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Assessment of *Toxoplasma gondii* levels in zebra mussel (*Dreissena polymorpha*) by real-time PCR: an organotropism study

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Abstract Water quality is a public health concern that calls for relevant biomonitoring programs. Molecular tools such as polymerase chain reaction (PCR) are progressively becoming more sensitive and more specific than conventional techniques to detect pathogens in environmental samples such as water and organisms. The zebra mussel (*Dreissena polymorpha*) has already been demonstrated to accumulate and concentrate various human waterborne pathogens. In this study, first, a spiking experiment to evaluate detection levels of *Toxoplasma gondii* DNA in zebra mussel organs using real-time PCR was conducted. Overall, lower DNA levels in the hemolymph, digestive gland, and remaining tissues (gonad and foot) were detected compared to mantle, muscle, and gills. Second, an in vivo experiment with 1000 *T. gondii* oocysts per mussel and per day for 21 consecutive days, followed by 14 days of depuration time in protozoa-free water was performed. *T. gondii* DNA was detected in all organs, but greatest concentrations were observed in hemolymph and mantle tissues compared to the others organs at the end of the depuration period. These results suggest that (i) the zebra mussel is a potential new tool for measuring *T. gondii* concentrations and (ii) real-time PCR is a suitable method for pathogen detection in complex matrices such as tissues.

Keywords Freshwater · Mollusk · Protozoa · Molecular detection · Spiking experiment · Hemolymph

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Introduction

Toxoplasma gondii is the main parasite responsible for zoonoses worldwide (Tenter et al. 2000). As a coccidian parasite, it has a complex life cycle. Asexual division occurs within felids and warm-blooded organisms, whereas sexual division occurs only in felids. *T. gondii* is the causative agent of toxoplasmosis. It has three infectious stages: the tachyzoite, the bradyzoite in tissue cysts, and the sporozoite under the form of environment-resistant oocysts (Dubey 2004). In immunocompetent people, toxoplasmosis is generally asymptomatic, but it can cause severe clinical symptoms in immunocompromised people. In the case of congenital toxoplasmosis, these symptoms can be blindness, mental retardation, encephalitis, or systemic infection of the fetus (Jones and Dubey 2010).

Analysis of water samples typically requires filtering of large quantities of water that are then concentrated and purified prior to analysis for the pathogen. Analysis of the parasite can be conducted using mouse inoculation (Villena et al. 2004) or through infection experiments with cultured cells (Miller et al. 2008). One sensitive assay that was recently developed for rapid detection of *T. gondii* in water is loop-mediated isothermal amplification (LAMP) (Sotiriadou and Karanis 2008). Quantitative methods such as flow cytometry, microscopy (Shapiro et al. 2010), or molecular analysis can also be used for the analysis of this parasite (Skotarczark 2010). Of these quantitative methods, polymerase chain reaction (PCR) represents a simple, reproducible, and specific technology that is increasingly used to detect pathogens within a few hours (Yang and Rothman 2004). For quantitative purposes, real-time PCR can be an advantageous approach

since the amplification and the detection of PCR products are coupled in the same step. To detect pathogens in watercourses to evaluate their potential sanitary impact on human health, PCR approaches provide a rapid response that allows for immediate action by local authorities (Botes et al. 2013). Aubert and Villena (2009) utilized the following strategy for the detection of *T. gondii* in natural water samples: filtration of water samples followed by real-time PCR for oocyst detection and a mouse bioassay to determine parasite infectivity. Using this approach, 7.7 % of the tested water samples were positive for *T. gondii* DNA, including drinking water, whereas none of these samples were positive according to the mouse bioassay. This suggests that real-time PCR was either more sensitive than the mouse bioassay or that the samples contained non-viable pathogens.

