PAPER

Impact of CeO_2 nanoparticles on the functions of freshwater ecosystems: a microcosm study†

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We investigated the impact of CeO_2 nanoparticles (NPs) with different sizes, shapes and coatings on the function of a freshwater experimental ecosystem. We hypothesized that the different types of NPs would have different effects on the organisms involved in leaf litter decomposition and could differentially affect this process. Experiments were conducted in microcosm under environmentally relevant conditions with low CeO_2 NP concentrations (1 mg L^{-1}). Leaf litter decomposition, leaf-associated fungal biomass, bacterial community diversity and toxicity on *Chironomus riparius* larvae were studied. A decrease in teratogenicity (mouthpart deformities) in chironomid larvae was observed with citrate-coated spherical NPs, suggesting a hormesis effect. In contrast, exposure to non-coated, spherical NPs led to increased teratogenicity in chironomids, changes in bacterial community diversity and decreased leaf litter decomposition. Large, non-coated plates induced changes in bacterial assemblages, whereas no effect on fungal biomass was observed. These results are discussed and several hypotheses are presented to explain the results. Leaf litter decomposition is a marker that is frequently used to assess freshwater ecosystems' health. Extending its use to nano-ecotoxicology enables the study of NP impact on the function of ecosystems. This study shows that leaf litter decomposition and mouthpart deformities in chironomid larvae are sensitive, congruent markers of the environmental impact of CeO_2 NPs under these experimental conditions.

Nano impact

Ecotoxicity studies conducted under environmentally relevant conditions are crucial to understanding the long-term fate of nanoparticles and their impact on ecosystems. In this study, the impacts of CeO_2 NPs on an experimental aquatic ecosystem are assessed in microcosm. The results provide evidence that CeO_2 NPs lead to teratogenicity in chironomid larvae and to a significant decrease in leaf litter decomposition. These effects might result in important impacts on aquatic ecosystems by decreasing the available organic matter used by numerous primary consumers that are the basis of many food webs. The results also show that effect endpoints, such as litter decomposition and teratogenicity in invertebrates, can be used as relevant and powerful markers of the long-term impact of NPs.

1. Introduction

Cerium dioxide nanoparticles (CeO_2 NPs) are largely used as fuel additives or wood coatings due to their catalytic properties.[1] Produced in large amounts in Europe,[2] their growing production and use are expected to result in environmental release,[3,4] making it crucial to determine their potential ecotoxicity. Once released, CeO_2 NPs are expected to be found in aquatic environments,[3] which represent the final sink for most contaminants. Furthermore, they tend to quickly aggregate and sediment in the water column,[5,6] and benthic organisms are predominantly exposed to these NPs. Disturbances in benthic systems could ramify and amplify throughout ecological networks and impact wider systems[7] because many benthic organisms, such as microorganisms or macro-invertebrates, are the basis of food webs or play a major role in litter decomposition and organic matter release.

Most studies are currently performed on single species and are poorly representative of the environmental conditions. Therefore, it is very important to use and develop integrative tools and methods to consider the complexity of ecosystems, as many biotic and abiotic parameters can modulate NP bioavailability and toxicity.[8] Microcosms and mesocosms

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are used to approximate environmental conditions or to mimic natural phenomena, such as tidal cycles.[9,10] They allow the establishment of ecological processes, such as species interactions (*e.g.*, predation and competition), biomass production and organic matter decomposition. Studying the impact of NPs on ecological processes would greatly expand our understanding of the potential ecosystem impact of NPs. Among the ecological processes, leaf litter decomposition has been shown to be a valuable tool for the evaluation of ecosystem function and freshwater ecosystems' health assessment.[11,12] Leaf litter decomposition is indeed a vital process in freshwater ecosystems[13] and involves microorganisms and macro-invertebrates.[7,14,15]

In this study, we investigate the impact of CeO_2 NP pollution on ecosystem function by focusing on the process of leaf litter decomposition. We hypothesized that CeO_2 NPs with different characteristics would differentially affect the organisms involved in leaf litter decomposition and therefore have a different impact on this process. For this purpose, we used three types of manufactured CeO_2 NPs to chronically contaminate aquatic indoor microcosms. Following long-term exposure, we assessed the NPs' impact on leaf litter decomposition and the associated decomposer organisms: microbial communities and larvae of the benthic invertebrate *Chironomus riparius*. The relative contributions of microorganisms and macro-invertebrates to litter decomposition were also evaluated. The originality of this approach lies in the use of litter decomposition as a marker of NP toxicity. Indeed, this marker is frequently used in the field of ecology as an indicator of the proper function of ecosystems, but it has been rarely used to assess the environmental impact of NPs.[16]

2. Materials and methods

2.1 Nanoparticles

Three types of CeO2 NPs, which are referred to as NP1, NP2 and NP3, were tested. NP1s are commercially available, small (2–5 nm) spherical NPs that are coated with a tri-ammonium citrate layer. NP2s are commercially available, small (2–5 nm), non-coated spherical NPs. NP3s are industrially produced, larger (20–60 nm), non-coated plates.

