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## Use of the bivalve *Dreissena polymorpha* as a biomonitoring tool to reflect the protozoan load in freshwater bodies

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1 Use of the bivalve *Dreissena polymorpha* as a biomonitoring tool to reflect the  
2 protozoan load in freshwater bodies.

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4 Elodie Géba<sup>1,2</sup>, Dominique Aubert<sup>2</sup>, Loïc Durand<sup>3</sup>, Sandy Escotte<sup>2</sup>, Stéphanie La  
5 Carbona<sup>3</sup>, Catherine Cazeaux<sup>3</sup>, Isabelle Bonnard<sup>1</sup>, Fanny Bastien<sup>1</sup>, Mélissa Palos  
6 Ladeiro<sup>1</sup>, Jitender P. Dubey<sup>4</sup>, Isabelle Villena<sup>2</sup>, Alain Geffard<sup>1</sup>, Aurélie Bigot-Clivot<sup>1</sup>

7  
8 <sup>1</sup> UMR-I 02 SEBIO (Stress Environnementaux et BIOSurveillance des milieux  
9 aquatiques), Université de Reims Champagne Ardenne, UFR Sciences Exactes et  
10 Naturelles, Campus Moulin de Housse, BP 1039, 51687 Reims cedex 2, France  
11 <sup>2</sup> EA7510, ESCAPE, Epidémiologie et Circulation des Parasites dans les  
12 Environnements, Université de Reims Champagne Ardenne, Faculté de Médecine, SFR  
13 Cap Santé Fed 4231, 51 Rue Cognacq Jay, 51096 Reims, France.

14 <sup>3</sup> ACTALIA Food Safety Department, 310 Rue Popielujko, 50000 Saint-Lô, France

15 <sup>4</sup> United States Department Agriculture, Agricultural Research Service, Beltsville  
16 Agricultural Research Center, Animal Parasitic Diseases Laboratory, building 1001,  
17 Beltsville, MD 20705 2350, USA

18

19 **Abstract:**

20 *Cryptosporidium parvum*, *Toxoplasma gondii* and *Giardia duodenalis* are worldwide  
21 pathogenic protozoa recognized as major causal agents of waterborne disease outbreaks.

22 To overcome the normative process (ISO 15553/2006) limitations of protozoa detection  
23 in aquatic systems, we propose to use the zebra mussel (*Dreissena polymorpha*), a  
24 freshwater bivalve mollusc, as a tool for biomonitoring protozoan contamination..

25 Mussels were exposed to three concentrations of *C. parvum* oocysts, *G. duodenalis*

26 cysts or *T. gondii* oocysts for 21 days followed by 21 days of depuration in clear water.  
27 *D. polymorpha* accumulated protozoa in its tissues and haemolymph. Concerning *T.*  
28 *gondii* and *G. duodenalis*, the percentage of protozoa positive mussels reflected the  
29 contamination level in water bodies. As for *C. parvum* detection, oocysts did  
30 accumulate in mussel tissues and haemolymph, but in small quantities, and the limit of  
31 detection was high (between 50 and 100 oocysts). Low levels of *T. gondii* (1-5 oocysts  
32 /mussel) and *G. duodenalis* (less than 1 cyst/mussel) were quantified in *D. polymorpha*  
33 tissues. The ability of zebra mussels to reflect contamination by the three protozoa for  
34 weeks after the contamination event makes them a good integrative matrix for the  
35 biomonitoring of aquatic ecosystems.

36

37 **Keywords:** *Toxoplasma gondii*, *Giardia duodenalis*, *Cryptosporidium parvum*, zebra  
38 mussel, biomonitoring

39

40

41 1. Introduction

42

43 *Cryptosporidium parvum* and *Giardia duodenalis* are the major causes of protozoal  
44 diarrhea in humans. Cryptosporidiosis and giardiasis can cause high morbidity and  
45 severe dehydration, and even death in immunocompromised hosts (Cacciò et al., 2005).  
46 *Toxoplasma gondii* is the causative agent of toxoplasmosis; it is usually asymptomatic  
47 in humans, but can cause severe clinical diseases in immunocompromised hosts or in  
48 case of congenital infection (Montoya and Liesenfeld, 2004). The minimal infectious  
49 doses for humans may be low: 9 to 1,042 *C. parvum* oocysts (Fayer et al., 2000) and  
50 down to 10 *G. duodenalis* cysts (Rendtorff, 1954). Although the minimal *T. gondii*  
51 infectious dose for humans has not been determined, based on animal experimentation

52 one single oocyst can infect humans (Dubey, 2010). The environment including food  
53 and water are contaminated by these protozoa via human or animal faeces: felids faeces  
54 for *T. gondii* or mammals' faeces for *C. parvum* and *G. duodenalis*. The consumption of  
55 fruit and vegetables (McKerr et al., 2015; Pönka et al., 2009) and untreated water are  
56 known vectors of the foodborne transmission cycle (Moreno et al., 2018). The  
57 environmental stages, i.e. *Giardia* cysts, and *T. gondii* and *Cryptosporidium* oocysts  
58 (hereafter designated as protozoa throughout this paper) are extremely resistant and can  
59 persist in aquatic ecosystems for months (Bingham et al., 1979; Lindsay and Dubey,  
60 2009; Olson et al., 1999). Protozoa have been found in several freshwater aquatic  
61 ecosystems such as surface water (Aubert and Villena, 2009; Bautista et al., 2018;  
62 Karanis et al., 2006; Vermeulen et al., 2019; Xiao et al., 2018) and underground water  
63 (Aubert and Villena, 2009; Hancock et al., 1998; Khaldi et al., 2011; Solo-Gabriele et  
64 al., 1998) but also in drinking water (Almeida et al., 2010; Aubert and Villena, 2009;  
65 Castro-Hermida et al., 2010; Cheun et al., 2013; Ware et al., 2010). Waterborne  
66 transmission to humans is the major cause of cryptosporidiosis (Putignani and  
67 Menichella, 2010) and giardiasis (Adam et al., 2016) outbreaks. *Cryptosporidium* spp.  
68 and *Giardia* spp. indeed caused 63% and 37% of waterborne parasitic disease outbreaks  
69 in Australia, North America and Europe between January 2011 and December 2016,  
70 respectively (Efstratiou et al., 2017), and *T. gondii* caused 2% between January 2004  
71 and December 2010 (Baldursson and Karanis, 2011).

