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HAL Authorization

1 **Title**

2 Evaluation of the accumulation of the iodinated contrast agents diatrizoic acid and iohexol in
3 *Dreissena polymorpha* mollusks

4

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22

23 **Abstract**

24 Mollusks are very sensitive to aquatic environmental alterations and then, are important bio-
25 indicators for monitoring the contamination of water bodies. Iodinated X-ray contrast media (ICMs)
26 are ubiquitously present in the aquatic environment, primarily due to their high consumption for
27 diagnosis purposes, high injection levels, low biodegradability, and low removal rates by wastewater
28 treatment plants. Although these compounds are assumed to be of low toxicity, aquatic organisms
29 are continuously exposed to these agents, which may result in adverse effects as ICMs can act as
30 iodine source and disrupt the endocrine system. Thus, the evaluation of their environmental risk,
31 especially on aquatic fauna is of great interest.

32 To this end, we first compared the accumulation behavior, based on iodine analysis, of two ICM
33 exhibiting different osmolality, diatrizoic acid and iohexol in *Dreissena polymorpha* bivalves exposed
34 under laboratory conditions at concentrations of 0, 100, and 1000 µg/L during 4 and 7 days. This
35 study was the first to provide information on iodine concentration in whole soft tissues and several
36 organs in control zebra mussels. Moreover, it showed, after exposure, an increase of iodine content
37 mainly in the digestive glands, followed by gills and gonads, highlighting that ICMs actually enter the
38 organisms. Thus, bioaccumulation of ICMs studies were then performed, by liquid chromatography
39 coupled to tandem mass spectrometry, on entire mollusks and digestive glands of organisms exposed
40 at 0, 10, 100, and 1000 µg/L of both ICMs during 21 days, followed by 4 days of depuration. These
41 first data on ICMs concentrations in zebra mussels, showed a clear accumulation of ICMs in mussels
42 as a function of relative exposure level, as well as a rapid depuration. Osmolality did not seem to
43 have a significant impact on the accumulation level, but a slight difference was observed on the
44 accumulation pattern between both ICMs.

45

46 **Keywords**

47 Iodinated contrast media, bioaccumulation, *Dreissena polymorpha*, ICP MS, LC-MS/MS

48 **1. Introduction**

49 The contamination of aquatic environments by pharmaceutical products is of high ecological concern
50 due to their potential health impacts on aquatic organisms. Among these compounds, iodinated X-
51 ray contrast media (ICMs) have been used since the 50s in medical imaging to artificially increase
52 contrast and improve investigations of organs, tissues, or tumors (Pérez and Barceló, 2017). The
53 annual production of ICMs is estimated at 5 000 tons, and 40 to 80 million doses (highly
54 concentrated aqueous solutions - up to 200 g/application) are injected annually, by the venous or
55 arterial route (Fabbri *et al.*, 2016). In order to reduce their harmful effects on health and to be
56 excreted in less than 24 hours, ICMs have been designed to be as inert and stable as possible (90% of
57 them are rejected by the human body in non-metabolized form) and highly hydrophilic. Most ICMs
58 are triiodobenzene derivatives incorporating highly polar functional groups to increase their water
59 solubility and lower their toxicity (Christiansen, 2005). They are generally classified on the basis of
60 their chemical structure, as ionic/non-ionic and monomer/dimer; diatrizoic acid being the most
61 common ionic ICM, whereas iohexol, iopamidol, iopromide, and iomeprol being non-ionic ones. ICMs
62 with low ionic strength and osmolality (as dimeric and non-ionic ones) exhibit lower intrinsic toxicity
63 (Christiansen, 2005; Kim *et al.*, 2015).

64

65 After being rejected by the human body in urine, ICMs are eliminated in wastewaters. However,
66 their removal rates in wastewater treatment plants (WWTPs) are low, due to both poor adsorption
67 to activated sludge and biological degradation. Their removal by ozonation vary from 35 to 55% or
68 even 20% for ionic ICMs; only iopromide is degraded up to 60 - 70% (Ternes *et al.*, 2003; Pal *et al.*,
69 2014; Fabbri *et al.*, 2016; Xu *et al.*, 2017). Thus, several µg/L of ICMs are found in natural waters,
70 groundwater, and drinking water (Sengar and Vijayanandan, 2021; Watanabe *et al.* 2016; Xu *et al.*,
71 2017; Zemmann *et al.*, 2014) with concentrations up to 12.9 µg/L, 9.6 µg/L, and 2.7 µg/L, respectively
72 (Sengar and Vijayanandan, 2021).

73

74 As a consequence, aquatic organisms are continuously exposed to these ICMs. However, if the
75 occurrence of these compounds in the environment is relatively well documented, very few studies
76 concern their potential impacts and fate in aquatic organisms. Indeed, no toxic effects were observed
77 after an exposure to 10 g/L of ICM, in fish, crustacean, or algae (Steger-Hartmann *et al.*, 1999), which
78 suggested that ICM were not of ecological concern (Williams *et al.*, 2017). Furthermore, it is
79 commonly believed that these compounds will not bioaccumulate in fish and wildlife due to their
80 negative log Kow values. Nevertheless, ICMs were recently detected in brain and gonads of fish,
81 which are essential organs for fish hormone regulation (Arnnok *et al.*, 2017). Moreover, ICMs have
82 structural similarities with the natural thyroid hormones thyroxine and triiodothyronine especially
83 involved in reproduction, brain, and body development (Venturi *et al.*, 2012). Indeed, Singh *et al.*
84 (2019) studied the potential binding of ICMs to nuclear receptors in the endocrine system based on
85 *in silico* calculations and showed stronger binding of iohexol compared to the natural hormones.

86

87 In addition, ICMs represent an iodine source that could potentially interact with the natural
88 regulation of iodine in aquatic organisms. Iodine is an essential trace element, and a wide range of
89 concentrations can be observed in aquatic organisms. Concentrations below limit of quantification
90 (LOQ : 0.02 µg/kg, ICP-MS) to 1210 µg/100 g (muscle) were measured in a large panel of fish species
91 (Bogard *et al.*, 2015; Nerthus *et al.*, 2018). Similarly, concentrations of 26-120 µg/100 g (raw edible
92 part : muscle) of iodine were measured in shrimp/prawn (Bogard *et al.*, 2015). Moniruzzaman *et al.*
93 (2021) observed high concentrations of iodine comprised between 58-66 mg/100 g in 4 mollusk
94 species of marine water (*Saccostrea cucullata*, *Crassostrea virginica*, *Meretrix meretrix*, *Andara*
95 *granosa*) and 50-53 mg/kg in 3 species of freshwater (*Pila globose*, *Lamellidens marginalis*,
96 *Lamellidens corrianus*). Moniruzzaman *et al.* (2021) underlined that significantly higher amount of
97 iodine was found in oyster species than the freshwater bivalves and snail. The iodine content in the
98 different species tends to be largely dependent on environmental conditions (FAO/WHO, 2004).
99 However, no information is currently available in the literature regarding the influence of ICMs

100 exposure on iodine regulation by aquatic organisms, particularly by species recognized as
101 bioaccumulators of essential or non-essential elements.

102

103 In this context, this study proposes to evaluate the impact of two ICMs of different osmolality
104 regularly detected in water, diatrizoic acid and iohexol, on the bivalve mollusk *Dreissena polymorpha*.