Fresh NP suspensions were prepared before each contamination. Stock suspensions were homogenized in an ultrasonic bath (Bioblock, type 570 HF, frequency 35 kHz) for 10 minutes and sampled to prepare fresh suspensions (93.4 mg L^{-1}) in ultrapure water.

Stock suspensions were characterized by transmission electron microscopy (TEM, Jeol Jem 2100, 200 kV, HR; see the ESI,† Fig. S1) to determine the primary size and shape of the NPs. The NPs were also characterized in microcosms throughout the experiment. The Ce concentrations were measured in water, in sediment and in organisms by ICP-MS (PerkinElmer, NexIon 300X; detection limit: 0.01 μg L^{-1}). Water samples were taken before the first NP addition (T0), before chironomid larvae introduction and one week later (T1 and T2, respectively), and at the end of the experiment (T4) and analyzed for total and dissolved Ce. Surface sediment (10 mm) was sampled at T4 to determine the total Ce concentrations after acidic digestion. Water, sediment and organism samples were prepared as previously described.[17] Ce concentrations were measured in triplicates (pools of 20 chironomid larvae).

2.2 Organisms and leaf litter

Microcosms contain microbial communities, diatoms and chironomid larvae. A microbial consortium was isolated from water filters of the freshwater Museum-Aquarium of Nancy (France). Microorganisms were centrifuged and suspended in 50% glycerol (2.7×10^{10} bacteria per mL), and then stored at −80 °C. The consortium was allowed to develop in mineral water (Volvic®) for 72 h prior to inoculation in the microcosms. Diatoms (*Nitzschia palea* and *Navicula pelliculosa*) were cultured at EcoLab laboratory in CHU 10 medium with Fe EDTA as iron source ($6.4 < pH < 6.6$). Chironomid larvae (*Chironomus riparius*) were obtained and grown at EcoLab laboratory according to standardized procedures.[18] The microcosms also contain laboratory-grown larvae of the amphibian species *Pleurodeles waltl.*, which were used for purposes beyond the scope of this paper; hence, they are not described here.

Alder leaves (*Alnus glutinosa* Gaertn.) were collected from trees at abscission in an uncontaminated site and oven dried for 72 h at 30 °C. Before the experiment, batches of identical weight were placed in deionized water to prevent breakage during handling and sterilized to limit the introduction of non-intended microbial species into the microcosms. The selected weight corresponds to a leaf surface covering approximately one-third of the microcosm surface.

2.3 Microcosm experiment set-up

An initial experiment was conducted using large indoor microcosms (glass tanks; 75 × 20 × 60 cm, $L \times W \times H$), as previously described.[19] Briefly, the microcosms were filled with reconstituted sediment (6.5 L; 89% silica sand, 10% kaolin, and 1% calcium carbonate) and Volvic® water (56 L). The microcosms were equipped with a water recirculating system. Natural light was provided under 16 : 8 light–dark cycles and a constant temperature was maintained (21 ± 1 °C) for organisms' needs and to prevent bias from temperature changes in litter decomposition. Temperature, conductivity, pH, redox potential, and dissolved oxygen were monitored continuously. The rates of NO_3^-, NO_2^- and NH_4^+ production were controlled every three days. The experimental conditions were analyzed in triplicate, with random assignation of the microcosms. Alder leaves, diatoms and microbial consortium were introduced in the systems first. A biofilm was allowed to develop for two weeks before NP contamination. Contamination consisted of the chronic addition of NPs: fresh NP suspensions (50 ml of 93.4 mg L^{-1}) were added 12 times over four weeks to obtain the final concentration of 1 mg L^{-1}. Deionized water was added to the control microcosms in place of

the NP suspension. After one week of contamination, 700 chironomid larvae aged 72 h were added to each microcosm. Amphibian larvae were added one week later and the experiment ended 12 days later. Alder leaves were then collected with microcosm water. The surface sediment was sampled for NP quantification and then sieved to collect remaining chironomid larvae, which were stored in 70% ethanol.