72 In the water matrix, *Cryptosporidium* oocysts and *Giardia* cysts are currently detected  
73 by immunofluorescence (IF) in filtered water sample (ISO 15553/2006). However, this  
74 method is time consuming, expensive and requires expert image analysis, so it is  
75 unsuitable for rapid parasite detection. It requires a large volume of filtered water and a  
76 high parasite concentration in the samples. Furthermore, the filtration and the

77 purification techniques can yield variable results depending on the water quality, the  
78 sampling period, the location and the water volume (Gallas-Lindemann et al., 2013;  
79 Karanis et al., 2006; Efstratiou et al., 2018). No normative process is available for *T.*  
80 *gondii* oocyst detection in water because, oocysts are present in limited numbers  
81 whereas *Giardia* and *Cryptosporidium* (oo)cysts are present in high numbers. This  
82 represents a limitation when applying a monitoring approach based on the water matrix  
83 and it seems necessary to investigate new methods for assessing water quality in terms  
84 of biological contamination.

85 Several studies have underlined the ability of filter-feeding species, such as bivalves, to  
86 accumulate and concentrate protozoa in their tissues (Aksoy et al., 2014; Arkush et al.,  
87 2003; Fayer et al., 2003; Gómez-Couso et al., 2004; Graczyk et al., 1999). Among  
88 bivalves, the freshwater zebra mussel – *Dreissena polymorpha* – has been particularly  
89 studied as a biomonitoring tool (Binelli et al., 2015). It is a sedentary species with a  
90 high filtration rate, (5 to 400 mL/h/mussel; Ackerman, 1999; Costa et al., 2008; Reeders  
91 et al., 1989). The ability of *D. polymorpha* to bioaccumulate *C. parvum*, *G. duodenalis*  
92 and *T. gondii* under laboratory conditions is well known (Graczyk et al., 2003; Palos  
93 Ladeiro et al., 2015, 2014). A few studies have already used this bivalve to recover and  
94 concentrate environmental *C. parvum* (Graczyk et al., 2008, 2004; Lucy et al., 2010,  
95 2008), *G. duodenalis* (Graczyk et al., 2008, 2004; Lucy et al., 2010, 2008) and *T. gondii*  
96 (Kerambrun et al., 2016). Using attached filter-feeding organisms allows for a  
97 representative measurement of protozoan contamination of the mussels living in water  
98 bodies and limits the variability of measurements by integrating temporal  
99 contamination. In addition, such a tool makes it possible to assess water contamination  
100 by *T. gondii*, *G. duodenalis* and *C. parvum* from only one sample. However, bivalves

101 are a complex matrix that render measurements difficult, it is therefore necessary to  
102 determine the limit of detection and the limit of quantification of the method.  
103 Before proposing *D. polymorpha* as a tool for biomonitoring protozoa, it is necessary to  
104 determine whether the parasite loads measured in zebra mussels are relevant to the  
105 water contamination levels. This study aimed to define for the first time the kinetics of  
106 *C. parvum*, *G. duodenalis* and *T. gondii* accumulation and depuration by *D. polymorpha*  
107 under control conditions, so as to propose recommendations for using *D. polymorpha* as  
108 a matrix to monitor the quality of watercourses. For this purpose, zebra mussels were  
109 exposed to three doses of these protozoa for 21 days. To characterize the integration of  
110 protozoa contamination by zebra mussels over time, the exposure period was followed  
111 by 21 days of depuration in clear water. Protozoa levels were measured in whole tissue  
112 and in the haemolymph because haemolymph collection can be considered as a  
113 nonlethal technique (Gustafson et al., 2005; McCartney and Wilbur, 2007) and the  
114 haemolymph bioaccumulates protozoa (Graczyk et al., 2004; Palos Ladeiro et al., 2015).  
115 In order to facilitate the use of zebra mussels as a biomonitoring tool, the three protozoa  
116 were detected by a molecular technique, and the same protocol of DNA extraction was  
117 used.

118

## 119 2. Materials and methods

### 120 2.1. Mussels and protozoa

121 Zebra mussels (*D. polymorpha*) were collected at around 5 m depth in Port de  
122 Nuisement (N 48°36'10.0728" E 4°44'57.408") at the Lac-du-Der-Chantecoq (Marne,  
123 France) in October 2015. Then the mussels measuring  $20 \pm 2$  mm were acclimated in  
124 the dark in aerated Cristaline® (Aurèle, France) drinking water at 12 °C for two weeks.  
125 The water was renewed twice a week to ensure that mussels were protozoa-free on

126 the first day of exposure. The mussels were fed *ad libitum* twice a week with 20,000  
127 cells of two microalgal species (*Chlorella pyrenoidosa* and *Scenedesmus obliquus*) per  
128 mussel and per day.

129 *T. gondii* oocysts of the strain ME49 genotype II were obtained as described previously  
130 (Dubey, 2010). Oocysts were sporulated in 2 %aqueous sulfuric acid and stored at 4°C  
131 until use. *C. parvum* oocyst and *G. duodenalis* cyst preparations were purchased from  
132 Waterborne™, Inc. (New Orleans, LA, USA) and kept at 4°C until use.

133

## 134 2.2. Exposure conditions

135 Before exposure, zebra mussels were randomly divided into 10 groups of 195 mussels  
136 each i.e., 1 control group and 9 exposed groups. Each group was placed in a tank  
137 containing 10 L of Cristaline® (Aurèle, France) water. Zebra mussels were exposed to 0;  
138 100; 1,000 or 10,000 protozoa per bivalve and per day for 21 days (supplementary  
139 data). The tank water was changed once a week and the volume of water was adapted to  
140 the number of remaining bivalves. The protozoa were spiked again after water changes  
141 to maintain the initial protozoa concentrations in the tanks. To determine the  
142 environmental equivalent for each exposure concentration the number of protozoa per  
143 litre (protozoa.L<sup>-1</sup>) in each tank was calculated for each condition:  $1.9 \times 10^3$ ,  $1.9 \times 10^4$   
144 and  $1.9 \times 10^5$  on day 1;  $3.6 \times 10^3$ ,  $3.6 \times 10^4$  and  $3.6 \times 10^5$  on day 2;  $5.3 \times 10^3$ ,  $5.3 \times 10^4$   
145 and  $5.3 \times 10^5$  on day 3;  $1.1 \times 10^4$ ,  $1.1 \times 10^5$  and  $1.1 \times 10^6$  on day 7;  $2.8 \times 10^4$ ,  $2.8 \times 10^5$   
146 and  $2.8 \times 10^6$  on day 14 and  $4.3 \times 10^4$ ,  $4.3 \times 10^5$  and  $4.3 \times 10^6$  on day 21 for 100; 1,000  
147 or 10,000 protozoa/bivalve/day respectively. Then, the mussels were transferred into  
148 new tanks filled with clean water for the depuration period. During the exposure and  
149 depuration periods, 10 mussels per condition were sampled on days 1, 2, 3, 7, 14, and  
150 21. Haemolymph was taken from the posterior adductor muscle, and whole tissue was

151 collected and, then stored at -80°C until analysis. Mussels were still fed *ad libitum*  
152 twice a week with *Chlorella pyrenoidosa* and *Scenedesmus obliquus*.  
153 Mortality was recorded daily: mortality of 1.62 % in the control tank, 1.08 % in the  
154 three tanks devoted to *T. gondii* exposure, 1.08 % in the three tanks devoted to *C.*  
155 *parvum* exposure and 0.72 % in three tanks devoted to *G. duodenalis* exposure were  
156 recorded throughout the experiment. No difference in mortality was observed between  
157 the different exposure concentrations.

