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Abstract:

Cryptosporidium parvum, Toxoplasma gondii and *Giardia duodenalis* are worldwide pathogenic protozoa recognized as major causal agents of waterborne disease outbreaks. To overcome the normative process (ISO 15553/2006) limitations of protozoa detection in aquatic systems, we propose to use the zebra mussel (*Dreissena polymorpha*), a freshwater bivalve mollusc, as a tool for biomonitoring protozoan contamination.. Mussels were exposed to three concentrations of *C. parvum* oocysts, *G. duodenalis* cysts or *T. gondii* oocysts for 21 days followed by 21 days of depuration in clear water. *D. polymorpha* accumulated protozoa in its tissues and haemolymph. Concerning *T. gondii* and *G. duodenalis,* the percentage of protozoa positive mussels reflected the contamination level in water bodies. As for *C. parvum* detection, oocysts did accumulate in mussel tissues and haemolymph, but in small quantities, and the limit of detection was high (between 50 and 100 oocysts). Low levels of *T. gondii* (1-5 oocysts /mussel) and *G. duodenalis* (less than 1 cyst/mussel) were quantified in *D. polymorpha* tissues The ability of zebra mussels to reflect contamination by the three protozoa for weeks after the contamination event makes them a good integrative matrix for the biomonitoring of aquatic ecosystems.

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

1. Introduction

Cryptosporidium parvum and *Giardia duodenalis* are the major causes of protozoal diarrhea in humans. Cryptosporidiosis and giardiasis can cause high morbidity and severe dehydration, and even death in immunocompromised hosts (Cacciò et al., 2005). *Toxoplasma gondii* is the causative agent of toxoplasmosis; it is usually asymptomatic in humans, but can cause severe clinical diseases in immunocompromised hosts or in case of congenital infection (Montoya and Liesenfeld, 2004). The minimal infectious doses for humans may be low: 9 to 1,042 *C. parvum* oocysts (Fayer et al., 2000) and down to 10 *G. duodenalis* cysts (Rendtorff, 1954). Although the minimal *T. gondii* infectious dose for humans has not been determined, based on animal experimentation one single oocyst can infect humans (Dubey, 2010). The environment including food and water are contaminated by these protozoa via human or animal faeces: felids faeces for *T. gondii* or mammals' faeces for *C. parvum* and *G. duodenalis*. The consumption of fruit and vegetables (McKerr et al., 2015; Pönka et al., 2009) and untreated water are known vectors of the foodborne transmission cycle (Moreno et al., 2018). The environmental stages, i.e. *Giardia* cysts, and *T. gondii* and *Cryptosporidium* oocysts (hereafter designated as protozoa throughout this paper) are extremely resistant and can persist in aquatic ecosystems for months (Bingham et al., 1979; Lindsay and Dubey, 2009; Olson et al., 1999). Protozoa have been found in several freshwater aquatic ecosystems such as surface water (Aubert and Villena, 2009; Bautista et al., 2018; Karanis et al., 2006; Vermeulen et al., 2019; Xiao et al., 2018) and underground water (Aubert and Villena, 2009; Hancock et al., 1998; Khaldi et al., 2011; Solo-Gabriele et al., 1998) but also in drinking water (Almeida et al., 2010; Aubert and Villena, 2009; Castro-Hermida et al., 2010; Cheun et al., 2013; Ware et al., 2010). Waterborne transmission to humans is the major cause of cryptosporidiosis (Putignani and Menichella, 2010) and giardiasis (Adam et al., 2016) outbreaks. *Cryptosporidium* spp. and *Giardia* spp. indeed caused 63% and 37% of waterborne parasitic disease outbreaks in Australia, North America and Europe between January 2011 and December 2016, respectively (Efstratiou et al., 2017), and *T. gondii* caused 2% between January 2004 and December 2010 (Baldursson and Karanis, 2011).

In the water matrix, *Cryptosporidium* oocysts and *Giardia* cysts are currently detected by immunofluorescence (IF) in filtered water sample (ISO 15553/2006). However, this method is time consuming, expensive and requires expert image analysis, so it is unsuitable for rapid parasite detection. It requires a large volume of filtered water and a high parasite concentration in the samples. Furthermore, the filtration and the purification techniques can yield variable results depending on the water quality, the sampling period, the location and the water volume (Gallas-Lindemann et al., 2013; Karanis et al., 2006; Efstratiou et al., 2018). No normative process is available for *T. gondii* oocyst detection in water because, oocysts are present in limited numbers whereas *Giardia* and *Cryptosporidium* (oo)cysts are present in high numbers. This represents a limitation when applying a monitoring approach based on the water matrix and it seems necessary to investigate new methods for assessing water quality in terms of biological contamination.