105 This sedentary mussel is commonly found in a wide habitat diversity in Europe and North America

106 (de Lafontaine *et al.*, 2000). In addition, this mussel has a high filtration rate and high

107 bioaccumulation potential that make it a widely used indicator for field biomonitoring studies and

108 laboratory experiments (Bourgeault *et al.*, 2010; Daniele *et al.*, 2016; Hani *et al.*, 2021). Based on

109 laboratory exposures, the first objective of this work concerned the study of the possible effects of

110 ICMs exposure on iodine concentration and its distribution into the different organism's tissues by

111 ICP-MS analysis. Based on these first results, the second aim was to study the accumulation kinetic of

112 both ICMs in whole soft tissues and in the digestive glands, the most accumulative organs, by LC-

113 MS/MS. This experiment was conducted during a 21-day exposure to different concentrations of

114 ICMs followed by a 4-day depuration step.

115

116 **2. Materials and Methods**

117 2.1. Chemicals and reagents

118 UPLC-MS grade acetonitrile (ACN) and methanol (MeOH) were purchased from Biosolve Chimie

119 (Dieuze, France). Tetramethylammonium hydroxide (TMAH 25%), formic acid (purity $\geq 99\%$) and

120 heptane (purity $\geq 90\%$) were acquired from Sigma-Aldrich (Saint-Quentin Fallavier, France). Water

121 (Optima LC/MS grade) was bought from Fisher Chemical (Illkirch, France) for LC-MS/MS analysis.

122 Ultrapure water (18.2 m Ω) produced from distilled water with a Milli-Q Elix water purification system

123 (Millipore, Molsheim, France) was used for the other experiments.

124 Analytical standards on diatrizoic acid (DTZ, purity \geq 98.0 %) and iohexol (IO, purity \geq 95.0 %) were
125 obtained from Merck. Phenacetin-ethoxy-1-¹³C (98 atom % ¹³C) used as LC-MS/MS injection standard
126 was purchased from Isotec (Ohio, USA). Iodate (calibrant) and tungsten (internal standard) standards
127 at 1000 mg/L (PlasmaCAL) used for ICP MS quantification were purchased from SCP Science
128 (Courtaboeuf, France). Certified Reference material (ERM BB422) of lyophilized fish muscle with $1.4 \pm$
129 0.4 mg/kg of iodine was obtained from LGC Standards (Molsheim, France).

130 2.2. Stock solutions and calibration standards

131 Standard stock solutions of DTZ and IO were prepared individually at 1000 μ g/L in MeOH and stored
132 at -20°C during 3 months. Appropriate dilutions of these solutions were used for quantification
133 purposes by LC-MS/MS. An 8-points matrix-matched calibration was performed by spiking “blank
134 matrices” free of target compounds to determine the ICM levels in the whole mollusk or digestive
135 glands.

136 For total iodine determination, stock elemental standards of iodine (I) and tungsten (W) at 500 and
137 25 μ g/L respectively were prepared in water. A calibration curve ranging from 0 – 5000 ng/L as I with
138 500 ng/L of internal standard (W) in 0.1% TMAH was used for quantification.

139

140 2.3. Zebra mussel maintenance and experimental design

141 For all experiments, adult zebra mussels (of 18–22 mm) were collected from the Lac du Der (51290
142 Giffaumont-Champaubert, France, 48°33'035" N; 4°45'011" E), considered as reference site, and
143 brought back to the laboratory where they were kept in 40 L glass tanks of aerated Cristalline Aurele
144 drinking water, at 14 °C. The acclimation period lasted three weeks in darkness. The mussels were
145 fed with two microalgae species (*Scenedesmus obliquus* and *Chlorella pyrenoidosa*, using 1,000,000
146 microalgae of each species per mussel) twice a week and the water was also changed twice a week.

147

148 Two consecutive exposure experiments of mussels to ICMs were conducted. The first one was
149 devoted to ICP-MS analysis of iodine and corresponded to a short exposition to ICMs during 7 days.
150 The second experiment, a chronic exposure during 21 days followed by 4-days of depuration, was
151 mainly devoted to the quantification of ICMs by LC-MS/MS. For both experiments, the same mussel
152 density (40 per liter) was used. A stock solution of 1 mg/ml of DTZ or IO in ultrapure water was used
153 for contamination and it was renewed each two days (experiment 1) or each week (experiment 2).
154 Water was sampled (5 mL) just before and after changes to monitor exposure concentration. At each
155 sampling date the volume of exposure medium was adjusted to the number of mussels remaining in
156 tanks. The mussels were fed twice a week as in the maintenance conditions (see before), a day
157 before water change. In both experiments, a larger number of zebra mussels were exposed than
158 those used for the study presented here. The remaining organisms were dedicated to other analyzes
159 not considered in this article. For all exposures, no mortality or biological effect on the organisms
160 were observed.

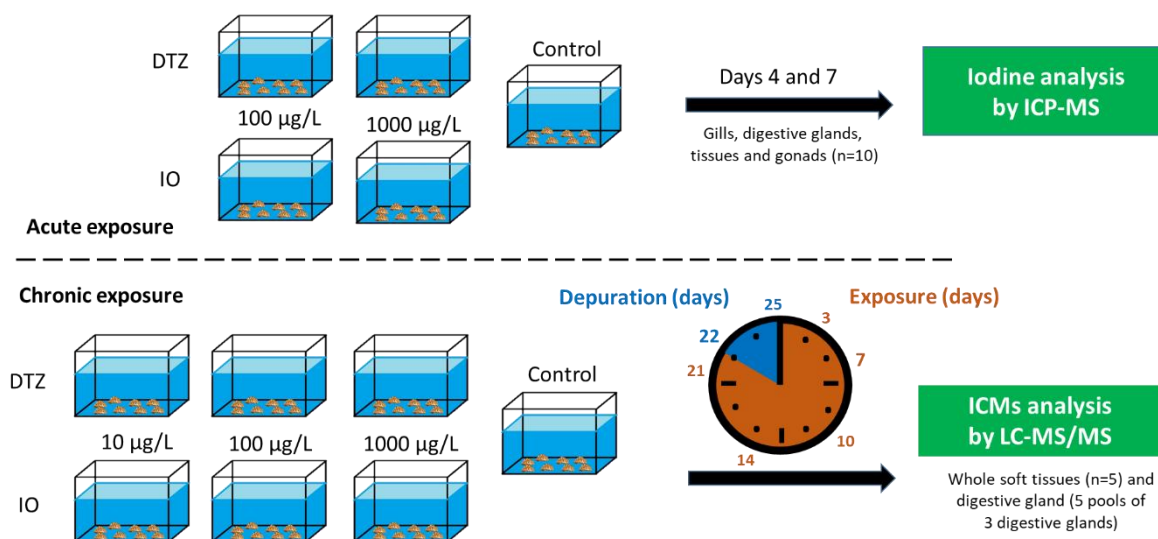
161

162 For the first experiment, zebra mussels were exposed in January 2019 to 0 and 100 µg/L of DTZ or IO.
163 Eighty mussels were placed in each tank (3: one control and two exposed). To complete the
164 experimental design, other mussels were exposed with the 1000 µg/L DTZ or IO. For both short
165 exposures, on day 4 and 7, 10 mussels per condition were sampled individually to recover gills,
166 digestive glands, gonads, and the remaining soft tissues. Samples were weighted (wet weight, ww),
167 shock frozen in liquid nitrogen, and stored at -80°C until analysis by ICP-MS (see 2.5).

