Ann. Occup. Hyg. 55, 980–988.

Wagner M et al. (2014) (Micro)plastics and the environment, Environmental sciences Europe 26(1):1-3

Wright S et al. (2013) The physical impacts of microplastics on marine organisms: a review, Environmental pollution, 178:483-492.

Zhang H et al. (1999) Diffusional characteristics of hydrogels used in DGT and DET techniques. Anal. Chim. Acta 398, 329–340.

Zhang P et al. (2012) Distribution and Bioavailability of Ceria Nanoparticles in an Aquatic Ecosystem Model. Chemosphere 89: 530-535.

Zhu X et al. (2010) Toxicity and Bioaccumulation of Tio2 Nanoparticle Aggregates in Daphnia Magna. Chemosphere 78: 209-215.