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

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

2. Materials and methods

2.1. Mussels and protozoa

Zebra mussels (*D. polymorpha*) were collected at around 5 m depth in Port de Nuisement (N 48°36’10.0728” E 4°44’57.408”) at the Lac-du-Der-Chantecoq (Marne, France) in October 2015. Then the mussels measuring 20 ± 2 mm were acclimated in the dark in aerated Cristaline® (Aurèle, France) drinking water at 12 °C for two weeks. The water was renewed twice a week to ensure that mussels were protozoa-free on the first day of exposure. The mussels were fed *ad libitum* twice a week with 20,000 cells of two microalgal species (*Chlorella pyrenoidosa* and *Scenedesmus obliquus*) per mussel and per day.

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

2.2. Exposure conditions

Before exposure, zebra mussels were randomly divided into 10 groups of 195 mussels each i.e., 1 control group and 9 exposed groups. Each group was placed in a tank containing 10 L of Cristaline® Aurèle, France) water. Zebra mussels were exposed to 0; 100; 1,000 or 10,000 protozoa per bivalve and per day for 21 days (supplementary data). The tank water was changed once a week and the volume of water was adapted to the number of remaining bivalves. The protozoa were spiked again after water changes to maintain the initial protozoa concentrations in the tanks. To determine the environmental equivalent for each exposure concentration the number of protozoa per litre (protozoa.L^{-1}) in each tank was calculated for each condition:1.9×10^3, 1.9×10^4 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 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 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 or 10,000 protozoa/bivalve/day respectively. Then, the mussels were transferred into new tanks filled with clean water for the depuration period. During the exposure and depuration periods, 10 mussels per condition were sampled on days1, 2, 3, 7, 14, and 21. Haemolymph was taken from the posterior adductor muscle, and whole tissue was collected and, then stored at -80°C until analysis. Mussels were still fed *ad libitum* twice a week with *Chlorella pyrenoidosa* and *Scenedesmus obliquus.* Mortality was recorded daily: mortality of 1.62 % in the control tank, 1.08 % in the three tanks devoted to *T. gondii* exposure, 1.08 % in the three tanks devoted to *C. parvum* exposure and 0.72 % in three tanks devoted to *G. duodenalis* exposure were recorded throughout the experiment. No difference in mortality was observed between the different exposure concentrations.

2.3 DNA extraction from different matrices

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

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

2.4. TaqMan qPCR

Parasite DNA detection was detected by TaqMan real-time qPCR in a CFX96 TOUCH™ thermocycler (Bio-Rad, USA) according to Palos Ladeiro et al. (2014). TaqMan real-time qPCR targeted specific *T. gondii* (AF487550.1; Lélu et al., 2011), *G. duodenalis* (M54878; Verweij et al., 2003) and *C. parvum/hominis* (AF188110; Fontaine and Guillot, 2002) genes (supplementary data). qPCR amplifications were performed as specified by the manufacturer's instructions in the following mixtures: 5 µL of extracted DNA sample were added to 20 µL of mix containing 2X iQ® supermix (Bio-Rad), 1 µL of BSA (10 $mg.mL^{-1}$; SIGMA, France), 400 nM of each primers and 200 nM of probe for *T. gondii* and *G. duodenalis* or 100 nM for *C. parvum*. A no-template control was added. DNA polymerase was activated after an initial 3-min denaturation step at 95 °C. Then, the samples were submitted to 45 cycles of 15 s at 95 °C and 1 min at 60 °C. The Cq values were collected, each correspond to the number of cycles for which fluorescence exceed a fixed threshold and allows for quantifying the amount of the target DNA. Each sample was analyzed in duplicate, and non-diluted and ten-fold diluted (10^{-1}) DNA extracts were analyzed to limit the inhibition effect. In case of inhibition, non-diluted Cq values were extrapolated from the Cq of the ten-fold dilution considering a Cq difference of 3.3 for a dilution factor equal to 10 and an optimal PCR efficacy equal to 2 (log2 (10)).

To determine the number of (oo)cyst in the samples, reference curves were established using a serial dilution ranging from 50,000 to 1 (oo)cyst(s) spiked in mussel tissues or added to the water suspensions. For tissues analysis, experiments were performed in quintuplicate for dilutions ranging from 100 to 1 protozoa (five dilutions) and in triplicate for dilutions ranging from 50,000 to 500 (oo)cysts (five dilutions too). For haemolymph analysis, samples were spiked in triplicate with dilutions ranging from 10,000 to 100 (oo)cysts (six dilutions). All spiked tissues and haemolymph samples were also used to determine the limit of quantification (LOQ) and the limit of detection (LOD_{95}). LOQ was determined as the lowest number of parasites for which a linear relationship was observed between the Cq and the number of parasites ($r^2 > 0.98$) and LOD_{95} was defined as the lowest number of parasites for which 95% of positive samples were detected.