168

169 For the chronic experiment, zebra mussels were exposed in January 2020 to 0, 10, 100, and 1000
170 µg/L of DTZ or IO. Four hundred mussels were placed in each tank (7: one control and six exposed).
171 Mussels were exposed during 21 days then placed in a new tank with clean water for a 4-day
172 depuration step (day 21 to day 25). At day 0 (control), day 3, 7, 10, 14, 21 (exposure step), and day 22
173 and 25 (depuration step), 20 mussels were sampled from each condition for LC-MS/MS analysis (see

174 2.6). Among these, whole soft tissues from five mussels were recovered individually, and digestive
 175 glands from the remaining 15 mussels were dissected and pooled by 3 (corresponding to 5 pools of 3
 176 glands). All samples were weighted (ww) shock frozen in liquid nitrogen and stored at -80°C until
 177 analysis. The design of the experiment is presented in Figure 1.
 178



179
 180 *Figure 1: Design of the experiment*

181

182 2.4. Sample preparation and extraction protocols

183 *Alkaline iodine extraction from tissues for total ICP MS analysis*

184 The protocol was adapted from Leufroy *et al.* (2015). Each organ (n= 10) were directly extracted
 185 individually in a 1.5 mL-ependorf tube. Organs were blended at 3000 rpm with an G50 electric
 186 homogenizer (G-Biosciences, VWR, France) in 0.25 mL of 5% TMAH for gonads, 0.5 mL of 5% TMAH
 187 for gills and digestive glands, and 0.5 mL or 0.75 mL for tissues depending of their mass (0.5 mL for
 188 tissue with mass < 200 mg and 0.75 mL for masses > 200 mg). Then, eppendorf tubes were placed
 189 individually into a water bath in 50-mL tubes for 3 hours at 90°C in a heating block (DigiPREP MS, SCP
 190 Science, Courtaboeuf, France). After cooling down, extracts were centrifuged at 10 000 rpm for 15
 191 min and the supernatant was diluted 50 fold in H₂O to adjust the concentration of TMAH to 0.1%,

192 after addition of W for a final concentration of 500 ng/L. Extracts were filtrated through a 0.45 µm
193 cellulose acetate membrane before ICP MS analysis.

194 To validate the extraction procedure and analysis, an aliquot of 50 mg of the Certified Reference
195 Material (ERM BB422) was extracted following the same protocol with 0.5 ml of 5% TMAH and
196 diluted 50-fold in H₂O before analysis.

197

198 *ICMs analysis in water*

199 DTZ and IO concentrations in water were monitored at each water change (every two days) to
200 measure actual exposure concentrations in the tanks. Water samples were thawed at room
201 temperature, homogenized and diluted 10 and 100 times respectively for the 100 and 1000 µg/L
202 exposures; water sample resulting from the exposure at 10 µg/L was injected without dilution. Then,
203 sample were analyzed by LC-MS/MS after addition of 50 µL of phenacetine-ethoxy-1-¹³C at 100 µg/L
204 as injection standard.

205

206 *Mollusk and digestive glands extractions for ICMs accumulation studies*

207 Extraction was performed on 5 individual mollusks or 5 pools of 3 digestive glands. An aliquot of 150
208 mg and 50 mg of grounded and homogenized fresh mollusk and digestive glands respectively, was
209 extracted by solid liquid microextraction in 2 mL-screw tubes with 1.2 mL ACN/MeOH (50:50, v/v)
210 and 500 µL of heptane. The tube was shaken 1 min with a Beadbug homogenizer (4000 rpm). Next,
211 the tube was centrifuged for 5 minutes at 10 000 rpm. Then, 800 µL of the supernatant were
212 transferred in a 15 mL-tube. A second extraction was performed with 1 mL of ACN/MeOH mixture,
213 and then 1 mL was removed and transferred in the 15 mL-tube. The gathered extract was vortexed
214 before being evaporated to dryness in a nitrogen flow at 40 °C. The dry residue was dissolved in 150

215 μL of H_2O containing the injection standard, and 5 μL of this mixture was injected in LC-MS/MS for
216 analysis.

217

218 2.5. ICP MS analysis

219 Analysis were performed on an 7700x ICP MS from Agilent Technology (Tokyo Japan) equipped with
220 a Scott chamber cooled at 2°C , a micromist nebulizer delivering 400 $\mu\text{L}/\text{min}$, nickel sampling and
221 skimmer cones, and a collision cell with H_2 gas (3.8 mL/min). The RF power was set to 1550 W. Lens
222 voltages, torch position and sample gas flow (c.a. 1.09 mL/min) were optimized daily. Quantification
223 was performed on ^{127}I , ^{182}W and ^{183}W (internal standard) isotopes with an integration time of 0.09
224 s/isotope.

225

226 2.6. Liquid chromatography tandem mass spectrometry (LC-MS/MS)

227 The analysis of DTZ and IO were performed using a liquid chromatographic system H-Class UPLC from
228 Waters (Saint-Quentin en Yvelines, France) coupled to a Xevo TQ-S triple-quadrupole mass
229 spectrometer (Waters) equipped with a StepWave ion guide. The chromatographic column was a
230 Kinetex PS-C18 (100 \times 2.1 mm; 2.6 μm) from Phenomenex (Le Pecq, France). The mobile phase was
231 composed of (A) 0.1% formic acid in ultrapure water and (B) ACN with 0.1% formic acid. The
232 following gradient was used for iohexol: 0-0.6 min, 1% B; 0.6-3.5 min, 1-52% B; 3.5-4.0 min, 52-100%
233 B, 4.0.-6.2 min, 100 %B; 6.2-9.0 min, 1% B. For diatrizoic acid the gradient was as follows: 0-0.6 min,
234 0% B; 0.6-3.5 min, 0-52% B; 3.5-4.0 min, 52-100% B, 4.0.-6.2 min, 100 %B; 6.2-9.0 min, 0% B. The flow
235 rate was maintained at 0.3 mL/min during 3.5 min, then increased up to 0.5 mL/min until 6.2 min,
236 before returning to its initial rate. The oven temperature was fixed at 45°C and the injection volume
237 was set at 5 μL and 10 μL for tissues and water samples, respectively.

238 The multiple reaction monitoring (MRM) mode was employed for the MS/MS detection to be more
239 sensitive. The electrospray source was set in the positive ionization mode (ESI+). The optimum ESI-
240 MS/MS settings were obtained by direct infusion of each individual ICM into the electrospray source.
241 The declustering potential (DP) and collision energy (CE) were optimized to get the highest signal
242 intensities for each ICM. Two transitions were selected in the MRM mode for each ICM (Table S1).
243 The transition that presented the most intense signal (MRM1) was chosen for quantification and the
244 second one (MRM2) for confirming the ICM detection. Ionization source parameters were optimized
245 for maximum intensity of the precursor ions as follows: capillary voltage 3200 V, desolvation
246 temperature 500°C (DTZ) and 550°C (IO), source offset 50 V, source temperature 150°C, nitrogen
247 desolvation and nebulizer gas flows of 900 L/h and 150 L/h, respectively.

248

249 **3. Results and discussion**

250 Due to their high occurrence in natural waters and to their structural similarities with the natural
251 thyroid hormones, ICMs represent a source of iodine that could potentially interact with the natural
252 regulation of iodine in aquatic organisms. Thus, the study of their accumulation in bioaccumulative
253 aquatic organisms such as bivalves is of great interest. First, we investigated the accumulation of
254 ICMs in a global approach, by studying the accumulation of iodine in the different organs. Then, we
255 studied the accumulation kinetics of the two ICMs both in whole soft tissues and in the most
256 accumulative organ.

257 3.1 Effects of ICMs exposure on iodine concentrations in mussels

258 To study the effect of ICMs exposure on organism's iodine concentration, *Dreissena polymorpha*
259 were exposed to acute and high concentrations of DTZ and IO (100 µg/L and 1000 µg/L). In the
260 exposure tanks, the average actual iodine concentrations measured were of 42.2 µg/L and 64.82
261 µg/L, 414.9 µg/L and 537.9 µg/L for the 100 µg/L and 1000 µg/L exposures to IO and DTZ,

262 respectively. For both experiments, a mean concentration of 3.8 µg/L of iodine was observed in the
263 control condition due to natural iodine.