158

### 159 2.3 DNA extraction from different matrices

160 Bivalve tissues were ground and digested using 1X Trypsin for 1h at 37°C under 90 rpm  
161 shaking. Each tissues homogenate was centrifuged at 5,000 g for 5min, and the pellet  
162 was stored at -20°C until DNA extraction. DNA was extracted using a FastDNA<sup>®</sup> SPIN  
163 Kit for Soil according to the manufacturer's instruction (MP Biomedicals, Illkirch-  
164 Graffenstaden, France). This kit was previously tested in our laboratory and appeared to  
165 be the most effective method for extracting the three protozoa from zebra mussel tissues  
166 (data not shown).

167 DNA was extracted from haemolymph samples using an optimized protocol based on  
168 Palos Ladeiro et al., (2014). Haemolymph samples were centrifuged at 5,000g for 5  
169 min. Pellets containing 100 µL of supernatant were submitted to heat shock cycles  
170 consisting of freezing at -80 °C for 5 min and thawing at 95 °C for 4 min. These heat  
171 shock cycles were repeated six times to break the wall and access protozoan DNA.  
172 Then, the samples were subjected to ultrasonic treatment at 37 Hz (Ultrasonics 88155)  
173 for 1 min for *C. parvum* and *G. duodenalis* samples and 10 min for *T. gondii* samples.  
174 DNA was extracted from the pellets using an InstaGene<sup>™</sup> Matrix kit following the  
175 manufacturer's instructions (Bio-Rad, Marnes-la-Coquette, France).



176

#### 177 2.4. TaqMan qPCR

178 Parasite DNA detection was detected by TaqMan real-time qPCR in a CFX96  
179 TOUCH™ thermocycler (Bio-Rad, USA) according to Palos Ladeiro et al. (2014).  
180 TaqMan real-time qPCR targeted specific *T. gondii* (AF487550.1; Lélou et al., 2011), *G.*  
181 *duodenalis* (M54878; Verweij et al., 2003) and *C. parvum/hominis* (AF188110;  
182 Fontaine and Guillot, 2002) genes (supplementary data). qPCR amplifications were  
183 performed as specified by the manufacturer's instructions in the following mixtures: 5  
184 µL of extracted DNA sample were added to 20 µL of mix containing 2X iQ® supermix  
185 (Bio-Rad), 1 µL of BSA (10 mg.mL<sup>-1</sup>; SIGMA, France), 400 nM of each primers and  
186 200 nM of probe for *T. gondii* and *G. duodenalis* or 100 nM for *C. parvum*. A no-  
187 template control was added. DNA polymerase was activated after an initial 3-min  
188 denaturation step at 95 °C. Then, the samples were submitted to 45 cycles of 15 s at 95  
189 °C and 1 min at 60 °C. The Cq values were collected, each correspond to the number of  
190 cycles for which fluorescence exceed a fixed threshold and allows for quantifying the  
191 amount of the target DNA. Each sample was analyzed in duplicate, and non-diluted and  
192 ten-fold diluted (10<sup>-1</sup>) DNA extracts were analyzed to limit the inhibition effect. In case  
193 of inhibition, non-diluted Cq values were extrapolated from the Cq of the ten-fold  
194 dilution considering a Cq difference of 3.3 for a dilution factor equal to 10 and an  
195 optimal PCR efficacy equal to 2 (log<sub>2</sub> (10)).

196 To determine the number of (oo)cyst in the samples, reference curves were established  
197 using a serial dilution ranging from 50,000 to 1 (oo)cyst(s) spiked in mussel tissues or  
198 added to the water suspensions. For tissues analysis, experiments were performed in  
199 quintuplicate for dilutions ranging from 100 to 1 protozoa (five dilutions) and in  
200 triplicate for dilutions ranging from 50,000 to 500 (oo)cysts (five dilutions too). For

201 haemolymph analysis, samples were spiked in triplicate with dilutions ranging from  
202 10,000 to 100 (oo)cysts (six dilutions). All spiked tissues and haemolymph samples  
203 were also used to determine the limit of quantification (LOQ) and the limit of detection  
204 (LOD<sub>95</sub>). LOQ was determined as the lowest number of parasites for which a linear  
205 relationship was observed between the Cq and the number of parasites ( $r^2 > 0.98$ ) and  
206 LOD<sub>95</sub> was defined as the lowest number of parasites for which 95% of positive  
207 samples were detected.

208

## 209 2.5. Statistical analysis

210 All statistical analyses were performed with R software (version 3.5.2). As  
211 physiological data did not comply with the parametric assumption of normality  
212 (Shapiro-Wilk test) and homogeneity of variance (Levene's tests), nonparametric tests  
213 were used. Kruskal-Wallis test and Nemenyi test for post hoc pairwise comparisons  
214 were used ( $\alpha \leq 0.05$ ). Only data above the LOQ was presented in the graphs.

215

## 216 3. Results

### 217 3.1. Determination of LOD<sub>95</sub> and LOQ

218 For the tissues samples, the performances of the method were determined using tissues  
219 spiked with 50,000 to 1 (oo)cyst(s) (Table 1). For *T. gondii* oocysts, a linear response ( $r^2$   
220  $> 0.98$ ) was observed from 50,000 to 5 oocysts, so LOQ was five oocysts in whole  
221 tissues. No amplification curve was obtained in 1/10 replicate corresponding to 1  
222 oocyst, so LOD<sub>95</sub> was between one ( $< 95\%$  of positive samples) and five (100% of  
223 positive samples) oocysts in whole tissues. The slope coefficient was -3.1193 with a  
224 squared correlation coefficient ( $r^2$ ) of 0.99 (supplementary data). For *G. duodenalis*  
225 cysts, a linear response ( $r^2 > 0.98$ ) was observed from 50,000 to 1 cyst(s), so LOQ was 1

226 cyst. An amplification curve was obtained in all replicates corresponding to 1 cyst, so  
227 LOD<sub>95</sub> was less than 1 cyst. The slope coefficient was -3.5093 with a squared  
228 correlation coefficient ( $r^2$ ) of 0.99 (supplementary data). For *C. parvum*, a linear  
229 response ( $r^2 > 0.98$ ) was observed from 50,000 to 100 oocysts, so LOQ was 100  
230 oocysts. An amplification curve was obtained in 90 % of the replicates corresponding to  
231 50 oocysts and in 100 % of the replicates corresponding to 100 oocysts, so LOD<sub>95</sub> was  
232 between 50 and 100 oocysts in whole tissues. The slope coefficient was -3.5673 with a  
233 squared correlation coefficient ( $r^2$ ) of 0.96 (supplementary data).