2.5. Statistical analysis

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

3. Results

3.1. Determination of LOD_{95} and LOQ

For the tissues samples, the performances of the method were determined using tissues spiked with 50,000 to 1 (oo)cyst(s) (Table 1). For *T. gondii* oocysts, a linear response ($r^2 > 0.98$) was observed from 50,000 to 5 oocysts, so LOQ was five oocysts in whole tissues. No amplification curve was obtained in 1/10 replicate corresponding to 1 oocyst, so LOD_{95} was between one (< 95 % of positive samples) and five (100 % of positive samples) oocysts in whole tissues. The slope coefficient was -3.1193 with a squared correlation coefficient (r^2) of 0.99 (supplementary data). For *G. duodenalis* cysts, a linear response ($r^2 > 0.98$) was observed from 50,000 to 1 cyst(s), so LOQ was 1 cyst. An amplification curve was obtained in all replicates corresponding to 1 cyst, so LOD_{95} was less than 1 cyst. The slope coefficient was -3.5093 with a squared correlation coefficient (r^2) of 0.99 (supplementary data). For *C. parvum*, a linear response ($r^2 > 0.98$) was observed from 50,000 to 100 oocysts, so LOQ was 100 oocysts. An amplification curve was obtained in 90 % of the replicates corresponding to 50 oocysts and in 100 % of the replicates corresponding to 100 oocysts, so LOD_{95} was between 50 and 100 oocysts in whole tissues. The slope coefficient was -3.5673 with a squared correlation coefficient (r^2) of 0.96 (supplementary data).

For the haemolymph analysis, samples were spiked in triplicate with dilutions ranging from 10,000 to 10 (oo)cysts (Table 1). For *T. gondii*, the slope coefficient was -3.2996, with a squared correlation coefficient (r^2) of 0.99 (supplementary data). A linear response ($r^2 > 0.98$) was observed from 50,000 to 100 oocysts per sample, so LOQ was 100 oocysts. An amplification curve was obtained in 83 % of the replicates corresponding to 10 oocysts and in 100 % of the replicates corresponding to 50 oocysts per sample, so LOD_{95} was between 10 and 50 oocysts. For *G. duodenalis*, the slope coefficient was -4.591, with a squared correlation coefficient (r^2) of 0.99 (supplementary data). A linear response ($r^2 > 0.98$) was observed from 50,000 to 10 cysts per sample, so LOQ was 10 cysts. An amplification curve was obtained in 67 % of the replicates corresponding to 5 cysts and in 100 % of the replicates corresponding to 10 cysts in the samples, so LOD_{95} was between 5 and 10 cysts. Lastly, for *C. parvum*, a linear response ($r^2 > 0.98$) was observed from 50,000 to 100 oocysts per sample, so LOQ was 100 oocysts. An amplification curve was obtained in 80 % of the replicates corresponding to 50 oocysts and in 100 % of the replicates corresponding to 100 oocysts in the samples, so LOD_{95} was between 50 and 100 oocysts. The slope coefficient was -3.2776, with a squared correlation coefficient (r^2) of 0.98 (supplementary data).

3.2. Protozoa levels in bivalves

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

3.2.1. Protozoa detection

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

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

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

3.2.2. Protozoa quantification

We failed to quantify part of the samples detected as protozoa positive because the amount of bioaccumulated protozoa was below the LOQ. The number of samples above the LOQ is summarized in Table 2. Quantification results are presented in graphs if at least 50% of the samples had values greater than the LOD_{95}, and if at least 30% of those samples had values greater than the LOQ per modality. Consequently, only the numbers of *T. gondii* oocysts bioaccumulated in tissues (at 1,000 and 10,000 oocysts/bivalve/day, Figure 2) and the numbers of *G. duodenalis* cysts bioaccumulated in the haemolymph (at 10,000 cysts/bivalve/day, Figure 3) and in tissues (at 1,000 and 10,000 cysts/bivalve/day, Figure 4) are presented in the graphs.