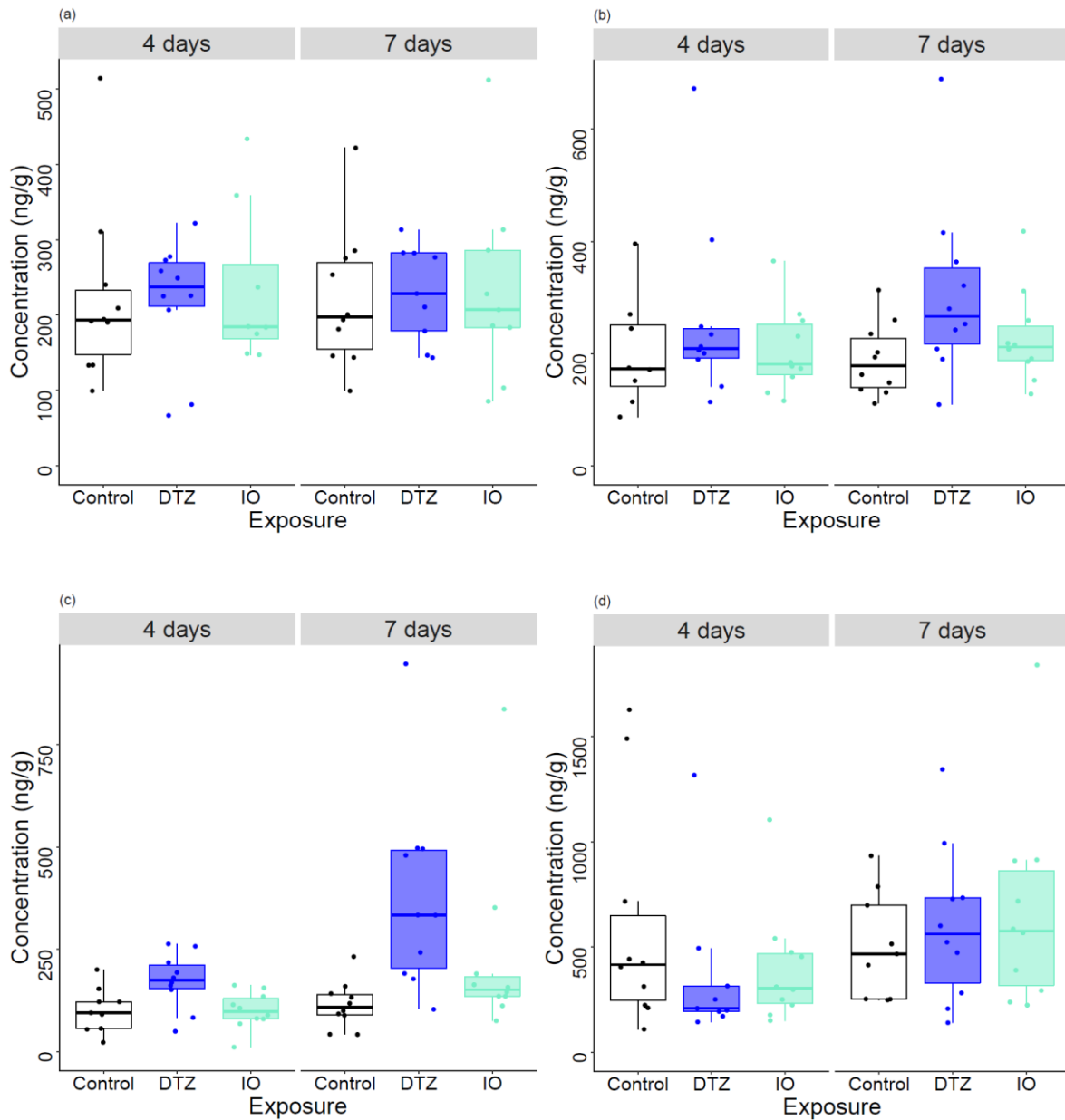
264 Figures 2 and 3 show the concentrations of iodine after 4 and 7 days of exposure to DTZ and IO at 0,
265 100 and 1000 µg/L, respectively in the four organs, i.e gills, digestive glands, gonads, and remaining
266 soft tissues. In this study basal iodine concentrations were determined in control organisms at $206 \pm$
267 104 , 158 ± 79 , 92 ± 105 , and 359 ± 334 ng/g for gill, digestive gland, gonad, and remaining tissues,
268 respectively, which corresponded to 260 ± 199 ng/g in the entire mussel. Actually, no information is
269 available in the literature regarding basal iodine concentrations in freshwater mussels, *Dreissena*
270 *polymorpha*. The iodine concentrations measured in the different organs/tissues are in accordance
271 with those registered in other shellfish or shrimp (ranged from 308 to 1300 ng/g, FAO/WHO 2004;
272 Oehlenschläger 2012; Broad *et al.*, 2015). However higher iodine concentrations were registered in
273 marine bivalves (Bay of Bengal : Moniruzzaman *et al.*, 2021; sea of Marmara : Özden *et al.*, 2009) and
274 freshwater ones from Bangladesh (Moniruzzaman *et al.*, 2021) with concentrations comprised
275 between 6.9 and 135.03 µg/g (wet weight).

276 Moreover, we can notice that among organs of control zebra mussels, similar iodine basal
277 concentrations are observed in gills and digestive glands and lower concentrations are observed in
278 the gonads (Figure 2 and 3). Digestive glands and gills are commonly considered in studies on metals
279 exposure, in link to their role in metals accumulation (according to the route of exposure: food vs
280 water), metals metabolism and detoxification. However, in this study, the highest concentrations of
281 iodine are observed in the remaining soft tissues. These are mainly composed of the gland of byssus,
282 muscle and mantle. Among these organs, the mantle could represent an iodine-rich organ according
283 to its rule in shell production.

284 In the exposed organisms, no accumulation of iodine was observed in gills during DTZ- and IO
285 exposures at 100 µg/L (Figure 2 and see Table 1). However, at 1000 µg/L, an iodine accumulation can
286 be observed, especially for DTZ exposure, with significant differences in iodine concentrations
287 compared to controls after 4 days of exposure. This could be explained by the high osmolality of DTZ

288 compared to IO which may migrate through membranes more easily. To note, the iodine
289 concentration is quite stable between 4 and 7 days of exposure to DTZ, suggesting an equilibrium of
290 the organisms with their exposure medium from day 4. In the digestive glands, the iodine
291 concentration increases with time for both molecules. Iodine accumulation can be significantly
292 detected in the digestive glands after 7 days of exposure at 100 µg/L of DTZ and from 4 days at 1000
293 µg/L for both DTZ and IO exposures. In the gonads, iodine accumulation can be detected significantly
294 (Table 1) during all DTZ exposure conditions. Iodine concentration in gonads increases over time
295 (between 4 and 7 days of exposure) whatever the concentration of DTZ in their medium.
296 Interestingly, in the remaining soft tissues (showing the highest iodine concentration in controls) no
297 iodine accumulation was observed during ICMs exposure whatever the condition (duration and/or
298 concentrations used). At 100 µg/L of ICM, corresponding to a 10- (IO) or 15- (DTZ) times increase in
299 iodine concentration in the medium compared to the control, the results suggest a lack of ICM
300 accumulation in the mussel or a regulation of iodine by the mussel to compensate for iodine supply
301 via the ICM. At 1000 µg/L of ICM, the results support the hypothesis of an ICM accumulation and an
302 excess of mussel homeostatic capacities leading to an increase in iodine concentrations in their
303 tissues. In the same way, despite the high basal iodine concentration in the remaining tissues
304 (including mantle), the results at the high exposure condition underline the role of the digestive
305 gland in contaminant accumulation/detoxification with a twofold increase in iodine concentration.
306 So, in the second part of this study, a particular interest was given to the digestive gland in addition
307 to whole soft tissues.

