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ORIGINAL ARTICLE

Zebra mussel as a new tool to show evidence of freshwater contamination by waterborne *Toxoplasma gondii*

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Abstract

Aims: The objective of this study was to evaluate if freshwater bivalves can be used to detect the presence of *Toxoplasma gondii* in water bodies.
Methods and Results: Zebra mussels (*Dreissena polymorpha)* were caged for 1 month upstream and downstream of the discharge points of wastewater treatment plants (WWTPs). Physiological status was assessed to assure good health of bivalves during transplantation. The presence of *T. gondii* was investigated in mussel tissues by qPCR. In autumn, *T. gondii* was detected in mussels caged downstream of the discharge points of two WWTPs. In spring, it was detected upstream of one WWTP.
Conclusions: For the first time, *T. gondii* DNA has been shown in a continental mollusc in environmental conditions. This highlights the interest of an active approach that could be applied independently of the presence or accessibility of autochthonous populations, and underlines the presence of *T. gondii* in natural waters under pressure of WWTP discharge at a certain time of the year.
Significance and Impact of the Study: This study shows that transplanted zebra mussels could be used as biosamplers to reveal contamination of freshwater systems by *T. gondii*.

Introduction

Providing pathogen-free drinking water supplies represents a key priority of the European Drinking Water Directive (Council Directive 98/83/EC). Despite current prevention and treatment methods, human populations are still impacted by many waterborne pathogens, especially by protozoa which remain highly persistent in water. This is particularly true for the coccidian *Toxoplasma gondii.* Many countries have been subject to human toxoplasmosis outbreaks due to contaminated water (Benenson *et al.* 1982; Bowie *et al.* 1997; Dardé *et al.* 1998; Bahia-Oliveira *et al.* 2003). There is now evidence that accumulation of *T. gondii* oocysts in the water environment poses a significant public health hazard (Torrey and Yolken 2003).

The oocyst stages of *T. gondii* are released into the water environment by cats. They are quite robust, so they can remain ubiquitous in aquatic habitats (Torrey and Yolken 2003). Wastewater treatment plants (WWTPs) are often ineffective to eliminate *T. gondii* oocysts from water because they are extremely resistant to chemical and physical disinfection (Wainwright *et al.* 2007). For example, in the eastern part of France, Aubert and Villena (2009) detected *T. gondii* in 8% of 482 environmental water samples, including public drinking water. At present, there are no specific regulations regarding methods to control *T. gondii* oocysts in drinking water supplies, and research in this area has been scant (Dubey *et al.* 1970; Lindsay *et al.* 2002, 2003). Moreover, most of the methods used to detect such protozoa in water have major limitations: they are time consuming, labour intensive and expensive (Toze

1999). In particular, large volumes of water are necessary to concentrate pathogens before their potential detection, and this can lead to a loss of the target organisms. From a monitoring point of view, pathogen assessment in water matrices appears poorly representative or not representative at all of the sampled sites, particularly in river systems.

New methods have recently emerged in water sanitary surveys. They take into account host-associated microorganisms as natural biosamplers (Roslev *et al.* 2010). In particular, special attention has been paid to bivalves because their intense filtering activity could lead to high accumulation of food and/or waterborne protozoan parasites (Graczyk *et al.* 2003; Palos Ladeiro *et al.* 2013). Studying bivalves can highlight pathogen contamination even when water analysis results appear to be negative (Ayres *et al.* 1978). In particular, the flagellate *Giardia duodenalis* and the coccidian *Cryptosporidium parvum* are the most widely detected protozoan parasites in several species of shellfish worldwide (Graczyk *et al.* 2003; Giangaspero *et al.* 2005; Miller *et al.* 2005; Molini *et al.* 2007; Putignani *et al.* 2011). As for *T. gondii*, laboratory studies have already shown that marine and freshwater bivalves can concentrate oocysts, but only few surveys of *T. gondii* in wild marine animals have been reported (Esmerini *et al.* 2010; Putignani *et al.* 2011; Aksoy *et al.* 2014; Zhang *et al.* 2014; Shapiro *et al.* in press). To our knowledge, no field study has reported the presence of *T. gondii* DNA in continental molluscs so far, although this protozoan is a terrestrial pathogen, more likely to be found in freshwater systems than in marine ones. Moreover, *T. gondii* oocysts seem to be more hydrophilic and negatively charged in freshwater systems than in estuarine or marine waters. This makes it easier for them to spread (Shapiro *et al.* 2009). As no accurate method is available, new tools are strongly needed to reveal their presence in freshwater environments.