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

The numbers of *G. duodenalis* cysts in the haemolymph significantly increased on day 21 of the exposure period (218.7 ± 143.3) compared to day 3 (15.8 ± 0.6) and day 7 (20.9 ± 7.7) of exposure (Figure 3). During the depuration period, the number of cysts per mussel significantly decreased after 1 day (32.7 ± 19.9) and cysts remained in the haemolymph for at least 21 days (36.1 ± 13.6). In tissues (Figure 4), the number of cysts per mussel was very variable, and no significant difference was shown at 1,000 cysts/mussel/day or between the two concentrations. At 10,000 cysts/mussel/day, the number of bioaccumulated cysts after 1 day of depuration (15.1 ± 19.9) was significantly higher than after 3 days (2.0 ± 2.2) and 7 days (2.5 ± 2.4) of exposure. Then, at 1,000 and 10,000 cysts/mussel/day, a non-significant decrease was observed after 3 days of depuration (1.8 ± 1.4 and 4.3 ± 4.9, respectively) compared to 2 days of depuration (12.5 ± 22.9 and 13.4 ± 18.7, respectively). The cyst level remained similar in tissues until 21 days of depuration with 15.9 (± 25.4) and 25.6 (± 46.0) cysts per mussel at 1,000 and 10,000 cysts/mussel/day, respectively.

4. Discussion

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

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

Moreover, the percentages of positive samples appeared to reflect contamination levels with different levels of sensitivity depending on the compartment (tissues or haemolymph). This may be related to LOD_{95}, which differed according to each protozoan and each mussel compartment. For *G. duodenalis*, contamination was detected in the haemolymph from 1 day of exposure, with 10%, 30% or 60% of positive samples for punctual contamination levels of 1.9×10^3, 1.9×10^4 and $1,9 \times 10^5$ cysts.L^{-1}, respectively. But contamination was not detected in the tissue samples. The percentage of positive haemolymph samples could reflect contamination by cysts, whether occasional or chronic (over a time span of 21 days): a percentage of 80 to 100% was representative of contamination ranging from 5.3×10^5 to more than 4.3×10^6 cysts.L^{-1}, while a percentage of 10 to 20% reflected a cyst level ranging from 1.9×10^3 to 5.3×10^4 cysts.L^{-1}. The percentage of positive mussels remained relatively stable throughout the depuration period (20% variation) and clearly distinct for each contamination level: 10 to 30%, 60 to 80% or 90 to 100% after chronic exposure to 4.3×10^4, 4.3×10^5 and 4.3×10^6 cysts.L^{-1}, respectively. The same patterns were observed in tissues, with later and lower detection percentages of 10% on average for each cyst concentration.

Contrary to *G. duodenalis*, *T. gondii* was detected earlier in tissues than in the haemolymph. The percentage of positive tissues reached 100% from 5.3×10^3 oocysts.L^{-1}, and remained stable for the 21 days of depuration after chronic exposure (21 days) at 4.3×10^5 oocysts.L^{-1}. *T. gondii* detection in the haemolymph samples occurred later, but still provided an assessment of *T. gondii* levels in water. All haemolymph samples were positive following exposure to only 4.3×10^6 oocysts.L^{-1}; 10 to 30% of positive samples reflected oocyst levels ranging from $1.1e^4$ to 1.1×10^5 oocysts.L^{-1}, while 80 to 90% of positive samples tended to reflect contamination by $2.8e^5$ to 4.3×10^5 oocysts.L^{-1}. Zebra mussels integrated protozoan contamination over the 21 days of depuration. This underlines their ability to reflect past contamination of the water environment. Previous studies also showed integration of contamination by *T. gondii* (Palos Ladeiro et al., 2015), *C. parvum* and *G. duodenalis* (Graczyk et al., 2003) for 14 days of depuration in zebra mussels. In marine species, *T. gondii* oocysts were still detected in *M. galloprovincialis* 21 days after inoculation (Arkush et al. 2003) and *C. parvum* oocysts remained detected in gills and haemocytes of *Crassostrea virginica* 12 days after exposure (Fayer et al., 1997). In agreement with our results, *C. parvum* oocysts persisted 14 days' after inoculation in the freshwater mollusc *Corbicula fluminea* (Graczyk et al. 1998).