308



309

310

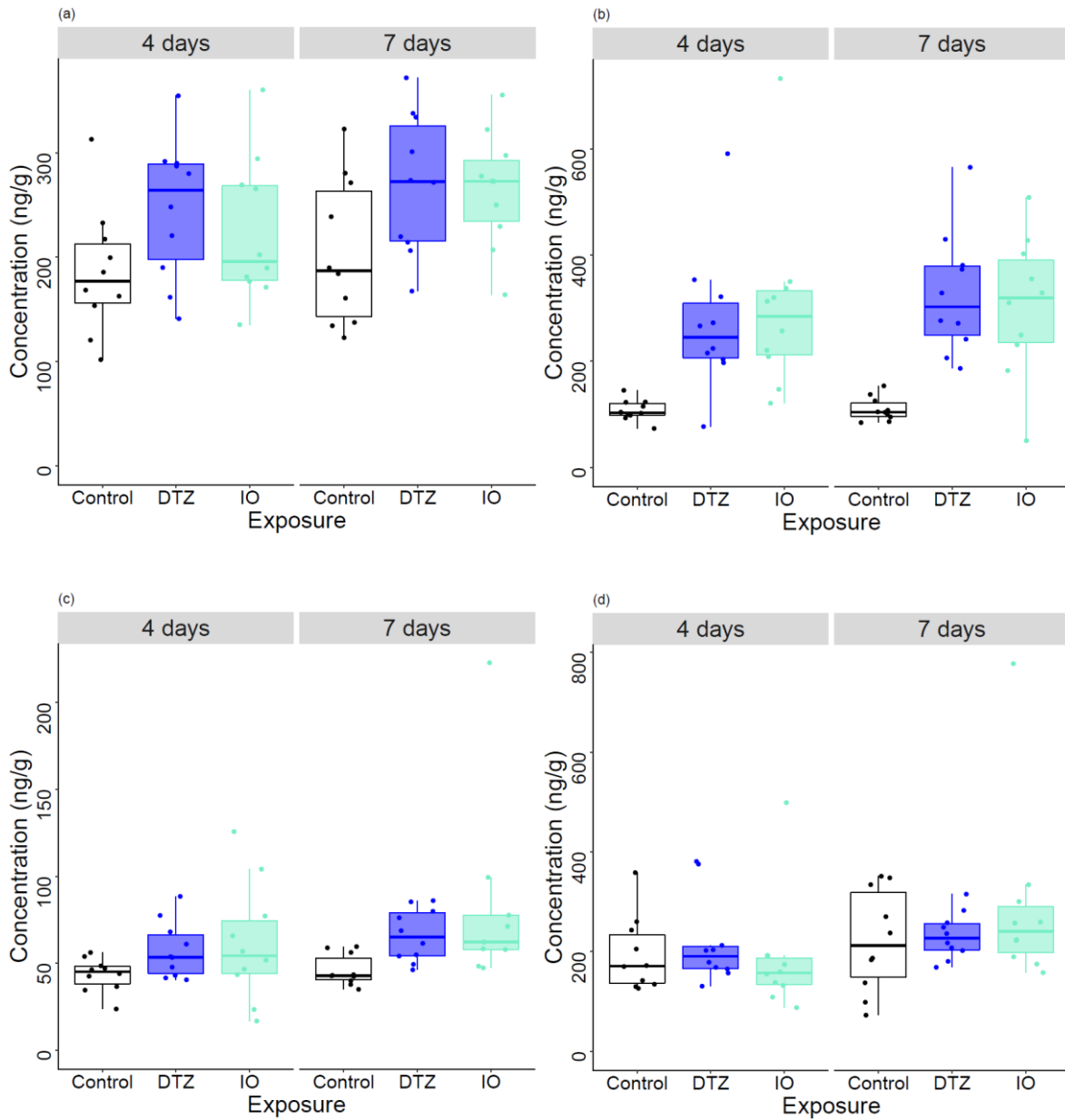
311 *Figure 2: Iodine concentration ($\mu\text{g}/\text{kg}$ wet weight) in (a) gills, (b) digestive glands, (c) gonads and (d)*
 312 *remaining soft tissues of *Dreissena polymorpha* after 4 and 7-days of exposure to 0 (Control, white)*
 313 *and 100 $\mu\text{g}/\text{L}$ of diatrizoic acid (DTZ, blue) and iohexol (IO, green) ($n=10$)*

314

315

316

317



318

319

320 *Figure 3: Iodine concentration ($\mu\text{g}/\text{kg}$ wet weight) in (a) gills, (b) digestive glands, (c) gonads and (d)*
 321 *remaining soft tissues of *Dreissena polymorpha* after 4 and 7-days exposure to 0 (Control, white) and*
 322 *1000 $\mu\text{g}/\text{L}$ of diatrizoic acid (DTZ, blue) and iohexol (IO, green) ($n=10$).*

323

324

325

326

Exposure		Diatrizoic acid				Iohexol			
Days	Concentration	Gills	Digestive glands	Gonads	Tissues	Gills	Digestive glands	Gonads	Tissues
4	100 µg/L	0.95	0.34	0.02	0.29	0.83	0.90	0.95	0.32
	1000 µg/L	0.05	<0.01	0.03	0.53	0.20	<0.01	0.14	0.82
7	100 µg/L	0.80	0.05	0.01	0.52	0.79	0.03	0.15	0.37
	1000 µg/L	0.05	<0.01	<0.01	0.79	0.05	<0.01	0.08	0.32

327 *Table 1: Student p-values relative to Dreissena polymorpha exposed 4 and 7-days to 100 and 1000*
328 *µg/L of diatrizoic acid and iohexol. p-values < 0.05 (grey cells) indicate significant differences*
329 *between exposed and control organs.*

330

331 3.2 Accumulation of diatrizoic acid and iohexol in Dreissena polymorpha

332 The second part of this work concerned the study of the accumulation of DTZ and IO in mussels
333 (whole soft tissues or digestive glands) during a 21 days-chronic exposure followed by a 4 days-
334 depuration phase to 0 (control), 10, 100, and 1000 µg/L (nominal concentration) of both ICMs.
335 Concentrations of DTZ and IO were stable throughout the exposure, so that bivalves were actually
336 exposed over 21 days to an average concentration of 9.5 ± 1.1 , 89.5 ± 4.9 , and 760 ± 60 µg/L for DTZ,
337 and of 9.3 ± 1.0 , 123 ± 11 , and 1032 ± 84 µg/L for IO at the 95% confidence level. A total of 5
338 individual bivalves and 5 pools of 3 bivalve digestive glands was extracted and analyzed at each
339 sampling time. Accumulation of both ICMs in whole soft tissues and digestive glands during
340 exposures to 0 (control), 10, 100, and 1000 µg/L are presented on Figures 4 and 5, respectively.
341 Whatever the sampling date, DTZ and IO were never detected in the organisms (whole soft tissues
342 and digestive glands) in the control condition (LQ <0.2 ng/g and <0.3 ng/g in whole tissues and
343 digestive glands, respectively).