Although water samples are relatively simple, easy-to-handle matrices, they often do not objectively reflect contamination in watercourses. Because of rainfalls, flow rates, and physicochemical parameters, the risk of false negatives due to sampling procedures represents a significant concern. Also, the literature highlights discrepancies in pathogen detection in water as a function of the detection method. Microscopy analyses yielded recovery levels of approximately 36 and 28 % using filtration, and of 15 and 64 % using flocculation for the protozoa *Cryptosporidium parvum* and *Giardia duodenalis*, respectively (Gallas-Lindemann et al. 2013). On the other hand, another study using TaqMan PCR or conventional PCR for detection revealed negative results for *T. gondii*-spiked freshwater (10 to 1000 oocysts per liter) (Shapiro et al. 2010). Pathogen behavior in water is characterized by settling, mobility, and interactions with particles (Dumètre et al. 2012), which depend on water characteristics such as temperature, pH, conductivity, and turbidity. In freshwater, *T. gondii* oocysts are hydrophilic, with negative charges that enable high mobility of oocysts, whereas charges are neutralized in saline waters (Shapiro et al. 2009). Consequently, dissemination of oocysts is higher in freshwater than in marine environments, which leads to flocculation and sedimentation of the pathogen. Evaluation of environmental contamination only based on water samples does not allow for analysis of spatial distribution of the pathogen and therefore cannot be used to reveal biological contamination events reliably. As water is a fluctuating medium, other more objective indicators such as parasite prevalence in sentinel species that are sedentary have to be developed to reliably reveal biological contamination of surface waters (Lindsay et al. 2001; Arkush et al. 2008; Roslev et al. 2010).

In aquatic environments, *T. gondii* is found in a large variety of marine mammals in California, which is surprising since marine mammals do not consume intermediate hosts. To evaluate how these organisms were infected, Miller et al. (2008) tested tissues collected from various animals along the California coast (terrestrial and marine wildlife and marine and estuarine invertebrates). They found that terrestrial carnivores and wild mussels were infected by the same strain of *T. gondii* (type X). The authors suggested that trophic transfer could be an important transmission route of *T. gondii*. Indeed, aquatic invertebrate accumulates and concentrates oocysts from freshwater runoff contaminated by feces from terrestrial wildlife. They then in turn become prey conveying *T. gondii* oocysts to marine wildlife. More recently, *T. gondii* was detected in shrimp, crayfish, and fish in various regions of China (Zhang et al. 2014) and in marine mussels in Turkey (Aksoy et al. 2014). Aquatic organisms can act as paratenic hosts by filtering, concentrating, and accumulating pathogens in their tissues. Many studies underline the accumulation of human waterborne pathogens by aquatic invertebrates under laboratory conditions or in field studies (Palos Ladeiro et al. 2013). In freshwater systems, the zebra mussel (*Dreissena polymorpha*) has previously been used successfully as a sentinel species for *C. parvum* and *G. duodenalis* (Lucy et al. 2008). *D. polymorpha* is sedentary, and it has a high filtration rate. It is commonly used in ecotoxicological studies as a sentinel species for biomonitoring to characterize the quality of freshwater ecosystems. Previous studies have shown accumulation of *T. gondii* by zebra mussels over a 1-week-long laboratory experiment, after exposure to low oocyst concentrations (20 oocysts per mussel and per day) (Palos Ladeiro et al. 2014). However, oocysts are hard to extract because they can interact with cells and be internalized in tissues. That is why their detection in environmental matrices such as plants or aquatic invertebrates is more complex than in water (Hohweyer et al. 2013). Thus, isolation and extraction are more complex in living organisms than in water, and selecting relevant organs is crucial to obtain accurate results (Willis et al. 2013).

The first aim of this study was to determine the sensitivity of quantitative real-time PCR (qPCR) to detect *T. gondii* DNA levels in zebra mussel tissues. For this purpose, hemolymph, muscle, digestive gland, gills, mantle, and remaining tissues (for the most part gonad and foot) were spiked with graded *T. gondii* oocyst doses. The second objective of this study was to determine whether zebra mussels could internalize *T. gondii* after exposure and depuration steps and which organs could be used in biomonitoring programs. For this purpose, zebra mussels were exposed to *T. gondii* oocysts in vivo under laboratory conditions for 21 days, followed by a 14-day depuration period. Also, oocysts were analyzed in water tanks by comparing the two matrices (water vs. zebra mussel organs).

Materials and methods

Detection levels

T. gondii oocysts were generously donated by J.P. Dubey (USDA, Beltsville, USA). The oocysts were obtained from

the feces of cats experimentally infected with the strain ME49 genotype II. They were stored at 4 °C in an aqueous solution containing 2 % H_2SO_4 until use.

To determine the influence of purification and quantification techniques on recovery of oocysts, six clean mussels per condition and replicate (N=6) were dissected organ by organ. A known oocyst concentration was spiked directly to the samples (organs and positive controls which represent oocyst-free in phosphate-buffered saline (PBS): Fig. 1). One hundred oocysts were added to each sample of the first group, 500 oocysts to each sample of the second group, and 1000 oocysts to each sample of the third group (according to Esmerini et al. (2010)). Purification and DNA detection were performed immediately after dissection, as described below.