234 For the haemolymph analysis, samples were spiked in triplicate with dilutions ranging  
235 from 10,000 to 10 (oo)cysts (Table 1). For *T. gondii*, the slope coefficient was -3.2996,  
236 with a squared correlation coefficient ( $r^2$ ) of 0.99 (supplementary data). A linear  
237 response ( $r^2 > 0.98$ ) was observed from 50,000 to 100 oocysts per sample, so LOQ was  
238 100 oocysts. An amplification curve was obtained in 83 % of the replicates  
239 corresponding to 10 oocysts and in 100 % of the replicates corresponding to 50 oocysts  
240 per sample, so LOD<sub>95</sub> was between 10 and 50 oocysts. For *G. duodenalis*, the slope  
241 coefficient was -4.591, with a squared correlation coefficient ( $r^2$ ) of 0.99  
242 (supplementary data). A linear response ( $r^2 > 0.98$ ) was observed from 50,000 to 10  
243 cysts per sample, so LOQ was 10 cysts. An amplification curve was obtained in 67 % of  
244 the replicates corresponding to 5 cysts and in 100 % of the replicates corresponding to  
245 10 cysts in the samples, so LOD<sub>95</sub> was between 5 and 10 cysts. Lastly, for *C. parvum*, a  
246 linear response ( $r^2 > 0.98$ ) was observed from 50,000 to 100 oocysts per sample, so  
247 LOQ was 100 oocysts. An amplification curve was obtained in 80 % of the replicates  
248 corresponding to 50 oocysts and in 100 % of the replicates corresponding to 100  
249 oocysts in the samples, so LOD<sub>95</sub> was between 50 and 100 oocysts. The slope

250 coefficient was -3.2776, with a squared correlation coefficient ( $r^2$ ) of 0.98  
251 (supplementary data).

252

### 253 3.2. Protozoa levels in bivalves

254 In the control samples, no protozoa was detected in the haemolymph. In whole tissues  
255 4.2% (5/120) of the samples were positive to *T. gondii* oocysts, with < 1 oocyst per  
256 mussel. No *C. parvum* oocyst or *G. duodenalis* cyst was detected in whole tissue  
257 samples.

258

#### 259 3.2.1. Protozoa detection

260 The number of mussels positive to *T. gondii* was higher in tissues than in the  
261 haemolymph (Figure 1). In the haemolymph samples, at 100 oocysts/mussel/day  
262 mussels were positive to *T. gondii* from day 7 of the exposure period until the end of the  
263 21 days of depuration (Figure 1 A). However, at higher concentrations, oocysts were  
264 detected in the haemolymph from 1 day of exposure ( $\geq 10\%$  of positive samples) and  
265 the number of positive mussels increased until 21 days of exposure and reached 100%  
266 for the highest dose. Mussels remained positive to *T. gondii* throughout the 21 days of  
267 the depuration period whatever the exposure dose. For each sampling time, the  
268 percentage of positive mussels increased with the protozoa concentration in water. In  
269 mussel tissues, *T. gondii* oocysts were consistently detected from day 1 of exposure  
270 period until the end of the day 21 of depuration at all oocyst concentrations (Figure 1  
271 B). During the accumulation phase, all samples were positive from 1 day of exposure at  
272 the highest concentration and from 3 days at the two-lower concentrations (100 and  
273 1,000 oocysts/mussel/day). During depuration, all mussels remained positive at the two  
274 highest doses while the percentage tended to decrease at 100 oocysts/mussel/day.

275 The percentage of mussels positive to *G. duodenalis* cysts increased with the protozoa  
276 concentration in water at each sampling time in the haemolymph and in tissues (Figure  
277 1). In the haemolymph, *G. duodenalis* cysts were detected from day 1 of the exposure  
278 period until the end of the 21 days of depuration (Figure 1 C). The percentage of  
279 positive haemolymph samples increased during the exposure period – it reached more  
280 than 50% of positive samples at the two lowest concentrations and 100% at the highest  
281 concentration – and remained relatively steady throughout the depuration period, only  
282 varying by 20%. Cysts were detected in tissues later than in the haemolymph (Figure 1  
283 D), i.e., after 7 days (10%), 2 days (40%) or 1 day (50%) of exposure to 100, 1,000 and  
284 10,000 cysts/mussel/day, respectively. The percentage of positive tissues increased until  
285 21 days of exposure, and cysts were still detected in mussel tissues after 21 days of  
286 depuration.

287 Few haemolymph and tissues samples positive to *C. parvum* were detected (Figure 1).  
288 In the haemolymph (Figure 1 E), the percentage of detection increased between 14 and  
289 21 days of exposure for the two highest concentrations and decreased during the  
290 depuration period. In tissues (Figure 1 F), detection slightly increased with the oocyst  
291 concentration at each sampling time. No clear trend was observed, and the percentage of  
292 positive mussels did not exceed 50% in the haemolymph or in tissues.

293

### 294 3.2.2. Protozoa quantification

295 We failed to quantify part of the samples detected as protozoa positive because the  
296 amount of bioaccumulated protozoa was below the LOQ. The number of samples above  
297 the LOQ is summarized in Table 2. Quantification results are presented in graphs if at  
298 least 50% of the samples had values greater than the LOD<sub>95</sub>, and if at least 30% of those  
299 samples had values greater than the LOQ per modality. Consequently, only the numbers

300 of *T. gondii* oocysts bioaccumulated in tissues (at 1,000 and 10,000 oocysts/bivalve/day,  
301 Figure 2) and the numbers of *G. duodenalis* cysts bioaccumulated in the haemolymph  
302 (at 10,000 cysts/bivalve/day, Figure 3) and in tissues (at 1,000 and 10,000  
303 cysts/bivalve/day, Figure 4) are presented in the graphs.

304 The number of *T. gondii* oocysts bioaccumulated in tissues (Figure 2) did not  
305 significantly vary throughout the exposure period, with  $10.2 (\pm 3.8)$  and  $166.4 (\pm 231.4)$   
306 oocysts at 1,000 and 10,000 oocysts/mussel/day respectively. Then, a non-significant  
307 decrease was observed after 3 days of depuration, with  $3.1 (\pm 2.6)$  and  $18.2 (\pm 10.8)$   
308 oocysts at 1,000 and 10,000 oocysts/mussel/day, respectively. Oocysts still accumulated  
309 in mussel tissues even after 21 days of depuration, with 7.7 (only one positive sample)  
310 and  $38.2 (\pm 61.5)$  oocysts, respectively. The quantity of bioaccumulated oocysts was not  
311 significantly different between the two concentrations all sampling times combined.