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

especially during the exposure period - despite a higher LOQ in tissues than in the haemolymph. These two protozoa could be managed differently by zebra mussel cells. *T. gondii* oocysts and *G. duodenalis* cysts have different sizes (10-13 µm and 12-15 µm, respectively) and wall structures (Adam, 2001; Dumètre et al., 2013). Moreover, some authors have shown the presence of lectins in the gills and labial palp mucus in *Crassostrea virginica* (Pales Espinosa et al., 2009) and *M. edulis* (Pales Espinosa et al., 2010). These lectins recognized some carbohydrates on the surfaces of filtered particles that is used for a biochemical selection of particles. This mechanism could explain the differences between the number of *T. gondii* oocysts and *G. duodenalis* cysts accumulated by *D. polymorpha*. The higher LOD_{95} for *C. parvum* oocysts might explain its low detection in zebra mussel matrix, whether haemolymph or tissue. Moreover, *C. parvum* oocysts have a small diameter (4-5 µm, Fayer et al., 2000) and their wall also has a different composition (Dumètre et al., 2012) than *T. gondii* oocysts and *G. duodenalis* cysts. Therefore, cellular management and biochemical selection of *C. parvum* oocysts by zebra mussels could lead to lower bioaccumulation or destruction of *C. parvum* oocysts. *In vitro* experiments showed that *C. parvum* oocysts could be internalized by *D. polymorpha* haemocytes (Palos Ladeiro et al., 2018), whereas *T. gondii* and *G. duodenalis* (oo)cysts were trapped in haemocyte aggregates owing to their large size (Le Guernic et al., 2018). These explanations are in agreement with the findings of other studies, in which *C. fluminea* haemocytes internalized *C. parvum* oocysts *in vivo* (Graczyk et al., 1998) and phagocytized them *in vitro* (Graczyk et al., 1997). This might explain the difference in the DNA quantities we detected in mussels, whether from *T. gondii/G. duodenalis* or *C. parvum* (oo)cysts.

Molecular techniques seemed to underline a higher accumulation of *T. gondii* oocysts than *G. duodenalis* cysts and *C. parvum* oocysts in zebra mussels as previously highlighted (Palos Ladeiro et al., 2014). This could be explained by the differences in LOD_{95} and LOQ observed for each parasite in tissues and in the haemolymph. The extraction method led to a sensitive LOD_{95} for *T. gondii* (1-5 oocysts/mussel) and *G. duodenalis* (< 1 cyst/mussel) in tissues, but was less efficient for *C. parvum* (50-100 oocysts/mussel). The literature does nor provide any data on the LOD of molecular techniques for *G. duodenalis* cyst detection in shellfish. However, our results highlighted a significant improvement in detection sensitivity for *T. gondii* and *C. parvum* oocysts in mussel tissues. Staggs et al. (2015) found a LOD ≥ 1,000 *T. gondii* and *C. parvum* oocysts per *Mytilus* spp. tissues by TaqMan qPCR targeting the repeat region gene, and Esmerini et al. (2010) found a LOD ranging between 100 and 1,000 *T. gondii* oocysts per sample in *Mytella guyanensis* and *C. rhiziphorae* by nested PCR targeting the B1 gene. The present study highlighted a LOD ranging between 10 and 50 *T. gondii* oocysts in mussel haemolymph samples. The same sensitivity was highlighted in *Perna canaliculus* haemolymph by qPCR targeting the repeat region gene (Coupe et al., 2019), but detection was more sensitive in *Mytilus* spp. haemolymph with a LOD ranging between 1 and 10 oocysts by TaqMan qPCR (repeat region gene, Staggs et al., 2015). For *C. parvum,* the LOD ranged between 50 and 100 oocysts in zebra mussel. Staggs et al. (2015) highlighted similar sensitivity in *Mytilus spp*.

The extraction technique should be improved for *C. parvum* in future experiments. The current extraction protocol (1) could be too drastic and might damage the DNA integrity of *C. parvum* oocysts, or on the contrary (2) might partially damage the integrity of the oocyst wall and reduce access to oocyst DNA. In order to verify these hypotheses, it would be necessary to test different parameters of the DNA extraction protocol (Ahmed and Karanis, 2018): trypsin digestion, heat shock cycles (Manore et al., 2019),

ultrasonic treatment, homogenization frequency. However, improving the method for *C. parvum* might alter the performance for *T. gondii* and *G. duodenalis*.
Zebra mussels could concentrate these two protozoa for a few weeks even during the depuration period, and could be used as an integrative matrix for biomonitoring aquatic ecosystems. The percentage of positive mussels used allows to limit the risk of false negatives compared to protozoa quantification in mussel matrix and reflect a health risk. The number of positive mussels could predict the water contamination level in water, therefore additional studies are needed to improve the sensitivity of this model. Our results underline higher numbers of positive samples and lower LOD_{95} and LOQ for tissue samples than for haemolymph samples for the tree protozoa. Thus, within the framework of protozoa biomonitoring in watercourses, it seems more appropriate to search for protozoa in tissue samples. In the light of these results, we propose to analyze whole zebra mussels without haemolymph punction. This method was previouly used in our laboratory and did not impact the quality of DNA extraction or protozoa quantification, the LOD_{95} and LOQ remained unchanged (data not shown). This simplifies handling, and handlers would not need any knowledge of zebra mussel anatomy or dissection. In addition, using pooled tissues (with pool of similar weights) could make the technique more reliable and limit inter-individual variation caused by zebra mussel size, for example (Lucy et al., 2010). *D. polymorpha* can be used for passive and active biomonitoring. The establishment of a reference population could provide a rapid and sustainable supply of zebra mussels to facilitate its caging. However, one should keep in mind that zebra mussel is an invasive species that cannot be used outside its distribution area. But since it is mainly present in Europe and North America, its use as a biomonitoring tool can still be applied in many countries. Moreover, this methodology is less expensive and time consuming than the current