344 Whatever the biological sample (whole soft tissues: Figure 4 or digestive glands: Figure 5), DTZ and IO
345 concentrations increased significantly ($p < 0.05$) according to the exposure concentration. In addition,
346 we can note that the ICM concentration in the digestive glands are 3-fold higher than in whole soft
347 tissues, especially for DTZ. This can be explained by the low accumulation of the ICMs in gills, gonads,

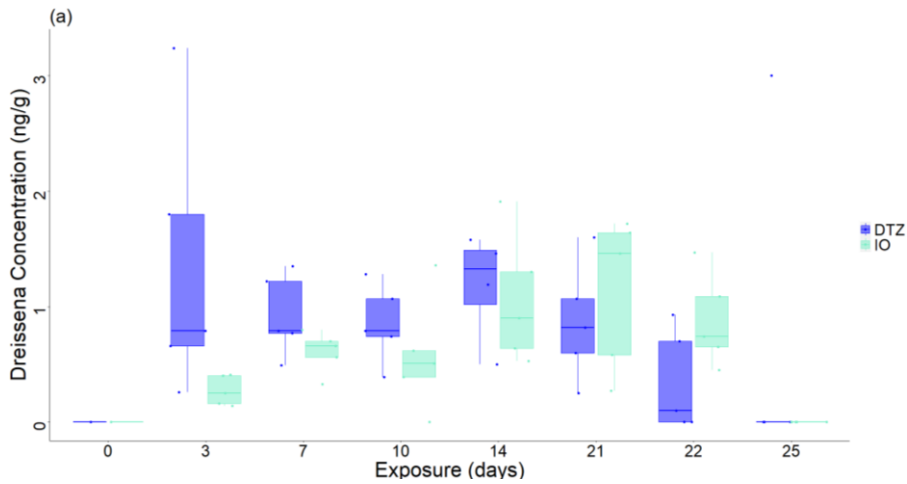
348 and remaining soft tissues which constitute the whole soft tissue compared to digestive glands alone
349 (dilution effect, see above). For DTZ, maximum mean concentrations (for one exposure date) in
350 whole soft tissues and digestive glands are 1.3 ± 1.2 and 3.3 ± 0.9 ng/g, 13 ± 4 and 29 ± 8 ng/g, $130 \pm$
351 58 and 249 ± 54 ng/g, respectively, for organisms exposed to 10, 100, and 1000 $\mu\text{g/L}$. For IO,
352 maximum mean concentrations in whole soft tissues and digestive glands are 1.1 ± 0.7 and 3.2 ± 0.9
353 ng/g, 17 ± 6 and 25 ± 8 ng/g, 254 ± 55 and 275 ± 51 ng/g, respectively, for organisms exposed to 10,
354 100, and 1000 $\mu\text{g/L}$. The higher concentration of IO compared to DTZ can be explained by a higher
355 measured concentration of this ICM in the exposed water for the nominal concentrations 100 and
356 1000 $\mu\text{g/L}$.

357 Moreover, in whole soft tissues (Figure 4), the accumulation patterns appear different according to
358 the ICM considered. Indeed, for all DTZ exposure concentrations, compared to control (Day 0) an
359 accumulation of DTZ was observed from day 3, then the concentrations were stable until the end of
360 the exposure (day 21). In the case of IO, the concentration increased significantly from day 3 to day
361 21 (comparison of mean concentrations between day 3 and day 21 for 10 $\mu\text{g/L}$: $p = 0.04$, for 100
362 $\mu\text{g/L}$: $p < 0.01$; and for 1000 $\mu\text{g/L}$: $p < 0.01$)(Table 2). In the digestive glands (Figure 5), a similar
363 pattern to that of whole soft tissues was observed for IO with a significant increase (except in 10 $\mu\text{g/L}$
364 condition) in concentration between day 3 and day 21 (100 $\mu\text{g/L}$: $p < 0.01$; 1000 $\mu\text{g/L}$: $p = 0.01$)(Table
365 3). For DTZ, after an accumulation observed on day 3 in the digestive glands, the concentration still
366 increased significantly (except for condition 1000 $\mu\text{g/L}$) after 14 days of exposure (highest
367 concentrations observed during the exposure step; 10 $\mu\text{g/L}$: $p < 0.01$; 100 $\mu\text{g/L}$: $p < 0.01$).

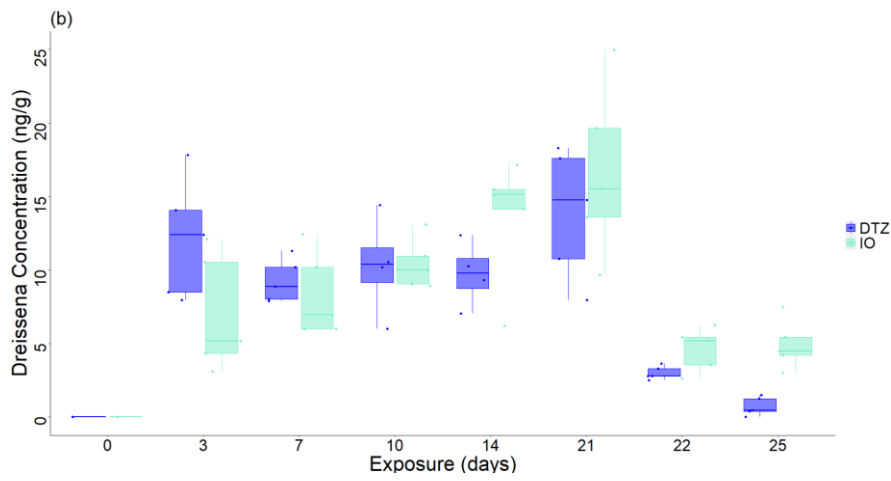
368 During the depuration step, we can observe an important and significant decrease (see Table 2 of p
369 values comparison between day 21 and day 22) in ICM concentration for both whole soft tissues
370 (Figure 4) and digestive glands (Figure 5) after only one day (day 22) of depuration. Nevertheless,
371 except for 10 $\mu\text{g/L}$ in whole soft tissues, the concentration of both ICM is relatively stable between
372 day 22 and day 25 ($p > 0.05$, see Table 2). Furthermore, we can note that for the 100 and 1000 $\mu\text{g/L}$
373 exposures, after the depuration phase, the concentrations of DZT in whole soft tissues (Figure 4) and

374 digestive glands (Figure 5) are closer to the concentrations of the controls compared to IO. These
375 results are in accordance with the half-lives of both ICMs that are evaluated at 121min for IO (Olsson
376 et al., 1993) and at about 30-60 min for DTZ (McEvoy, 2005) in humans.