Zebra mussels (*Dreissena polymorpha)* have been largely used for freshwater biomonitoring because they are an abundant bivalve with a worldwide distribution in lakes and rivers (Binelli *et al.* 2001; Guerlet *et al.* 2007; Bacchetta and Mantecca 2009; Bourgeault *et al.* 2010). They offer strong tolerance to transplantation, which makes them an interesting sentinel species for the active monitoring of various types of water bodies. In North America, zebra mussels already serve as a biological indicator of water quality in the Great Lakes (Brieger and Hunter 1993; De Lafontaine *et al.* 1999; Horgan and Mills 1999). They can concentrate *C. parvum* and *G. duodenalis* very efficiently, even at low ambient concentrations (Graczyk *et al.* 2003), and already serve as good indicators of *C. parvum* contamination in the St. Lawrence River (Graczyk *et al.* 2001). Their ability to concentrate *T. gondii* has been demonstrated experimentally (Palos Ladeiro *et al.* 2014). This study evidenced that mussels could bioaccumulate *T. gondii* oocysts proportionally to ambient contamination.

As water is a fluctuating medium, indicators such as parasite prevalence in sedentary sentinel species have to be developed to reliably reveal biological contamination of surface waters. We tested the hypothesis that zebra mussels could be used to track the presence of *T. gondii* pathogens in freshwater systems as natural biosamplers. However, according to the water bodies to be monitored and to their depth, autochthonous populations were not always present or easily available. So we used an active approach to study water bodies independently of the presence/accessibility of autochthonous populations, and assessed the presence of pathogens at precise time-periods. Mussels were transplanted nearby WWTPs, in rivers located in eastern France. The experiments were carried out in fall and spring. Mussel morphometric condition indices and digestive enzyme activity levels were assessed to evaluate their physiological status during the caging periods.

Materials and methods

Experimental caging technique

The 1-month-long caging trials nearby WWTPs were carried out in October 2013 and March 2014. Zebra mussels with shell lengths of 18–25 mm were collected from a common site, the Lake of Der (eastern France). The sampled mussels were distributed into 2-mm-mesh polyethylene experimental cages (25 × 15 cm) in laboratory (40 and 30 mussels per cage in October and March respectively). The cages were then placed in tanks containing aerated clean water for 1 week. Before transplantation, four pools of whole tissue of three mussels were realized and frozen at −20°C to check for the absence of *T. gondii* in their tissues. The digestive glands of 10 other mussels were sampled and stored at −80°C to establish the reference digestive activity level.

Five WWTPs from five big towns in the Picardie and Champagne-Ardenne regions were selected (Fig. 1). The WWTPs of Creil, Soissons, Epernay, and Reims discharge their treated wastewater into rivers which drain the Seine Basin: the Oise, Aisne, Marne and Vesle rivers respectively. The last WWTP is located in the town of Charleville-Mézières, which is crossed by the Meuse river.

Mussel cages were transplanted upstream and downstream of the discharge points of the WWTPs of the five towns mentioned above (Table 1), therefore to 10 different sites (five upstream and five downstream). At each exposure site ($n = 10$), two experimental cages were

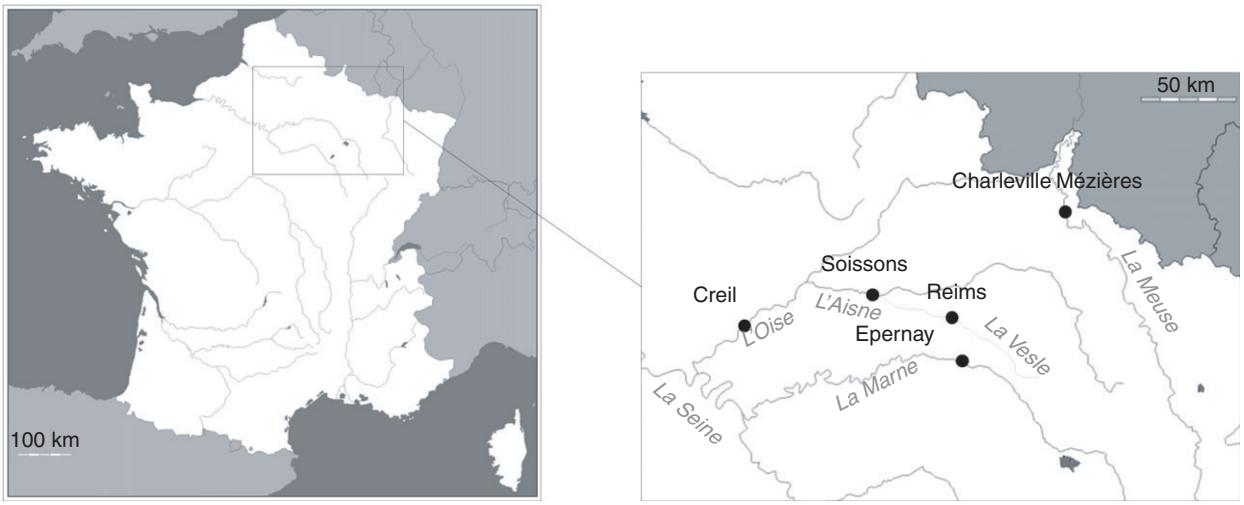

Figure 1 Geographical locations of the caging sites in relation to the wastewater treatment plants of the five towns.