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

5. Conclusion

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

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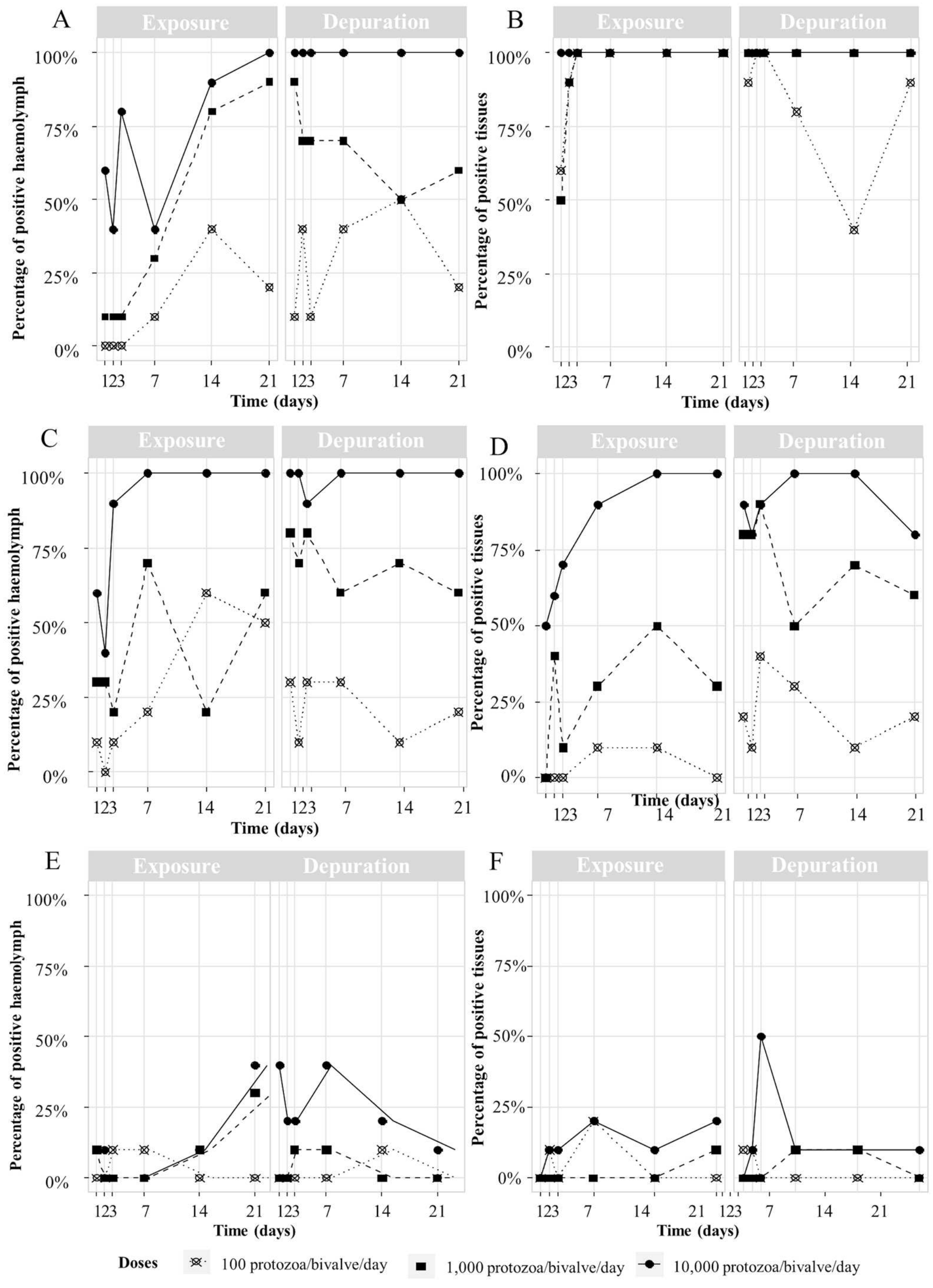
Figure 1 – Percentages of positive samples (n=10) to *T. gondii* (A: haemolymph and B: tissues), *G. duodenalis* (C: haemolymph and D: tissues) and *C. parvum* (E: haemolymph and F: tissues) (oo)cysts per condition.



