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Fate, subcellular distribution and biological effects of rare earth elements in a freshwater bivalve under complex exposure

Nicolas Lachaux^{*ab}, Alba Otero-Fariña^{ac}, Laetitia Minguez^a, Bénédicte Sohm^a, Julie Rétif^{cd}, Amélie Chatel^c, Laurence Poirier^d, Simon Devin^{ab}, Sandrine Pain-Devin^{ab}, Elisabeth M. Gross^{ab}, Laure Giamberini^{ab}

^aUniversité de Lorraine, CNRS, LIEC, F-57000, Metz, France

^bLTSER-Zone Atelier Moselle, F-57000, Metz, France

^cUniversité Catholique de l'Ouest, Laboratoire Mer, Biologie des Organismes, Stress, Santé, Environnement (BIOSSE), F-49000 Angers Cedex 01, France

^dNantes University, Institut des Substances et Organismes de La Mer, ISOMer, UR 2160, F-44000, Nantes, France

1 **Abstract**

2 Rare earth elements (REE) are emerging contaminants due to their increased use in
3 diverse applications including cutting-edge and green-technologies. Their environmental
4 concerns and contradicting results concerning their biological effects require an extensive
5 understanding of REE ecotoxicology. Thus, we have studied the fate, bioaccumulation and
6 biological effects of three representative REE, neodymium (Nd), gadolinium (Gd) and
7 ytterbium (Yb), individually and in mixture, using the freshwater bivalve *Corbicula fluminea*.
8 The organisms were exposed for 96 h at 1 mg L⁻¹ REE in the absence and presence of dissolved
9 organic matter (DOM) reproducing an environmental contamination. Combined analysis of the
10 fate, distribution and effects of REE at tissue and subcellular levels allowed a comprehensive
11 understanding of their behaviour, which would help improving their environmental risk
12 assessment. The bivalves accumulated significant concentrations of Nd, Gd and Yb, which were

13 decreased in the presence of DOM likely due to the formation of REE-DOM complexes that
14 reduced REE bioavailability. The accumulation of Nd, Gd and Yb differed between tissues,
15 with gills > digestive gland \geq rest of soft tissues > hemolymph. In the gills and in the digestive
16 gland, Nd, Gd and Yb were mostly (>90 %) distributed among metal sensitive organelles,
17 cellular debris and detoxified metal-rich granules. Gadolinium, Yb and especially Nd decreased
18 lysosome size in the digestive gland and disturbed osmo- and iono-regulation of *C. fluminea* by
19 decreasing Na concentrations in the hemolymph and Ca²⁺ ATPase activity in the gills.
20 Individual and mixed Nd, Gd and Yb exhibited numerous similarities and some differences in
21 terms of fate, accumulation and biological effects, possibly because they have common abiotic
22 and biotic ligands but different affinities for the latter. In most cases, individual and mixed
23 effects of Nd, Gd, Yb were similar suggesting that additivity approach is suitable for the
24 environmental risk assessment of REE mixtures.

25 **Keywords:** lanthanide; mixture; ecotoxicity; bioaccumulation; dissolved organic matter,
26 *Corbicula fluminea*

27 **1. Introduction**

28 Rare earth elements (REE) are a group of seventeen metals composed of scandium (Sc),
29 yttrium (Y) and the lanthanide series, which include lanthanum (La), cerium (Ce),
30 praseodymium (Pr), neodymium (Nd), samarium (Sm), europium (Eu), gadolinium (Gd),
31 terbium (Tb), dysprosium (Dy), holmium (Ho), erbium (Er), thulium (Tm), ytterbium (Yb) and
32 lutetium (Lu). These elements share similar physicochemical properties, including electronic
33 structure, oxidation state (+III) and atomic radius size. Based on their molecular weight, REE
34 are divided in three groups: light REE (LREE) from La to Nd; medium REE (MREE) from Sm
35 to Gd and heavy REE (HREE) from Tb to Lu. The ionic radius size of lanthanides is relatively
36 close but it decreases progressively from La to Lu due to a phenomenon called “lanthanide
37 contraction”. Due to their specific physicochemical properties, REE production and uses in
38 diverse applications (cutting-edge technologies, renewable energy production, industry,
39 medicine...) are growing, which is expected to continue in the future (Gregoir, 2022). From
40 mining extraction through production and use to e-waste management, REE are released into
41 all compartments of the environment, which raised concerns because of their potential
42 accumulation and toxicity, notably towards aquatic organisms (Gwenzi et al., 2018). Despite
43 the increasing amount of ecotoxicological studies, several knowledge gaps remain to be filled
44 concerning their fate, bioaccumulation and biological effects (Arienzo et al., 2022; Blinova et
45 al., 2020), as the ones described below. It is not clear whether all REE have a common pattern
46 of biological effects. Some authors reported a similar toxicity among REE (Blinova et al., 2018;
47 Huang et al., 2022; Lachaux et al., 2022b; Morel et al., 2021; Tai et al., 2010), which may be
48 related to their similar physicochemical properties that influence their chemical behaviour,
49 bioaccumulation pathway and toxicity mechanisms. On the contrary, other studies showed a
50 higher toxicity or ecotoxicological risk of HREE (González et al., 2015; Gu et al., 2023, 2022,
51 2020; Manusadžianas et al., 2020) or LREE (Blaise et al., 2018; Lin et al., 2022). Environmental

52 risk assessment (ERA) of REE on freshwater ecosystems is limited because ecotoxicological
53 data focus on standard species and because existing studies are poorly representative of
54 environmental complexity. For instance, exposure media of standard tests are exclusively
55 mineral omitting the ubiquitous dissolved organic matter (DOM), which is likely to interact
56 with REE and thus influence their speciation (Davranche et al., 2017; Tang and Johannesson,
57 2003), bioaccumulation and toxicity (Lachaux et al., 2022a).

58 In the present study, we analyse REE from their distribution in water and organisms until
59 their biological effects at different levels of organization. This integrated approach aims at a
60 comprehensive understanding of the mechanisms running REE bioaccumulation and toxicity
61 using the bivalve *Corbicula fluminea*. This widespread filter-feeding organism represents a
62 relevant part of freshwater ecosystems with often high biomass being essential for regulation
63 of phytoplankton density and water depuration among others, which make this species a
64 relevant model organism (Araujo et al., 1993). In addition, it is worldwide distributed allowing
65 for comparisons of biological responses at a global scale (Pigneur et al., 2014). Its filtration
66 activity leads to the bioaccumulation of environmental contaminants to which it is sensitive,
67 making *C. fluminea* especially suitable for biomonitoring and assessing pollutants effects (Guo
68 and Feng, 2018). Its use in this study aimed to provide new sublethal ecotoxicological data of
69 REE on freshwater bivalves and more globally on benthic species, which despite their role in
70 ecosystem processes have been rarely used for regulatory purposes. Thus, *C. fluminea* was
71 exposed to Nd, Gd and Yb, which are representative of LREE, MREE and HREE, respectively.
72 These three REE are of specific concern because of their increasing uses (Nd in permanent
73 magnet), their occurrence in freshwaters (Gd is worldwide distributed) and their lack of
74 ecotoxicological data (Yb is one of the least studied REE) (Blinova et al., 2020; Gregoir, 2022;
75 Trapasso et al., 2021). We have compared their fate, bioaccumulation and biological effects to
76 decipher whether REE represent a uniform group. The three REE have been tested individually

77 and in mixture, in the absence and presence of DOM to reproduce complex exposure being
78 closer to environmental conditions because REE and DOM occur together in natural
79 freshwaters. In addition, the use of DOM can modify REE speciation, which is important to
80 assess its influence on REE bioaccumulation and toxicity in an environmentally realistic way.
81 In most previous works, REE bioaccumulation was measured in the whole soft tissues of the
82 marine bivalve *Mytilus galloprovincialis* (Akagi and Edanami, 2017; Cunha et al., 2022; Freitas
83 et al., 2020b; Henriques et al., 2019; Pinto et al., 2019). In the present study, Nd, Gd and Yb
84 accumulation was investigated at the tissue (gills, digestive gland, rest of soft tissues,
85 hemolymph) and subcellular (five sub-compartments of the gills and digestive gland) levels.
86 This represents the most exhaustive description of REE distribution in a bivalve species so far.
87 At the same time, a battery of biomarkers was measured in the gills, digestive gland and in the
88 hemolymph in order to analyse energetic reserves and metabolism, osmo- and iono-regulation,
89 nervous function, antitoxic and antioxidant defences and cellular damages in *C. fluminea*.

