Environ Sci Pollut Res (2013) 20:778–789
DOI 10.1007/s11356-012-1189-1

REVIEW ARTICLE

Protozoa interaction with aquatic invertebrate: interest for watercourses biomonitoring

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Received: 2 July 2012 / Accepted: 6 September 2012 / Published online: 22 September 2012

Abstract *Toxoplasma gondii*, *Cryptosporidium parvum*, and *Giardia duodenalis* are human waterborne protozoa. These worldwide parasites had been detected in various watercourses as recreational, surface, drinking, river, and seawater. As of today, water protozoa detection was based on large water filtration and on sample concentration. Another tool like aquatic invertebrate parasitism could be used for sanitary and environmental biomonitoring. In fact, organisms like filter feeders could already filtrate and concentrate protozoa directly in their tissues in proportion to ambient concentration. So molluscan shellfish can be used as a bioindicator of protozoa contamination level in a site since they were sedentary. Nevertheless, only a few researches had focused on nonspecific parasitism like protozoa infection on aquatic invertebrates. Objectives of this review are twofold: Firstly, an overview of protozoa in worldwide water was presented. Secondly, current knowledge of protozoa parasitism on aquatic invertebrates was detailed and the lack of data of their biological impact was pointed out.

Keywords *Toxoplasma gondii* · *Cryptosporidium* spp. · *Giardia* spp. · Aquatic invertebrate · Interaction · Biomonitoring

Abbreviations

FISH	Fluorescence in situ hybridization
IF	Immunofluorescence technique
IFAT	Indirect fluorescent antibody technique
IMS	Immunomagnetic separation
IP	Propidium iodide
PCR	Polymerase chain reaction

Introduction

Water quality has becoming an increasing problem for health and environmental authorities. In fact, microbial pathogens are considered as human health risks but only viruses and bacteria were widely studied in literature in spite of protozoa. However, one in two person of the world was affected by waterborne or foodborne parasitic zoonoses (Macpherson 2005). Protozoa were considered as human health risk because they could be transmitted by drinking water and by recreational water such as lakes and streams. Moreover, foodborne transmission was also considered at risk because undercooked meats or raw meals could be vehicle for enteric protozoa, although it was more difficult to link source and disease (Thompson et al. 2005). Here, we discuss three of them which were the main parasites associated with waterborne outbreaks: *Toxoplasma gondii, Cryptosporidium* spp., and *Giardia* spp. (Fayer et al.

Responsible editor: Philippe Garrigues

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2004b; Villena et al. 2004). Indeed, cryptosporidiosis and giardiasis constituted the most common causes of human waterborne infection leading to high morbidity in developed and developing countries and severe dehydration and death in immunocompromised hosts (Cacciò et al. 2005). Toxoplasmosis may be considered as reemerging parasitic zoonoses. In fact, childbearing women were less and less immune against toxoplasmosis which could pose a worrying scenario for their offspring.