Table 1 Characterization of the caging procedure: sites, GPS coordinates, duration, and numbers of live mussels at the end of the experiments

					October 2013			March 2014		
						Live mussels (/40)			Live mussels (/30)	
Town	River	Site	GPS coordinates		Caging days	Cage 1	Cage 2	Caging days	Cage 1	Cage 2
Charleville M.	Meuse	Downstream	E 004°41′32·2″	N 49°45′27·2″	37	38	38	34	30	30
		Upstream	E 004°44′38·2″	N 49°46′14·7″		l.c.	l.c.		30	27
Creil	Oise	Downstream	E 002°28′13·0″	N 49°15′37·0″	36	38	39	35	30	27
		Upstream	E 002°25′24·5″	N 49°12′48·8″		37	40		l.c.	l.c.
Epernay	Marne	Downstream	E 003°58′10·7″	N 49°02′35·3″	41	31	39	37	27	28
		Upstream	E 003°54′22·7″	N 49°03′44·4″		40	l.c.		30	28
Reims	Vesle	Downstream	E 003°58′10·7″	N 49°02′35·3″	35	37	36	37	–	–
		Upstream	E 003°54′22·7″	N 49°03′44·4″		38	35			
Soissons	Aisne	Downstream	E 003°18′27·1″	N 49°23′29·7″	36	40	38	35	28	30
		Upstream	E 003°16′06·2″	N 49°23′22·8″		40	36		30	25

l.c., lost cage.

ballasted and immersed to an 0·2–1·0 m depth, so 20 cages were used. Temperature and conductivity were recorded at the beginning and at the end of each experiment. In October, temperature ranged between 11·9 and 16·6°C, and conductivity between 515 and 718 μS cm^{-1}. In March, temperature ranged between 8 and 13·3°C, and conductivity between 494 and 690 μS cm^{-1}.

At the end of the month, the cages were sampled and transported to the laboratory. Mortality rates were below 8% for all cages. The tissues of nine mussels from the cages from each upstream and downstream site were pooled by three and frozen at −20°C for protozoa detection (three pools per cage = six pools per site). Five mussels per cage (n = 10 per site) were weighed with their shell (total weight) and without it (body weight), and each digestive gland was dissected and stored at −80°C until individual analysis of amylase and cellulase activity levels.

Physiological status

The condition index (CI) of the sampled mussels was calculated using the following formula:

$$\text{CI} = \text{body weight}/\text{total weight}$$

Digestive glands were ground in a glass mortar and homogenized in phosphate buffer (0·01 mol l^{-1}, pH 6·5) for amylase and cellulase activity measurements. Samples were centrifuged at 4°C and 15 000 ***g*** for 30 min, and the supernatants were collected. Enzyme activity levels

were measured using the 3,5-dinitrosalicylic reagent method (Bernfeld 1955), according to the procedure described in Palais *et al.* (2010). The protein content of each supernatant was measured using the method of Bradford (1976). Activity levels were then expressed in μg of maltose released per minute and per mg of protein (μg min^{-1} mg^{-1} protein).

Protozoa detection

Sample purification

Tissues from whole mussels were ground in a glass mortar with trypsin 1×, and incubated at 37°C for 90 min for the tissues to be digested to facilitate oocyst extraction. Then the mixes were centrifuged at 1200 ***g*** for 5 min, and the pellets were collected. A series of 15-min heat shock cycles consisting in freezing at −80°C and thawing at 95°C was repeated six times, and followed by ultrasonic treatment for 10 min, to break the oocyst wall and to access protozoan DNA.