A second experiment was performed using the same exposure systems without NPs to study the relative contributions of microbial species and invertebrates to litter decomposition. Two conditions were tested: "condition 1" comprised all of the species cited above (microbial communities, chironomid larvae and amphibian larvae), and "condition 2" comprised the same species, with the exception of chironomid larvae. The kinetics of organism introduction/biofilm development were the same as described above.

Table 1 summarizes the experimental conditions and objectives.

2.4 Leaf decomposition assessment

Leaf litter decomposition was assessed as described in Cornut *et al.*[20] After collection, leaves were gently rinsed with water from their respective microcosm to remove the sediment. Sets of five discs (12 mm diameter) were cut from the leaves of each microcosm, avoiding the central vein, and promptly frozen at −20 °C until they were processed for ergosterol extraction. The remaining leaf litter was dried at 105 °C to a constant mass and weighed to the nearest 0.01 g. The leaf material was then ground and portions of leaf material of approximately 500 mg were ashed (4 h at 550 °C) and weighed to determine the organic matter content. The remaining leaf mass was expressed as the ratio of the ash-free dry mass (AFDM) between the final and the initial leaf litter. Four unexposed batches of leaf litter were used to determine the initial AFDM according to the procedures described above.

2.5 Fungal biomass and bacterial diversity assessment

Leaf-associated mycelial biomass was assessed through its ergosterol contents, as previously described.[20,21] Leaf material was lyophilized and weighed to the nearest 0.1 mg, and then lipids were extracted with alkaline methanol and heated at 80 °C for 30 min. The extracts were purified using solid-phase extraction cartridges (Oasis HLB, 60 mg, 3 cc, Waters, Milford, MA, USA) and ergosterol was quantified by high-performance liquid chromatography. The ergosterol concentration was corrected for the extraction efficiency (87–100%), which was measured for each sample series of controls to which known amounts of ergosterol were added. The ergosterol concentration was converted into fungal biomass using a conversion factor of 5.5 mg ergosterol per g of fungal dry mass.[22]

After DNA extraction, the bacterial community structure in the water column was assessed by PCR-DGGE, as described in Clivot *et al.*[23] Pelagic microorganisms were sampled every week by water filtration (total volume of 100 ml, filtered at 0.45 μm). Detailed information about the DNA extraction and DGGE analysis procedures are provided in the ESI.† Briefly, DNA was extracted from the filters using an isolation kit and then PCR amplified. The fragments were analyzed on a polyacrylamide gel. Software analysis was performed to normalize and compare DGGE profiles. Data were analyzed by NMDS and ANOSIM.

2.6 Toxicity on *C. riparius*

Larval growth was determined by measuring the body length (ImageJ® software). Cephalic capsules of each larva were also measured to determine the larval instars[24] and assess potential delays in development. Teratogenicity was evaluated in the cephalic capsules as mouthpart deformities, as described by Dias *et al.*[25] Briefly, the cephalic capsules were placed in 15% potassium hydroxide (Sigma, France), heated for 12 minutes at 95 °C, and then incubated in 70% ethanol overnight to stop the reaction. The capsules were then mounted with Eukitt® mounting medium (03989, Fluka, France) and observed under a microscope (Olympus CX41). Mouthpart deformities were assessed and rated according to the methods described by Warwick and Tisdale[26] and Vermeulen *et al.*[27] The treatment groups were compared based on the incidence (individual or total deformities) and severity of the deformities. The latter is evaluated by ratings based on the impacted mouthpart surfaces. Briefly, each tooth is virtually divided into four areas and the number of areas covered by a deformity is used as the tooth rate.

2.7 Data analysis

Differences in the AFDM contents and fungal biomass between groups were tested for significance using a one-way analysis of variance (ANOVA) test followed by Tukey's test.

Table 1 Experimental conditions and objectives

Experiment 1			Experiment 2		
Conditions		Objectives	Conditions (no NP)		Objectives
Control (no NP)	NP contamination (NP1, NP2 or NP3)		Condition 1	Condition 2	
Leaf litter Microorganisms Chironomids Amphibians	Leaf litter Microorganisms Chironomids Amphibians	Assess NP effects on leaf litter, micro-organisms and chironomids	Leaf litter Microorganisms Chironomids Amphibians	Leaf litter Microorganisms Amphibians	Determine the relative contribution of micro-organisms and invertebrates on leaf litter decomposition

The differences in the body sizes and mouthpart deformity ratings of the chironomid larvae between groups were tested for significance using a Kruskal–Wallis one-way analysis of variance on ranks, followed by Dunn's test. The differences in the incidence of mouthpart deformities were tested for significance using a chi-square test. All of the statistical analyses were performed with SigmaPlot 12.0 software. The analysis of the DGGE profiles is described in the ESI.†

3. Results

3.1 Analysis of the physico-chemical parameters

No significant differences in the physico-chemical parameters of the system were observed between conditions (see the ESI,† Fig. S2). Temperature was maintained at 21 ± 1 °C. Conductivity ranged between 207 and 235 μS cm^{-1} (mean values) at the beginning and at the end of the experiment, respectively. The mean pH was 8.5, with a slight decrease (7.8) during the second week of NP contamination. The oxygen rates ranged between 110% and 95% (mean values) and were no less than 70% (mean values) throughout the experiment and in every condition.