However, pathogen detection methods in water are still expensive, inaccurate, and time consuming (Toze 1999). As a result, legislation had chosen to use indicators like *Escherichia coli* and another fecal and total coliforms to follow biological water contamination (Chauret et al. 1995; Field and Samadpour 2007; Figueras and Borrego 2010) although they can be rapidly removed from the environment and are more sensitive to environmental stress and disinfection treatment (Chauret et al. 1995). Moreover, various studies showed that neither *Cryptosporidium* oocysts nor *Giardia* cysts were correlated with total or fecal coliforms (Rose et al. 1988; Chauret et al. 1995; Bonadonna et al. 2002). In fact, Helmi et al. (2011) had highlighted some protozoa-positive sites although they were fecal bacteria indicators negative and, at opposite, some samples were bacterial indicators positive and protozoa negative. Moreover, there are different limits according to (oo)cyst concentration in water which depend on the country legislation. For the US EPA, the risk value considered to be acceptable is 10^{-4} infection per person per year. Countries such England, Canada, and New Zealand had also implemented a national drinking water regulation to avoid outbreaks of protozoan diseases (Castro-Hermida et al. 2010). In fact, in the UK, the legal limit of *Cryptosporidium* in drinking water was 0.1 oocysts/L (Coffey et al. 2010). In EU, the European Drinking Water Directive (Council Directive 98/83/EC) sets the order of pathogenic organism-free water. Nevertheless, in Spain, there were no regulations according to *Cryptosporidium* oocyst limits (Castro-Hermida et al. 2010). This lack of real legislation could be explained by the fact that the scientific community was disagreeing with the (oo)cyst dose response inducing a disease. For example, when various studies had suggested that cryptosporidiosis infectious dose was reached with one oocyst (Coffey et al. 2010; Gale 2001), another investigations underscored an infectious dose with ten oocysts (Castro-Hermida et al. 2010; Helmi et al. 2011) or with a median dose of 130 oocysts (Fayer et al. 2000). Furthermore, pathogen detection in water is complex since it was necessary to filter a large water amount and concentrate parasites in the sample for analysis. Expensive and time consuming, these methods did not allow a rapid detection in routine. Also, filtration and purification techniques from water supplies could highlight different results depending on water quality, sampling period, locality, and quantity (Karanis et al. 2006).

Even so, protozoa were considered as reemerging zoonoses since they were abundant around the world in a large number of watercourses and could infect human by recreational or drinking water. Moreover, they could persist in fresh and even in seawater for several months (Tamburrini and Pozio 1999; Lindsay et al. 2003) and could contaminate aquatic invertebrates which played an important role in the aquatic food chain and may act as a vector to human waterborne parasites (Slifko et al. 2000; Gajadhar and Allen 2004; Graczyk et al. 2004; Appelbee et al. 2005; Dawson 2005).

In an environmental biomonitoring program, organisms like filter feeders were used to reveal chemical contamination because they already could concentrate xenobiotics in their tissues. They were sedentary; thus, they could be relevant of site contamination and in proportion to the ambient pollution (Geffard et al. 2001; Palais et al. 2012). Invertebrates, and particularly bivalves, are considered as useful sentinel organisms in various biomonitoring programs (Viarengo et al. 2007). In Europe, the UNEP Mediterranean Biomonitoring Program is responsible for the follow-up work related to the protocol for the protection of the Mediterranean Sea against pollution from land-based source and activities, and the OSPAR Commission attempts to protect the marine environment of the North-East Atlantic with mollusc and fish as bioindicators tools. More recently, the European Water Framework Directive had made water pollution as a priority and would get polluted water clean again and ensure clean waters are kept clean. Thus, invertebrate community had been used to discriminate contaminated sites by endocrine disruptors (Oetken et al. 2004; Peck et al. 2007; Matozzo et al. 2008), by trace metals (Rainbow 2002; Amiard et al. 2006; Voets et al. 2009), by organic pollutants (Porte et al. 2006), and even by pesticide exposure (Fulton and Key 2001). As filter-feeding species, contaminants could be accumulated and concentrated in strong quantity in their tissues. Their wide distribution, their sedentary comportment, and their suitability for caging and laboratory experiments made them bioindicators of choice for site analysis. As another tool had to be developed to describe protozoan contamination state, aquatic invertebrates could be used since they could accumulate and concentrate protozoa directly in their tissues. Moreover, since parasitism could be considered as a confounding factor and could contribute to misinterpreted results in ecotoxicological studies (Minguez et al. 2009), it seems important to understand protozoa behavior in watercourses and their interaction with aquatic invertebrates as regard to biological responses.

Objectives of this review are to highlight the presence of protozoa, firstly, in aquatic environment and, secondly, in

aquatic invertebrates trying to understand how aquatic organisms could contribute to misinterpreted results in ecotoxicological studies.

Protozoa

Cryptosporidium spp.

