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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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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 ± 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⁻¹) in each tank was calculated for each condition: 1.9×10^3 , 1.9×10^4
144 and 1.9×10^5 on day 1; 3.6×10^3 , 3.6×10^4 and 3.6×10^5 on day 2; 5.3×10^3 , 5.3×10^4
145 and 5.3×10^5 on day 3; 1.1×10^4 , 1.1×10^5 and 1.1×10^6 on day 7; 2.8×10^4 , 2.8×10^5
146 and 2.8×10^6 on day 14 and 4.3×10^4 , 4.3×10^5 and 4.3×10^6 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[®] 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[™] 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⁻¹; 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 (10⁻¹) 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 (log₂ (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 (LOD₉₅). 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 LOD₉₅ 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 LOD₉₅ 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 LOD₉₅ 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 LOD₉₅ 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 LOD₉₅ 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 LOD₉₅ 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 LOD₉₅ 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 LOD₉₅ 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 LOD₉₅, 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 ± 143.3) compared to day 3 (15.8 ± 0.6) and day 7
314 (20.9 ± 7.7) of exposure (Figure 3). During the depuration period, the number of cysts
315 per mussel significantly decreased after 1 day (32.7 ± 19.9) and cysts remained in the
316 haemolymph for at least 21 days (36.1 ± 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 ± 19.9) was
320 significantly higher than after 3 days (2.0 ± 2.2) and 7 days (2.5 ± 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 ± 1.4 and 4.3 ± 4.9 , respectively) compared to 2 days of