The Ce concentrations in water increased over time. NP3s were rapidly removed from the water column, whereas NP1s were the most stable in the column (Fig. 1A). No differences were observed in the Ce concentrations in sediment between conditions (Fig. 1B).

3.2 Impacts of the CeO_2 NPs on leaf litter decomposition and organisms

After six weeks of incubation in the microcosms, the rates of alder leaf decomposition were rather high, with mean AFDM values of less than 15% at the end of the experiment (Fig. 2). A significant decrease ($p < 0.05$) in litter decomposition was observed in the microcosms contaminated with NP2 compared to the control. The NP1 and NP3 conditions were not significantly different from the control condition.

The ergosterol contents showed that the fungal biomass on alder leaves was important in every condition at the end of the experiment. No significant differences were observed between conditions (Fig. 3).

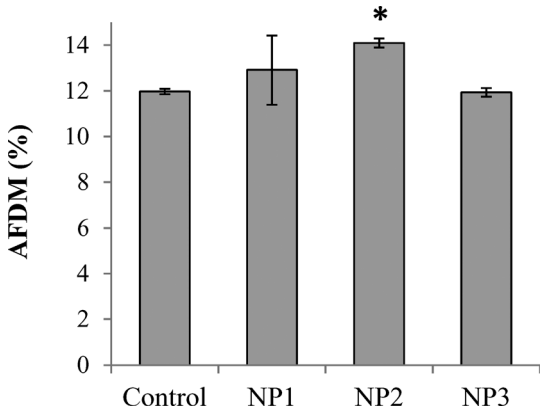

Fig. 2 Remaining organic matter from leaf litter incubated in mesocosms exposed to CeO_2 NPs (mean values ± standard error). n = 3. *Statistically different from control ($p < 0.05$).

The DGGE analysis revealed slight changes in the bacterial communities that were exposed to NP2 or NP3. Although no differences were observed between groups at the beginning of the experiment, the bacterial community structures from NP2 and NP3 groups tended to differ from that of the control group, starting from the third week of contamination (Fig. 4). No changes in community diversity were observed between NP1 and the control group.

No significant differences were observed in the numbers of chironomid larvae observed at the end of the experiment, with a mean number of 79 ± 16 larvae out of the 700 that were initially introduced. No effects on growth and development were observed. No differences were observed in the mean larvae body sizes at the end of the experiment between groups (ESI,† Fig. S3). The measurements of the cephalic capsules showed that with few exceptions (2–3 larvae per condition), all of the larvae had reached the fourth developmental instar in every condition, indicating that there was no developmental delay. The teratogenicity results obtained from the study of mouthpart deformities are presented in Table 2. The teratogenicity observed in the presence of NP1 was significantly less ($p < 0.05$) than that in the control condition; both

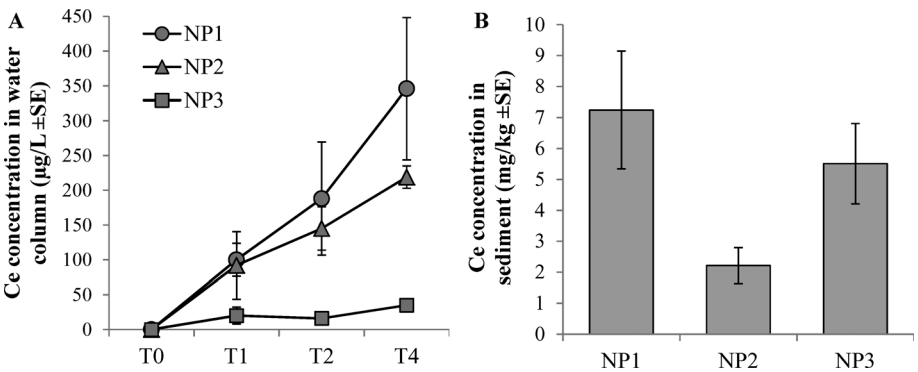

Fig. 1 (A) Ce concentration in the water column throughout the experiment (n = 3). (B) Ce concentration in sediment at the end of the experiment (n = 3). Data are corrected from background concentration determined in the control group.

