Can photocatalytic and magnetic nanoparticles be a threat to aquatic detrital food webs?

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ABSTRACT

Freshwaters are likely to serve as reservoirs for engineered nanomaterials (ENMs) due to their accelerated production and usage, increasing the relevance of assessing their impacts on aquatic biota and the ecosystem processes they drive. Stream-dwelling microbes, particularly fungi, and invertebrate shredders play an essential role in the decomposition of organic matter and transfer of energy to higher trophic levels. We assessed the impacts of two photocatalytic (nano-TiO_2 and nano-Er:TiO_2) and one magnetic (nano-$CoFe_2O_4$) ENMs on detrital-based food webs in freshwaters by exposing chestnut leaves, colonized by stream-dwelling microbes, to a series of concentrations (0.25–150 mg L^{-1}) of these ENMs. Microbial decomposition and biomass of fungal communities, associated with leaves, were not affected by the ENMs. However, the activities of antioxidant enzymes of microbial decomposers were stimulated by ENMs in a concentration-dependent way, suggesting oxidative stress in stream microbial communities. The stronger responses of these stress biomarkers against nano-TiO_2 suggest a higher toxicity of this ENM comparing to the others. To determine whether the effects could be transferred across trophic levels, the invertebrate shredder *Sericostoma* sp. was exposed to ENMs (1 and 50 mg L^{-1}) for 5 days either via contaminated water or contaminated food (leaf litter). Leaf consumption rate by shredders decreased with increasing concentrations of ENMs via food or water; the effects were more pronounced when exposure occurred via contaminated food. Overall, the tested photocatalytic and magnetic ENMs can be harmful to microbes and invertebrates that drive detrital food webs in streams at predicted environmentally relevant concentrations.

Keywords: photocatalytic and magnetic nanoparticles, stream microbial decomposers, stress biomarkers, invertebrate shredders, trophic interactions

47

48 **1. INTRODUCTION**

49 Recent developments in nanotechnology led to an increased worldwide production and
50 application of engineered nanomaterials (ENMs) (Stark et al. 2015). The TiO_2 nanoparticles
51 (nano-TiO_2) are among the most extensively used ENMs with a wide range of applications as
52 in supercapacitors, photocatalysis, sensors, personal care products, biomedicine, dye-
53 sensitized solar cells, lithium batteries, paints and food products (Chen and Mao 2007; Weir
54 et al. 2012; Tian et al. 2014). In July 2016, the European Commission allowed the application
55 of nano-TiO_2 as a UV-filter in sunscreens at a concentration up to 25% (European
56 Commission, 2016), which may further enhance the commercial use of these ENMs in
57 Europe. The estimated global production of nano-TiO_2 was about 10^4 tonnes per year, and
58 might even be higher in Europe (Piccinno et al. 2012).

59 Nanoparticles of TiO_2 are often applied in wastewater effluent treatments and chlorine-free
60 disinfection due to their photocatalytic properties (Rickerby 2014). However, the nonporous
61 structure of the bare nano-TiO_2 and their aggregation capacity in water may limit the
62 photocatalytic and adsorption of organic contaminants in aquatic environments. Nano-TiO_2,
63 doped with rare earth metals, like erbium (nano-Er:TiO_2), can enhance the photocatalytic
64 performance because of the vacant f-orbitals of Er^{3+} that allow intermediate energy states
65 (reducing the band gap), improving the adsorption of various molecules (*e.g.* amines,
66 alcohols, aldehydes, amines thiols) from contaminants onto the nanoparticle surface (Gomez
67 et al. 2012; Martins et al. 2014). On the other hand, due to suitable physicochemical and
68 magnetic properties, cobalt-ferrite nanoparticles (nano-$CoFe_2O_4$) undergo increasing
69 applications in biomedical engineering, including drug delivery, magnetic separation and
70 purification, biosensor, magnetic resonance imaging, cancer therapy and hyperthermia
71 (Cardoso et al. 2018; Srinivasan et al. 2018). Nanoparticles of $CoFe_2O_4$ have potential to

remove anionic dyes (Yavari et al. 2016) and to treat metal-rich industrial effluents or wastewaters (Srivastava et al. 2016).

Due to the vast applications and use of these ENMs, they are likely to be present in significant amounts in aquatic environments. Indeed, nanoparticles of TiO_2 were detected in groundwater and drinking water as a consequence of their release from house facades into the nearby stream or from urban runoffs (Kaegi et al. 2008; Kiser et al. 2009; Westerhoff et al. 2011). Adverse effects of TiO_2 on aquatic organisms including bacteria, microalgae, invertebrates and vertebrates have been reported (Federici et al. 2007; Li et al. 2014b; Schaumann et al. 2015; Girardello et al. 2016). Although bare or Er-doped nano-TiO_2 are expected to be biocompatible to humans (Martins et al. 2014; European Commission 2016), the impacts of nano-Er:TiO_2 on aquatic organisms are unknown. On the other hand, the few ecotoxicological studies with nano-$CoFe_2O_4$ showed toxicity against plant-pathogenic fungi (Sharma et al. 2017) and to freshwater algae and fish (Ahmad et al. 2015a; Ahmad et al. 2015b).