323 depuration (12.5 ± 22.9 and 13.4 ± 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 LOD₉₅, 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 1.9×10^3 , 1.9×10^4 and 1.9×10^5 cysts.L⁻¹,
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 5.3×10^5 to more than 4.3×10^6 cysts.L⁻¹,
359 while a percentage of 10 to 20% reflected a cyst level ranging from 1.9×10^3 to $5.3 \times$
360 10^4 cysts.L⁻¹. 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 4.3×10^4 , 4.3×10^5 and
363 4.3×10^6 cysts.L⁻¹, 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 5.3×10^3 oocysts.L⁻¹,
367 and remained stable for the 21 days of depuration after chronic exposure (21 days) at
368 4.3×10^5 oocysts.L⁻¹. *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 4.3×10^6 oocysts.L⁻¹; 10 to 30% of positive samples
371 reflected oocyst levels ranging from 1.1×10^4 to 1.1×10^5 oocysts.L⁻¹, while 80 to 90% of
372 positive samples tended to reflect contamination by 2.8×10^5 to 4.3×10^5 oocysts.L⁻¹. 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 μm and 12-15 μm ,
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 LOD₉₅ 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 μm , 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 LOD₉₅ and LOQ observed for each parasite in tissues and in the haemolymph. The
426 extraction method led to a sensitive LOD₉₅ 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 LOD₉₅ 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 LOD₉₅ 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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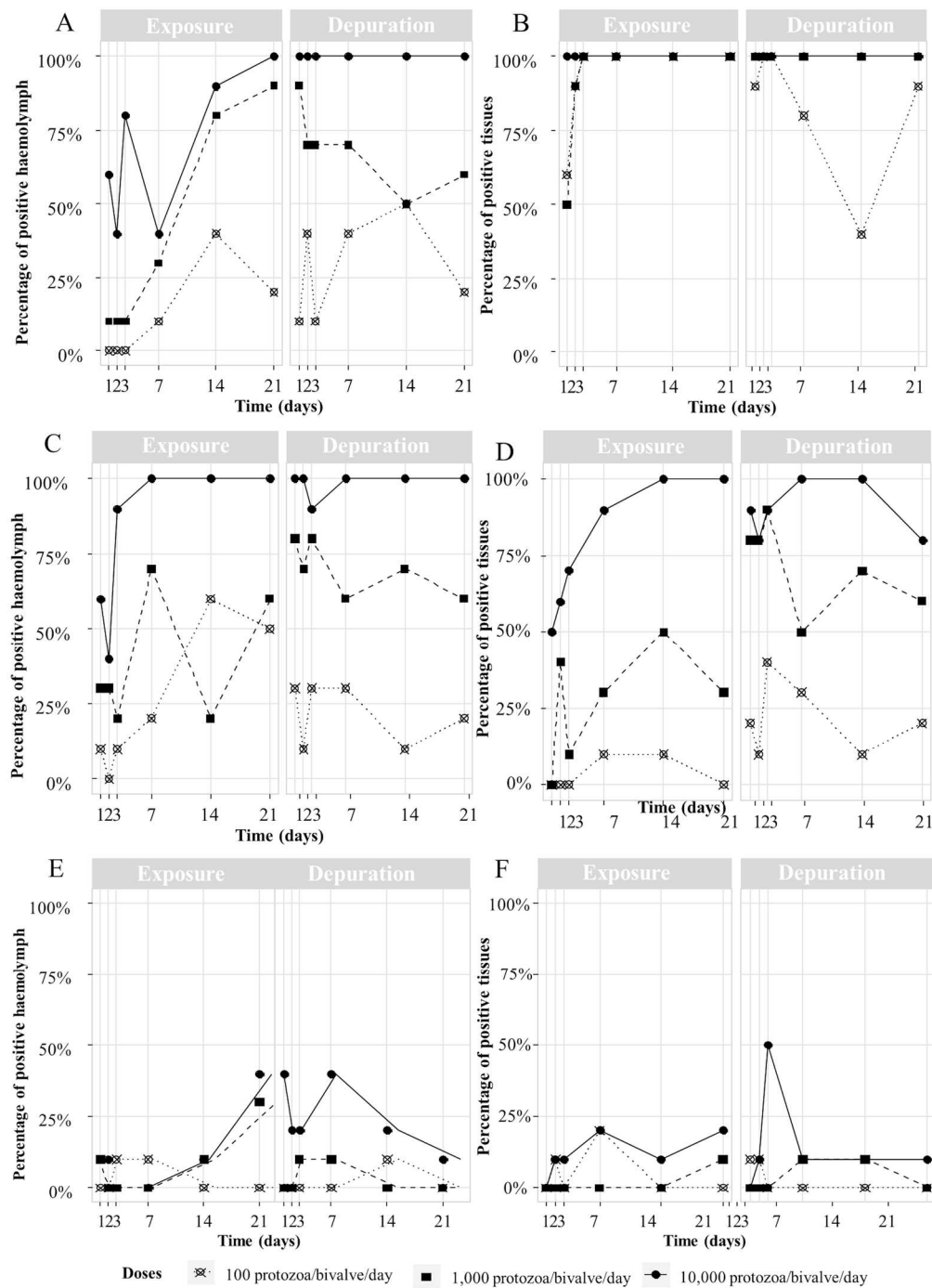
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Figure 1 – Percentages of positive samples (n=10) to *T. gondii* (A: haemolymph and B: tissues), *G. duodenalis* (C: haemolymph and D: tissues) and *C. parvum* (E: haemolymph and F: tissues) (oo)cysts per condition.