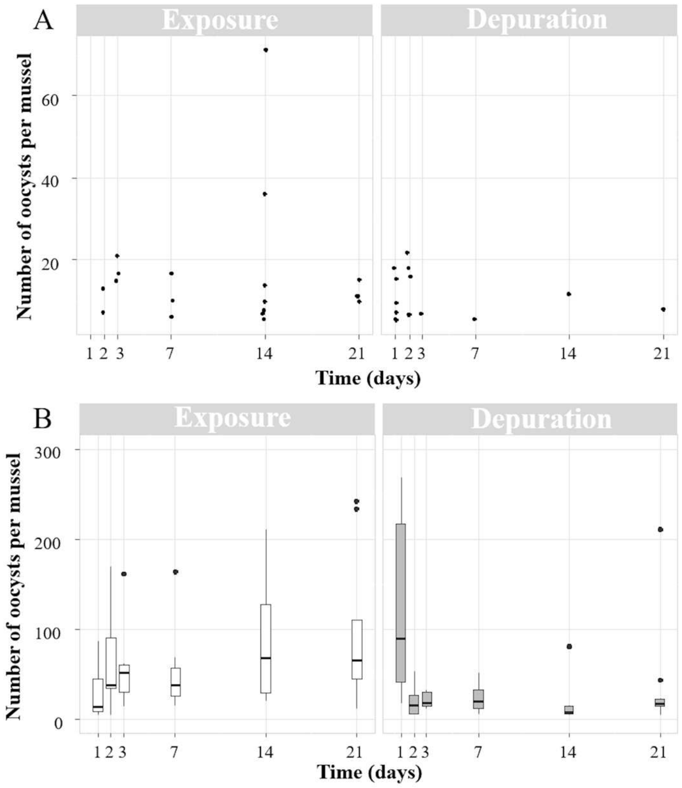


Figure 2 - Number of *T. gondii* oocysts in mussel tissues (scatterplot or boxplot with median, quartiles and outliers) after exposure to 1,000 oocysts/day/mussel (A) or 10,000 oocysts/day/mussel (B).

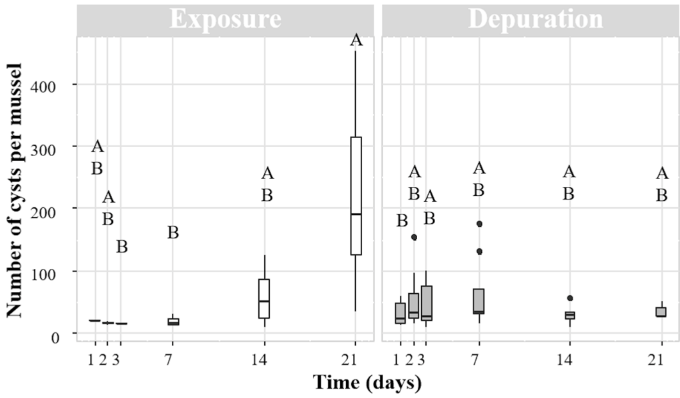


Figure 3 - Number of *G. duodenalis* cysts in mussel haemolymph (boxplot with median, quartiles and outliers) after exposure to 10,000 cysts/day/mussel. Groups with no common letters are statistically different from each other (Kruskal-Wallis, post-hoc Nemeyi, α≤ 0.05).