In forest streams, plant litter breakdown is a key ecosystem process driven by microbes, predominantly fungi, and invertebrate shredders that transfer nutrients and energy from plant litter of riparian trees to higher trophic levels (Graça 2001). Invertebrate shredders generally prefer to feed on leaf litter colonized by stream microbial communities because microbial activities and biomass improve leaf litter quality and its palatability (Graça 2001). However, the knowledge on the impacts of nano-TiO_2, nano-Er:TiO_2 and nano-$CoFe_2O_4$ on detritus-based food webs is lacking.

The current study aims to evaluate the effects of nano-TiO_2, nano-Er:TiO_2 and nano-$CoFe_2O_4$ on stream microbial decomposer communities and invertebrate shredders using the detrital model system, which has proven sensitive to various contaminants (Pradhan et al. 2011; Pradhan et al. 2015a; Tlili et al. 2016). We hypothesized that ENMs would i) reduce

97 microbial decomposition and the biomass of leaf-associated fungi; ii) induce oxidative stress
98 in microbial decomposer communities; and iii) decrease leaf litter consumption by
99 invertebrate shredders, mainly when animals were exposed via contaminated food.
100
101 **2. MATERIALS AND METHODS**
102 **2.1. Synthesis and physicochemical characterization of ENMs**
103 Titanium dioxide nanoparticles (nano-TiO_2 P25; ~21 nm, ≥99.5%, CAS No. 13463-67-7) and
104 Cobalt ferrite nanoparticles (nano-$CoFe_2O_4$; 35-55 nm, 98%, density: ~5.3 g cm^{-3}, CAS No.
105 12052-28-7) were purchased from Evonik (Evonik Industries AG, Essen, Germany) and
106 Nanoamor (Nanostructured & Amorphous Materials Inc, Katy, USA), respectively.
107 The TiO_2 nanoparticles doped with erbium (nano-Er:TiO_2) were synthesised according to
108 Gomez et al. (2012) and Martins et al. (2014). Briefly, titanium(IV) isopropoxide (97%,
109 Sigma-Aldrich) was mixed (in 1:15 v:v ratio) with analytical grade absolute ethanol
110 (Panreac). Afterwards, acetic acid (1:10 v:v; Panreac) and Er(III) nitrate pentahydrate (14.7
111 mg; Sigma-Aldrich) were added to get Er:TiO_2 atomic ratios of 0.005 (0.5% Er). Deionised
112 water (5 mL) was added after 5 min (under magnetic stirring) and the solution was shifted to
113 a Teflon-lined steel autoclave, heated in a microwave oven (15 min, 120°C). The produced
114 ENMs were centrifuged (4536 × g, for 20 min) to remove debris and resuspended in absolute
115 ethanol (for 3 min) in sonication bath (42 kHz, 100 W, Branson 2510, Danbury, CT, USA).
116 This process was repeated twice and the nano-Er:TiO_2 were placed overnight (at 80°C) in
117 oven (Gomez et al. 2012; Martins et al. 2014).
118 The morphology of the primary particles was monitored by transmission electron microscopy
119 (TEM, Tecnai T20, FEI). The ENMs were sonicated for 5 min to achieve a homogeneous
120 dispersion; a drop of the solution was placed on a copper grid and dried at room temperature
121 (RT). The crystallinity of the ENMs was assessed by X-ray powder diffraction (XRD) with

Philips X'Pert instrument equipped with Cu K_α radiation (λ = 1.54178Å) at 40 kV/50 mA. The hydrodynamic diameter and zeta potential (ζ) of the ENMs were determined using Zetasizer (NANO ZS-ZEN3600, Malvern Instruments Limited, UK), in backscatter mode (173°). The analyses were performed at 25°C by dispersing 10 mg of ENMs in 100 mL of ultra-pure water (to avoid multicasting). The suspension was sonicated for 30 min, and aliquots were used to estimate the mean hydrodynamic diameter from the intensity-weighted distributions (Zeta-average), as well as the polydispersity index (PdI), and Zeta-potential values (Zetasizer 6.20 software).

2.2. Stream microbial colonization on leaves

Leaves of *Castanea sativa* (L.) (chestnut) were collected during autumn and air-dried at RT. Chestnut is one of the dominant riparian plant species in Northwest Portugal. The chestnut leaves were cut into discs (12-mm) and placed into fine-mesh (0.5-mm) bags (to minimize the access of benthic macroinvertebrates), and immersed for 12 days in Algeriz Stream (41°35'24.56"N, 8°22'36.96"W) to allow colonization by stream-dwelling microbes. The stream was situated in a low populated area. At the sampling site, the width and depth of the stream were 0.5–0.8 m and 0.3–0.4 m, respectively; the geological substratum was composed mostly of sand and pebbles.