Cryptosporidium spp. is an apicomplexan protozoan parasite. There are 11 species within the genus *Cryptosporidium* but two are considered as human health risk: *Cryptosporidium parvum* and *Cryptosporidium hominis*. Transmission could be person to person direct or indirect, animal to person, waterborne, foodborne, and probably airborne (Fayer et al. 2000). Although any person can develop a cryptosporidiosis, high-risk people are immunodeficient hosts who include HIV/AIDS patients and children under 2 years old who could suffer from severe dehydration and increased diarrhea (Slifko et al. 2000). Moreover, to date, there are no effective therapy for both immunocompetent and immunocompromised patients (Graczyk et al. 2011). Their life cycles are complex but rapid and autoinfective to provide a low number of oocysts to cause cryptosporidiosis (Carey et al. 2004). Oocysts (4–5 μm) are resistant forms which could survive in the environment for several months and could resist to the water treatment commonly used (Slifko et al. 2000). Proof of infectivity was highlighted in the USA in the spring of 1993. *Cryptosporidium* spp. contamination of municipal water induces the largest outbreak in the USA history with 403,000 cases (Marshall et al. 1997; Macpherson 2005). Fayer underscores that not only surface water but also groundwater samples were tested positive for *Cryptosporidium* spp. in the USA (Fayer 2004). Concentration in surface water could vary from 0.001 to 100 oocysts per liter (Table 1).

Giardia spp.

Giardia is a flagellated unicellular eukaryotic microorganism that causes intestinal infections in mammals (*Giardia duodenalis*), birds, reptiles (*Giardia muris* and *G. duodenalis*), and amphibians (*Giardia agilis*) (Adam 2001; Thompson 2004). *Giardia* cysts (12–15 μm) can be transmitted directly from one infected person to another and indirectly via environment, water, and food. Diarrhea, malaise, flatulence, greasy stools, and abdominal cramps are clinical symptoms although the majority of infected patients are asymptomatic (Gardner et al. 2001). *Giardia* has a simple life cycle which is easily transmitted from individual to another. Discharges can contaminate the environment through food and drinking water (Cacciò et al. 2005). Risk factors are almost the same than cryptosporidiosis but effective treatment exists (Magne et al. 1996). Their resistant cyst permits an important waterborne transmission. Each year, an estimated 2.8×10^8 cases of giardiasis occur around the world. Likewise, giardiasis is one of the most important waterborne diseases along with cryptosporidiosis (Thompson 2004). In fact, *Giardia* cysts are detected in recreational river water, raw surface water, groundwater, in drinking, waste-, tap, well, sewage, and bottle water (Table 1).

T. gondii

T. gondii, the causative agent of toxoplasmosis, is an obligate intracellular protozoan parasite that infects more than one-third of the world's human population. Toxoplasma transmission can be both horizontal (person to person) or vertical (mother to fetus) (Aubert and Villena 2009). Most infections are asymptomatic in humans, but *T. gondii* can cause severe clinical diseases such as encephalitis or systemic infection in immunocompromised patients, particularly individuals with HIV infection and in cases of congenital toxoplasmosis (Villena et al. 2004; Hide et al. 2009). Infection is mainly acquired by ingestion of food or water that is contaminated with oocysts shed by cats or by eating undercooked or raw meat containing tissue cysts. Oocysts (10–12 μm) are excreted by felids which are the definitive hosts contributing to the sexual division, and warm-blooded animals are intermediate hosts and the seat of the asexual division (Jones and Dubey 2010). Oocysts are formed only in felids which shed unsporulated oocysts. Sporulation occurs within 1–5 days and contain two sporocysts with four sporozoites each (Dubey 2004). A single cat can shed from 2 to 20×10^6 oocysts per day and cat feces are generally disposed of down toilets by their owners. Thus, environment and water could be infected through storm runoff or sewage (Cole et al. 2000; Miller et al. 2002; Fayer et al. 2004a).