For the October samples, DNA was extracted using an InstaGene™ Matrix kit (Bio-Rad). Briefly, samples were incubated with a matrix (v/v) for 35 min at 56°C. Then the cells were lysed by boiling for 8 min at 99°C, and samples were centrifuged at 11 000 ***g*** for 3 min, and then the supernatants were stored at −80°C until detection by qPCR. DNA extraction was optimized between October and March, as suggested in Faucher *et al.* (2012). Using this last method, sample purification is more efficiently and final volume is reduced of about 10 fold which improve the sensitivity of *Toxoplasma* PCR. For the March samples, DNA extraction was performed on an automatic NucliSENS easyMAG system (Biomérieux). DNA samples were treated with proteinase K for 30 min at 56°C (with Tris HCl 20 mmol l^{-1} and SDS 0·5%). After centrifugation at 1200 ***g*** for 5 min, supernatants were purified with a NucliSENS easyMAG system, following the manufacturer's Specific B protocol, with a 50-μl final elution volume.

TaqMan® qPCR

Parasite detection was carried out by TaqMan® real-time PCR in a Chromo4 detector (Bio-Rad), as described by Palos Ladeiro *et al.* (2014). Primers, probe and GenBank accession number are summarized in Table 2. Mixtures were levelled to 25 μl final volume made of 5 μl of extracted DNA sample and 20 μl of mix containing 2X iQ™ supermix (Bio-Rad), 400 nmol l^{-1} of each primer, and 200 nmol l^{-1} of probe. Thermal cycling parameters were 3 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. Each sample was analysed in duplicate. A sample was considered as positive if at least one well transmitted a signal. Results were expressed relative to the cycle threshold (C_t), defined as the number of cycles required for the fluorescent signal to cross the threshold.

Table 2 Primers and probe used for *Toxoplasma gondii* detection by TaqMan® real-time PCR (Reischl *et al.* 2003)

	Sequences	GenBank accession numbers
Forward primer	5′-AGAGACACCG GAATGCGATCT-3′	AF 487550.1
Reverse primer	5′-CCCTCTTCTCCA CTCTTCAATTCT-3′	
Probe	Cy3-ACGCTTTCCTC GTGGTGATGGCG-BHQ2	

Positive and negative controls

The effectiveness of the qPCR method used to detect protozoa in zebra mussel tissue was investigated in previous studies (Palos Ladeiro *et al.* 2014, 2015). In particular, in that last study, spiking and *in vivo* experiments were conducted to evaluate the detection levels of *T. gondii* DNA in zebra mussel organs. In the present study, positive controls composed of free oocysts in phosphate-buffered saline were prepared. *T. gondii* oocysts (ME 49 strain) were generously gifted by J.P. Dubey (USDA, Beltsville, MD). DNA from the oocyst solution was extracted in the same way as from mussels. For each qPCR, four series of 10-fold dilutions of the solution were used to establish a standard curve and then evaluate the number of oocysts found in the mussel samples. C_T values ranged from 26 to 40 with InstaGene™ extraction with a PCR efficiency of 0·82 and from 23 to 35 with NucliSENS extraction with a PCR efficiency of 1·24. Four pools of mussel tissue were also spiked with *T. gondii* oocysts before extraction to ensure appropriate detection in tissues. Negative controls were analysed with distilled water; results were negative for all runs.

Statistical analysis

Statistical analyses were performed with XLSTAT 2007. As physiological data did not comply with the parametric assumption of normality (Shapiro-Wilk tests) and homogeneity of variance (Levene tests), nonparametric tests were used. Comparisons of physiological parameters between values measured at the beginning and the end of experiments, and between downstream and upstream sites were performed using Kruskal–Wallis test and Mann–Whitney U test for *post hoc* pairwise comparisons.

Results

Physiological status

The mean mussel CI was 0·129 ± 0·034 in October, and 0·276 ± 0·062 in March (Fig. 2). Over the two periods and at all sites, we did not note any significant difference in CI values between the beginning and the end of experiments (1 month exposure), except upstream of Creil WWTP where the CI significantly increased in March as compared to T_0 (Fig. 2). In all towns and whatever the season, no significant difference was noted between the upstream and downstream sites of the discharge points of the WWTPs.

Mean amylase activity levels were 94·1 ± 34·2 μg min^{-1} mg^{-1} protein in October, and 40·4 ± 34·7 μg min^{-1} mg^{-1} protein in March (Fig. 3). In October, no significant difference in amylase activity was noted between the beginning and the end of the experiments at any site, except upstream of Epernay WWTP where amylase activity significantly increased in March as compared to T_0 (Fig. 3a). In March, amylase activity levels were significantly higher at the end of the experiment than at T_0 at all sites, except upstream of Creil WWTP where there was no significant difference (Fig. 3b). No significant difference was noted between the upstream and downstream sites of the discharge points of the WWTPs whatever the town or the season.

Mean cellulase activity levels were 11·4 ±4·3 μg min^{-1} mg^{-1} protein in October, and 23·4 ±11·4 μg min^{-1} mg^{-1} protein in March (Fig. 4). In October, no upstream and downstream, whatever the site (Fig. 4a). In March, cellulase activity levels were significantly higher at the end of the experiments than at T_0 at both the upstream and downstream sites in Soissons and at the downstream sites of Epernay and Charleville M. WWTPs (Fig. 4b).