90 2. Material and Methods

91 2.1. Preparation of test medium, rare earth elements (REE) and dissolved organic 92 matter (DOM)

93 The test medium was a reconstituted water composed of : 294 mg L⁻¹ CaCl₂, 2H₂O; 123
94 mg L⁻¹ MgSO₄, 7H₂O; 65 mg L⁻¹ NaHCO₃; 6 mg L⁻¹ KCl. It was previously oxygenated with
95 an air pump and the temperature was adjusted to 9 ± 1°C to stay close to the temperature
96 measured *in situ* during the bivalve sampling. The pH was lowered and adjusted at 7.3 (using
97 HCl 3 %) to enhance REE solubility and exposure while remaining in the range of pH values
98 measured at the bivalve sampling location. In order to limit losses of REE during the experiment
99 due to their possible adsorption on glass walls, the flasks used to prepare the test medium and
100 the test flasks (glass beaker, 600 mL capacity) were previously filled with the test medium and
101 REE during at least 12 h and then emptied (Lachaux et al., 2022a). At the test beginning, the
102 flasks were filled again with the test medium spiked with REE and DOM as described below.

103 REE stock solutions (5 g L⁻¹ REE salt) were prepared by dissolution in MilliQ water of
104 chemicals (purity > 99 %) purchased from Sigma Aldrich, precisely: neodymium nitrate
105 (Nd(NO₃)₃, 6 H₂O), gadolinium chloride (GdCl₃, 6 H₂O) and ytterbium chloride (YbCl₃, 6
106 H₂O). REE exposure concentration was obtained by dilution of the stock solution in the test
107 medium.

108 The used DOM had been prepared at Wageningen University, the Netherlands, by
109 purification of local groundwater to be used as drinking water using membrane filtration.
110 Composition of the DOM stock solution in fulvic acids (>80 %), humic acids (>2 %) and
111 hydrophilic acids (≈10 %) was determined using the batch procedure described in van Zomeren
112 and Comans (2007). The tested dissolved organic carbon (DOC) concentration was 8 mg L⁻¹ C

113 because it is widely representative of rivers and lakes (Thurman, 1985). This concentration was
114 reached by dilution of the DOM solution in the test medium (Lachaux et al., 2022a).

115 **2.2. Experimental design and conditions**

116 *Corbicula fluminea* individuals (2-2.5 cm) were collected in the Meurthe river, in
117 Rosières-aux-Salines (Northeast of France), in February 2019. They were gradually
118 acclimatised to lab conditions (test medium, temperature) and fed with green microalgae
119 (*Raphidocelis subcapitata*, *Chlorella vulgaris* and *Scenedesmus subspicatus* at approximately
120 1.5×10^7 cells ind⁻¹ day⁻¹) for 10 days.

121 Afterwards, the organisms were exposed for 96 h in a beaker filled with 500 mL of test
122 medium spiked with 1 mg L⁻¹ REE. These exposure concentrations and durations are
123 representative of acute REE contamination due to mining (Liang et al., 2014) or industrial
124 (Kulaksız and Bau, 2011) activities. Nd, Gd and Yb were tested individually and in ternary
125 mixture (1/3 mg L⁻¹ of each REE) in order to assess the potential interactions between REE.
126 Two different media were tested: in the absence (0 mg L⁻¹ C) or presence (8 mg L⁻¹ C) of DOM
127 in order to estimate the influence of DOM on REE fate, bioaccumulation and biological effects.
128 Controls (no REE) with and without DOM were included to the experiment in order to verify
129 any direct influence of DOM (alone) on the results because DOM may affect organisms
130 (Steinberg et al., 2008). For each experimental group (C, COM, Nd, NdOM, Gd, GdOM, Yb,
131 YbOM, Mix, MixOM where C = control, Mix = mixture and OM = organic matter = 8 mg L⁻¹
132 C), 30 individuals were divided into 6 beakers (5 individuals per beaker).

133 The test medium was changed daily to renew dissolved oxygen concentration and ensure
134 semi-static exposure because REE concentrations tend to decrease significantly over time
135 (Blinova et al., 2018; González et al., 2015; Romero-Freire et al., 2019). The pH was monitored
136 for 24 h and confirmed that it was maintained at 7.3 ± 0.1 . The photoperiod was natural

137 corresponding to 11 h light / 13 h dark (in February in North East France). The bivalves were
138 not fed during the experiment.

139 **2.3. Analysed physiological functions**

140 After 96 h of exposure and depending on biomarkers between six and 12 bivalves (per
141 experimental group) were taken from the six beakers (= six replicates) and dissected to collect
142 the gills, the digestive gland and the rest of soft tissues, which were immediately stored at -
143 80°C until analyses. REE biological effects were assessed by measuring a battery of biomarkers
144 in the gills and in the digestive gland of the bivalves (Figure 1). The samples were prepared
145 according to Koehlé-Divo et al. (2018). Briefly, the organs were thawed and crushed with
146 metallic beads with a beadbeater (30 sec) in phosphate buffer (50 mM, pH 7.6) supplemented
147 with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM L-serine borate mixture as
148 protease inhibitor at 8:1 volume:weight ratio. The preparation was then centrifuged for 5
149 minutes (min) at 4°C at 250 g. A part of the obtained supernatant (denoted homogenate) was
150 used to measure protein ($PROT_{hom}$), triglyceride (TRIGLY) and lipid hydroperoxide (LOOH)
151 concentrations, acid phosphatase (ACP) and electron transport system (ETS) activities. The
152 remaining supernatant was firstly centrifuged at 1000 g for 20 min and then at 20000 g for 50
153 min at 4°C. The resulting supernatant corresponds to the cytosolic fraction used to measure
154 protein ($PROT_{cyt}$) and total antioxidant capacity (TAC) concentrations, lactate dehydrogenase
155 (LDH), total glutathione peroxidase (GPx), superoxide dismutase (SOD), glutathione-S-
156 transferase (GST) and caspase-3 (CSP-3) activities. These analyses were performed using the
157 automated spectrophotometer analyser Konelab 20 XTi (Thermo Scientific) adapted from the
158 protocols detailed by Garaud et al. (2016) and Bertrand et al. (2016).

159 2.3.1. Energetic reserves and metabolism and nervous system

160 Reserves represented by protein and triglyceride concentrations were measured using
161 Thermo-Scientific Konelab ready-to-use reagents. ETS activity was determined according to
162 the method described by Owens and King (1975), by monitoring formazan production at 480
163 nm resulting to the reduction of p-iodonitrotetrazolium as final electron acceptor in
164 mitochondrial respiratory chain instead of O₂. LDH was determined by monitoring lactate to
165 pyruvate oxidation by LDH, which reduces NAD to NADH, the latter being measured at 340
166 nm. ETS and LDH are indicators of aerobic and anaerobic metabolism, respectively.

167 Cholinesterase (ChE) enzymes catalyse the breakdown of some choline esters
168 (neurotransmitters) after nervous influx, which is essential for the well-functioning of nervous
169 cells. REE are likely to influence nervous influx by replacing calcium (Ca) in the voltage
170 operated Ca channels in nervous cells due the similarities of ionic radii and coordination
171 numbers between REE and Ca (Valcheva-Traykova et al., 2014). This can lead to an inhibition
172 of ChE activity (Das et al., 1988) as previously observed in *Mytilus galloprovincialis* exposed
173 to La (Pinto et al., 2019) and Gd (Henriques et al., 2019). Prior to the experiment, we measured
174 the activity of different pseudocholinesterase enzymes (acetylcholinesterase,
175 butyrylthiocholinesterase, propionyl-thiocholinesterase) in *C. fluminea* gills. A higher signal
176 was detected for propionyl-thiocholinesterase (PrSChE) activity, which was then selected for
177 the rest of analyses. PrSChE activity was measured in the absence and presence of several ChE
178 inhibitors (Iso-OMPA, serine, BW) in order to check the specificity of measured ChE activity.
179 PrSChE activity was determined in the gills by photometry according to a protocol adapted
180 from Ellman et al. (1961). Briefly, the gills were thawed and crushed with metallic beads using
181 a bead beater (30 sec) in phosphate buffer (0.1 M, pH 8). The preparation was centrifuged at
182 1000 g (20 min) and then at 10000 g (30 min). PrSChE activity was determined in the final
183 supernatant by measuring the absorbance of dithiobisnitrobenzoate (DNTB)-thiocholine

184 complex after propionylthiocholine cleavage by PrSChE. PrSChE activity was expressed
185 according to protein concentration determined using the method from Bradford (1976), which
186 consists in measuring the absorbance at 595 nm of Coomassie blue complexed to proteins.