The physicochemical properties of stream water, measured *in situ* using multiparametric field probes (Multiline F/set 3 no. 400327, WTW, Weilheim, Germany), were: pH, 6.4 ± 0.2; temperature, 13.5 ± 0.2°C; dissolved oxygen, 9.2 ± 0.1 mg L^{-1}; and conductivity, 31 µS cm^{-1}. Concentrations of NO_3^-–N (0.14 ± 0.01 mg L^{-1}; HACH, programme 355), PO_4^{3-}–P (0.02 ± 0.001 mg L^{-1}; HACH kit, programme 480) and NH_3–N (0 mg L^{-1}; HACH kit, programme 385) were determined with a HACH DR/2000 (HACH, Loveland, CO, USA) in the laboratory.

2.3. Exposure in microcosms

147 After 12 days, leaf bags were retrieved from the stream and taken to the laboratory where the
148 leaf discs were carefully washed and allocated to 150-mL Erlenmeyer flasks (microcosms).
149 Stock suspensions (1500 mg L^{-1}) of nano-TiO_2, nano-Er:TiO_2 and nano-$CoFe_2O_4$ were
150 prepared in mineral water followed by sonication (Pradhan et al. 2011). Composition of the
151 mineral water was: pH 5.8 ± 0.2, silica 9.5 ± 2 mg L^{-1}, sodium 4.1 ± 0.4 mg L^{-1}, potassium
152 0.6 ± 0.1 mg L^{-1}, calcium 1.3 ± 0.3 mg L^{-1}, chloride 4.1 ± 0.5 mg L^{-1}, sulphate 1 ± 0.2 mg L^{-1}
153 , and bicarbonate 8 ± 0.6 mg L^{-1} (Fastio®, Gerês Mountain, Portugal). A gradient of
154 concentrations of each type of ENMs (0.25, 1, 10, 50 and 150 mg L^{-1}) was prepared by
155 diluting the stock suspension with mineral water to get 90 mL of final volume in each
156 microcosm. Mineral water without ENMs was used as controls. Three replicates were used
157 per treatment. All microcosms were incubated at 14°C for 21 days under shaking (140 rpm),
158 and water suspensions were renewed every 7 days. The experiment was performed in the
159 absence of light, because TiO_2 is photosensitive, especially reactive to ultraviolet radiation
160 (Rickerby 2014; Li et al. 2014b).

161 **2.4. Loss of leaf mass**

162 The mass loss of chestnut leaves was estimated by weighing (up to 0.001 mg) lyophilized
163 (Christ alpha 2–4, B. Braun, Germany) leaf discs before and after the microbial colonization
164 in Algeriz Stream, and after the microcosm experiment. Initial leaf mass was determined by
165 immersing 3 leaf bags in the stream for 30 min, and the leaf discs were subsequently
166 lyophilized and weighed.

167 **2.5. Fungal biomass**

168 To determine fungal biomass, ergosterol, a sterol present in fungal cell membranes, was
169 quantified by ultra-high-performance liquid chromatography (UltiMate 3000, Thermo
170 Scientific UHPLC system) using a LiChrospher 100 RP18 (5 μm) column (Merck) in 6
171 lyophilized chestnut leaf discs per replicate. Lipid extraction was carried out from the

chestnut leaf discs by heating (80°C, 45 min) in KOH-methanol (0.8%), before purified by solid-phase extraction and eluted in isopropanol (Sigma-Aldrich, analytical grade). Ergosterol peaks were monitored at 282 nm and eluted (at 1.4 mL min^{-1}) with methanol (Sigma-Aldrich, HPLC-grade). The concentrations of ergosterol from the samples were computed using a standard curve (Sigma-Aldrich) in isopropanol. The extracted ergosterol was converted to fungal biomass considering the factor of 5.5 μg of ergosterol per mg dry biomass (Gessner and Chauvet 1993).

2.6. Activities of antioxidant enzymes

For determining the activities of antioxidant enzymes (glutathione peroxidase: GPx, glutathione S-transferase: GST, and catalase: CAT) in microbial communities on chestnut leaves, 15 leaf discs from each microcosm were retrieved, washed thrice with ultrapure water, and frozen in liquid nitrogen (to prevent biological activities). Leaf discs were homogenised (Utratratrax T 25, IKA, Staufen, Germany) using potassium phosphate (K-phosphate, 0.1 M, pH 7.4) buffer (1:10 w:v) and PMSF (phenylmethylsulfonyl fluoride as protease inhibitor, 1 mM) at 4°C. The leaf homogenates were centrifuged (10,000 × g, 20 min, 4°C) and the supernatants (cell-free extract: CFE) were separated and frozen at –80°C in several aliquots till the measurement of the activities of antioxidant enzymes.