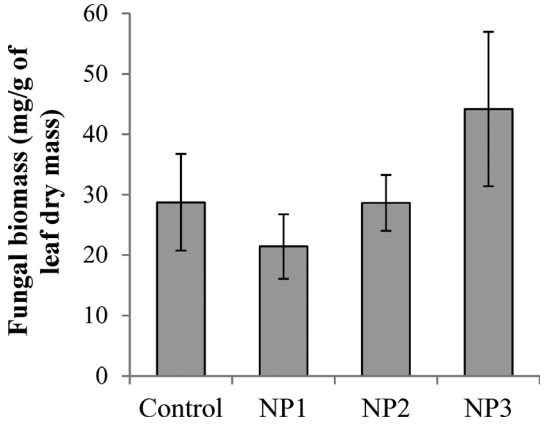

Fig. 3 Fungal biomass on alder leaves incubated in mesocosms exposed to CeO_2 NPs (mean values ± standard error). n = 3.

the frequency and the seriousness of deformities were reduced in the NP1 condition compared to the control. When considering all of the assessed deformities as a whole, the NP2 and NP3 conditions were not significantly different from the control, either in the frequency or in the seriousness of the deformities. However, a significantly larger ($p < 0.05$) number of larvae lacking one or more teeth (Fig. 5) was observed in the NP2 condition compared to the other groups. No similar phenomenon has been reported for other individually studied deformities. The NP concentrations in chironomid larvae ranged between 266 and 606 mg kg^{-1} (Table 3).

Table 2 Mouthpart deformities in *C. riparius* larvae exposed to NPs

	Deformity frequency (%)	Deformity ratings[a]	Frequency of "lacking tooth/teeth" deformity (%)
Control	28.1	0.64 ± 0.15	7.8
NP1	21.1[b]	0.32 ± 0.06[b]	8.5
NP2	41.0	1.43 ± 0.25	23.0[b]
NP3	34.1	1.24 ± 0.19	20.6

[a] Mean value ± standard error. [b] Statistically different from control ($p < 0.05$).

3.3 Relative contributions of microorganisms and invertebrates to leaf litter decomposition

A patently increased decomposition was observed in the presence of chironomid larvae; most of the alder leaves had been eaten, and only the hard parts of the leaves (*i.e.*, veins) remained. In contrast, leaves that were incubated without chironomid larvae were still intact at the end of the experiment (ESI,† Fig. S4). The assessment of remaining organic matter from the leaves showed that the AFDM was significantly reduced ($p < 0.01$) in the presence of chironomids (Fig. 6), indicating a greater decomposition rate.

4. Discussion

4.1 NP effects on microorganisms

The fungal biomass present on decomposing alder leaves was not impacted after four weeks of NP contamination with any