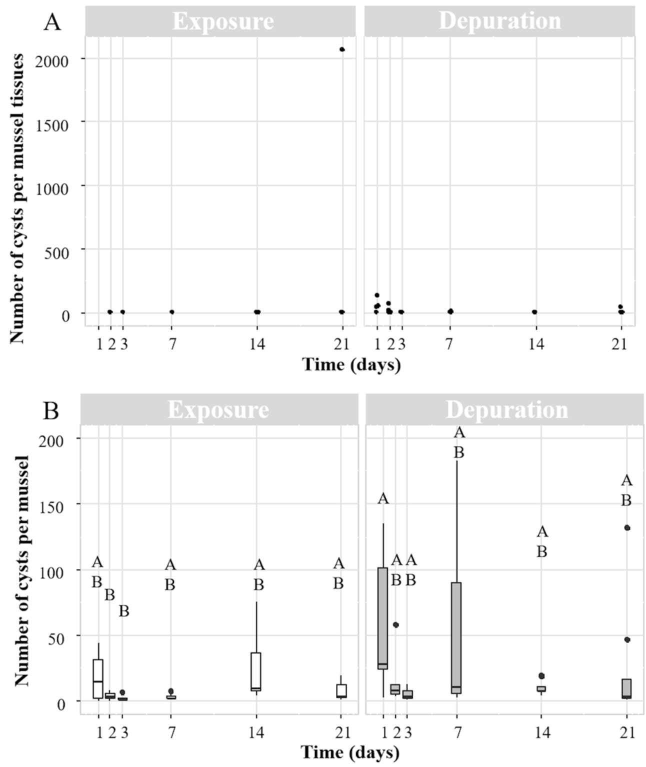


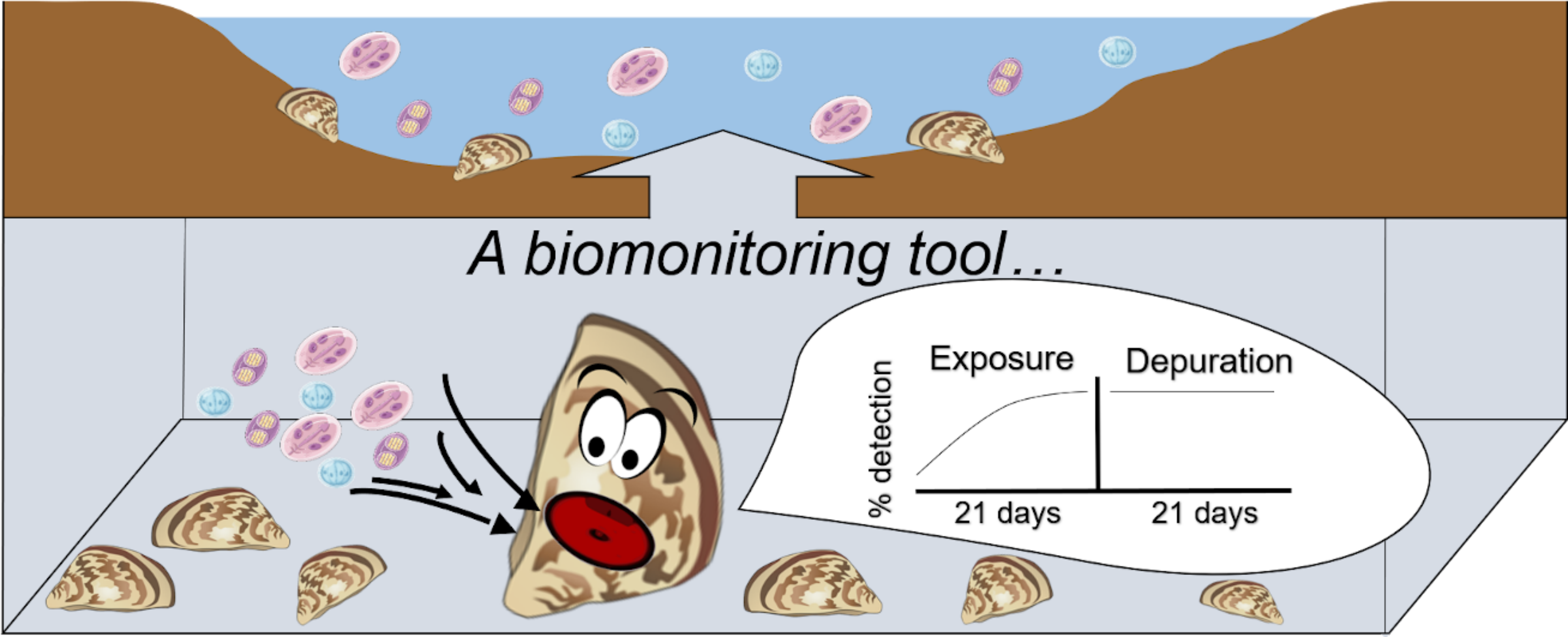
Figure 4 - Number of *G. duodenalis* cysts in mussel tissues (scatterplot or boxplot with median and quartiles) after exposure to 1,000 cysts/day/mussel (A) or 10,000 cysts/day/mussel (B). Groups with no common letters are statistically different from each other (Kruskal-Wallis, post-hoc Nemeyi, α≤ 0.05).

Table 1 - Summary of LOD_{95} and LOQ of *T. gondii*, *G. duodenalis* and *C. parvum* in *D. polymorpha* haemolymph and tissues. LOD_{95} and LOQ are expressed in (oo)cyst number per bivalve.

		Haemolymph	**Tissues**
T. gondii	LOD_{95}	10-50	1-5
	LOQ	100	5
G. duodenalis	LOD_{95}	5-10	<1
	LOQ	10	1
C. parvum	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**
T. gondii	100	0/24	6/105
	1,000	0/64	35/114
	10,000	9/101	102/120
G. duodenalis	100	2/28	8/15
	1,000	11/65	39/60
	10,000	70/108	86/101
C. parvum	100	1/3	4/4
	1,000	1/7	3/3
	10,000	3/28	16/16


A biomonitoring tool…
Exposure
Depuration
% detection
21 days
21 days