In vivo experiment

Zebra mussels between 20- and 25-mm long were collected at Commercy (northeastern France) along the Meuse east channel (N 48° 45′ 26.13″, E 5° 36′ 14.51″). They were acclimated in tanks for 2 weeks before exposure. Four pools of three mussels were analyzed randomly to ensure that they were free of *T. gondii* oocysts on the sampling day and on the first exposure day (D0). Tanks were filled with Cristaline Aurèle (Jandun, France) drinking water, at a constant temperature of 14±1 °C and with controlled aeration (conductivity, 382± 8 μS/cm; nitrates, 27±17 mg/L; nitrites, 0.6±0.4 mg/L; ammonium, 2±1.4 mg/L). Mussels were fed ad libitum twice a week with a mixture of two species of microalgae, *Chlorella pyrenoidosa* and *Scenedesmus obliquus*. Tank water was removed weekly and replaced by fresh water. Oocyst amounts were spiked again after water changes to maintain the previous oocyst concentrations in the tanks.

Mussels were randomly divided into two groups (control and exposed) in duplicate tanks of 70 specimens each. Each group was placed in a 5-L tank that contained 3 L of Cristaline Aurèle water for the first week of exposure, and then 2 L until the end of the experiment, to ensure the same quantity of oocysts per organism and per liter. For 21 days, the mussels were exposed to zero (negative control) or 1000 *T. gondii* oocysts per mussel and per day. Then, the mussels were transferred into new tanks filled with clean water and left there for 14 days for the depuration period.

Mussel tissue analyses were performed on four pools of three organs per condition and per sampling event. Hemolymph was withdrawn from the posterior adductor muscle. Then muscle, gills, digestive gland, mantle, and remaining tissues (gonads and foot) were dissected (i) after 1, 7, 14, and 21 days of exposure and (ii) after 7 and 14 days of depuration to assess *T. gondii* DNA levels organ by organ. Water filtration was previously described by Villena et al. (2004). Briefly, tank water was entirely filtered after 8, 15, and 22 days of exposure using Mini Profile Capsules (Pall Corporation, USA). Elution of particulate matter was performed over 24 h in an elution buffer (10 mM Tris, 1 mM EDTA, 3.5 mM sodium lauryl sulfate) and centrifuged at 1500×g for 15 min without brake. Pellets were stored at −80 °C until purification and DNA detection.

Sample purification

Immediately after dissection, organs (except hemolymph and water samples) were ground with 1X trypsin (Sigma-Aldrich, Saint-Quentin Fallavier, France) to allow tissue digestion and to facilitate oocyst extraction. Samples were incubated at 37 °C for 90 min. Suspensions were centrifuged at 1250×g for 5 min, washed, and then kept at −80 °C. The oocyst wall is a complex protective barrier that allows for the long-term survival of protozoa in adverse environments. Therefore, before DNA extraction, a series of heat shock cycles consisting in freezing at −80 °C for at least 2 h and thawing at 95 °C for 15 min was repeated six times to break the wall and to access *T. gondii* DNA (Villena et al. 2004). Then, the samples were subjected to ultrasonic treatment for 1 min at 37 Hz (Ultrasonics 88155), and then pelleted by centrifugation at 92×g. DNA was extracted from the pellets using an InstaGene™ Matrix kit according to the manufacturer's instructions (Bio-Rad, Marnes-la-Coquette, France). The pellets

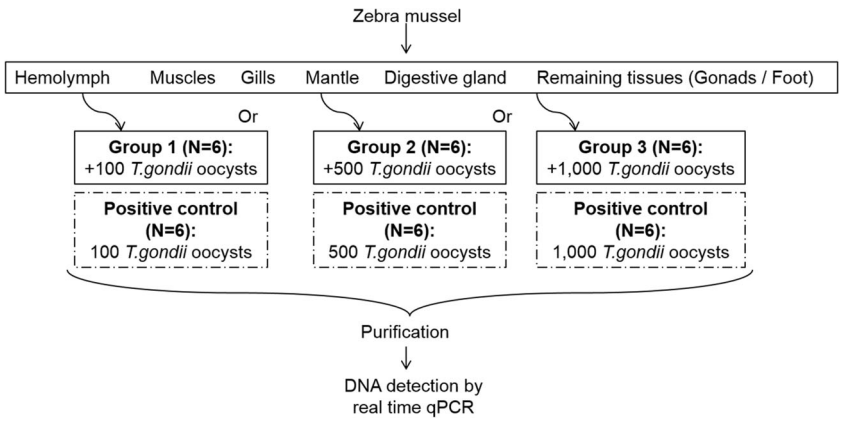

Fig. 1 Detection level protocol: after dissection of zebra mussel tissues, spiked experiments were performed on each sample (N=6 per condition) with 100 *T. gondii* oocysts (group 1), 500 oocysts (group 2), or 1000 oocysts (group 3). Sample purification and then detection by real-time PCR were performed to determine DNA levels in each organ