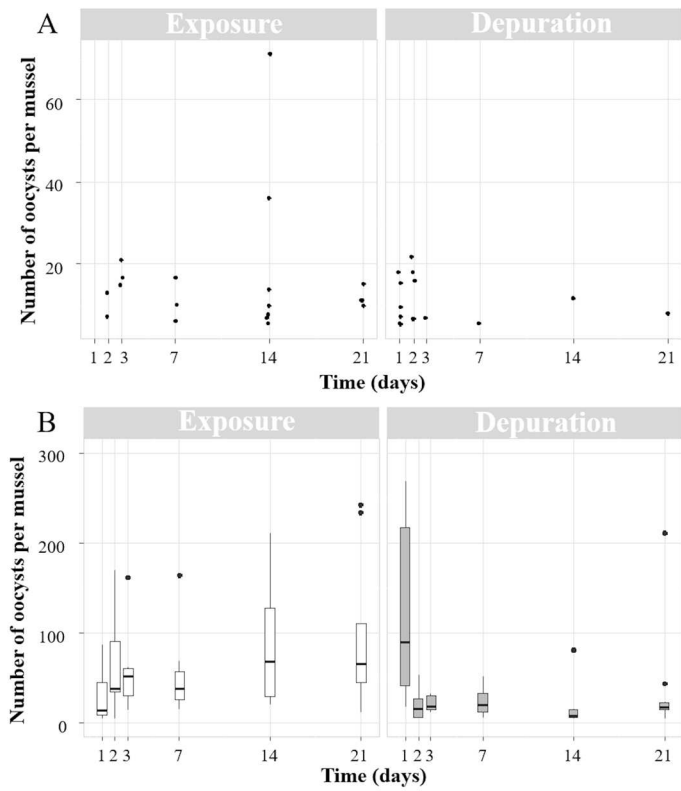


Figure 2 - Number of *T. gondii* oocysts in mussel tissues (scatterplot or boxplot with median, quartiles and outliers) after exposure to 1,000 oocysts/day/mussel (A) or 10,000 oocysts/day/mussel (B).

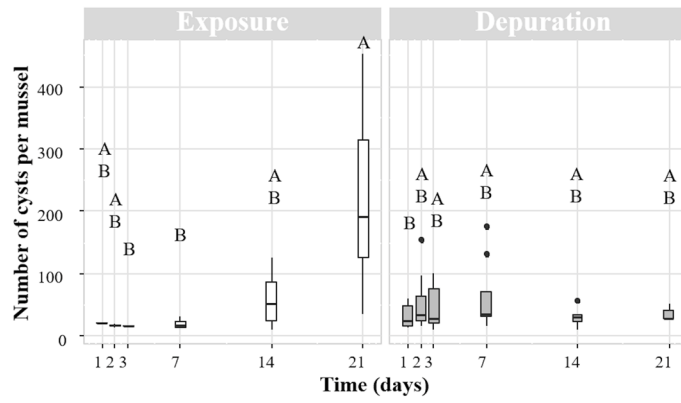


Figure 3 - Number of *G. duodenalis* cysts in mussel haemolymph (boxplot with median, quartiles and outliers) after exposure to 10,000 cysts/day/mussel. Groups with no common letters are statistically different from each other (Kruskal-Wallis, post-hoc Nemeyi, $\alpha \leq 0.05$).

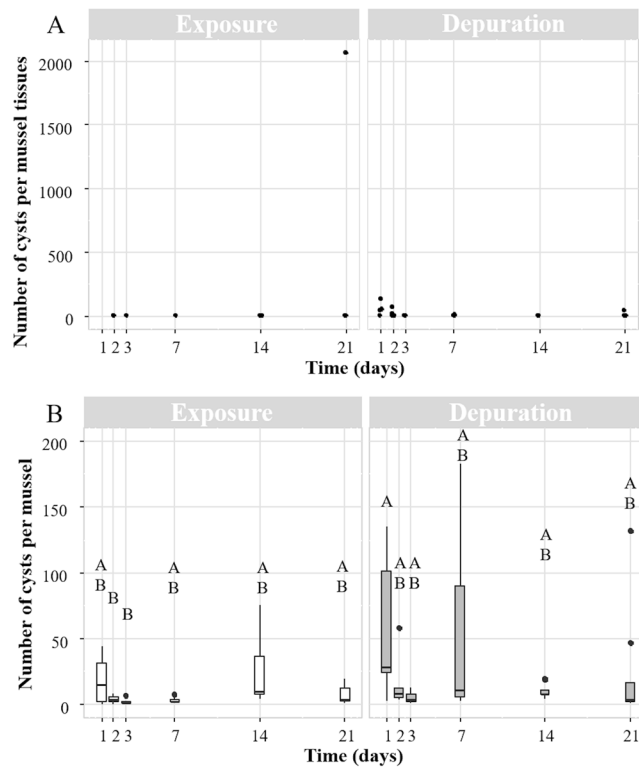


Figure 4 - Number of *G. duodenalis* cysts in mussel tissues (scatterplot or boxplot with median and quartiles) after exposure to 1,000 cysts/day/mussel (A) or 10,000 cysts/day/mussel (B). Groups with no common letters are statistically different from each other (Kruskal-Wallis, post-hoc Nemeyi, $\alpha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

Table 1 - Summary of LOD₉₅ and LOQ of *T. gondii*, *G. duodenalis* and *C. parvum* in *D. polymorpha* haemolymph and tissues. LOD₉₅ and LOQ are expressed in (oo)cyst number per bivalve.

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

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