187 2.3.2. Osmo- and iono-regulation

188 $\text{Na}^+\text{-K}^+$ ATPase and plasma membrane Ca^{2+} ATPase (PMCA) were measured on total
189 protein extract in the digestive gland and on enriched membrane extracts in gills. Digestive
190 glands were suspended in 6 mL mg^{-1} of fresh weight (FW) of Tris buffer (25 mM Tris pH 7.4,
191 1 mM PMSF) and crushed with metallic beads in a bead beater (30 sec). Homogenates were
192 centrifuged at 2000 g for 10 min at 4°C, and then supernatants were centrifuged at 10000 g for
193 20 min at 4°C. The S10000 fractions were used to measure straight away protein concentration
194 and ATPase activities. Membrane extracts were done according to the protocol of Santini et al.
195 (2011). Briefly, gills were suspended in 6 mL mg^{-1} FW in HEPES buffer (10 mM HEPES pH
196 7.2, 250 mM Sucrose, 1 mM EDTA, 1 mM PMSF) and crushed with metallic beads in a bead
197 beater (30 sec). Gill's homogenate was centrifuged at 2000 g for 10 min at 4°C, then S2000
198 fraction was centrifuged at 10000 g for 20 min at 4°C and S10000 fraction was ultracentrifuged
199 at 76000 g for 1 h at 4°C. Final pellet containing enriched membrane fraction was resuspended
200 in 4 mL mg^{-1} FW of Tris buffer. Biomarkers were measured straight away on the automated
201 chemistry analyser Konelab 20 Xti. Protein concentrations were measured using Thermo-
202 Scientific Konelab ready-to-use reagent. PMCA activity was measured on a modified protocol
203 from Santini et al. (2011). Membrane fraction (6 μL) were incubated in a final volume of 120
204 μL for 20 min at 37°C in a buffer containing 25 mM Tris (pH 7.4), 100 mM NaCl, 20 mM KCl,
205 4 mM $\text{Na}_2\text{-ATP}$, 0.5 mM NaN_3 , 0.2 mM ouabain and 10 mM of CaCl_2 for PMCA activity or 2
206 mM of EDTA to chelate Ca^{2+} free ion. The amount of Pi released was quantified at 700 nm by
207 adding 120 μL of a solution containing 560 mM de H_2SO_4 , 8,1 mM ammonium molybdate, 176

208 mM FeSO₄. A standard curve of Na₂HPO₄ dilution is used to assess the phosphate
209 concentration. The enzyme specific activity was expressed in mM Pi mg⁻¹ protein h⁻¹.

210 Na⁺-K⁺ ATPase activity was assessed as the difference of ATP hydrolysis in the presence
211 and absence of potassium, according to a modified protocol from Mosher et al. (2012).
212 Membrane fractions (6 μL) were incubated at 37°C for 20 min in a final volume of 120 μL
213 reaction mix (10 mM HEPES pH 7.2, 50 mM NaCl, 2.5 mM MgCl₂, 5 mM Na₂ ATP) with and
214 without 10.5 mM KCl. Phosphate released was assessed as described above for PMCA activity.
215 The enzyme specific activity was expressed in μM Pi mg⁻¹ protein h⁻¹.

216 Bivalve hemolymph was sampled using minicaps (5 μL) and stored at -20°C until
217 analyses. Sodium (Na), calcium (Ca), potassium (K) and magnesium (Mg) were quantified by
218 inductively coupled plasma mass spectrometry (ICP-MS, iCAP-TQ, ThermoScientific) after
219 hemolymph thawing and dilution in Milli-Q water.

220 2.3.3. Antioxidant, antitoxic defences and cellular damages

221 Several antitoxic defences were analysed to highlight any potential toxic stress induced
222 by REE. GST activity was determined by monitoring, at 340 nm, CNDB decrease resulting to
223 its conjugation with GSH being induced by GST (Habig et al., 1974). ACP was determined by
224 measuring p-nitrophenol complexation at 400-420 nm resulting to 4-nitrophenol phosphate
225 hydrolysis by ACP.

226 Metallothionein (MT) concentrations were measured by polarography using the apparatus
227 797 VA Computrace (Metrohm®) based on the protocol from Mouneyrac et al. (1998). Briefly,
228 tissues were ground on ice in two times the volume with the grinding tampon (Trizma® base +
229 NaCl, pH 8.6). Then, they were centrifuged at 14000 rpm for 55 min at 4°C. 350 μL of
230 supernatant (S1) were collected for the metal assay while the unsuspended pellet is frozen with
231 the remaining supernatant. Tubes with S1 were then placed in a water bath for 15 min at 75°C

232 and immediately incubated on ice (between 10 min and 1 h). Samples were then centrifuged for
233 10 min at 13000 rpm, 4°C and the supernatant was transferred into a new microtube and frozen
234 until analysis.

235 The digestive lysosomal system morphology was analysed by histochemical revelation
236 of β -glucuronidase in unfixed cryostat sections according to Giambérini and Cajaraville (2005).
237 Image analysis was performed on digestive tissue sections (8 μ m thick) using a microscope.
238 Four stereological parameters were calculated: surface density ($S = S_L/V_C$), volume density (V
239 $= V_L/V_C$), surface to volume ratio ($S/V = S_L/V_L$, interpreted as the inverse of lysosome size)
240 and numerical density ($N = N_L/V_C$) of lysosomes, with lower S, V and N refer to surface,
241 volume and number respectively and L, c correspond to lysosomes and cytoplasm, respectively.

242 Activities of antioxidant enzymes were measured to decipher any oxidative stress. GPx
243 activity was measured by monitoring NADPH consumption at 340 nm according to Lawrence
244 (2012). SOD activity was determined by monitoring formazan production (inversely
245 proportional to SOD activity). TAC activity was measured by monitoring AzBTS⁺ reduction
246 by all antioxidant species including enzymes (GSH reductase, catalase, peroxidase), small
247 molecules (ascorbate, uric acid, GSH, vitamin E...) and proteins (e.g. albumin, transferrin)
248 (Erel, 2004).

249 Lipid peroxidation is a cellular damage provoked by oxidative stress. It was measured by
250 monitoring Fe²⁺ oxidation by lipid hydroperoxides according to Arab and Steghens (2004).
251 CSP-3 activity was determined by measuring the fluorescence (emission 400 nm; excitation
252 505 nm) of 7-amino-4-trifluoromethylcoumarin (AFC) cleaved from Asp-Glu-Val-Asp
253 (DEVD) by CSP-3. The latter indicates cellular death.

254 Some biomarkers were not measured in the gills because of an insufficient biomass.
255 Figure 1 summarizes the design of performed analyses.

256 **2.4. REE concentration measurements and analysis**

257 Nd, Gd and Yb were quantified in the test medium and in the bivalves by ICP-MS (Nexion
258 350x PerkinElmer). Details concerning ICP-MS analyses can be found in Lachaux et al.
259 (2022a).

260 For one renewal cycle of the test medium (24h), REE concentrations were measured in
261 water immediately (t0), 1 h (t1), 6 h (t6) and 24 h (t24) after the successive addition of DOM,
262 REE and bivalves. For each experimental group, the test medium of three beakers (replicates)
263 was filtered at 0.45 μm using a mixed cellulose ester filter to dissociate the particulate phase
264 kept on the filter and the dissolved phase representing the filtrate. Determination of measured
265 dissolved ($<0.45 \mu\text{m}$, denoted [REE_{diss}]) and particulate ($>0.45 \mu\text{m}$, [REE_{part}]) concentrations
266 of REE is described in Lachaux et al. (2022a). We considered the sum of dissolved and
267 particulate concentrations as the total REE concentration ([REE_{tot}]).