312 The numbers of *G. duodenalis* cysts in the haemolymph significantly increased on day  
313 21 of the exposure period ( $218.7 \pm 143.3$ ) compared to day 3 ( $15.8 \pm 0.6$ ) and day 7  
314 ( $20.9 \pm 7.7$ ) of exposure (Figure 3). During the depuration period, the number of cysts  
315 per mussel significantly decreased after 1 day ( $32.7 \pm 19.9$ ) and cysts remained in the  
316 haemolymph for at least 21 days ( $36.1 \pm 13.6$ ). In tissues (Figure 4), the number of cysts  
317 per mussel was very variable, and no significant difference was shown at 1,000  
318 cysts/mussel/day or between the two concentrations. At 10,000 cysts/mussel/day, the  
319 number of bioaccumulated cysts after 1 day of depuration ( $15.1 \pm 19.9$ ) was  
320 significantly higher than after 3 days ( $2.0 \pm 2.2$ ) and 7 days ( $2.5 \pm 2.4$ ) of exposure.

321 Then, at 1,000 and 10,000 cysts/mussel/day, a non-significant decrease was observed  
322 after 3 days of depuration ( $1.8 \pm 1.4$  and  $4.3 \pm 4.9$ , respectively) compared to 2 days of  
323 depuration ( $12.5 \pm 22.9$  and  $13.4 \pm 18.7$ , respectively). The cyst level remained similar

324 in tissues until 21 days of depuration with 15.9 ( $\pm$  25.4) and 25.6 ( $\pm$  46.0) cysts per  
325 mussel at 1,000 and 10,000 cysts/mussel/day, respectively.

326

#### 327 4. Discussion

328 The waterborne transmission of *C. parvum* and *G. duodenalis* is well documented.  
329 Adam et al. (2016) identified 242 giardiasis outbreaks including 74.6 % with a  
330 waterborne origin between 1972 and 2011. Likewise, among the 70 cryptosporidiosis  
331 outbreaks reported between 2000 and 2010, 56.4% had a waterborne origin (Putignani  
332 and Menichella, 2010). The prevalence of *T. gondii* oocysts in watercourses has been  
333 studied only recently. Between 2004 and 2010 out of 325 waterborne protozoa  
334 outbreaks reported, 2 % were caused by *T. gondii* (Baldursson and Karanis, 2011). For  
335 example, *T. gondii* oocysts were found in 27.2% (n= 114) of drinking water samples in  
336 Poland (Sroka et al., 2006). *C. parvum* oocysts were detected in 10.2% (n= 167), 32.7%  
337 (n= 52) and 35% (n= 26) and *G. duodenalis* in 8.4% (n=167), 36.5% (n= 52) and 12%  
338 (n= 26) of drinking water samples in Portugal (Almeida et al., 2010), Spain (Castro-  
339 Hermida et al., 2010) and Japan (Hashimoto et al., 2002), respectively. Considering the  
340 low doses required for these parasites to infect humans, positive detection in water  
341 reflects a health risk.

342 This study addresses for the first time exposure of zebra mussels to *G. duodenalis* and  
343 *T. gondii* for 21 days and the subsequent depuration kinetics. The percentages of  
344 detection provided relevant results for assessing the presence of protozoa loads in  
345 aquatic environments. Detection of *T. gondii* and *G. duodenalis* in zebra mussel tissues  
346 or haemolymph indeed provided early diagnosis, i.e., as early as after 1 day of exposure  
347 to 100 (oo)cysts/mussel/day. (Oo)cysts were still detected after 21 days of depuration,  
348 therefore the method could reflect present or recent contamination of water bodies.

349 Moreover, the percentages of positive samples appeared to reflect contamination levels  
350 with different levels of sensitivity depending on the compartment (tissues or  
351 haemolymph). This may be related to LOD<sub>95</sub>, which differed according to each  
352 protozoan and each mussel compartment. For *G. duodenalis*, contamination was  
353 detected in the haemolymph from 1 day of exposure, with 10%, 30% or 60% of positive  
354 samples for punctual contamination levels of  $1.9 \times 10^3$ ,  $1.9 \times 10^4$  and  $1.9 \times 10^5$  cysts.L<sup>-1</sup>,  
355 respectively. But contamination was not detected in the tissue samples. The percentage  
356 of positive haemolymph samples could reflect contamination by cysts, whether  
357 occasional or chronic (over a time span of 21 days): a percentage of 80 to 100% was  
358 representative of contamination ranging from  $5.3 \times 10^5$  to more than  $4.3 \times 10^6$  cysts.L<sup>-1</sup>,  
359 while a percentage of 10 to 20% reflected a cyst level ranging from  $1.9 \times 10^3$  to  $5.3 \times$   
360  $10^4$  cysts.L<sup>-1</sup>. The percentage of positive mussels remained relatively stable throughout  
361 the depuration period (20% variation) and clearly distinct for each contamination level:  
362 10 to 30%, 60 to 80% or 90 to 100% after chronic exposure to  $4.3 \times 10^4$ ,  $4.3 \times 10^5$  and  
363  $4.3 \times 10^6$  cysts.L<sup>-1</sup>, respectively. The same patterns were observed in tissues, with later  
364 and lower detection percentages of 10% on average for each cyst concentration.

365 Contrary to *G. duodenalis*, *T. gondii* was detected earlier in tissues than in the  
366 haemolymph. The percentage of positive tissues reached 100% from  $5.3 \times 10^3$  oocysts.L<sup>-1</sup>,  
367 and remained stable for the 21 days of depuration after chronic exposure (21 days) at  
368  $4.3 \times 10^5$  oocysts.L<sup>-1</sup>. *T. gondii* detection in the haemolymph samples occurred later, but  
369 still provided an assessment of *T. gondii* levels in water. All haemolymph samples were  
370 positive following exposure to only  $4.3 \times 10^6$  oocysts.L<sup>-1</sup>; 10 to 30% of positive samples  
371 reflected oocyst levels ranging from  $1.1 \times 10^4$  to  $1.1 \times 10^5$  oocysts.L<sup>-1</sup>, while 80 to 90% of  
372 positive samples tended to reflect contamination by  $2.8 \times 10^5$  to  $4.3 \times 10^5$  oocysts.L<sup>-1</sup>. Zebra  
373 mussels integrated protozoan contamination over the 21 days of depuration. This



374 underlines their ability to reflect past contamination of the water environment. Previous  
375 studies also showed integration of contamination by *T. gondii* (Palos Ladeiro et al.,  
376 2015), *C. parvum* and *G. duodenalis* (Graczyk et al., 2003) for 14 days of depuration in  
377 zebra mussels. In marine species, *T. gondii* oocysts were still detected in *M.*  
378 *galloprovincialis* 21 days after inoculation (Arkush et al. 2003) and *C. parvum* oocysts  
379 remained detected in gills and haemocytes of *Crassostrea virginica* 12 days after  
380 exposure (Fayer et al., 1997). In agreement with our results, *C. parvum* oocysts  
381 persisted 14 days' after inoculation in the freshwater mollusc *Corbicula fluminea*  
382 (Graczyk et al. 1998).