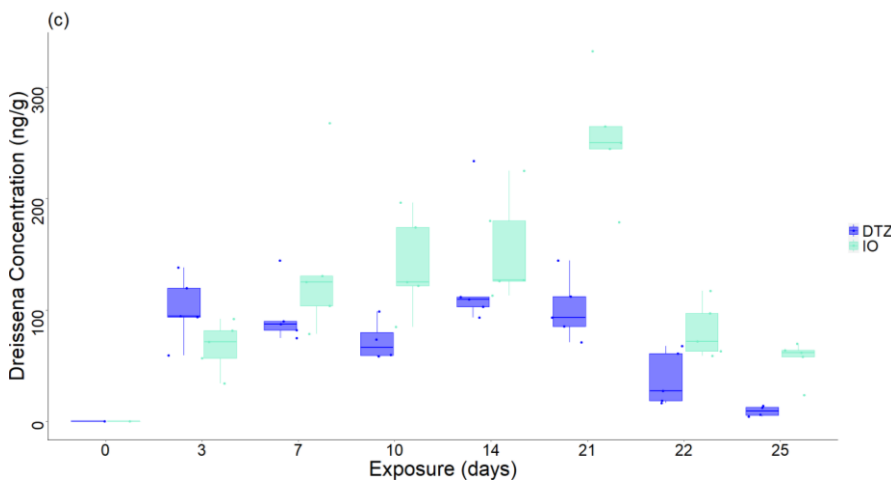
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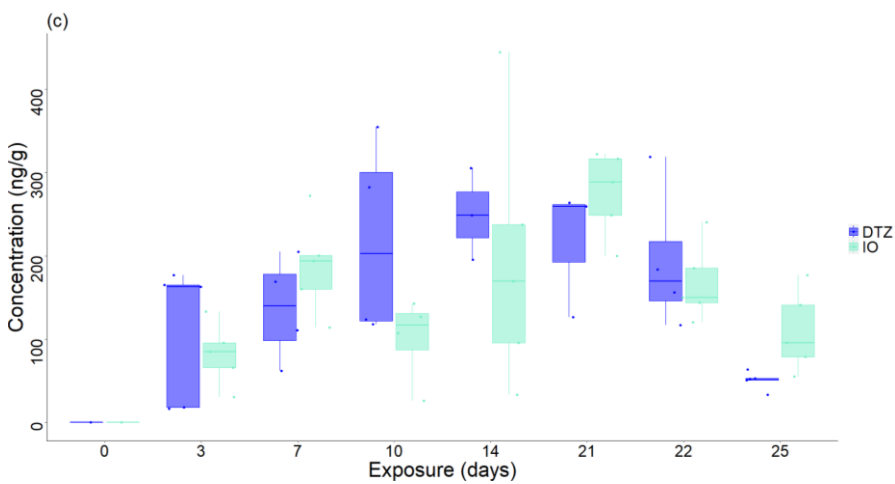
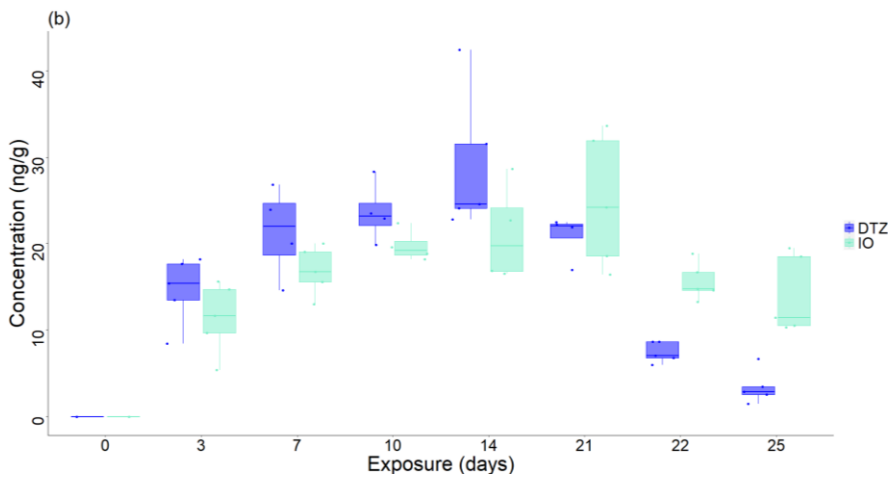
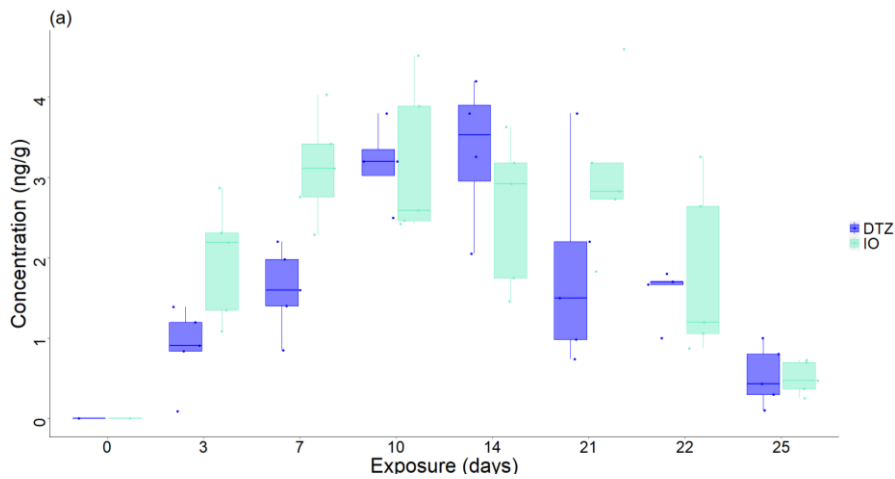
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380

381 Figure 4: Concentrations of diatrizoic acid (DTZ, blue) and iohexol (IO, green) in *Dreissena polymorpha*
382 whole soft tissues (n=5 individuals) during the chronic exposure (21 days) at 10 (a), 100 (b), and 1000
383 (c) $\mu\text{g/L}$ of each molecule and depuration phase (day 22 and day 25)

384



388 Figure 5: Concentrations of diatrizoic acid (DTZ, blue) and iohexol (IO, green) in *Dreissena polymorpha*
 389 digestive glands (n=5 pools of 3 glands) during the chronic exposure (21 days) at 10 (a), 100 (b) and
 390 1000 (c) µg/L of each molecule and depuration phase (day 22 and day 25)

391

		Diatrizoic acid			Iohexol		
		10	100	1000	10	100	1000
Between days	7-3	0.97	0.67	1.00	0.85	1.00	0.16
	10-3	0.95	0.95	0.84	0.90	0.73	0.17
	14-3	1.00	0.86	0.78	0.08	0.08	0.06
	21-3	0.95	0.96	1.00	0.04	< 0.01	< 0.01
	22-3	0.42	< 0.01	0.06	0.27	0.92	1.00
	25-3	0.73	< 0.01	< 0.01	0.94	0.96	1.00
	10-7	1.00	1.00	0.93	1.00	0.96	1.00
	14-7	1.00	1.00	0.62	0.63	0.23	1.00
	21-7	1.00	0.17	1.00	0.44	0.01	0.01
	22-7	0.90	0.02	0.11	0.94	0.63	0.38
	25-7	0.99	< 0.01	0.01	0.27	0.72	0.07
	14-10	1.00	1.00	0.15	0.54	0.77	1.00
	21-10	1.00	0.50	0.83	0.37	0.10	0.01
	22-10	0.94	0.01	0.69	0.90	0.15	0.39
	25-10	1.00	< 0.01	0.12	0.33	0.20	0.07
	21-14	1.00	0.34	0.78	1.00	0.80	0.02
	22-14	0.68	0.02	< 0.01	0.99	0.01	0.18
	25-14	0.92	< 0.01	< 0.01	0.01	0.01	0.02
	22-21	0.93	< 0.01	0.06	0.96	< 0.01	< 0.01
	25-21	1.00	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
25-22	1.00	0.85	0.83	0.03	1.00	0.97	

392 Table 2: Day-by-day comparison of Student *p*-values relative to whole soft tissues of *Dreissena*
 393 *polymorpha* exposed to 10, 100, and 1000 µg/L of diatrizoic acid and iohexol. *p*-values < 0.05 (grey
 394 cells) indicate significant differences between two conditions.

395

396

		Diatrizoic acid			Iohexol		
		10 µg/L	100 µg/L	1000 µg/L	10 µg/L	100 µg/L	1000 µg/L
Between days	7-3	0.66	0.31	1.00	0.34	0.51	0.33
	10-3	< 0.01	0.07	0.35	0.29	0.13	1.00
	14-3	< 0.01	< 0.01	0.20	0.90	0.05	0.25
	21-3	0.34	0.39	0.48	0.43	< 0.01	< 0.01
	22-3	0.70	0.18	0.64	1.00	0.77	0.57
	25-3	0.98	< 0.01	0.89	0.13	0.97	1.00
	10-7	0.03	0.99	0.72	1.00	0.96	0.62
	14-7	0.02	0.17	0.48	0.95	0.80	1.00
	21-7	1.00	1.00	0.81	1.00	0.12	0.56
	22-7	1.00	< 0.01	0.93	0.21	1.00	1.00
	25-7	0.22	< 0.01	0.64	< 0.01	0.96	0.66
	14-10	1.00	0.55	1.00	0.92	1.00	0.52
	21-10	0.10	0.97	1.00	1.00	0.63	0.03
	22-10	0.03	< 0.01	1.00	0.18	0.83	0.84
	25-10	< 0.01	< 0.01	0.05	< 0.01	0.53	1.00
	21-14	0.05	0.13	1.00	0.98	0.88	0.66
	22-14	0.01	< 0.01	0.96	0.76	0.56	1.00
	25-14	< 0.01	< 0.01	0.03	< 0.01	0.27	0.56
	22-21	1.00	< 0.01	1.00	0.28	0.05	0.32
	25-21	0.08	< 0.01	0.09	< 0.01	0.01	0.03
25-22	0.25	0.78	0.12	0.22	1.00	0.88	

398 Table 3: Day-by-day comparison of Student *p*-values relative to digestive glands of *Dreissena*
399 *polymorpha* exposed to 10, 100, and 1000 µg/L of diatrizoic acid and iohexol. *p*-values < 0.05 (grey
400 cells) indicate significant differences between two conditions.