Protein concentration was measured in the CFE according to Bradford (1976) in 96-well flat-bottomed microplates and expressed per unit mass of leaves. The activities of the antioxidant enzymes were measured in CFE using a spectrophotometer (SpectraMax Plus 384 Microplate Reader, Molecular Devices) and normalized to the protein concentration. The activity of GST was determined by measuring the formation of 1-glutathione-2,4-dinitrobenzene resulting from the conjugation of GSH with the substrate 1-chloro-2,4-dinitrobenzene (CDNB) (Habig et al.; Barros et al. 2019a). The cell-free extract was added to the reaction mixture (1:3 v:v) containing K-phosphate (0.1 M, pH 6.5) buffer, GSH (1.5 mM) and CDNB

(1.5 mM). The GST activity was computed from the slope of absorbance curve (at 340 nm, ε = 9.6 mM^{-1} cm^{-1}).

For CAT activity, the CFE was added to a reaction mixture (1:11 v:v) containing K-phosphate (0.05 M, pH 7.0) buffer and H_2O_2 (30 mM). The CAT activity was calculated from the slope of decrease in absorbance (at 240 nm, ε = 0.04 mM^{-1} cm^{-1}) due to the dismutation of H_2O_2 (Claiborne 1985; Barros et al. 2019a).

For activity of GPx, the CFE was added to a reaction mixture (1:29 v:v) containing K-phosphate (0.05 M, pH 7.0) buffer, EDTA (1 mM), GSH (reduced glutathione, 1 mM), NaN_3 (1 mM), NADPH (reduced nicotinamide adenine dinucleotide phosphate, 0.24 mM), H_2O_2 (0.25 mM) and GR (0.2 U). H_2O_2 served as substrate and NaN_3 prevented CAT activity. When GR reduced the GSSG (oxidized glutathione) to GSH, the oxidation of NADPH was monitored from absorbance (at 340 nm, ε = 6.2 mM^{-1} cm^{-1}) and the GPx activity was computed from the slope (Flohé and Günzler 1984; Barros et al. 2019a).

2.7. Invertebrate collection and exposure to nanoparticles

Sericostoma sp. (Latreville) is an invertebrate shredder (Trichoptera, Sericostomatidae) common in low-order streams in Southwest Europe (Bonada et al. 2008; Varandas and Cortes 2010) with good water quality. Early-stage larvae (1.1 ± 0.1 cm) of *Sericostoma* sp. were collected in upstream of the Cávado River (Northwest Portugal) and brought to the laboratory in a cold box. Shredders were placed in aquaria with mineral water (Fastio®, Gerês Mountain, Portugal) and sterilized (121°C, 20 min) sand and maintained under aeration at 16°C, with a photoperiod (12h/12h: light/dark). Shredders were allowed to feed on chestnut leaves for 28 days before the feeding experiment. To assess the potential effects of nano-TiO_2, nano-Er:TiO_2 and nano-$CoFe_2O_4$, the shredders were exposed to contaminated water or contaminated chestnut leaves for 5 days in microcosms. For exposure via water, the microcosms with mineral water (Fastio®) were supplemented with nano-TiO_2, nano-Er:TiO_2

or nano-$CoFe_2O_4$ at 1 mg L^{-1} or 50 mg L^{-1} and shredders were allowed to feed on microbially-colonized leaf discs not exposed to ENMs. For exposure via food (leaves), the microcosms were supplemented with mineral water (Fastio®) and shredders were allowed to feed on microbially-colonized leaf discs previously exposed (for 21 days) to the same concentrations of ENMs (see section 2.3). Same number of pre-exposed or unexposed microbially-colonized leaf discs enclosed in fine-mesh bags was also placed in each microcosm of the respective treatment to determine the contribution of stream-dwelling microorganisms to leaf litter breakdown during 5 days.

2.8. Rate of invertebrate feeding

The feeding rate of the shredders on chestnut leaves was determined as F_e / (S_f × t), in which F_e is the leaf consumption by shredders; S_f is the dry mass of shredders at time t (5 days). The leaf consumption by shredders was calculated as $F_e = (F_i - F_f) - (F_i \times (D_i - D_f) / D_i)$, where F_i and F_f are the initial and final dry mass of the microbially-colonized chestnut leaves provided to shredders; and D_i and D_f are the initial and the final dry mass of microbially-colonized chestnut leaves inaccessible to shredders (Pradhan et al. 2015a).

2.9. Statistical analyses

Two-way ANOVAs were applied to evaluate the effects of the concentration (0.25, 1, 10, 50 and 150 mg L^{-1}) and type of ENMs (nano-TiO_2, nano-Er:TiO_2 or nano-$CoFe_2O_4$) on leaf mass loss, fungal biomass and activities of antioxidant enzymes of microbial communities on leaves. Two-way ANOVAs were also applied to analyse the effects of the concentration (1 and 50 mg L^{-1}) and type of ENMs on the feeding rate of the shredders. ANOVAs were followed by Tukey's multiple comparisons post-hoc tests. The analyses were performed with Prism 7.0 (GraphPad software Inc., San Diego, CA, USA).