383 Many samples were not quantifiable, with protozoa loads in mussels was no  
384 representative of the water contamination level. Nevertheless, our experiment provided  
385 further details on bioaccumulation of (oo)cysts between 1 and 3 days of depuration. The  
386 number of *T. gondii* oocysts bioaccumulated in zebra mussel tissues dropped between 1  
387 and 2 days of depuration, and then a minimal quantity per mussel seemed to persist up  
388 to 21 days of depuration. Palos Ladeiro et al. (2015) also showed a sharp decrease in the  
389 number of bioaccumulated oocysts after one week of depuration, which then remained  
390 the same the 2 following weeks. For *G. duodenalis*, an increasing trend was observed in  
391 tissues after 1 day in clear water compared to the exposure period. Simultaneously in  
392 the haemolymph, a significant increase of the number of cysts was observed after 21  
393 days of exposure, followed by a decrease after 1 day in clear water. Therefore, zebra  
394 mussels appeared to transfer cysts from the haemolymph to tissues in the depuration  
395 period which explained the concentration of *G. duodenalis* cysts after 1 day in clear  
396 water. As previously observed (Palos Ladeiro et al., 2015), *T. gondii* oocysts seemed to  
397 be less detected in the haemolymph than in tissues. As opposed to *T. gondii* oocysts, *G.*  
398 *duodenalis* cysts seemed to be more accumulated in the haemolymph than in tissues -

399 especially during the exposure period - despite a higher LOQ in tissues than in the  
400 haemolymph. These two protozoa could be managed differently by zebra mussel cells.  
401 *T. gondii* oocysts and *G. duodenalis* cysts have different sizes (10-13  $\mu\text{m}$  and 12-15  $\mu\text{m}$ ,  
402 respectively) and wall structures (Adam, 2001; Dumètre et al., 2013). Moreover, some  
403 authors have shown the presence of lectins in the gills and labial palp mucus in  
404 *Crassostrea virginica* (Pales Espinosa et al., 2009) and *M. edulis* (Pales Espinosa et al.,  
405 2010). These lectins recognized some carbohydrates on the surfaces of filtered particles  
406 that is used for a biochemical selection of particles. This mechanism could explain the  
407 differences between the number of *T. gondii* oocysts and *G. duodenalis* cysts  
408 accumulated by *D. polymorpha*. The higher LOD<sub>95</sub> for *C. parvum* oocysts might explain  
409 its low detection in zebra mussel matrix, whether haemolymph or tissue. Moreover, *C.*  
410 *parvum* oocysts have a small diameter (4-5  $\mu\text{m}$ , Fayer et al., 2000) and their wall also  
411 has a different composition (Dumètre et al., 2012) than *T. gondii* oocysts and *G.*  
412 *duodenalis* cysts. Therefore, cellular management and biochemical selection of *C.*  
413 *parvum* oocysts by zebra mussels could lead to lower bioaccumulation or destruction of  
414 *C. parvum* oocysts. *In vitro* experiments showed that *C. parvum* oocysts could be  
415 internalized by *D. polymorpha* haemocytes (Palos Ladeiro et al., 2018), whereas *T.*  
416 *gondii* and *G. duodenalis* (oo)cysts were trapped in haemocyte aggregates owing to  
417 their large size (Le Guernic et al., 2018). These explanations are in agreement with the  
418 findings of other studies, in which *C. fluminea* haemocytes internalized *C. parvum*  
419 oocysts *in vivo* (Graczyk et al., 1998) and phagocytized them *in vitro* (Graczyk et al.,  
420 1997). This might explain the difference in the DNA quantities we detected in mussels,  
421 whether from *T. gondii*/*G. duodenalis* or *C. parvum* (oo)cysts.  
422 Molecular techniques seemed to underline a higher accumulation of *T. gondii* oocysts  
423 than *G. duodenalis* cysts and *C. parvum* oocysts in zebra mussels as previously

424 highlighted (Palos Ladeiro et al., 2014). This could be explained by the differences in  
425 LOD<sub>95</sub> and LOQ observed for each parasite in tissues and in the haemolymph. The  
426 extraction method led to a sensitive LOD<sub>95</sub> for *T. gondii* (1-5 oocysts/mussel) and *G.*  
427 *duodenalis* (< 1 cyst/mussel) in tissues, but was less efficient for *C. parvum* (50-100  
428 oocysts/mussel). The literature does not provide any data on the LOD of molecular  
429 techniques for *G. duodenalis* cyst detection in shellfish. However, our results  
430 highlighted a significant improvement in detection sensitivity for *T. gondii* and *C.*  
431 *parvum* oocysts in mussel tissues. Staggs et al. (2015) found a LOD  $\geq$  1,000 *T. gondii*  
432 and *C. parvum* oocysts per *Mytilus* spp. tissues by TaqMan qPCR targeting the repeat  
433 region gene, and Esmerini et al. (2010) found a LOD ranging between 100 and 1,000 *T.*  
434 *gondii* oocysts per sample in *Mytella guyanensis* and *C. rhizophorae* by nested PCR  
435 targeting the B1 gene. The present study highlighted a LOD ranging between 10 and 50  
436 *T. gondii* oocysts in mussel haemolymph samples. The same sensitivity was highlighted  
437 in *Perna canaliculus* haemolymph by qPCR targeting the repeat region gene (Coupe et  
438 al., 2019), but detection was more sensitive in *Mytilus* spp. haemolymph with a LOD  
439 ranging between 1 and 10 oocysts by TaqMan qPCR (repeat region gene, Staggs et al.,  
440 2015). For *C. parvum*, the LOD ranged between 50 and 100 oocysts in zebra mussel.  
441 Staggs et al. (2015) highlighted similar sensitivity in *Mytilus* spp.  
442 The extraction technique should be improved for *C. parvum* in future experiments. The  
443 current extraction protocol (1) could be too drastic and might damage the DNA integrity  
444 of *C. parvum* oocysts, or on the contrary (2) might partially damage the integrity of the  
445 oocyst wall and reduce access to oocyst DNA. In order to verify these hypotheses, it  
446 would be necessary to test different parameters of the DNA extraction protocol (Ahmed  
447 and Karanis, 2018): trypsin digestion, heat shock cycles (Manore et al., 2019),

448 ultrasonic treatment, homogenization frequency. However, improving the method for *C.*  
449 *parvum* might alter the performance for *T. gondii* and *G. duodenalis*.

450 Zebra mussels could concentrate these two protozoa for a few weeks even during the  
451 depuration period, and could be used as an integrative matrix for biomonitoring aquatic  
452 ecosystems. The percentage of positive mussels used allows to limit the risk of false  
453 negatives compared to protozoa quantification in mussel matrix and reflect a health risk.