were incubated with the matrix for 35 min at 56 °C, and then vortexed for 10 s to ensure that sample and solution kit are in contact. After a boiling cell lysis step for 8 min at 99 °C, the matrix absorbed the cell lysis products that could interfere with the PCR process. The samples were centrifuged at 11,093×*g* for 3 min, and then the supernatants were carefully transferred to new tubes and stored at −80 °C until detection by real-time PCR.

Parasite quantification by real-time PCR

DNA detection was carried out by TaqMan real-time PCR in a CFX96 detector (Bio-Rad). Specific primers and detection probes were chosen according to a partial sequence of *T. gondii* strain RH repeat region (GenBank accession number AF 487550.1) (Reischl et al. 2003). Forward primer Toxoplasma F (5′-AGAGACACCGGAATGCGATCT-3′), reverse primer Toxoplasma R (5′-CCCTCTTCTCCACT CTTCAATTCT-3′), and probe Toxoplasma P (Cy3-ACGCTTTCCTCGTGGTGATGGCG-BHQ2) were used (Palos Ladeiro et al. 2014).

Real-time PCR amplifications were performed as specified in the manufacturer's instructions using the following mixtures: 5 μL of extracted DNA sample was added to 20 μL of mix containing 2X iQ^{TM} supermix (Bio-Rad), 400 nM of each primer, and 200 nM of probe. A no-template control was prepared. 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. Each sample was analyzed in duplicate. To determine the numbers of *T. gondii* DNA copies in our samples, a known DNA concentration of the positive control was diluted tenfold, starting at a 1:5 dilution, to establish a reference curve. Real-time PCR results were reported to the reference to determine oocysts number in our samples.

Statistical analyses

The results are expressed in number of oocysts per sample. Data from the positive control and experimental groups were compared using the non-parametric Mann-Whitney *U* test. Correlations between the exposure/depuration steps were determined using Spearman Rank correlation analysis. All statistical tests were performed using SPSS software for Windows v10.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant when $p<0.05$.

Results and discussion

Detection levels

In this spiking study, varying *T. gondii* levels were detected after direct injection of oocysts into *D. polymorpha* organs (Fig. 2). Low recoveries of oocytes occurred in the first group spiked with 100 oocysts, even in the positive control sample with a mean of 5.3 (±3.9) oocysts detected; 21.2 (±29.4) oocysts were detected in mantle, 32.44 (±19.2) in muscle, 17 (±12.4) in gills, 3.7 (±6.8) in hemolymph, 11.17 (±20.5) in digestive gland, and 5.9 (±3.0) in remaining tissue. In the second group spiked with 500 oocysts, a mean of 88.8 (±43.9) oocysts were detected in mantle, 71.9 (±42.0) in gills, and 65.1 (±60.1) in muscle. These results did not differ from the positive control, which displayed 91.4 (±26.2) oocysts. However, significantly lower concentration were observed in hemolymph, digestive gland and in the remaining tissues with