268 To investigate REE accumulation and organ distribution in the bivalves, Nd, Gd and Yb
269 were quantified in gills, digestive gland, rest of soft tissues and in the hemolymph by ICP-MS.
270 After 96 h of exposure, the bivalves were depurated for 96 h (Figure 1) in clean water (test
271 medium without REE) in order to remove the potential REE that are weakly bound to the
272 tissues. After dissection, the gills, digestive gland and the rest of soft tissues were immediately
273 stored at -80°C until analysis. Before analysis, the tissues were thawed and digested with 1 mL
274 of HNO_3 65 % at 80°C .

275 In addition, REE were quantified in five subcellular fractions in the gills and the digestive
276 gland. Cellular debris (membranes, tissue fragments), metal rich granules (MRG), organelles
277 (i.e. nuclear, mitochondria, microsomes, lysosomes), heat-sensitive proteins (including a large
278 variety of enzymes) and heat-stable proteins (MT and MT-like proteins) were isolated based on
279 the protocol from Wallace et al. (2003). To do so, each tissue from individual bivalves was

280 weighed in Eppendorf tubes and grinded (~1:5 heaviest tissue to buffer ratio,) using cold TRIS
281 buffer. The obtained homogenized tissue was first fractionated by centrifugation at 1450 g for
282 15 min producing a pellet (P1) containing tissue fragments and other cellular debris (e.g.
283 membranes, nuclear, MRG) and a supernatant (S1') containing the cytosol. MRG were isolated
284 from P1 through digestion of tissue fragments with NaOH and centrifugation at 5000 g (P2).
285 The resulting supernatant (S2) contained metal associated with dissolved tissues. The 1450 g
286 supernatants (S1') were collected in special ultracentrifuge tubes and ultracentrifuged at 100
287 000 g for 1 h to produce a pellet (P3) containing various organelles (i.e. nuclear, mitochondria
288 and microsomes); the 100 000 g supernatant (S3) contained the cytosol which is collected in
289 special ultracentrifuge tubes. The cytosol was then heat-denatured (80°C) for 10-15 min and
290 then ultracentrifuged at 30 000 g for 10 min. The final pellet (P4) contained heat-sensitive
291 proteins ('enzymes'), while the supernatant (S4) contained heat-stable proteins (MT and MT-
292 like proteins). Each fraction was stored at -80°C until measurement. Investigation of subcellular
293 distribution of metals affords to assess the part of accumulated metals that is likely to induce
294 toxicity (Otero-Fariña et al., 2022). Organelles and heat-sensitive proteins (denoted "enzymes")
295 are considered as "metal-sensitive fractions" (MSF) because metal accumulation in these
296 fractions can lead to harmful effects (Wallace et al., 2003). On the contrary, metals accumulated
297 in MRG and heat-stable proteins (denoted "MT") are considered "biologically detoxified
298 metals" (BDM) (Wallace et al., 2003). It is still not clear whether metals accumulated in cellular
299 debris should be included in MSF or BDM fractions because they contain tissue fragments, cell
300 membranes and other cellular components of unknown consequence (Cardon et al., 2019).

301 The bioconcentration factor (BCF) was calculated in order to compare bioaccumulation
302 between the different experimental groups using the formula:

$$303 \quad BCF = \frac{[REE]_{ORG}}{[REE]_{diss}^{mean}} \quad (2)$$

304 with $[REE]_{ORG}$ being the total REE concentration measured in the bivalve tissues (gills +
305 digestive gland + rest of soft tissues).

306 REE mass balance was calculated in order to determine the distribution (proportion) of
307 REE among the water (dissolved and particulate phases) and the bivalves as a function of total
308 REE mass measured at the end of the experiment (Lachaux et al., 2022b).

309 Recovery of REE was estimated by calculating the ratio between the total mass of REE
310 measured at the end of the experiment and the total mass theoretically introduced at t_0 . We
311 consider the difference between theoretical (nominal) and real (measured) concentrations as
312 significant if recovery is lower than 80 % or higher than 120 % (ISO, 2012). In that case,
313 exposure concentrations of REE were corrected by determining the geometric mean
314 concentration in accordance with Romero-Freire et al. (2019):

$$315 \quad [REE]_{mean} = \sqrt{(C_{t0} \times C_{t24})} \quad (1)$$

316 with C_{t0} and C_{t24} being the concentrations measured in the water at the beginning and at the end
317 of medium renewal cycle (24 h), respectively. When using total concentration to calculate the
318 geometric mean, the latter is denoted “[REE_{tot}]mean” and when using dissolved concentration,
319 it refers to “[REE_{diss}]mean”.

320 **2.5. Data analysis**

321 Statistical analyses were conducted with R software (version 3.6.1). Data from the
322 different experimental groups were compared performing analysis of variance (AOV) when
323 normality and homoscedasticity conditions were respected (parametric test) otherwise by
324 performing Kruskal-Wallis (KW) test (non-parametric-test) in order to discriminate REE and/or
325 DOM effect on a given variable (biomarker). Tukey HSD (Miller, 1981) and the multiple
326 comparisons test from Siegel and Castellan (1988) (for parametric and non-parametric tests,
327 respectively) were conducted as post-hoc tests to identify exactly which groups differ from each

328 other. Data normality and homoscedasticity were checked with Shapiro-Wilk and Bartlett tests
329 respectively. In addition, Hedge's g statistic was calculated based on the mean and standard
330 deviation of each variable (biomarker) with the package "metafor" (Viechtbauer, 2010). Effect
331 size represents the magnitude of difference between experimental groups and was thus used as
332 a complement to AOV and KW tests to quantify the difference between groups (Sullivan and
333 Feinn, 2012). Effect sizes were calculated between exposed (Nd, NdOM, Gd, GdOM, Yb,
334 YbOM, Mix, MixOM) and control groups (C, COM) to analyse REE effects respect to the
335 control. Effect sizes were also calculated to compare mixed (Mix, MixOM) and individual
336 effects of Nd, Gd and Yb. A given effect size is considered as very small if $0 \leq g < 0.2$; small
337 if $0.2 \leq g < 0.5$; moderate if $0.5 \leq g < 0.8$; large if $0.8 \leq g < 1.3$; very large if $1.3 \leq g$ (Sullivan
338 and Feinn, 2012). The effect size is significant when confidence interval of g does not include
339 0. Data concerning subcellular distribution of REE was analysed by comparing medians of the
340 different experimental groups and calculating their respective 95 % confidence intervals (CI)
341 (BCa method, $R=1000$), using bootstrap resampling (Canty and Ripley, 2016). This method is
342 more suitable and accurate for small samples ($n = 3$) (Cline, 2019). Differences in median
343 values between groups were considered statistically significant when the 95 % CI excluded
344 zero. Relationship between two variables was evaluated by performing a linear regression test
345 of Pearson (when data normality is respected) otherwise Spearman using the whole data set for
346 each variable ($n = 60-120$ individuals). A difference was considered as statistically significant
347 in case of a p -value < 0.05 . A principal component analysis (PCA) was performed to compare
348 biological effects observed in the different experimental groups. The criterion of Kaiser-Meyer-
349 Olkin (Kaiser and Rice, 1974) was calculated ($KMO = 0.72$) and confirmed that the used dataset
350 was suitable to run factorial analysis. The PCA was based on the mean of biomarkers exhibiting
351 significant differences between experimental groups (in AOV, KW or effect size tests) in order
352 to enhance the variance explained by PCA factors (axis).

353 **3. Results**

354 **3.1. REE in the water**

355 REE concentrations measured at 0 h and 1 h were not significantly different. Then, we
356 considered REE concentration at the beginning of the renewal cycle (t_0) as the mean of the
357 concentrations measured at 0 h and 1 h.

358 The measured concentrations of Nd, Gd and Yb in water were significantly lower (<80
359 %) than nominal ones (cf. section 2.4). Total (dissolved + particulate) concentrations measured
360 at the end of medium renewal cycle (t_{24}) represented less than 80 % of nominal concentrations
361 (Table 1). REE exposure concentrations were thus corrected by calculating the geometric mean
362 of measured concentrations (= REE mean) (cf. section 2.4).