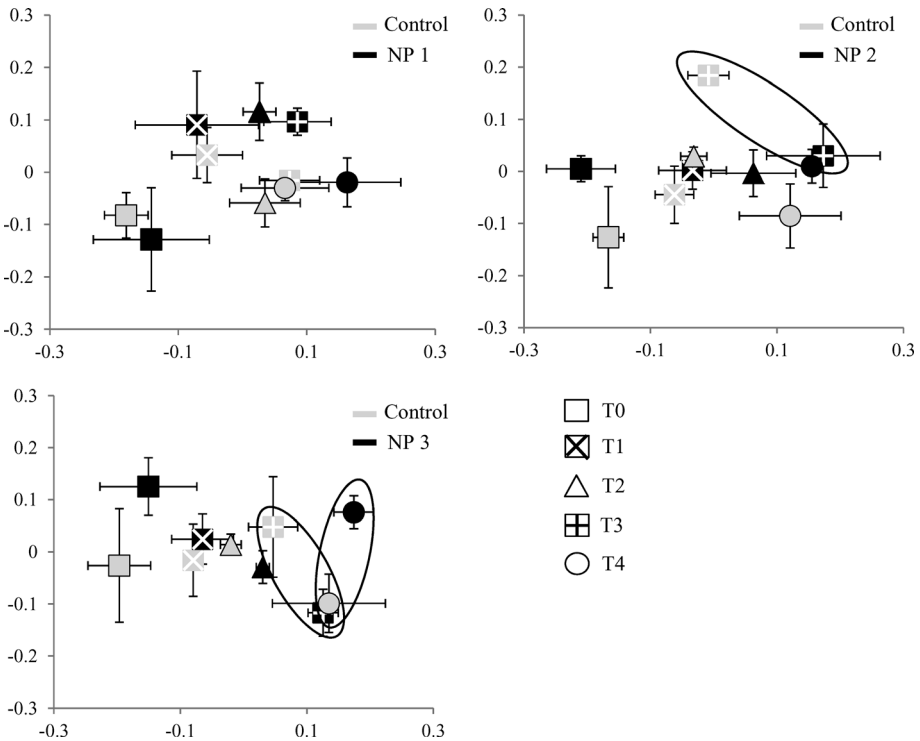

Fig. 4 NMDS plots of DGGE pelagic bacterial community profiles over time from control (grey icons) and NP (black icons) conditions (n = 3). T0: before NP contamination, T1: after one week of contamination, T2: after two weeks of contamination T3: after three weeks of contamination, T4: after four weeks of contamination. Circles indicate the most important differences between control and NP groups.

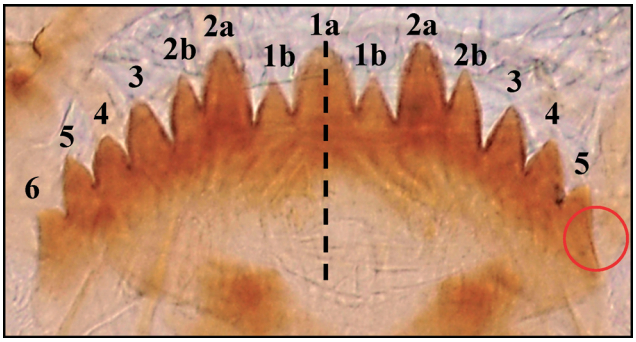

Fig. 5 Microscopic observation of *C. riparius* larvae mouthparts (×400). The dashed line represents the horizontal symmetry axis and the red circle indicates the absence of a lateral tooth.

Table 3 Toxicity on *C. riparius* and NP body concentrations

	Growth	Development	Teratogenicity	NP concentration (mg kg^{-1})
NP1	No effect	No effect	Lowered teratogenicity	606[a]
NP2	No effect	No effect	Teratogenicity	282[b]
NP3	No effect	No effect	No effect	266[b]

(a, b) Statistically different groups.

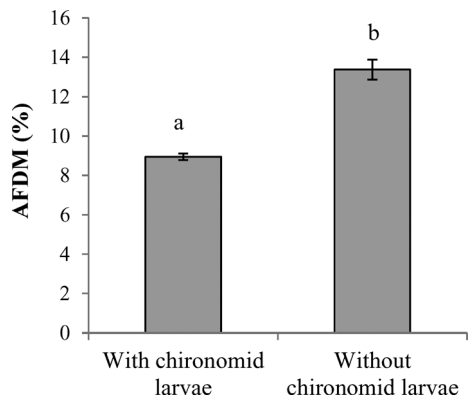

Fig. 6 Remaining organic matter from leaf litter in the presence or absence of chironomid larvae (mean values ± standard error). n = 3. Letters (a, b) indicate statistically different groups ($p < 0.01$).

type of CeO_2 NP. However, given the important fungal biomass observed in all the conditions, it is possible that slight differences between conditions occurred but were not statistically detected, given the low statistical power related to the relatively low replication. Bacterial communities were impacted by the CeO_2 NPs. The analysis of the DGGE profiles showed that communities exposed to NP2 or NP3 tended to be different from the control group at the end of the experiment. However, despite the strong tendencies, these differences are at the limits of statistical significance due to the relatively low replication within conditions. The differences observed between conditions could be related to the NP concentrations in the water column, with low concentrations resulting in a more important impact. Indeed, the Ce concentrations were reduced for NP2 and NP3 (Fig. 1A). However, a single NP concentration has been tested in this study, and dose–response studies would be necessary to confirm this hypothesis. Another hypothesis to explain these differences is that the impact of the NPs on the bacterial community is related to the NP shape and coating.

Previous studies have shown that CeO_2 NPs are cytotoxic, and contact between bacteria and the CeO_2 NPs may result in lipid peroxidation, membrane damage and reactive oxygen species (ROS) production.[28,29] It has also been shown that NPs with rough surfaces, corners and edges are more biologically and chemically reactive.[30,31] This result suggests that NP2 and, to a greater extent, NP3, which presents many corners and edges, may have been cytotoxic and induced cellular damage and ROS production in bacteria. Therefore, the changes in the bacterial community may be due to the different sensitivities of different species to CeO_2 NP cytotoxicity. For NP1, the citrate coating may have prevented direct contact between the bacteria and the CeO_2 NPs and, therefore, may have hindered ROS production and cellular membrane damage. The late response of the communities can be explained by different causes. The tested CeO_2 NPs could impact bacterial communities only over a long period of time, and/or the NP concentrations were not high enough to impact bacteria in the first weeks of contamination. Indeed, the repeated addition of NPs in the microcosms results in an increase in NP concentration over the course of the experiment.