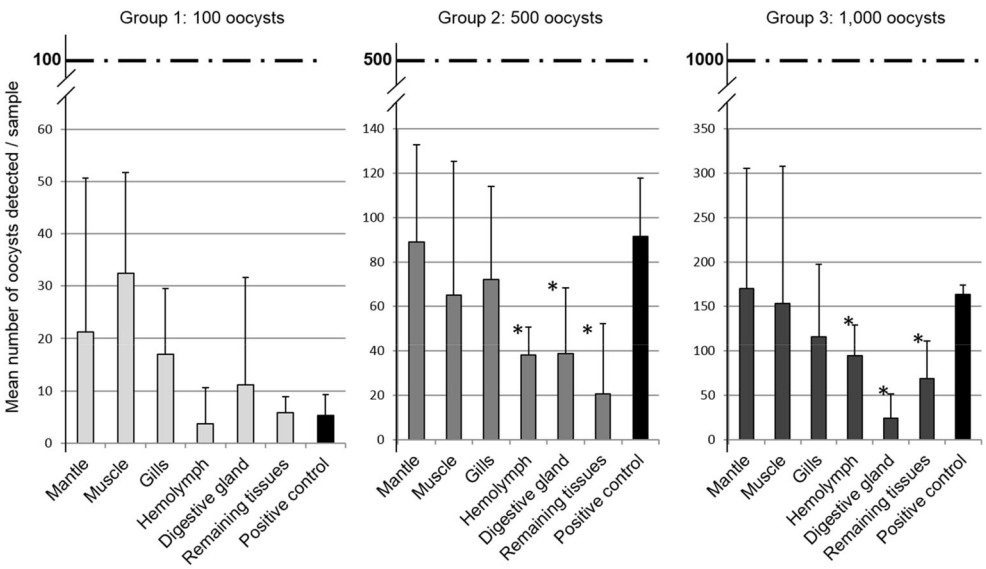

Fig 2 Mean numbers and standard deviations of *T. gondii* oocysts detected in zebra mussel organs and positive control samples ($N=6$ per condition) after being spiked with 100 (group 1), 500 (group 2), or 1000 (group 3) oocysts. *$p<0.05$ level of significant differences with the positive control, using the Mann-Whitney *U* test

38.1 (±12.4), 38.6 (±29.6), and 20.6 (±31.6) oocysts detected, respectively, according to the positive control. The same pattern was observed in the third group spiked with 1000 oocysts where 170.1 (±135.38) oocysts were detected in mantle, 153.3 (±154.4) in muscle, 115.8 (±81.5) in gills, 94.7 (±34.4) in hemolymph, 24.03 (±27.6) in digestive gland, and 68.5 (±42.7) in remaining tissues. Thus, as compared to the positive control (163.4±10.7), detection was better in mantle, gills, and muscle tissues. Conversely, the hemolymph, digestive gland, and remaining tissues displayed significantly lower detection levels than control.

The significant differences in oocyst numbers observed between tissue pools exposed under the same conditions is likely due to loss of oocysts during tissue preparation and DNA extraction. In fact, a large number of steps is needed to extract oocysts from mussel tissues and then to extract DNA from very resilient environmental forms such as oocysts of coccidia genera. The number of oocysts detected in positive controls were 5.3 (±3.9), 91.4 (±26.2), and 163.4 (±10.7) for the first, second, and third group, respectively, which represent only 5.3, 18.28, and 16.34 % of the total recovery, respectively, for the first, second, and third group. These results are very surprising since positive controls represent free oocysts in PBS, without any substances that could interfere with detection, and are probably due to incomplete breakdown of oocyst walls at the end of the lysis steps. A series of six heat shock cycles was performed in this study (freezing at −80 °C and thawing at 95 °C), plus an ultrasonic treatment, to break the oocyst wall and to access to *T. gondii* DNA. Previous studies had reported a degradation of oocyst walls due to this treatment, but healthy oocysts were still observed (data not shown). Improving the extraction step is therefore essential to reach a relevant recovery level in real-time PCR detection. Longer thawing times and ultrasonic treatments, freezing in liquid nitrogen, or including beads during vortexing steps would make it possible to thoroughly break down oocyst walls in the samples. Nevertheless, it is interesting to note that hemolymph, digestive gland, and remaining tissues display significantly lower detection than positive controls for the second and the third condition. This observation could highlight a matrix effect rather than a technical issue. These three particular organs may present a high level of PCR inhibitors that could result in poor DNA recovery (Loge et al. 2002; Sidhu et al. 2012). There are no data in the literature about levels of PCR inhibitors in organisms such as *D. polymorpha* or specific organs. However, it has been hypothesized that unwanted substances such as suspended organic matter could interfere with DNA extraction and amplification and, thus, impair the accuracy and sensitivity of the method (Green and Field 2012). New techniques based on computational methods called kinetic outlier detection (KOD) have recently been developed to detect PCR inhibition (Bar et al. 2012). To alleviate the effects of PCR inhibition, various approaches could be used such as (1) adding a purification step before DNA extraction (flotation method, use of continuous or discontinuous gradient of sucrose) to separate oocysts from other unwanted elements, (2) removing of inhibitors during the extraction step (chemical flocculation, use of chelating substances), or (3) adding PCR facilitators (bovine serum albumin, T4 gene 32 protein) to the PCR mix (Jiang et al. 2005). In the case of matrix effects, the first two methods will supplement the current procedure already overloaded with a lot of steps, which could be associated with loss of oocysts/DNA. Further studies have to be conducted under laboratory conditions to evaluate the benefit of PCR facilitators on oocyst detection and to determine if the divergent results obtained from mussel organs are due to different levels of PCR inhibitors or due to incomplete/inefficient purification and extraction steps in these particular matrices (hemolymph, digestive gland, and remaining tissues).