363 Total and dissolved concentrations of Nd, Gd and Yb (individually and in mixture)
364 decreased by 12-43 % from t_0 to t_{24} , whereas particulate concentrations increased by 37-130
365 % (Figure S1). The three REE (individually and in mixture) were mainly (77-93 %) measured
366 in the dissolved phase and in a lesser extent in the particulate phase (6-20 %) (Figure S2). Total
367 and dissolved concentrations of Gd were significantly lower than those of Nd and Yb. Dissolved
368 concentrations of Yb were significantly higher than those of Nd and Gd whereas particulate Yb
369 was lower than Nd. These results were observed with REE tested both individually (Table 1)
370 and in mixture (Table S1) in the absence and presence of DOM.

371 Recovery, total and dissolved concentrations of Nd, Gd and Yb tested individually (Table
372 1) and in mixture (Table S1) were significantly higher in the presence than in the absence of
373 DOM. Particulate concentrations of REE were lower in the presence of DOM respect to in its
374 absence (significant for Nd) (Table 1).

375 **3.2. REE concentrations in *C. fluminea***

376 Significant concentrations of Nd, Gd and Yb were measured in the exposed bivalves
377 compared to the controls (not exposed to REE) ranging from 58 to 85 $\mu\text{g g}^{-1}$ and from 33 to 58
378 $\mu\text{g g}^{-1}$ in the absence and presence of DOM, respectively (Table 1), which represent 1-4 % of
379 total REE measured at the end of the experiment (Figure S2). Bioaccumulation of the three REE
380 were significantly lower in the presence of DOM compared to in its absence. Bioconcentration
381 factors of Nd, Gd and Yb tested individually (Table 1) and in mixture (Table S1) were 2-3-fold
382 lower in the presence of DOM.

383 In the absence and presence of DOM, Yb bioaccumulation was significantly lower than
384 the one of Nd (Table 1). No significant differences were observed between Nd and Gd uptake.
385 Bioaccumulated concentrations of REE in mixture (73 and 38 $\mu\text{g g}^{-1}$ without and with DOM,
386 respectively) were relatively close to the average of individual accumulations of Nd, Gd and
387 Yb (73 and 48 $\mu\text{g g}^{-1}$ without and with DOM, respectively).

388 **3.3. Tissue and subcellular distribution of REE in *C. fluminea***

389 In the absence of DOM, Nd, Gd and Yb (individually and in mixture) were mostly
390 distributed in the gills (63-74 %), then in the digestive gland (11-15 %), in the rest of soft tissues
391 (14-25 %) and in a lesser extent in the hemolymph (<1 %) (Figure 2; Table 1). Similar results
392 were observed in the presence of DOM. In all studied tissues, the concentrations of the three
393 REE were lower with than without DOM (Figure 2; Table 1).

394 In the gills and in the digestive gland, Nd, Gd and Yb (individually and in mixture) were
395 mainly (>90 %) distributed among the organelles, MRG and cellular debris (Figure 3). This
396 was observed in both the absence and presence of DOM. Less than 10 % of REE were
397 accumulated in the enzymes and metallothioneins (MT).

398 The proportion of REE in the organelles was lower in the digestive gland compared to
399 the gills whereas REE proportion in MT was increased. The three REE were more accumulated
400 in MT and less in organelles (in the digestive gland) in the presence of DOM than in its absence
401 (significant for Nd). In the presence of DOM, REE were less accumulated in MRG and more
402 in cellular debris than in the absence of DOM (significant for Nd and Yb). Neodymium was
403 significantly less distributed in cellular debris (in the gills and digestive gland), enzymes (gills)
404 and more in MRG (digestive gland) compared to Gd and Yb. Ytterbium (in the absence of
405 DOM) had significantly higher concentrations in MT (in the gills and digestive gland) and
406 enzymes (gills) than Nd and Gd. In the gills, Gd accumulated significantly more in MRG (with
407 and without DOM) and less in organelles (with DOM) than Yb and Nd. No significant
408 differences were observed between Nd, Gd and Yb tested in mixture. REE accumulated more
409 in organelles and less in MRG when tested in mixture than individually (significant for Gd).

410 **3.4. Biological effects of REE and DOM**

411 Energetic reserves and metabolism, osmo- and iono-regulation and antitoxic defences
412 were modulated in the bivalves exposed to REE compared to the controls (not exposed to REE)
413 (Figure 4).

414 Protein concentrations were lower in the gills of bivalves exposed to Nd and Gd in the
415 presence of DOM. Bivalves exposed to Nd without DOM had lower triglyceride concentrations
416 and an increased LDH activity in the gills. On the contrary, LDH activity was decreased in the
417 bivalves exposed to the mixture, both with and without DOM.

418 Neodymium, Gd and Yb, individually and in mixture (with and without DOM), decreased
419 Na concentration in the hemolymph by 14 % in average (Table S2). The latter was negatively
420 correlated to the REE concentrations measured in bivalve tissues and in the organelles of the
421 digestive gland. Concentrations of K, Mg and Ca in the hemolymph were positively correlated

422 to REE concentrations measured in the hemolymph and in the tissues. Ca^{2+} ATPase activity was
423 3-fold higher in the gills than in the digestive gland and $\text{Na}^{+}\text{-K}^{+}$ ATPase activity was detected
424 only in the gills. Ca^{2+} ATPase activity was significantly decreased by 17 % in the gills of the
425 bivalves exposed to Nd compared to the controls.

426 GPx activity and MT concentrations were lower in bivalves exposed to Gd without DOM.
427 In the presence of DOM, Nd, Gd and Mix increased or decreased MT concentrations in the gills
428 and in the digestive gland. Metallothionein concentrations were positively correlated to REE
429 concentrations in bivalve tissues. Neodymium, Yb, Mix and to a lesser extent Gd decreased
430 lysosome volume (V) and/or surface (S) in the presence of DOM. Surface to volume ratio of
431 lysosomes (S/V) was significantly higher in the bivalves exposed to Nd and Gd indicating a
432 reduced size of organelles. V and S were positively correlated to the number of lysosomes (N)
433 and negatively correlated to REE concentrations measured in bivalve tissues and in the enzymes
434 and organelles of the digestive gland.

435 Neither neurological function (PrSchE), antioxidant defences (except GPx) nor cellular
436 damages were significantly affected in the bivalves exposed for 96 h to Nd, Gd and Yb. All
437 results concerning percentage of biological effect (Table S2), biomarker measurements,
438 statistical (Table S3) and correlation tests (Tables S4-S6) are available in Supplementary
439 material.

440 The principal component analysis (PCA) shows that V, S, S/V of lysosomal system and
441 Na concentration in the hemolymph are the biomarkers that most differentiate control bivalves
442 from the ones exposed to REE, especially to Nd (Figure 5). The gap between Nd and control is
443 enhanced by Ca^{2+} ATPase activity and TRIGLY concentrations along the horizontal axis that
444 explains most of data variance. The difference between Nd and control in the presence of DOM
445 is also visible along the vertical axis driven by PROT and MT concentrations (Figure 5).

446 The difference between mixed and individual effects of Nd, Gd and Yb was very small,
447 small, moderate, large and very large for 25, 32, 21, 14 and 8 % of the 174 calculated effect
448 sizes. Ten percent of the latter were significant corresponding to a lower LDH activity, S/V
449 ratio, MT concentration and higher Ca^{2+} ATPase, GPx activities, triglycerides, MT
450 concentrations and lysosome volume (V) in the bivalves exposed to the mixture compared to
451 individual Nd, Gd and Yb (Figure 6).

452 The presence of DOM influenced significantly biomarker responses by increasing ETS
453 activity, PROT and Na concentrations and by decreasing GPx activity, S/V, Ca and MT
454 concentrations respect to the absence of DOM (Table S3), which is illustrated by the PCA
455 (Figure 5). Effects of REE on lysosomal system (V, S, V/S) and Na concentrations in the
456 hemolymph (described above) were stronger in the presence of DOM (Figure 4).