3. RESULTS

3.1. Characterization of ENMs

The TEM images (Fig. 1A-C) showed that i) the nano-TiO_2 exhibited an irregular morphology with an average size of the primary particles (PPs) ~20 nm (Fig. 1A); ii) the nano-Er:TiO_2 also had an irregular morphology (rectangular flat-face structure) with an average ~10 nm size of the PPs (Fig. 1B); and iii) the nano-$CoFe_2O_4$ were circular and irregular forming agglomerates with an estimated average size of ~200 nm (Fig. 1C). The XRD diffractogram showed intense peaks at $2\theta \approx 25.39°$, 37.13°, 37.89°, 38.65°, 48.09°, 53.99°, 55.15° and 62.81°, with respective assigned planes (101), (103), (004), (112), (200), (105), (211) and (118) ascribed to anatase phase (Joint Committee on Powder Diffraction Standard - JCPDS Card no. 21-1272). Additionally, the reflexes with distributed planes at $2\theta \approx 27.49°$ (110), 36.15° (101) and 56.65° (220) suggested the standard spectrum of rutile (R) phase (JCPDS Card no. 88-1175). Similarly, for nano-Er:TiO_2, the obtained reflexes with distributed planes at $2\theta = 25.3°$ (101), 37.8° (004), 48.0° (200), 53.9° (105), 55.1° (204) and 62.7° (116) (Fig. 1E) corresponded to the titanium crystal structure characteristics of anatase phase in agreement with JCPDS (2000) (Martins et al. 2014). The nano-$CoFe_2O_4$ showed reflexes with distributed planes at $2\theta = 18.6^{o}$ (111), 30.4^{o} (220), 35.7^{o} (311), 43.4^{o} (400), 53.8^{o} (422), 57.2^{o} (511) and 62.9^{o} (440) (JCPDS Card no. 22-1086) (Fig. 1F), corresponding to a cubic phase (Habibi and Parhizkar 2015). The DLS revealed that the average hydrodynamic diameter (HDD) of the nano-TiO_2 and nano-Er:TiO_2 were 222.1 ± 4 nm and 241.5 ± 23 nm, respectively, whereas the mean HDD of the nano-$CoFe_2O_4$ was 472.6 ± 48 nm (Fig. 2A-C). The polydispersity index (PdI) of the nano-TiO_2 was 0.243 ± 0.011; whereas the PdI of the nano-Er:TiO_2 and the nano-$CoFe_2O_4$ were 0.449 ± 0.019 and 0.467 ± 0.047, respectively. The mean zeta potential (ζ) of the ENMs varied with pH (Fig. 2D-F); at the exposure pH (5.8), the zeta potential of the nano-TiO_2, nano-Er:TiO_2 and nano-$CoFe_2O_4$ were 17.5 mV, -16 mV and 20 mV, respectively (Fig. 2D-F).

3.2. Effects of ENMs on decomposition of chestnut leaves and fungal biomass

After 3 weeks of exposure, the concentration and type of ENMs did not show any significant effect on the leaf mass loss driven by microbes, as the remaining mass under treatments did not differ from control (two-way ANOVA, $P>0.05$) (Fig. 3A). Also, fungal biomass was not significantly affected by the concentration or type of ENMs (two-way ANOVA, $P>0.05$) (Fig. 3B).

3.3. Responses of antioxidant enzymes of microbial decomposer communities

In control microcosms, CAT activity of microbial communities on leaves was 11.5 μmol min^{-1} mg^{-1} protein (Fig. 4A). The concentration and type of ENMs significantly affected the activity of CAT (two-way ANOVA, $P<0.0001$). CAT activity strongly increased at all concentrations of all ENMs ($P<0.05$), except for the lowest concentration (0.25 mg L^{-1}) of nano-Er:TiO_2 ($P>0.05$). The maximum increase was obtained at the highest concentration (150 mg L^{-1}) of nano-TiO_2 (837.5%), followed by nano-$CoFe_2O_4$ (693.9%) and nano-Er:TiO_2 (589.6%) (Fig. 4A).

In the control, the GPx activity of microbial decomposers was 139.5 nmol min^{-1} mg^{-1} protein. The activity increased significantly upon exposure to different concentrations and types of ENMs (two-way ANOVA, $P<0.0001$; Fig. 4B). At the lowest concentration, the GPx activity significantly increased under exposure to nano-$CoFe_2O_4$ (230.5%; $P<0.05$) and nano-TiO_2 (173.2%; $P<0.05$), but not to nano-Er:TiO_2 ($P>0.05$). At the highest concentration, nano-TiO_2 led to the maximum increase in GPx activity (1546.8%), while nano-Er:TiO_2 and nano-$CoFe_2O_4$ increased the activity to 503.1% and 496.3%, respectively (Fig. 4B).