In vivo experiment

Previous studies underline the capacity of zebra mussels to bioaccumulate and concentrate human waterborne pathogens such as *C. parvum* and *G. duodenalis* in their tissues and, thus, have the potential to serve as a good bioindicator species for these protozoans (Graczyk 2008; Lucy et al. 2008). Recently, results of laboratory exposures underscore that zebra mussels could also bioaccumulate and concentrate *T. gondii* oocysts in higher concentrations than *C. parvum* and *G. duodenalis* during simultaneous or single-week exposure experiments (Palos Ladeiro et al. 2014). Moreover, *T. gondii* DNA was detected in significantly higher amounts in muscle tissue than in digestive gland and gills. In this paper, existing data were completed by a longer term experiment consisting of 21 days of exposure with an additional depuration period of 2 weeks, using the same protocol as in the previous study by Palos Ladeiro et al. (2014). The results of in vivo exposure of zebra mussels to 1000 *T. gondii* oocysts underline a time-response relationship with regard to oocyst accumulation. The mean numbers of oocysts detected in whole tissues on days 1, 7, 14, and 21 were 1.8 (±1.7), 4 (±2.02), 6.6 (±1.6), and 55.3 (±32.4) oocysts per mussel, respectively (Fig. 3). As a result, a significant positive correlation was observed between exposure time and dose (r_s=0.897, p<0.001). Moreover, 9.7 (±3.5) *T. gondii* oocysts were also detected at the end of the depuration time, and a significant negative correlation was found between the maximum exposure level and the end of the depuration time (r_s=−0.887, p<0.001). At the end of the experiment, oocysts were still detected in all organs. As water was changed twice a week, and tanks were also interchanged between the exposure and the depuration time, oocysts recovered in tissues could not been attributed to excess of oocysts in tanks but perchance to an interaction with organs, even if this hypothesis have to be reinforced. None of the negative controls yielded positive

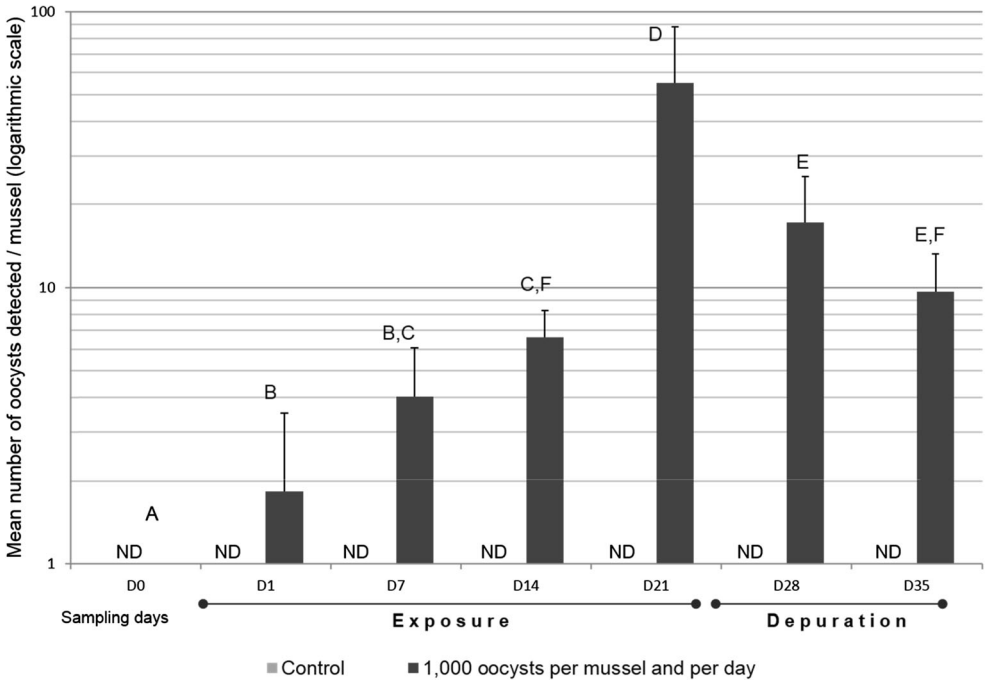

Fig 3 Mean numbers and standard deviations of *T. gondii* oocysts determined per mussel (N=4 pools of 3) after 21 days of exposure to 0 (control) and 1000 protozoa per mussel and per day, followed by 14 days of depuration in clean water. ND=not detected. *Letters* represent significant differences between exposure times. Mann-Whitney U test $p<0.05$

results in the real-time PCR assessment. Results underline important variations between pools under the same exposure condition (four pools of 3 organs per condition and per sampling event), which were mainly due to biological variation. The main cause for this variability is like due to changes in filtration activity, which can vary significantly depending on water parameters (temperature, pH), and particle concentration and, in this case, which also vary daily between individuals in a great extent relative to their health status (Bourgeault et al. 2010).