457 **4. Discussion**

458 **4.1. REE in the water**

459 The concomitant decrease of dissolved and increase of particulate REE in the test medium
460 should be a consequence of REE precipitation as observed in numerous studies (Blinova et al.,
461 2018; González et al., 2015; Romero-Freire et al., 2019). The difference between nominal and
462 measured concentrations is probably related to REE precipitation but also to REE adsorption
463 on glass walls (Crémazy et al., 2014; Weltje, 2002) despite the flask preconditioning (Lachaux
464 et al., 2022a). These observations highlight the relevance to consider measured concentrations
465 to characterize REE exposure and to avoid misinterpretation of observed biological effects
466 (Lachaux et al., 2022b). Precipitation and adsorption of REE could happen due to the high
467 reactivity of trivalent REE and to the low solubility of their complexes with carbonates,
468 phosphates, or hydroxides (Johannesson et al., 1995; Liu and Byrne, 1997).

469 The higher dissolved concentrations of Nd, Gd and Yb in the presence of DOM was also
470 demonstrated in previous works using similar medium composition (Lachaux et al., 2022a,
471 2022b). It is explained by REE-DOM complexation that maintain REE in the
472 dissolved/colloidal phase of the water enhancing their solubility (Johannesson et al., 2004;
473 Vukov et al., 2016). The influence of DOM on REE fate in freshwater is described elsewhere
474 (Catrouillet et al., 2019; Gangloff et al., 2014; Marsac et al., 2011, 2010; Sonke and Salters,
475 2006).

476 The three REE showed a common fate in the water balanced by some differences mostly
477 between Nd and Yb, which may be related to their atomic number. The higher dissolved
478 concentration and lower particulate concentration of Yb compared to Nd can be linked to the
479 higher solubility of HREE-carbonates than LREE-carbonate complexes (Elderfield et al., 1990;
480 Sholkovitz, 1995).

481 **4.2. REE concentrations in *C. fluminea***

482 The amount of REE accumulated by the bivalves after 96 h demonstrates that a part of
483 REE was bioavailable despite the potential REE complexation and precipitation, which are
484 known to reduce their bioavailability (González et al., 2015; Romero-Freire et al., 2019).
485 Bioaccumulated concentrations of Nd, Gd and Yb determined in this study are relatively close
486 to the concentrations of Gd ($45 \mu\text{g g}^{-1}$) and samarium (Sm) ($47 \mu\text{g g}^{-1}$) measured in the
487 freshwater bivalve, *Dreissena polymorpha*, exposed to 1.25 mg L^{-1} of Gd or Sm for 14 days
488 (Hanana et al., 2018, 2017). Similar results were reported in *Mytilus galloprovincialis*, which
489 accumulated $119 \mu\text{g g}^{-1}$ ($\text{BCF} = 142 \text{ L kg}^{-1}$) of La after 28 days of exposure at 1 mg L^{-1} La (Pinto
490 et al., 2019). These results show that bivalves accumulate concentrations of different REE in
491 the same order of magnitude when they are exposed to similar concentration. The similar
492 bioaccumulated concentrations observed after different exposure durations (4, 14 and 28 days)
493 show that bivalves quickly accumulate REE from the beginning of the exposure period.
494 However, it should be noted that the different bivalve species were not exposed in the same
495 conditions (test medium, etc.).

496 The presence of DOM mitigated Nd, Gd and Yb (individually and in mixture) uptake due
497 to the formation of REE-DOM complexes, which reduced REE bioavailability, as previously
498 demonstrated with *D. magna* (Lachaux et al., 2022a) and *D. rerio* (Lachaux et al., 2022b). To
499 our knowledge, no other studies investigated the influence of DOM on measured
500 bioaccumulation of REE in animal species.

501 The lower bioaccumulation of Yb respect to Nd supports the lower accumulation of
502 HREE compared to LREE and MREE reported in several species during lab experiments (Khan,
503 2017; Lachaux et al., 2022a; Qiang et al., 1994; Yang et al., 1999) and *in situ* (Amyot et al.,
504 2017; Li et al., 2016; MacMillan et al., 2017). This could be linked to the higher stability of
505 HREE complexes, especially HREE-carbonate complexes (Pourret et al., 2007), which would

506 decrease HREE bioavailability relative to LREE (Neira et al., 2022). Another hypothesis may
507 imply a different mode of bioaccumulation (e.g. different transporters) of HREE compared to
508 LREE. The investigation of the distribution of Nd, Gd and Yb at tissue and subcellular levels
509 could help for a better understanding of REE internalisation pathway.

510 **4.3. Tissue and subcellular distributions of REE in *C. fluminea***

511 Neodymium, Gd and Yb have a similar organotropism in *C. fluminea* that suggests a
512 common pattern of bioaccumulation among the different REE, which has also been reported in
513 fish (Hao et al., 1996). Neodymium, Gd and Yb were preferentially accumulated in the gills
514 because these organs are directly exposed to the medium *via* filtration activity. Water was the
515 only route of exposure (because bivalves were not fed during the experiment), which enhanced
516 metal accumulation in the gills (Marigómez et al., 2002). Most of studies with bivalve species
517 measured REE accumulation in the whole soft tissues and we found only two that quantified
518 REE in different organs. In the freshwater bivalve, *Hyriopsis cumingii*, all REE were mainly
519 distributed in the gills (Xu et al., 2022) whereas *C. fluminea* and *Dreissena rostriformis*
520 *bugensis* accumulated higher concentrations of Gd in the digestive gland (Perrat, 2017).
521 However, the different form of Gd tested in the latest study (contrast-agent for magnetic
522 resonance imaging against GdCl₃ in the present study) has probably influenced its distribution
523 in bivalves. We have also detected significant concentrations of Nd, Gd and Yb in the rest of
524 soft tissues (including mantle, muscles, foot, siphon, etc.). Among these tissues, REE were
525 mainly located in the mantle of *H. cumingii* (Xu et al., 2022) probably because it is the tissue
526 of shell formation and calcification (Marigómez et al., 2002) where REE are likely to displace
527 Ca due to their similar ionic radius size (0.83-1.03 pm) with Ca (1 pm) (Valcheva-Traykova et
528 al., 2014). REE may be accumulated more precisely in inorganic calcium concretions
529 (carbonates and/ or phosphates) used to store Ca for shell formation. Indeed, the presence of
530 REE has been observed in the shell of *C. fluminea* (Merschel and Bau, 2015). In the present

531 study, we have observed that REE also occur in the hemolymph of *C. fluminea* being, to our
532 knowledge, the first demonstration of the presence of REE in internal fluids of aquatic animal.
533 Bivalves have an opened circulatory system where the organs are immersed in hemolymph.
534 Metals are transferred from an organ to another and eliminated *via* the hemolymph and
535 circulating cells (Marigómez et al., 2002) explaining the presence of REE in the latter. The
536 occurrence of REE in all studied compartments show that they are dispersed in the whole body
537 of *C. fluminea*.

538 At subcellular level, the dominant distribution among the organelles (MSF), MRG
539 (BDM) and cellular debris shared by Nd, Gd and Yb suggests that they have common biotic
540 ligands. It is likely that the three REE do not have the same affinity for these ligands explaining
541 some differences in their distribution. All REE belong to the same category (class “a”) from the
542 empirical classification based on the atomic properties and the solution chemistry of metal ions
543 (Nieboer and Richardson, 1980). This classification separates the periodical table into three
544 categories: class “a” elements (REE, Ca, Na, K, Mg etc.) having a higher affinity for O-
545 containing ligands (OH^- , CO_3^{2-} , HPO_4^{2-} , SO_4^{2-} , etc.), class “b” elements (Hg, Cu, Pb, Ag, etc.)
546 binding preferentially to S-containing ligands and the third class corresponds to borderline
547 elements (Ni, Zn, Cd, As, etc.). Due to their similar physicochemical properties, an element
548 belonging to class “a” is able to replace another class “a” element from its endogenous binding
549 site (Nieboer and Richardson, 1980). Then, in MRG, REE can substitute Ca^{2+} due to their
550 similar ionic radius size (Brown et al., 1990; Lansman, 1990; Pałasz and Czekaj, 2000;
551 Valcheva-Traykova et al., 2014). As Nd (0.98 pm) and Gd (0.94 pm) have an ionic radius closer
552 to the one of Ca (1 pm) than Yb (0.87 pm), they may have a greater affinity for Ca^{2+} -receptors
553 in the MRG, which could explain why they tend to be more accumulated than Yb in MRG
554 causing their depletion in other fractions.