454 The number of positive mussels could predict the water contamination level in water,  
455 therefore additional studies are needed to improve the sensitivity of this model. Our  
456 results underline higher numbers of positive samples and lower LOD<sub>95</sub> and LOQ for  
457 tissue samples than for haemolymph samples for the tree protozoa. Thus, within the  
458 framework of protozoa biomonitoring in watercourses, it seems more appropriate to  
459 search for protozoa in tissue samples. In the light of these results, we propose to analyze  
460 whole zebra mussels without haemolymph puncture. This method was previously used in  
461 our laboratory and did not impact the quality of DNA extraction or protozoa  
462 quantification, the LOD<sub>95</sub> and LOQ remained unchanged (data not shown). This  
463 simplifies handling, and handlers would not need any knowledge of zebra mussel  
464 anatomy or dissection. In addition, using pooled tissues (with pool of similar weights)  
465 could make the technique more reliable and limit inter-individual variation caused by  
466 zebra mussel size, for example (Lucy et al., 2010). *D. polymorpha* can be used for  
467 passive and active biomonitoring. The establishment of a reference population could  
468 provide a rapid and sustainable supply of zebra mussels to facilitate its caging.

469 However, one should keep in mind that zebra mussel is an invasive species that cannot  
470 be used outside its distribution area. But since it is mainly present in Europe and North  
471 America, its use as a biomonitoring tool can still be applied in many countries.

472 Moreover, this methodology is less expensive and time consuming than the current

473 normative process (ISO 15553/2006) and makes it possible to monitor contamination by  
474 protozoan (oo)cysts and reduce the variability of the results (induced by water quality,  
475 the sampling period, physicochemical parameters ...) using protozoa concentration in  
476 mussel tissues.

477

## 478 5. Conclusion

479

480 This study highlights a high sensitivity of molecular techniques for detecting *G.*  
481 *duodenalis* and *T. gondii* in mussel matrix, i.e., 1 to 5 *T. gondii* oocysts /mussel and less  
482 than 1 *G. duodenalis* cyst /mussel. The percentage of detection in mussels could  
483 highlight present or past protozoa contamination and reflect the contamination level of  
484 water bodies. *D. polymorpha* also concentrated *C. parvum* oocysts, but our technique  
485 was less sensitive and still needs to be improved. Zebra mussels are sedentary  
486 organisms with a worldwide distribution allowing for the monitoring of a wide range of  
487 water bodies. Implementing this biomonitoring would be easier than the current  
488 normative process (ISO 15553/2006) and may limit result variation caused by water  
489 quality, the sampling period or physicochemical parameters. The use of zebra mussels  
490 as an integrative tool may allow for assessing water quality in terms of protozoa  
491 contamination and could consequently help to estimate the health risk associated with  
492 contaminated water potentially (re-)used for crop irrigation or as recreational water.

493

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502

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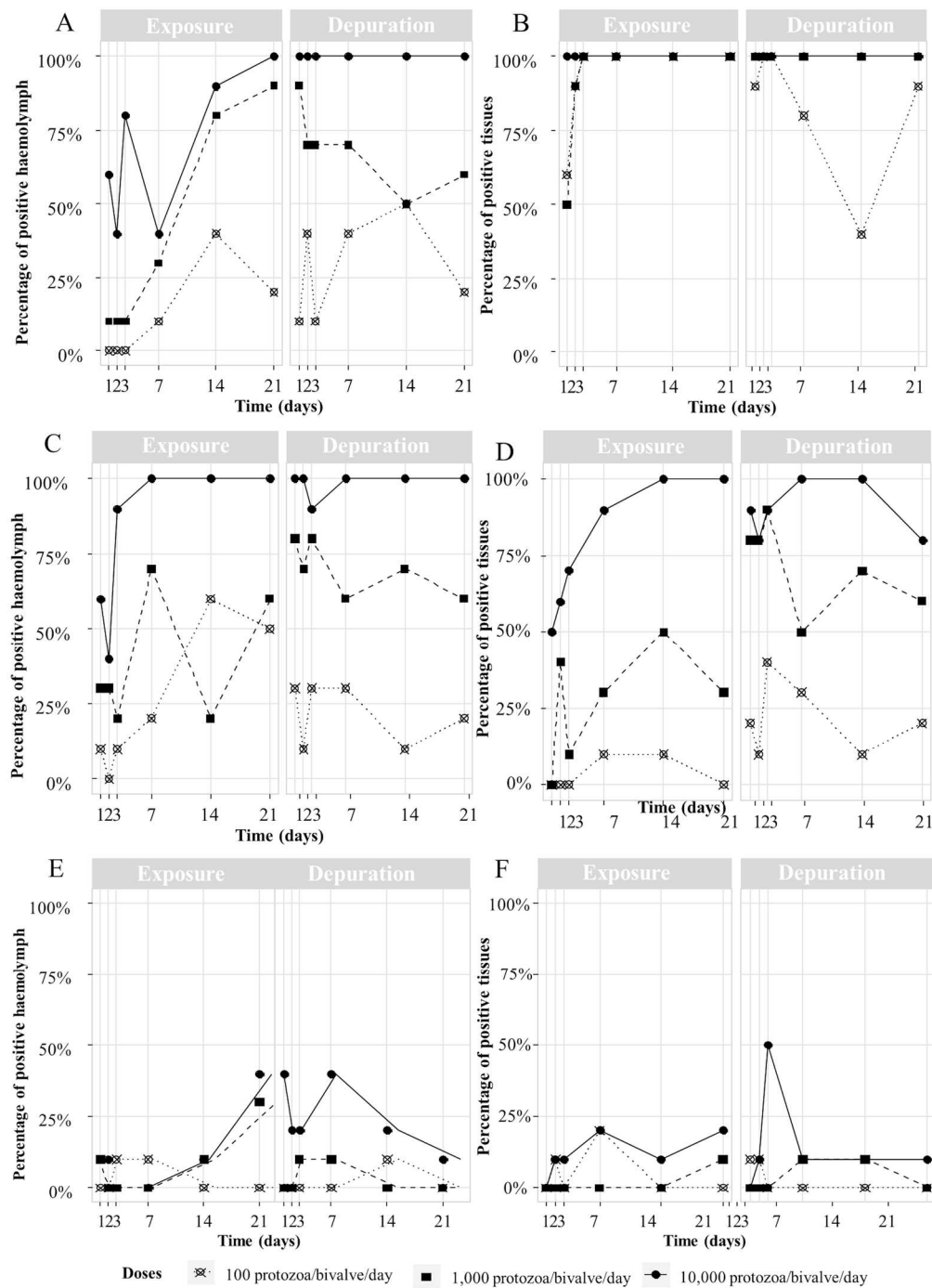
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Figure 1 – Percentages of positive samples (n=10) to *T. gondii* (A: haemolymph and B: tissues), *G. duodenalis* (C: haemolymph and D: tissues) and *C. parvum* (E: haemolymph and F: tissues) (oo)cysts per condition.