Throughout the exposure, oocysts were detected in all organs of zebra mussels (Table 1). At the end of the exposure period, the number of oocysts was higher in all organs in comparison to the beginning of the exposure. Nonetheless,

Table 1 Mean numbers and standard deviations of *T. gondii* oocysts determined in zebra mussel organs (N=4 pools of 3) after 21 days of in vivo exposure to 1000 oocysts per mussel and per day, and then 14 days of depuration in clean water

Day/organs		Mantle		Hemolymph		Gills		Muscle		Digestive		Remaining tissue (gonads/foot)	
	0	ND	a	ND	a	ND	a	ND	a	ND	a	ND	a
			A		A		A		A		A		A
Exposure	1	0.66±1.33	a,b	0.86±0.62	b	0.05±0.11	a,c	0.23±0.3	a.b,d	0.03±0.03	a,b	ND	a
			A,B		A		A,B		A,B		A,B		B
	7	1.63±1.42	b,c	1.62±3.05	b,c	0.03±0.06	a,f	0.16±0.21	b	0.19±0.78	a,b	0.19±0.39	a,c
			A		A,B		B		b		A,B		A,B
	14	1.52±1.06	b	0.77±0.72	b	1.72±1.84	b,d	1.89±0.5	c,d	0.01±0.02	a,b	0.71±1 02	b,c,d
			A,b		A		A,B		B		C		A,B,C
	21	23.28±25.31	c	15.19±14.14	c	5.75±7.18	b,e	4.57±4.46	b,c	2.94±2.79	b	3.62±3.01	d
			A		A,B		A,B		A,B		B		B
Depuration	28	8.12±1.78	c	2.27±1.96	b	1.27±1.45	c,d,e	2.13±2.01	c	3.22±5.85	b	0.28±0.5	b,c
			A		B		B,C		B,C		A,B,C		C
	35	6.93±2.59	c	2.16±1.2	b	0.02±0.22	c,d,f	0.21±0.22	b	0.16±0.26	a,b	0.01±0.01	b,c
			A		D		B,C		B		B,C		C
Negative control*	ND		ND		ND		ND		ND		ND		

Lower case letters represent significant differences between exposure times whereas uppercase ones represent significant differences between organs. Mann-Whitney U test $p<0.05$

ND not detected throughout the experiment

some organs presented higher accumulation rates of oocysts. For example, 23.28 (±25.31) oocysts were detected in mantle whereas only 2.94 (±2.79) oocysts were observed in digestive gland ($p=0.021$). This observation clearly indicates that organotropism and transport of oocysts in organisms represents an important factor to be further characterized in further studies. In fact, *T. gondii* was recently detected in aquatic organisms in China, i.e., shrimps, crayfish, and fish, but only 6 out of 3432 organisms were *T. gondii*-positive after DNA extraction from digestive tracts and PCR detection (Zhang et al. 2014). The authors concluded that *T. gondii* levels in Chinese waters are low. However, this assessment may represent an underestimation of the actual contamination scenario since DNA detection was evaluated only in digestive tracts. Even if organotropism could differ in others organisms, the present results underscore low levels of *T. gondii* DNA in the digestive glands of mollusks after in vivo exposure.

At the end of the depuration time, *T. gondii* DNA were detected by real-time PCR in all zebra mussel organs, with significantly higher quantities in mantle ($p<0.02$) and hemolymph ($p<0.02$) than all others organs considered with 6.93 (±2.59) and 2.16 (±1.2) oocysts detected, respectively (Table 1). However, direct comparison of organs was unlikely to be highly accurate and must be adjusted taken into consideration the size of organs, since mantle is the largest tissue of zebra mussel. To harmonize the result expression, results should be expressed as *number of oocysts per gram of tissue*. However, it is technically difficult to weigh all organs because zebra mussel is a small-sized organism (between 20 and 25-mm long). One limitation of our study is that we did not consider pathogens in mussel pseudofeces. Filter-feeder species use pseudofeces to get rid of unwanted particles, and a recent study underlines that pseudofeces discharge areas are important infection routes (Allam et al. 2013).