555 Subcellular distribution of REE in a bivalve species has never been analysed before.
556 Cardon et al. (2019) showed in the crustacean *D. magna* that yttrium was mostly (>75 %)
557 recovered in the detoxified MRG, 6-18 % in the organelles (mitochondria, lysosomes) and the
558 remaining yttrium was located in cellular debris and MT. The fish, *O. mykiss* exhibited a
559 different distribution with most of yttrium in MSF, among which more than 50 % in the
560 organelles (mitochondria, lysosomes and microsomes) and around 20 % in the fraction relative
561 to enzymes. Less than 15 % of accumulated yttrium were associated to the detoxified MRG and
562 MT and the rest was recovered in cellular debris. It has been demonstrated that *D. magna*
563 accumulates more REE than fish species (Cardon et al., 2019; Lachaux et al., 2022b).
564 Crustaceans may not limit REE uptake as most of accumulated metal can be detoxified whereas
565 fish might regulate REE uptake as low levels of accumulated metal can be toxic. In the present
566 study, the freshwater bivalve exhibited an intermediate pattern between crustacean and fish in
567 terms of REE accumulated concentrations (Lachaux et al., 2022b) and subcellular distribution
568 (Cardon et al., 2019) showing that species have different strategies to handle REE. The analysis
569 of internal distribution of metals is useful for understanding the way they are handle by
570 organisms and for deciphering their potential toxic mechanisms (Otero-Fariña et al., 2022).

571 **4.4. Biological effects of REE and DOM**

572 4.4.1. REE detoxification

573 A relevant part of Nd, Gd and Yb was detoxified being accumulated in MRG (BDM).
574 Freshwater mollusks have numerous and diverse mineral granules mainly composed of
575 carbonates, phosphates, Ca, K and Mg. They perform several functions such as metal
576 detoxification and regulation of pH and ion homeostasis, especially the one of calcium which
577 is an essential element in bivalves (Marigómez et al., 2002). The high accumulation of REE in

578 MRG is probably due to the presence of strong affinity ligands (carbonates, phosphates, Ca²⁺
579 receptors).

580 The lower accumulation of REE in the organelles (MSF) of the digestive gland compared
581 to the gills is related to the higher detoxification capacity of the digestive gland, which can
582 implement diverse detoxification and elimination mechanisms preventing metal accumulation
583 in MSF (Marigómez et al., 2002). This would explain the higher proportion of REE linked to
584 metallothioneins (MT) in the digestive gland compared to the gills. However, less than 7 % of
585 Nd, Gd and Yb were accumulated in MT, demonstrating that REE have less affinity for thiols
586 groups (contained in MT) than class “b” metals (Marigómez et al., 2002). Metallothionein
587 exhibited a contrasting pattern of response being increased or decreased in the presence of REE.
588 It has been demonstrated that MT induction by *C. fluminea* can be related to metal exposure
589 (Doherty et al., 1987), which is supported by the positive correlation that we observed between
590 MT and REE concentrations. However, Hanana et al. (2017) reported that MT expression in *D.*
591 *polymorpha* was stimulated at low concentration (50 µg L⁻¹) and inhibited at high concentration
592 (1.25 mg L⁻¹) of Gd. The authors suggested that MT inhibition was due to a strong interaction
593 of REE (especially HREE and MREE) with GSH of MT, which would prevent GSSG formation
594 and thus MT induction (Hanana et al., 2021).

595 In the present study, other defence enzymes (against toxic and oxidative stress) were not
596 induced after 4 days of exposure. After 28 days of exposure, REE enhanced enzymatic defence
597 mechanisms such as GST, SOD, GPx and catalase in *M. galloprovincialis* (Andrade et al., 2023;
598 Freitas et al., 2020b, 2020a; Pinto et al., 2019) and in *D. polymorpha* (Hanana et al., 2018,
599 2017). These results suggest that REE sequestration in MRG is an early response to REE
600 exposure, which is implemented in bivalves before enzymatic responses. However, it seems
601 that this detoxification process did not prevent the occurrence of some biological effects.

602 4.4.2. REE effects on osmo- and iono-regulation

603 Homeostasis and osmoregulation are tightly regulated functions that maintain the well-
604 functioning of organisms. Even slight deviations of these constants from normal values
605 represent a disturbance. The decrease of hemolymphatic concentrations of Na (dominant cation
606 of osmoregulation) and the decreased Ca^{2+} ATPase activity in the gills of exposed bivalves are
607 probably related to the accumulation of Nd, Gd and Yb in the gills. Indeed, gills represent the
608 main organ of osmo- and iono-regulation in aquatic animals (Griffith, 2017), which was
609 illustrated by the higher activities of Ca^{2+} ATPase and $\text{Na}^{+}\text{-K}^{+}$ ATPase measured in the gills
610 compared to the digestive gland. The presence of REE in cell debris suggests that they can be
611 present in cell membranes. As cell membranes contain numerous and diverse transporters
612 (including ion exchangers, cotransporters, ionic channels, ATPase proteins), with which REE
613 may have interacted, it could explain the observed effects on iono- and osmo-regulation. The
614 presence of REE in cells membranes can also be explained by REE binding to phosphate
615 moieties in membrane phospholipids (Nieboer and Richardson, 1980).

616 The decrease of Ca^{2+} ATPase activity in the gills was statistically significant only for Nd
617 possibly because of its closer ionic radius size with Ca compared to Gd and Yb leading to a
618 stronger interaction of Nd with Ca^{2+} receptors. The decrease of Na concentration in the
619 hemolymph of exposed bivalves can be also related to the close ionic radius size of REE (0.86-
620 1.03 pm) with Na (1.02 pm) leading to a disruption of Na receptors. A potential mechanism for
621 the lower Na concentrations in the hemolymph is a downregulation of the $\text{Na}^{+}\text{K}^{+}$ -ATPase
622 activity, yet our results showed a decrease of these enzymes but it is not statistically significant
623 due to a high data variability.

624 Effects of REE on osmo- and iono-regulation deserve more attention because they can
625 lead to a disruption of the metabolism of essential elements (namely Na and Ca) and affect the

626 well-functioning of the organism as already described in mollusc species exposed to other
627 metals (Griffith, 2017; Mosher et al., 2012; Vercauteren and Blust, 1999). However, such
628 effects have been poorly studied in aquatic animals exposed to REE, thus mechanisms remain
629 to be deciphered as numerous and diverse processes are involved (ion channel, exchanger,
630 carbonic anhydrase and lysosome activities, membrane properties, etc.).

631 4.4.3. Biological effects potentially associated to REE in metal sensitive fraction

632 A significant part of Nd, Gd and Yb was accumulated in the metal sensitive fraction
633 relative to organelles (including nuclei, mitochondria, lysosomes, ribosomes), which induce
634 harmful effects in bivalves.

635 Lysosomes perform essential functions in cells such as cellular waste digestion and
636 homeostasis. They respond to intra- and extra-cellular stimuli adjusting continuously their
637 number, position and size. The present results indicated a decrease of lysosomal system due to
638 smaller lysosomes, which can be explained by the accumulation of Nd, Gd and Yb in lysosomes
639 (retrieved in the organelle fraction) and their subsequent effects. Indeed, class “a” metals are
640 present in lysosomes of mollusks (Marigómez et al., 2002). REE have possibly interacted with
641 Ca^{2+} channels located in lysosome membranes, which regulate lysosome size (de Araujo et al.,
642 2020). Thus, interactions of REE with lysosomes should disturb their turnover and especially
643 the lysosome fusion process affecting their well-functioning (de Araujo et al., 2020).
644 Consequently, the fundamental functions of lysosomes including signal transduction, vesicular
645 trafficking, autophagy, exocytosis and membrane reparation (Feng and Yang, 2016) could have
646 been disrupted inducing cellular stress and damages. Lysosome sensitivity to contaminants,
647 including metals, has been described elsewhere (Giamberini, 1993; Regoli, 1992; Yancheva,
648 2017) but never in aquatic animals exposed to REE.