Protozoa detection

Toxoplasma gondii DNA was detected in two pools of mussels transplanted in October and one pool trans-

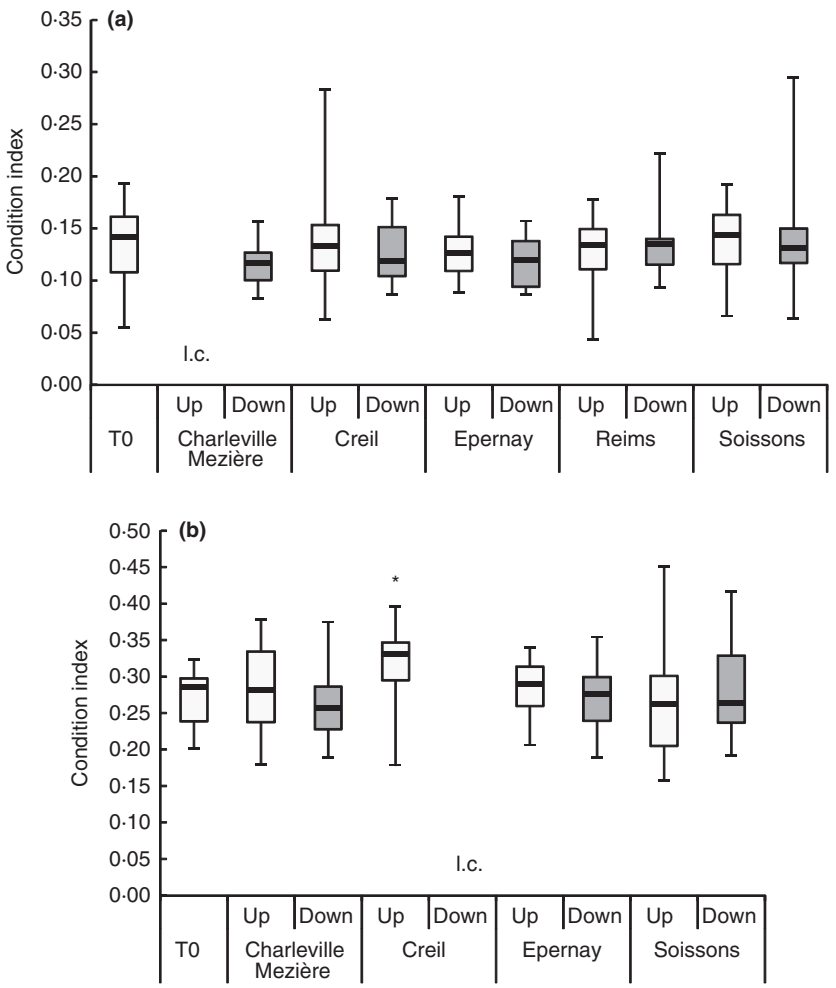

Figure 2 Comparison of condition indices (max., median, min.) of zebra mussels (*Dreissena polymorpha*) caged upstream (□) and downstream (▨) of the discharge points of the wastewater treatment plants of five towns in October 2013 (a) and March 2014 (b). *significant difference compared to T_0 ($P < 0·05$, $n = 20$). l.c., lost cage.