401

402 The bioaccumulation factor (BAF) was calculated for each ICM, in whole soft tissues and digestive
403 glands (Table 4). It reflects the body's ability to bioaccumulate a given compound from the
404 environment, through its respiratory and skin surfaces, excluding exposure through food. It was
405 calculated as follows:

$$BAF (L/kg) = \frac{C_{tissue}}{C_{water}}$$

406 where C_{tissue} ($\mu\text{g}/\text{kg}$) is the mean ICM concentration determined for wet whole soft tissues or
 407 digestive glands during the exposition (day 3 to day 21), and C_{water} ($\mu\text{g}/\text{L}$) is the mean water
 408 concentration measured during the corresponding exposition.

409

		DTZ		IO	
BAF (L/kg)		Whole soft tissues	Digestive glands	Whole soft tissues	Digestive glands
Exposure concentration	10 $\mu\text{g}/\text{L}$	0.10	0.23	0.08	0.30
	100 $\mu\text{g}/\text{L}$	0.12	0.24	0.09	0.15
	1000 $\mu\text{g}/\text{L}$	0.12	0.24	0.15	0.16

410 *Table 4: Bioaccumulation factors (BAF) in Dreissena polymorpha's whole soft tissues or digestive glands after*
 411 *21-days exposure to 10, 100, and 1000 $\mu\text{g}/\text{L}$ of diatrizoic acid and iohexol*

412

413 For both ICMs and whatever the experimental condition (exposure concentration and organ/tissue
 414 considered) the BAF are low and lower than 1, underlying the absence of bioaccumulation of these
 415 molecules in the studied bivalves. Nevertheless, we can notice a slightly higher BAF for DTZ in
 416 digestive glands, which is in accordance with the greater iodine accumulation observed in the acute
 417 exposition (see section 3.1). These results confirm the generally accepted view that these
 418 compounds do not bioaccumulate in fish and wildlife because of their negative log Kow values (-1.28
 419 to -3.05) reinforced by the fact that to date, no environmental risks have been associated with these
 420 compounds in humans and wildlife (Steger-Hartmann et al., 1999; Williams *et al.*, 2017). However, a
 421 recent study unexpectedly detected high levels of the highly polar chemical iopamidol in brain and
 422 gonad tissues of various fish species caught in the Niagara River with concentrations as high as 1059
 423 ng/g (dry basis), resulting in a bioaccumulation factor of approximately 46,000 (Arnnok *et al.*, 2017).
 424 This suggests that the bioaccumulation of ICMs is receptor-mediated and, based on a *in silico*

425 approach, that they may act as endocrine disrupting chemicals in the environment (Singh *et al.*,
426 2019). Nevertheless, in our study, if bioaccumulation is not observed, the accumulation curves show
427 clearly that there is an intake of these ICMs by the organisms, with higher registered concentrations
428 in digestive glands. Although the accumulation pattern seems to differ in whole soft tissues
429 depending to the ICM and DTZ shows a slightly higher bioaccumulation factor, no clear difference
430 appears depending on the osmolality of the two ICMs studied.

431 For the experiments at 10 and 100µg/L of ICMs, analysis by LC-MS/MS showed the intake of ICMs by
432 mussels, particularly in the digestive glands, without an effect on the iodine concentration in this
433 organ (see section 3.1). With the total ICP MS analysis, not only the natural iodine background in the
434 organs did not allow to detect any significant accumulation at low exposure concentrations, but as
435 iodine is an essential element, the intake of iodine at low ICMs exposure concentrations, could be
436 compensated by a loss of native iodine by homeostasis. At the highest exposure condition (1000
437 µg/L), the ICMs quantification by LC-MS/MS, showed a change in the iodine concentration (an
438 increase), particularly in the digestive glands. This increase in the iodine concentration could reflect
439 an effect of ICMs exposure on iodine regulation/metabolism of the mussel. For both iodine and ICMs
440 analyses, the original results obtained in this study highlight an accumulation of ICMs in aquatic
441 organisms. The lack of information actually in the literature concerning the ICMs ecotoxicity using
442 early biological markers (molecular responses) makes it difficult to propose an hypothesis on direct
443 toxic effects of accumulated ICMs on mussels or indirect effects on the base of iodine metabolism
444 disturbance. In this way it would be interesting to study the potential effect of ICMs on mussels by
445 omics approaches, i.e metabolomics and metallomics. Initially developed in the clinical and
446 biomedical fields, metabolomics has gained interest in ecotoxicology during the last decade (Bundy
447 *et al.*, 2009; Bonnefille *et al.*, 2018; Hani *et al.*, 2021). The aim of this approach is the characterization
448 of a set of metabolites, or metabolome, which is the collection of low-molecular weight molecules
449 (typically with mass lower than 1500 Da) present in a cell, tissue or organism at a given time. Being
450 the result of gene expression and the end-product of the metabolism, the metabolome is considered

451 representative of the physiological state of an organism (Weckwerth, 2003). In parallel, potential
452 biological effects of ICMs could be assessed by the study of their interaction and functional
453 connections with metabolites, proteins and other biomolecules within organisms and cells. This type
454 of study defined as metallomics (Haraguchi, 2004; Mounicou *et al.*, 2009; Williams, 2001), will help us
455 in understanding the homeostasis of iodine as well as other endogenous elements.

456

457 **4. Conclusion**

458 This study was the first to provide iodine concentration in the whole soft tissues or organs of control
459 freshwater zebra mussels. A basal iodine concentration of 260 ± 199 ng/g was determined in the
460 whole mussel *Dreissena polymorpha*, shared among the gills, digestive glands, gonads, and remaining
461 tissues (composed of the gland of byssus, muscle, and mantle) at 206 ± 104 , 158 ± 79 , 92 ± 105 , and
462 359 ± 334 ng/g, respectively. Iodine concentration increased in gills, digestive glands, and gonads
463 when bivalves were exposed to high concentrations of ICMs, showing that both high and low
464 osmolality ICMs can effectively penetrate the tissues of filter-feeding organisms such as the bivalves
465 *Dreissena polymorpha*. Despite the high concentration of iodine in the remaining tissues, no
466 accumulation of iodine was observed in these tissues in the exposed organism. Further studies are
467 necessary to precise the potential role of mantle in the high iodine concentrations in control
468 organism without accumulation during ICMs exposure. Based on the total iodine analysis on the
469 different organs, the digestive gland was chosen for accumulation studies as it exhibits the highest
470 iodine accumulation via ICMs exposure. This study offers the first determination of ICM
471 concentrations in both mollusk's whole soft tissues and digestive glands by selective and sensitive LC-
472 MS/MS analysis. The level of accumulation was quite similar for diatrizoic acid and iohexol in whole
473 soft tissues and in digestive glands in a manner dependent on the relative level of exposure, with
474 however a slightly different accumulation pattern in whole soft tissues, and dependent of the
475 molecule. Although bioaccumulation factors are low (inferior to 1), the present findings show that

476 ICMs can enter in bivalves that may use ICM as iodine source. Further studies are needed to finely
477 study biological effects at molecular level through metallomic and metabolomic approaches.

478

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