649 The decreased protein concentration could be related to REE accumulation in
650 endoplasmic reticulum, Golgi apparatus or nuclei (retrieved in organelles), which may disturb
651 protein synthesis. Protein decrease by REE has been never reported before contrary to previous
652 studies showing that La and Gd increased protein concentration in *M. galloprovincialis* (Cunha
653 et al., 2022; Henriques et al., 2019; Pinto et al., 2019) due to the production of enzymatic
654 defences (GST, GPx, SOD, catalase) that do not seem to be activated in the present study.

655 Aerobic metabolism (ETS activity located in mitochondria membranes) was not affected
656 but REE modulated anaerobic metabolism by increasing or decreasing LDH activity. An
657 increase of LDH activity could be associated to valve closure in order to limit REE exposure
658 and prevent any stress. An increase of LDH can also indicate cellular and tissue damages (Chan
659 et al., 2013), which would be in line with protein concentration decrease.

660 4.4.4. REE mixture effects

661 In most cases, the biological effects of Nd, Gd and Yb in mixture were similar to
662 individual effects, which is related to their relatively close subcellular distribution when tested
663 individually and in mixture. The higher and lower proportions of individual REE in MRG
664 (BDM) and organelles, respectively, compared to those of REE in mixture, reflect an enhanced
665 detoxification of individual REE, which can be linked to the higher concentrations of Nd, Gd
666 and Yb tested individually (1 mg L^{-1}) than in mixture (0.333 mg L^{-1}). The very close subcellular
667 distribution of Nd, Gd and Yb in mixture suggests that they have common molecular targets.
668 This would explain the similarity between individual and mixture effects suggesting additive
669 effects of REE in mixture, which have previously been observed on *D. rerio* (Kang et al., 2022),
670 *D. magna* and *R. subcapitata* (Lachaux et al., 2022b).

671 Yet, our results show also some differences between individual and mixed effects. The
672 higher GPx activity and MT concentrations indicate an increase of defences and stress in the

673 bivalves exposed to the mixture compared to individual Gd and Yb, which could mean
674 synergistic effects. On the contrary, the increase of Ca²⁺ATPase activity, S/V ratio, and the
675 decrease of lysosome volume in the bivalve exposed to the mixture compared to individual REE
676 suggest a lower impact of the mixture on osmoregulation and lysosomal system. Moreover, the
677 lowered LDH activity, MT concentration and the higher TRIGLY concentrations observed in
678 the bivalves exposed to the mixture is linked with a lower metal exposure and stress. These
679 results indicate mitigation of harmful effects of REE in mixture, mainly in the presence of
680 DOM, which is notable on the PCA. Antagonist interactions can be caused by a competition
681 between the different REE for binding to common biotic ligands, which is supported by the
682 similar subcellular distribution of Nd, Gd and Yb in mixture. A similar hypothesis had been
683 suggested to explain antagonist effects of REE towards *Hydra vulgaris* (Hanana et al., 2022)
684 and *C. reinhardtii* (Morel et al., 2021).

685 4.4.5. DOM effects

686 Our results on the morphology of the lysosomal system indicate that lysosomes were
687 larger in the presence of DOM suggesting that DOM was taken up by the bivalves and digested
688 by lysosomes. The differences in REE subcellular distribution in the absence and presence of
689 DOM suggest that at least a part of the REE-DOM complexes was accumulated. This would be
690 consistent with the lower accumulation of REE in MRG (in the presence of DOM) because
691 REE-DOM complexes are not likely to bind to carbonates, phosphates and/or Ca²⁺ receptors as
692 much as free ion REE. REE-DOM complexes have possibly formed ternary complexes in cell
693 membranes (Zhao and Wilkinson, 2015), which would support the higher accumulation of REE
694 in cellular debris (including cell membranes) in the presence of DOM.

695 The presence of DOM and its possible uptake by the bivalves had direct effects on
696 measured biomarkers. Digestion of DOM could increase metabolism explaining the higher

697 protein concentration and ETS activity in the digestive gland in the presence compared to the
698 absence of DOM. The decrease of GPx activity in the presence of DOM can be related to its
699 capacity to isolate superoxide radicals and other reactive oxygen species outside and inside cells
700 (Cardenas et al., 2011). A similar mechanism could explain MT decrease in the presence of
701 DOM with the capacity of the latter to scavenge metals, which would reduce metal abundance
702 (compared to in the absence of DOM) and thus the concentration of MT. These protective
703 effects of DOM against metal toxicity and oxidative stress can thus be considered as beneficial
704 for the organisms. The scavenging capacity of DOM for REE and other metals may also explain
705 the decrease of Ca concentration in the hemolymph. Complexation between Ca and DOM might
706 decrease Ca bioavailability and uptake. Indeed, the strong affinity of Ca for DOM lead to a
707 competition between Ca and REE for DOM complexation (Kautenburger, 2009). The increase
708 in Na concentration with DOM may be related to DOM interaction with Na or Na receptors.
709 Such an increase in the presence of DOM was also observed in *D. magna* (Glover and Wood,
710 2005) and *D. rerio* (Al-Reasi, 2012) and has been explained by Na uptake stimulation due to
711 DOM interactions on Na membrane transporters.

712 Dissolved organic matter can have an indirect effect on the observed REE effects. The
713 stronger decrease of Na concentration and lysosomal system size observed in bivalves exposed
714 to REE in the presence of DOM is probably due to the increase of lysosome volume and Na
715 concentration in the hemolymph induced by DOM (without REE) rather than an enhancement
716 of REE toxicity directly caused by DOM.

717 **5. Conclusion**

718 The combined analysis of REE distribution in the medium, in the organisms and their
719 biological effects at organ and subcellular levels allowed a thorough understanding of the
720 underlying mechanisms of REE accumulation and biological effects. We demonstrated by using

721 media with and without DOM that medium composition influences REE bioavailability and
722 bioaccumulation, which highlights the relevance to consider speciation of REE in order to
723 understand their bioaccumulation. The detailed analysis of REE accumulation, organotropism
724 and subcellular distribution in the organisms allowed to explain the observed biological effects.
725 The presence of REE in gills, hemolymph and more precisely in cell membranes explain the
726 observed effects on osmo- and iono-regulation, illustrated by the decrease of both Na
727 concentrations in the hemolymph and Ca²⁺ATPase activity in the gills. These effects can affect
728 the metabolism of Na and Ca, which are essential elements in bivalve physiology. Further, REE
729 accumulation in the organelles of the digestive gland is related to the decreased size of
730 lysosomal system, which could disturb lysosome and cell functioning. These early
731 physiological effects of REE have so far been little described and deserve more attention.
732 Cellular and molecular approaches would be consistent to decipher the exact mechanisms of
733 toxicity and the potential impacts on organisms. Concerning REE detoxification, sequestration
734 in MRG appears to be an early process implemented in bivalves.

735 Our study on the fate, bioaccumulation and effects of Nd, Gd and Yb revealed several
736 similarities between LREE, MREE and HREE such as 1) a close distribution in water and in
737 organisms (measured concentrations of the three REE did not vary by a factor > 2); 2) their fate
738 and bioaccumulation were similarly affected by the presence of DOM; 3) they accumulate
739 mainly in the gills, MRG, organelles and cellular debris; 4) they induced common biological
740 effects on lysosomes and Na concentration in the hemolymph. These similarities in their fate,
741 bioaccumulation and biological effects suggest that REE share common abiotic and biotic
742 ligands (phosphates, carbonates, hydroxides, Ca receptors, etc.) which is supported by different
743 studies (Blinova et al., 2020; Huang et al., 2022; Morel et al., 2021; Tang and Johannesson,
744 2003; Wood, 1990). Abiotic ligands of REE determine their speciation and thus their
745 bioavailability whereas biotic ligands influence their uptake and internal distribution, which are

746 directly linked to their biological targets and subsequent effects. It is likely that Nd, Gd and Yb
747 have different affinities for these ligands, in relation with their physicochemical properties
748 (including their ionic radius size), which could explain the observed differences between the
749 three REE. The relatively close fate, bioaccumulation and biological effects between individual
750 and mixed REE suggest limited interactions between Nd, Gd and Yb. Occasionally, mixture
751 effects were lower than individual effects suggesting antagonism, which could be due to
752 competition between the three REE for their common biotic ligands. These results show that an
753 additivity approach should be appropriately protective in a context of environmental risk
754 assessment of REE mixture.

755

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