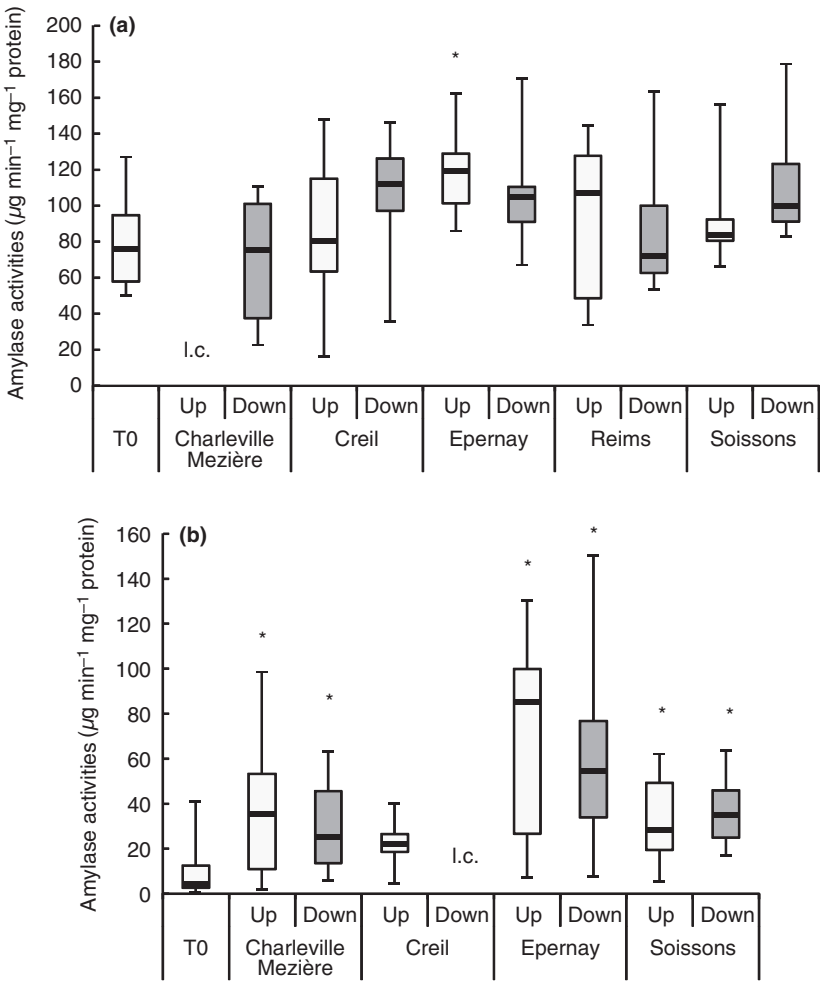

Figure 3 Comparison of amylase activity levels (max., median, min.) of zebra mussels (*Dreissena polymorpha*) caged upstream (□) and downstream (▨) of the discharge points of the wastewater treatment plants of five towns in October 2013 (a) and March 2014 (b). *Significant difference compared to T_0 ($P < 0{\cdot}05$, $n = 10$). l.c., lost cage.

planted in March (Table 3). In October, *T. gondii* was detected downstream of the discharge points of Creil and Soissons WWTPs. For Creil, the two PCR replicates were positive, with a mean C_t of 40·7 (1·3 oocysts). For Soissons, only one replicate was positive, with a C_t of 40·7 (1·4 oocysts). In March, *T. gondii* was detected upstream of the discharge point of Creil WWTP, with only one positive PCR replicate, with a C_t of 39·1 (0·1 oocyst).

Discussion

Zebra mussels are frequently transplanted into the environment for ecotoxicological studies and biomonitoring processes (Mersh and Beauvais 1997; Roper *et al.* 2001; Palais *et al.* 2012; Châtel *et al.* 2014). In particular, a recent review by Binelli *et al.* (2015) suggests that they could be a reference organism for the active biomonitoring of freshwater ecosystems. The main limitation to their use is that although they are present in many locations, they are not found in all freshwater ecosystems. However, previously cited authors underline that the invasive behaviour of this species should not hinder its use as a reference organism, provided that its use is restricted to sites that the species has already colonized. In this study, we measured condition indices and digestive activity levels to assess the physiological status of mussels after their transplantation into the environment. Using digestive enzyme activity levels as biomarkers of the energy metabolism appeared relevant because a relationship is commonly observed between the digestive activity of organisms and their access to energy through food (Amiard-Triquet *et al.* 2013). We did not note any difference in these physiological biomarkers between the upstream and downstream sites of the discharge points of the WWTPs. Moreover, whatever the caging site, condition indices and digestive enzyme activity levels were equally strong, and sometimes even higher than at the beginning of the experiment. This shows that mussels did not suffer during the experiment. Similar enzyme activity values were recorded by Palais *et al.* (2012) at a similar season. These