In the control, the activity of GST in microbial communities was 11.2 nmol min^{-1} mg^{-1} protein. GST activity was significantly stimulated by increased concentration and varied with the type of ENMs (two-way ANOVA, $P<0.0001$; Fig. 4C). The activity of GST increased in a dose-dependent manner under exposure to all ENMs ($P<0.05$), except at the lowest

297 concentration of nano-Er:TiO_2 (P>0.05). The maximum increase in GST activity was
298 observed upon exposure to the highest concentration of nano-TiO_2 (1154.6%), followed by
299 nano-$CoFe_2O_4$ (814.6%) and nano-Er:TiO_2 (539.9%) (Fig. 4C).

300 **3.4. Effects of ENMs on the feeding rate of invertebrate shredders**

301 After 5 days, in the absence of ENMs, the feeding rate of invertebrate shredders on
302 microbially-colonized leaves was 0.15 mg leaf mass mg^{-1} animal mass day^{-1} (Fig. 5). The
303 shredder feeding rate was affected significantly by the concentration of ENMs (two-way
304 ANOVA, P<0.0001) irrespective of their type (P>0.05), when animals were fed on
305 contaminated leaves (via food, Fig 5A). The exposure to 1 mg L^{-1} of nano-Er:TiO_2, nano-
306 TiO_2 and nano-$CoFe_2O_4$ via food led to 86.3%, 88.8% and 89.3% inhibition (P<0.05) in the
307 feeding rates, respectively. The exposure at 50 mg L^{-1} also led to a severe inhibition (P<0.05)
308 in the feeding rate by nano-Er:TiO_2 (99.3%), followed by nano-$CoFe_2O_4$ (90.7%) and nano-
309 TiO_2 (90.3%) (Fig. 5A). When exposure occurred via contaminated water, the feeding rate of
310 shredders was affected by the concentration of ENMs (two-way ANOVA, P<0.0005), but not
311 by the ENM type (P>0.05) (Fig. 5B). The waterborne exposure to ENMs at 1 mg L^{-1} led to a
312 significant decrease (P<0.05) in the feeding rate of shredders (up to 77.8% for nano-Er:TiO_2),
313 and the inhibition was maximum when shredders were exposed to 50 mg L^{-1} of nano-
314 $CoFe_2O_4$ (84%) (Fig. 5B).

315

316 **4. DISCUSSION**

317 Our study showed for the first time that photocatalytic and magnetic ENMs can affect key
318 players involved in organic matter breakdown in streams, such as the microbial decomposers
319 of plant litter and the invertebrate shredders. Stimulation of antioxidant enzymatic activities
320 in microbial communities was found, but the effects depended on the dose and type of the
321 ENMs. However, unlike hypothesized, the biomass of fungal communities and leaf litter

322 decomposition driven by microbes were not affected by ENMs. The absence of effects on the
323 biomass of fungal communities was found earlier after short- and long-term exposure to
324 metals (Duarte et al. 2004; Duarte et al. 2009) or nanometals (Pradhan et al. 2011). These
325 results might be the consequence of i) triggering physiological acclimation mechanisms in
326 fungi, ii) decreasing the direct contact between fungal mycelia and ENMs with the plant litter
327 tissues acting as a physical barrier, and/or iii) shifting towards a better adapted microbial
328 community (Fernandes et al. 2009; Pradhan et al. 2014). In our study, the absence of effects
329 of ENMs on microbial decomposition might be explained by the non-effects on fungal
330 biomass since fungi are considered the major microbial decomposers of plant litter (Graça
331 2001; Pascoal and Cássio 2004).
332 Despite the minimal effects of nano-TiO_2, nano-Er:TiO_2 and nano-$CoFe_2O_4$ on fungal
333 biomass or microbial decomposition, our study clearly unravelled sublethal effects of these
334 ENMs on microbial decomposers of plant litter. The strong responses of enzymatic stress
335 biomarkers in microbial communities suggest that they were under oxidative stress. The
336 activities of antioxidant enzymes from the ascorbate-glutathione cycle play active role in
337 cellular defense against reactive oxygen species (ROS), preventing the cellular damage and
338 maintaining the cellular redox homeostasis (Ayer et al. 2014); hence our results reinforce the
339 role of these enzymes as early warning biomarkers of oxidative stress induced by EMNs
340 (nano-CuO: Pradhan et el. 2015b; nano-Ag: Barros et al. 2019a; Barros et al. 2019b). In our
341 study, metal oxide nanoparticles significantly induced the activities of CAT, GPx and GST in
342 microbial decomposer communities at $\geq$ 0.5 mg L^{-1}. Microbial decomposers exposed to
343 nano-TiO_2 exhibited the highest enzymatic activities, suggesting intense oxidative stress.
344 Negative impacts of nano-TiO_2 on freshwater planktonic and biofilm communities were
345 associated with increased activities of stress biomarkers, and damages in cell-membrane and
346 DNA due to intracellular accumulation of ROS under light (Battin et al. 2009; Wang et al.