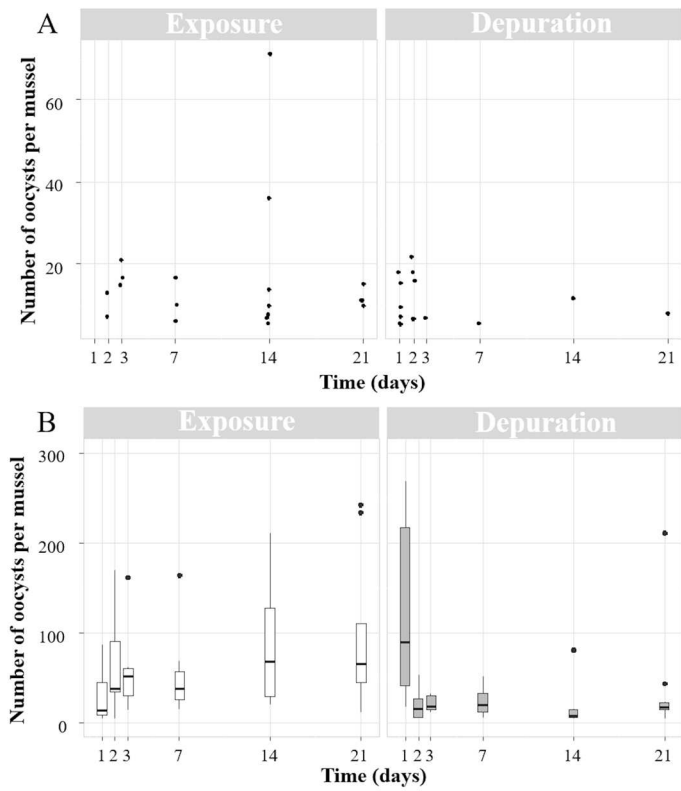


Figure 2 - Number of *T. gondii* oocysts in mussel tissues (scatterplot or boxplot with median, quartiles and outliers) after exposure to 1,000 oocysts/day/mussel (A) or 10,000 oocysts/day/mussel (B).

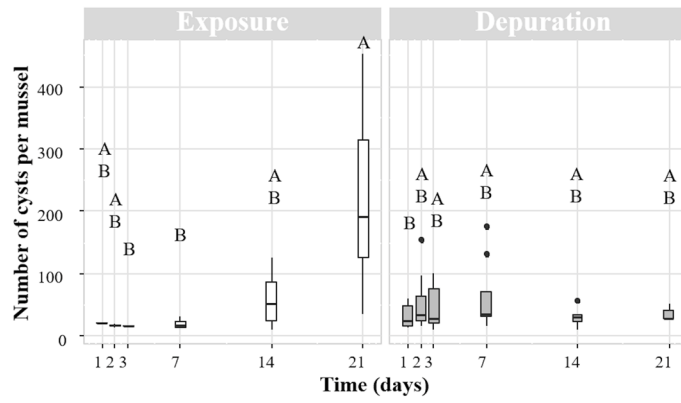


Figure 3 - Number of *G. duodenalis* cysts in mussel haemolymph (boxplot with median, quartiles and outliers) after exposure to 10,000 cysts/day/mussel. Groups with no common letters are statistically different from each other (Kruskal-Wallis, post-hoc Nemeyi,  $\alpha \leq 0.05$ ).

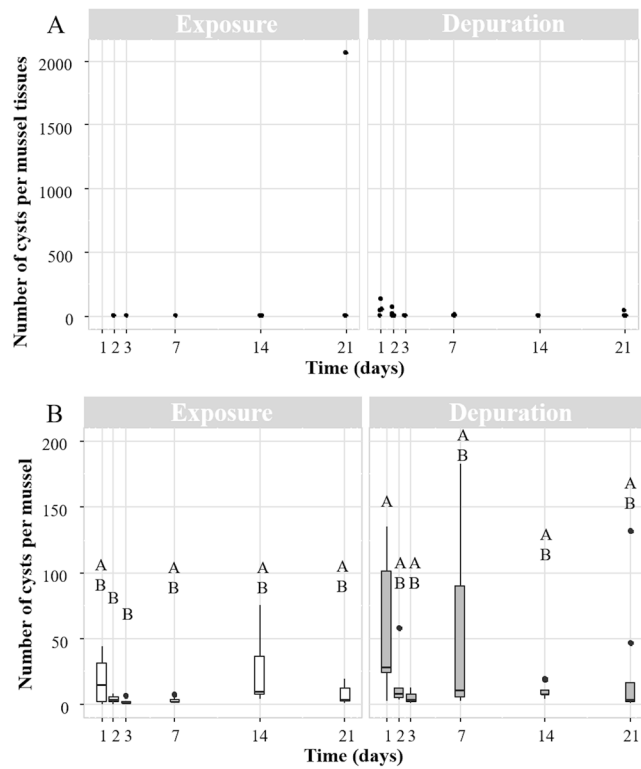


Figure 4 - Number of *G. duodenalis* cysts in mussel tissues (scatterplot or boxplot with median and quartiles) after exposure to 1,000 cysts/day/mussel (A) or 10,000 cysts/day/mussel (B). Groups with no common letters are statistically different from each other (Kruskal-Wallis, post-hoc Nemeyi,  $\alpha \leq 0.05$ ).

Table 1 - Summary of LOD<sub>95</sub> and LOQ of *T. gondii*, *G. duodenalis* and *C. parvum* in *D. polymorpha* haemolymph and tissues. LOD<sub>95</sub> and LOQ are expressed in (oo)cyst number per bivalve.

		<b>Haemolymph</b>	<b>Tissues</b>
<i>T. gondii</i>	LOD <sub>95</sub>	10-50	1-5
	LOQ	100	5
<i>G. duodenalis</i>	LOD <sub>95</sub>	5-10	<1
	LOQ	10	1
<i>C. parvum</i>	LOD <sub>95</sub>	50-100	50-100
	LOQ	100	100

Table 2 - Number of quantifiable haemolymph and tissue samples for each exposure condition.

	Number of (oo)cysts per bivalve per day added	Number of quantifiable samples per positive samples	
		Haemolymph	Tissues
<i>T. gondii</i>	100	0/24	6/105
	1,000	0/64	35/114
	10,000	9/101	102/120
<i>G. duodenalis</i>	100	2/28	8/15
	1,000	11/65	39/60
	10,000	70/108	86/101
<i>C. parvum</i>	100	1/3	4/4
	1,000	1/7	3/3
	10,000	3/28	16/16

*A biomonitoring tool...*