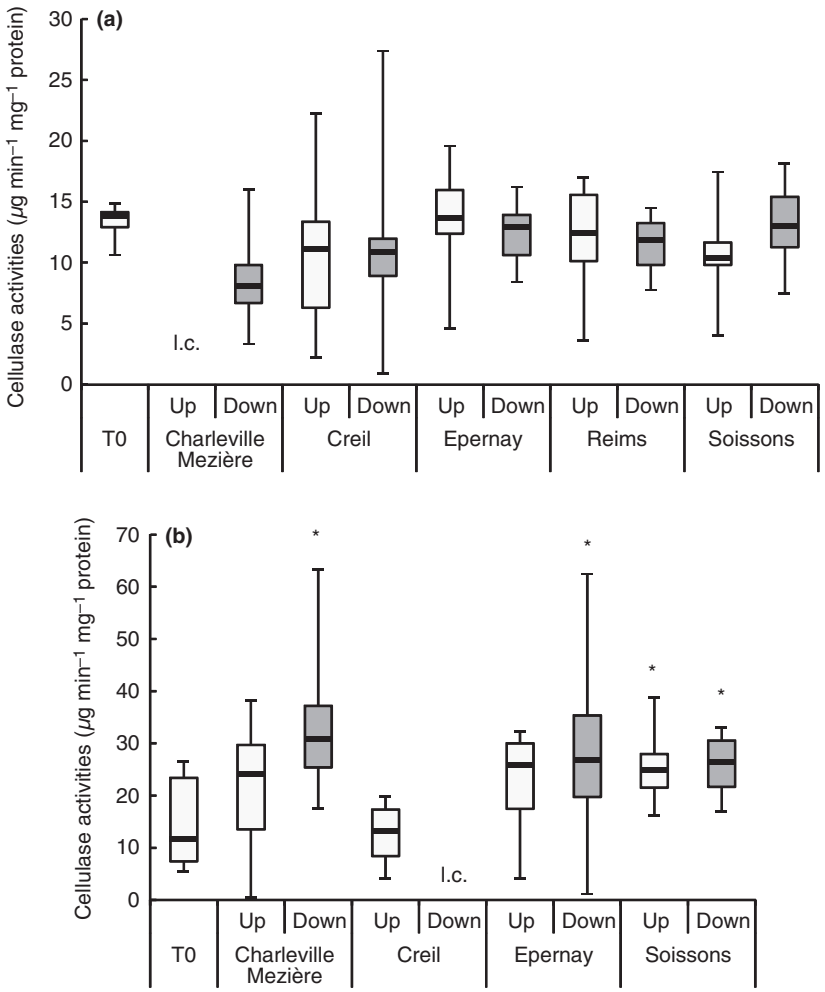

Figure 4 Comparison of cellulase activity levels (max., median, min.) of zebra mussels (*Dreissena polymorpha*) caged upstream (□) and downstream (▣) of the discharge points of the WWTPs of five towns in October 2013 (a) and March 2014 (b). *Significant difference compared to T_0 ($P < 0{\cdot}05$, $n = 10$). l.c., lost cage.

authors support the idea that the induction of digestive activity underlines the implementation of an energy-optimizing strategy in transplanted mussels. We noted differences in condition indices between October and March, which could be related to the development and maturation of gametes in spring (Palais *et al.* 2012). The low mortality rates and the good physiological status demonstrated by the energy biomarkers confirm that the mussels were in good health and support that they may be transferred for biomonitoring purposes, even at the potentially stressful discharge points of WWTPs.

This is the first time that *T. gondii* DNA has been detected in a freshwater mollusc in environmental conditions. *Toxoplasma gondii* DNA was detected in mussels at the two time-periods after only 1 month of exposure. Marine and freshwater bivalves can concentrate *T. gondii* oocysts in laboratory conditions (Lindsay *et al.* 2001; Arkush *et al.* 2003; Palos Ladeiro *et al.* 2014), but surveys in wild invertebrates are scarce. We detected *T. gondii* DNA in 3·1% of 96 pools of three mussels. This is consistent with results obtained by Miller *et al.* (2008), who sampled 1396 marine and estuarine invertebrates and found a TaqMan® positive in only 1 sample of the mussel *Mytilus californianus*. The oyster *Crassostrea rhizophorae* harboured *T. gondii* DNA in its tissues in 3·3% of 60 pools of five oysters (Esmerini *et al.* 2010). Putignani *et al.* (2011) evaluated the presence of *T. gondii* DNA to be 3·2% of 62 pools of 30 marine shellfish specimens (one positive sample in *Crassostrea gigas* and one in *Tapes decussatus*). *Toxoplasma gondii* DNA was found in 0·2% of freshwater animals, out of 3432 aquatic animals covering eight species (five positive samples from two shrimps, *Procambarus clarkia* and *Macrobranchium nipposense*, and one positive sample from the fish *Hypophthalmichthys molitrix*) (Zhang *et al.* 2014). Shapiro *et al.* (2014) highlighted the presence of *T. gondii* DNA in 1·4% of 959 *Mytilus californianus* (13 positive samples). *Toxoplasma gondi* DNA was found in 9·4% of 53 pools of 15 specimens of *Mytilus galloprovincialis* from the west coast of Turkey (Aksoy *et al.* 2014). We detected

Table 3 Number of positive detections of *Toxoplasma gondii*, in whole tissues of zebra mussels caged upstream and downstream of the discharge points of the wastewater treatment plants of five towns in October 2013 and March 2014. The C_t is only given when detection was positive

			T. gondii	
Town	River	Site	October	March
Charleville M.	Meuse	Upstream	0/6	0/6
		Downstream	l.c.	0/6
Creil	Oise	Upstream	0/6	1/6 (C_t: 39·1)
		Downstream	1/6 (C_t: 40·7)	l.c.
Epernay	Marne	Upstream	0/6	0/6
		Downstream	0/6	0/6
Reims	Vesle	Upstream	0/6	–
		Downstream	0/6	–
Soissons	Aisne	Upstream	0/6	0/6
		Downstream	1/6 (C_t: 40·7)	0/6

l.c., lost cage.