4.2 NP effects on *C. riparius*

In our conditions, the small number of chironomid larvae remaining at the end of the experiment is largely explained by predation from the amphibian larvae.[19] Similar to previous studies,[32,33] no growth inhibition or developmental delays were observed in the chironomids regardless of the type of CeO_2 NP tested. However, differences in teratogenicity were observed between conditions in the microcosms, which were different from the observations after single-species exposure for 7 days.[32] The induction of mouthpart deformities has previously been shown in chironomid larvae that were exposed to uranium,[25] organic compounds[34] and heavy metals,[35–38] but to the best of our knowledge, this is the first study to observed teratogenic effects on chironomids exposed to NPs. Teratogenicity was more important in the presence of NP2 compared to the control group because the NP2 group had a significantly larger number of missing teeth. In contrast, a significant decrease in teratogenicity was observed with NP1 compared to the control group. The teratogenicity observed in the control group represents a normal, naturally occurring background in chironomidae.[35] Moreover, laboratory breeding increases the natural background of teratogenicity.[25,27] The significantly decreased teratogenicity observed with NP1 suggests that this type of NP could have a protective action on *C. riparius* larvae. As no differences in NP concentrations were observed in the sediment where larvae are found, the differences in teratogenicity are mainly attributed to the NP characteristics. Ce quantification shows that the concentrations of NP3 in the water column are 5- to 10-fold less than

those of NP1 and NP2, suggesting a greater amount of aggregation and sedimentation.[5,39] Thus, it can be hypothesized that NP3s, in the form of large aggregates, are less bioavailable for chironomid larvae once ingested. In contrast, NP2s are small, less likely to be aggregated, and may be more easily internalized, although further work is required to confirm their passage through the intestinal membrane. It can also be hypothesized that once ingested, NP2s clog the gut, as has been demonstrated for TiO_2 NPs,[40] carbon nanotubes[41] and colloidal clay,[42] thus producing decreased nutrient absorption and physiological disturbances. In contrast, NP1s have a positive impact on teratogenicity. In a previous study, the authors observed a small number of chironomid larvae with deformities, possibly because mortality due to uranium exposure eliminated the larvae with the lowest fitness, which are the most likely to present deformities.[25] In our study, this hypothesis can be ruled out as we previously showed that NP1s are not acutely toxic to *C. riparius*.[32] Therefore, the explanation of the differences observed between the CeO_2 NPs might lie in the mechanisms involved in deformity induction. Deformities are defined as morphological features that depart from the normal chironomid larvae configuration.[43] Broken teeth, which are easily recognizable by their chipped or rough edges, are not included in deformities. These deformities are due to physiological disturbances during molting.[44] Because molting is regulated by hormones,[34] it can be hypothesized that CeO_2 NPs positively or negatively impact hormonal processes, depending on the NP type. Other studies suggest that phthalate-induced upregulation of the HSP 40 and 90 genes might be correlated with the increased occurrence of deformities in *C. riparius* larvae.[45] It has also been shown that NPs induce changes in gene expression.[46–48] Together, these results suggest that the effects of the CeO_2 NPs on teratogenicity could be due to CeO_2 NP-induced changes in gene expression. Mouthpart deformities can also be considered as fluctuating asymmetry.[49] Some authors underscore the possibility that stress in organisms reduces energy reserves, and as the control over growth processes is energetically costly, the energy allocated for developmental control decreases, likely leading to an increase in developmental stability under stress.[50] Therefore, in our study, the mouthpart deformities might be due to decreased energy resources related to stress induced by NP2 exposure. As previously mentioned, gut clogging by NPs could also limit nutrient absorption and decrease the energy resources. Concerning the results observed with NP1, it can be hypothesized that a hormesis phenomenon[51] occurred, and overcompensation following NP1-induced stress resulted in increased developmental stability. Another hypothesis would be that citrate uptake subsequently increased the energy resources for the chironomids. The different hypotheses still need to be confirmed and have opened many perspectives for future research.