2019). ROS can be generated from the surface of the photoexcited nano-TiO_2 (Li et al. 2014a). However, that was not the case in our study as we clearly showed that the induced stress by nano-TiO_2 to microbial decomposers occurred in the dark, without any photocatalytic interference. Also, nano-TiO_2 was able to induce oxidative stress and lipid peroxidation in bacteria in the absence of light (Kumari et al. 2014; Erdem et al. 2015).

In the present study, nano-$CoFe_2O_4$ and nano-Er:TiO_2 also increased enzymatic biomarker activities in microbial decomposers, denoting oxidative stress. The information on the behaviour of nano-$CoFe_2O_4$ and nano-Er:TiO_2 in freshwater environments is scarce. However, the adsorption of nano-$CoFe_2O_4$ to the microalgae *Chlorella vulgaris* caused severe oxidative damage through the production of intracellular ROS leading to accelerated lipid peroxidation and increased activities of CAT and GST (Ahmad et al. 2015b). In our study, the lowest oxidative stress was induced by Er-doped nano-TiO_2 as indicated by the level of biomarker activities in freshwater microbes. However, the nano-Er:TiO_2 is likely to perform higher photocatalytic activity than non-doped nano-TiO_2 (Martins et al. 2014), which in turn may cause severe oxidative damage in the presence of light.

In our study, metal ions released from the surface of ENMs might have played a role in inducing oxidative stress; however, the underlying mechanisms in the absence of light are not clear. Depending on the environmental conditions, nano-TiO_2 can release Ti^{4+} ions from the nanoparticle surface. In fact, enhanced attachment of nano-TiO_2 to the microbial cell surface may occur in dark (Dalai et al. 2012) which may lead to the release of Ti^{4+} from nanoparticles outside the cells (Dasari and Hwang 2013). In our study, physicochemical characterization (based on TEM and XRD) showed that the primary particles of nano-Er:TiO_2 and nano-TiO_2 were smaller than nano-$CoFe_2O_4$; whereas the hydrodynamic size, PdI and zeta potential data indicated relatively lower agglomeration and higher dispersity and stability of nano-TiO_2 in suspensions, explaining the strongest effects of these nanoparticles among all tested ENMs.

In our study, the possible action mechanisms of nano-TiO_2 might have involved the following steps: i) interaction and adsorption of nanoparticles to microbes, ii) release of Ti^{4+} ions from surface of the outer membrane-localized nanoparticles and internalization of the ions by the cells, iii) partial internalization of the nanoparticles, iv) release of Ti^{4+} ions in acidic condition of the lysosome-like organelles, and v) reduction of Ti^{4+} to Ti^{3+} by peroxides via pseudo-Fenton-type reaction and reoxidation ($Ti^{4+} + H_2O_2 \rightarrow Ti^{3+} + OH^- + \bullet OH$; $Ti^{3+} + O_2 \rightarrow Ti^{4+} + \bullet O_2^-$), resulting in ROS generation that induced oxidative stress (Dodd and Jha 2011; Dalai et al. 2012; Pradhan et al. 2015b; Liu et al. 2017). Similar mechanisms are expected for nano-Er:TiO_2; but their relatively lesser stability and higher agglomeration compared to the bare nano-TiO_2 might have contributed to induce less oxidative stress in microbial decomposer communities. Moreover, the doping with Er might have decreased the surface release of Ti^{4+} ions. On the other hand, relatively greater primary particle size and higher agglomeration of nano-$CoFe_2O_4$ might have led to the less negative effects of these nanoparticles. These magnetic nanoparticles might have been attached to microbial cells, and Co^{2+} and Fe^{3+} ions released from the surface of the nanoparticles could be internalized by the cells where the ions might have undergone pseudo-Fenton-type reactions to generate ROS and induce oxidative stress (Novak et al. 2013; Ahmad et al. 2015b; Pradhan et al. 2015b). Co^{2+} ions appeared to be more toxic than nano-$CoFe_2O_4$, and intracellular accumulation of Co^{2+} have been shown while nano-$CoFe_2O_4$ were not retained *in vivo* (Novak et al. 2013).

Our results also showed that photocatalytic and magnetic ENMs can affect stream invertebrate shredder performances. Negative effects of nano-TiO_2 on freshwater invertebrates were reported earlier (Menard et al. 2011; Girardello et al. 2016). Changes in the feeding activity of invertebrates may have dramatic ecological consequences and have often been used to assess sublethal effects of nano-metal oxides (Buffet et al. 2011; Pradhan et al. 2012; Pradhan et al. 2015a). In the present study, the feeding rate of *Sericostoma* sp. on

microbially colonized leaves in the absence of ENMs was within the conventional range (0.04-0.5 mg leaf mass mg^{-1}animal mass day^{-1}) documented for invertebrate shredders in streams (Arsuffi and Suberkropp 1989). The feeding rate decreased significantly upon exposure to all ENMs, even at the lowest concentration (1 mg L^{-1}) via contaminated food or water. The lowest observed effect concentration on shredder feeding rate in our study was similar to the hazard concentration (HC_{50}: 1.1 mg L^{-1}) of nano-TiO_2 estimated for freshwater secondary consumers, predominantly invertebrates (Semenzin et al. 2015).