T. gondii DNA within a similar range (3·1% of samples) to previous studies. These results underline the potential interest of *D. polymorpha* for monitoring the contamination level of water bodies. What is more, the active methodology applied in this study makes it possible to be more precise about the period of water body contamination by *T. gondii*. Therefore, it offers a substantial advantage for biomonitoring programs.

We found <2 *T. gondii* oocysts per mussel pool. This detection level seems low, but compared to laboratory studies, it corresponds to the level found during the first 2 weeks of a 21-day *in vivo* exposure to 1000 oocysts per mussel and per day (Palos Ladeiro *et al.* 2015). The sensitivity of the PCR method may have been affected by PCR inhibitors because shellfish homogenates are often laden with soil, sediment, organic matter, as well as chemical and biological pollutants (Castro-Hermida *et al.* 2010). Another hypothesis could be that oocysts did not resist digestion and disintegrated inside the shellfish tissues. If parasites are inactivated or disintegrate inside shellfish, they will have limited applications in biomonitoring processes (Graczyk *et al.* 2003). In this transplantation study, we chose to focus on *T. gondii* detection. However, from a biomonitoring point of view, it could be interesting to propose a single approach encompassing several parasites, with simultaneous detection on the same individuals. However, there exists a wide range of methods for extracting and purifying protozoan DNA from complex matrices like shellfish tissue (Willis *et al.* 2013), and further research would be necessary to define a single broad-spectrum method.

To test the approach of mussel transplantation, we chose sites nearby WWTPs because protozoan pathogens are extremely resistant to disinfection techniques used in WWTPs. Wainwright *et al.* (2007) showed that *T. gondii* oocysts remained viable and infectious for mice when subjected to hypochlorite and ozone, even at high concentrations (Finch *et al.* 1993). Dumètre *et al.* (2008) suggested that UV treatment could be an effective disinfection method to inactivate *T. gondii* oocysts in drinking water. Cutting-edge WWTPs use filtration for the physical removal of pathogens, including *T. gondii* oocysts (Dubey 1998). However, in small towns, this technique is not really relevant considering the different steps necessary to a successful filtration (Bowie *et al.* 1997; Betancourt and Rose 2004; Wainwright *et al.* 2007).

A significant seasonal pattern is commonly observed in protozoa occurrence in water. Terrestrial pathogens are likely to reach fluvial waters following heavy surface runoff events that are driven by rainfall (Shapiro *et al.* 2009). Previous studies reported a higher persistence of oocysts at low temperatures (Fayer 2004; Helmi *et al.* 2011). The odds of detecting *T. gondii* are more than 12 times higher in California mussels sampled during the wet season as compared to the dry season (Shapiro *et al.* 2014). The application of cattle manure on land and heavy rainfalls during wet weather spells could explain this trend, as they could facilitate transport of cat faeces into ponds and rivers (Atherholt *et al.* 1998; Curriero *et al.* 2001; Fayer 2004; Carmena *et al.* 2007). To define a precise seasonal pattern and the ecodynamics of protozoa, particularly *T. gondii*, an active approach applied over a large geographical (spatial) scale and at different seasons appears to be a particularly cogent strategy. By transplanting individuals from a same population to the study sites, the precise locations and duration of exposure are controlled, while the influence of biological parameters (physiology, tolerance, adaptation...) on bioaccumulation capacities is limited. This improves the comparison of contamination levels between water bodies.

This study shows for the first time that zebra mussels can accumulate *T. gondii* under *in situ* conditions, and suggests their potential to be used as biosamplers to monitor freshwater systems. Although further studies are necessary, the positive results obtained at two different time-periods despite a relatively short time of exposure (1 month) suggest that this active approach is of interest to survey the sanitary status of freshwater bodies, but also to study the ecodynamics of these pathogens more precisely.

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Conflict of Interest

No conflict of Interest has to be declared for this study.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 PCR amplification curves obtained in *Toxoplasma gondii* oocysts standard samples following 4 series of 10-fold dilution (full line) and in three mussel samples (stippled line).

Figure S2 Standard curves obtained following extraction using an InstaGene™ Matrix kit (a) and performed on an automatic NucliSENS easyMAG system (b).