At the end of the experiment, mantle and hemolymph seem to be the most suitable organs to highlight current or previous water contamination events at least 2 weeks after the occurrence of the event. As mantle is the largest organ in mussels and is directly in contact with water, it could accumulate contaminants in higher proportions than other organs. Since all organs are bathed in hemolymph, we can hypothesize that higher pathogen quantities in the hemolymph could result from the fact that mussels protect themselves against pathogens through organ washes until pathogens are entirely eliminated by phagocytosis or encapsulation. Phagocytosis plays an important role in pathogen retention: hemocytes of *Corbicula fluminea* retained 81.6 % of a spiked dose of 1.13×10^5 *C. parvum* after 120 min of incubation in an in vitro study (Graczyk et al. 1997). We did not detect any pathogens in our tank water, so we cannot rule out a detrimental effect of mussel defenses on the pathogens. Allam and Paillard (1998) highlighted functional hemocytes in extrapallial fluid of clam, *Ruditapes philippinarum*, as one of the first lines of defense against pathogens. Villalba et al. (2004) reviewed host-pathogen interactions in the case of oyster infection by *Perkinsus* spp. They highlighted that hemolymph could facilitate parasite dispersal among mollusk organisms. For biomonitoring purposes, we need to develop simple, rapid, and convenient tools for field applications. The hemolymph could fulfill these conditions. Although we emphasized a low detection level in the hemolymph, it could reflect past or present contamination events by protozoa, and it is time integrative. Hemolymph sampling is rapid, non-invasive for organisms, and directly available for protozoa extraction since no purification step with trypsin is needed.

T. gondii does not multiply in cold-blooded organisms such as mussels. However, passive interaction of protozoa with a sentinel species such as the zebra mussel raises acute biological questions concerning impacts on hemocytes, key cells of invertebrate immunity. Hemocytes play a major role in homeostasis. They are involved in immunity responses, wound and shell repair, and nutrient transport (Cheng 1981). Besides, throughout our in vivo experiment, *T. gondii* DNA levels were extremely low compared with the theoretical spiked conditions of 21×10^3 per mussel. Surprisingly, over the entire exposure period, no *T. gondii* DNA was detected in tank water by the here utilized qPCR method. This observation is in agreement with other laboratory experiments. For instance, a mismatch was observed between the quantities of oocysts recovered from tissues and the amounts of oocysts spiked in tanks, and no oocysts were found in water or sediment after a 5-week-long exposure to *C. parvum* and *G. duodenalis* (Graczyk et al. 2003). The differences between the concentrations of oocysts spiked in tanks and final recovery values raised some questions about the pathogens' accessibility by organisms under laboratory conditions. Interactions with glass tanks and with organic components (algae and biofilms) seem important factors that should be assessed in this context by further studies to understand whether all spiked protozoa were truly available to the test organisms. Nevertheless, regardless of the matrices tested, no study achieved 100 % pathogen recovery. Therefore, it is all the more necessary to improve purification and extraction steps to accurately reveal contamination events in a water monitoring context.

Conclusion

Water characteristics, rainfall, and pathogen mobility in water make it difficult to characterize protozoa dispersal in surface waters. Additionally, contamination by protozoa is a public health issue that has been rising over the years. As a result, other tools than traditional water sampling are needed to more objectively characterize water contamination with pathogens such as *T. gondii*. Zebra mussels are widely used for chemical biomonitoring purposes and for detecting other human

waterborne pathogens, i.e., *C. parvum* and *G. duodenalis*. Thus, it seems to represent a promising tool to trace *T. gondii* contamination in surface waters. Our results underscore not only the capacity of zebra mussels to accumulate *T. gondii* proportionally to water contamination but also identified the presence of protozoa in their tissues for up to 14 days post inoculation. Furthermore, hemolymph appears to be one of the tissues that concentrated the highest quantities of oocysts during in vivo experiments. In our experiment, DNA extraction was not in perfect agreement with nominal *T. gondii* doses because extraction efficiencies need to be improved to better detect pathogens in complex matrices. Regardless, this study indicated that the zebra mussel seems to represent an interesting and relevant tool to reveal biological contamination in watercourses.

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Ethical considerations In France, experiments conducted with *Dreissena polymorpha* do not require authorization. This specie is not protected and their use in scientific research does not require a specific permit. However, every effort will be made to reduce the suffering of animals.

Conflict of interest The authors declare no competing conflict of interest.

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