The reduced feeding rate of the shredders probably resulted from the food avoidance behaviour (Wilding and Maltby 2006; Pradhan et al. 2012; Pradhan et al. 2015a). In our study, the effects of ENMs on feeding rate via contaminated leaves were more pronounced than via contaminated water, which was probably due to the decreased quality and palatability of the chestnut leaves after 21 days of exposure to the ENMs. The exposure to ENMs might have led to high adsorption and accumulation of metals and/or nanoparticles to leaves (Pradhan et al. 2012) and aquatic fungi (Barros et al. 2019b). Indeed, an earlier study on trophic transfer of nano-TiO_2 in freshwaters demonstrated that, comparing to aqueous exposure, the dietary intake could constitute the main route of ENM exposure to higher trophic levels (Zhu et al. 2010). In addition to the decrease in food quality, the aqueous or dietary exposure of shredders to ENMs probably led to their accumulation in the gut, inducing oxidative stress to the invertebrate shredders (Pradhan et al. 2015a; Girardello et al. 2016).

In our study, the adverse effects of ENMs on microbial decomposers and invertebrate shredders in stream detrital food web were observed even at concentrations predicted to be environmentally relevant (Gottschalk et al. 2013; Xia et al. 2017). On the other hand, the effects of ENMs at higher concentrations may mimic the conditions of wastewaters, mine-

421 drainage streams or accidental spills and, therefore, are also relevant to be considered for
422 environmental safety.
423
424 **5. CONCLUSIONS**
425 Overall, the responses of enzymatic biomarkers revealed that nano-TiO_2, nano-Er:TiO_2, and
426 nano-$CoFe_2O_4$ induced oxidative stress in microbial decomposer communities involved in the
427 decomposition of plant litter in streams. The effects increased in a dose-dependent manner for
428 all ENMs, although the effects of nano-TiO_2 were the most pronounced. All three ENMs
429 were able to decrease the feeding rate of the invertebrate shredder *Sericostoma* sp. via
430 aqueous and dietary exposure. The effects on the feeding rate were stronger when the
431 shredders were exposed to ENMs via contaminated food (leaves). To our knowledge, our
432 study is the first to show the harmful effects of erbium-doped nano-TiO_2 and nano-$CoFe_2O_4$
433 on microbial decomposer communities and invertebrate shredders with a key role in detrital
434 food webs in streams. Our study also provided evidence that photocatalytic and magnetic
435 ENMs can induce negative effects even in the absence of light at predicted environmentally
436 relevant concentrations. These findings pinpoint that stream detrital food webs may have
437 potential for ecological risk assessment of emergent contaminants in complex realistic
438 environments.
439
440 **Declaration of competing interests**
441 The authors declare that they have no known competing financial interests or personal
442 relationships that could have appeared to influence the work reported in this paper.
443 **Acknowledgement**

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672

673 **FIGURE LEGENDS:**

674 **Figure 1** TEM micrographs of nano-TiO_2 (A), nano-Er:TiO_2 (B), and nano-$CoFe_2O_4$ (C).
675 XRD patterns of nano-TiO_2 (D), nano-Er:TiO_2 (E), and nano-$CoFe_2O_4$ (F).

676 **Figure 2** Hydrodynamic size distributions of nano-TiO_2 (A), nano-Er:TiO_2 (B), and nano-
677 $CoFe_2O_4$(C) based on dynamic light scattering. Zeta potential of nano-TiO_2 (D), nano-
678 Er:TiO_2 (E), and nano-$CoFe_2O_4$ (F) at varying pH.

679 **Figure 3** Dry mass of decomposing chestnut leaves after exposure (21 days) to different
680 concentrations of nano-TiO_2, nano-Er:TiO_2, and nano-$CoFe_2O_4$ (A). Fungal biomass on
681 decomposing chestnut leaves after exposure (for 21 days) to different concentrations of nano-
682 TiO_2, nano-Er:TiO_2, and nano-$CoFe_2O_4$ (B). Mean ± SEM, n = 3.

683 **Figure 4** Activities of CAT (A), GPx (B) and GST (C) in microbial decomposer communities
684 on chestnut leaves, after exposure (21 days) to different concentrations of nano-TiO_2, nano-
685 Er:TiO_2, and nano-$CoFe_2O_4$ (B). Different letters suggest significant differences (P<0.05).
686 Mean ± SEM, n = 3.

687 **Figure 5** Feeding rate of the stream invertebrate shredder *Sericostoma* sp. after exposure (5
688 days) to different concentrations of nano-TiO_2, nano-Er:TiO_2, and nano-$CoFe_2O_4$ via
689 contaminated leaves (A) or via contaminated water (B). Different letters suggest significant
690 differences (P<0.05). Mean ± SEM, n = 3.