4.3 NP effects on leaf litter decomposition

Decreases in leaf litter decomposition have been observed in heavy metal-contaminated aquatic systems as a side effect of metal toxicity on microbial communities and macroinvertebrates.[13,52–54] Despite the relevance of this phenomenon, litter decomposition has only been studied once as a marker of NP impact on freshwater ecosystems.[16] In our study, a significant decrease in alder leaf decomposition was observed in microcosms exposed to NP2. This decomposition may result from microbial and/or chironomid larval activity. Indeed, many studies have shown that microbial communities are involved in litter decomposition and that contaminant toxicity could result in decreased litter decomposition rates.[13,16,52–54] Several authors also reported that macro-invertebrates, particularly shredders, are also involved in leaf litter decomposition.[7,14,15,54–56] Chironomid larvae are collector–grazers and preferentially feed on fine particulate organic matter or graze on biofilms at the surface of sediments. They can still decompose and directly feed on leaf litter, particularly in the absence of shredders.[13,57,58] To determine the relative contributions of microorganisms and invertebrates to leaf litter decomposition, the second experiment was performed in the presence or absence of chironomid larvae. The patent decrease in litter decomposition observed in the absence of chironomids indicates that these organisms are mainly responsible for leaf litter decomposition in our experimental conditions. The results observed with the CeO_2 NPs are consistent with this finding. Decreased leaf litter decomposition was only observed with NP2, and a negative impact on chironomids was also observed with NP2. In contrast, the observed shift in the bacterial communities in the presence of NP2 could partially explain the effects on litter decomposition, but litter decomposition was not altered with NP3, although a shift in the bacterial communities was observed with this type of NPs. Therefore, in contrast to what was observed with CuO NPs,[16] the impact of CeO_2 NPs on litter decomposition is not related to the biocidal effects of the NPs, but rather to their impact on macro-invertebrates. Teratogenicity and leaf litter decomposition were both impacted in the presence of NP2. Although no statistical correlation could be established between these two endpoints, likely due to the low statistical power, we cannot exclude the possibility that they are related. It can be hypothesized that mouthpart deformities hamper the chironomids' grazing activity and, therefore, lead to a decrease in litter decomposition. Another hypothesis would be that a disturbance in feeding behavior was related to the ingestion of NP2. As previously mentioned, gut clogging by NPs could interfere with the digestive process, affect larval feeding behavior and result in decreased decomposition rates, as previously reported with heavy metal.[59] More work is still needed to explore and confirm these hypotheses.

Many studies have reported that leaf litter decomposition is a sensitive marker of natural stream contamination.[11,16,35,52–54,60,61] Similarly, the induction of mouthpart deformities in chironomid larvae has proven to be a sensitive bioindicator of contamination in natural freshwater ecosystems.[35,37,38,62] In our study, despite the absence of a direct statistical link between mouthpart deformities in chironomids and leaf litter decomposition, the results observed with NP2 indicate that these two bioindicators are congruent concerning the effects of

CeO_2 NPs. Similarly, MacDonald and Taylor[35] reported that leaf litter decomposition was congruent with mouthpart deformities in chironomids in freshwater ecosystems exposed to municipal sewage effluent. Therefore, the present study shows that these markers can be used as valuable tools in microcosm experiments, particularly for the assessment of NP ecotoxicity. Moreover, they appeared to be more sensitive than other markers, such as growth inhibition or developmental delays.

5. Conclusion

This study aimed to investigate how CeO_2 NPs affect organisms involved in leaf litter decomposition and impact this process. The most important impacts were observed with the small, uncoated spheres, which impacted the bacterial communities and teratogenicity on chironomid larvae and decreased litter decomposition. The small, citrate-coated spheres did not impact bacterial communities, but a significantly decreased teratogenicity was observed on chironomids, suggesting a hormesis effect. The large, uncoated, cubic NPs only affected the bacterial communities. This study suggests that the differences in the observed effects are due to specific characteristics and behaviors of the NPs. The observed effects on bacteria could be due to ROS production, resulting in membrane damage. The teratogenicity observed on chironomid larvae could be the result of gene expression or hormonal disturbances or to decreased energy resources due to general stress or limited nutrient absorption related to gut clogging by the NPs. We observed that toxicity in chironomids, which are the main decomposers in this system, has a significant impact on litter decomposition, a vital process in freshwater ecosystems. Therefore, this study highlights the potential long-term, severe impact of CeO_2 NPs on aquatic environments, resulting from sub-lethal toxicity to decomposer species. It also highlights that leaf litter decomposition and mouthpart deformities in chironomids are sensitive, congruent markers, as well as the advantages of using microcosms for NP ecotoxicity assessments. Thus, the simultaneous use of microcosms and sensitive markers of toxicity should be more widely used in the field of nanoecotoxicology.

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