Protozoa prevalence in watercourses

High protozoa density in water was correlated with water receiving sewage effluents (Chauret et al. 1995). *Cryptosporidium* oocysts and *Giardia* cysts were often detected in various watercourses around the world, in raw surface water in the USA (Rose et al. 1988), Canada (Chauret et al. 1995), Italy (Giangaspero et al. 2009), Luxembourg (Helmi et al. 2011), in groundwater in Honduras (Solo-Gabriele et al. 1998), in underground water in France (Aubert and Villena 2009), and in drinking and recreational water in Spain (Castro-Hermida et al. 2010) (Table 1). In 166 water supplies in eastern Europe, Karanis et al. reported *Cryptosporidium* oocysts in 18.1 % of samples and *Giardia* cysts in 9.6 % and they also highlighted

Table 1 Protozoa prevalence in different watercourses

References	Country	Protozoa	Medium	Results: % of positive samples (mean (oo)cysts/L)	Techniques
Rose et al. (1988)	USA	*C. parvum* *G. duodenalis*	Raw surface water	72 % (0.00835 oocysts/L) 31 % (0.0015 cysts/L)	Water filtration, sucrose gradient, IF
Chauret et al. (1995)	Canada	*C. parvum* *G. duodenalis*	Raw surface water	78.8 % (0.19 oocysts/L) 75 % (0.08 cysts/L)	Water filtration, IF
Solo-Gabriele et al. (1998)	Honduras	*C. parvum*	Surface water Groundwater	0.58–2.6 oocysts/L 0.26 oocyst/L	IF
		G. duodenalis	Surface water Groundwater	3.8–21 cysts/L 0.06 cyst/L	
Karanis et al. (2006)	Bulgaria, Russia	*C. parvum* *G. duodenalis*	Tap, river, well, sewage, and bottle water	18.1 % (river=56.5 oocysts/L) 9.6 % (river=178.5 cysts/L, well=255 cysts/L, bottle=0.5 cyst/L)	Water filtration or flocculation, sucrose gradient, IF
Castro-Hermida et al. (2010)	Spain	*C. parvum* *G. duodenalis*	Recreational river, drinking, and wastewater	57.1 % of recreational water (1–60 oocysts/L), 40 % of drinking water (1–13 oocysts/L), 32.7 % of wastewater (1–4 oocysts/L) 60 % of recreational water (1–160 cysts/L), 42.3 % of drinking water (1–7 cysts/L), 36.5 % of wastewater (1–5 cysts/L)	IMS, IFAT, IP
Helmi et al. (2011)	Luxembourg	*C. parvum* *G. duodenalis*	Surface water	81 % (1.4 oocysts/L), 53 % (0.1 oocysts/L) 81 % (7.6 oocysts/L), 53 % (0.2 oocysts/L)	IMS, IF, PCR

Giardia cysts in bottled water (Karanis et al. 2006). In most studies, concentrations in water varied from 0.1 to 56 oocysts/L and from 0.2 to 178 cysts/L. Chauret et al. had underscored in the raw surface water sample, detection of 78.8 and 75 % of *Cryptosporidium* spp. and *Giardia* spp., respectively, with an average of 0.19 oocysts and 0.08 cysts per liter (Chauret et al. 1995). As we know, *Cryptosporidium* oocysts were more resistant to environmental stress and were more prevalent in animals than *Giardia* cysts (Helmi et al. 2011). It was probably the reason why some studies had detected more *Cryptosporidium* oocysts than *Giardia* cysts in the environment. Moreover, *Cryptosporidium*, *Toxoplasma* oocysts, and *Giardia* cysts could survive and persist in the environment (Aubert and Villena 2009), and various investigations had underscored oocysts' ability to sporulate in seawater and remain infective for several months (Tamburrini and Pozio 1999; Lindsay et al. 2003; Lélu et al. 2012). As a result, these protozoa have been considered as public health risks since they are responsible of the main waterborne outbreaks. Karanis et al. had reported 325 outbreaks related with protozoa parasite, and majority of them were associated with *G. duodenalis* for 40.6 % and with *C. parvum* for 50.8 % (Karanis et al. 2006).

Protozoa prevalence in aquatic invertebrates

Various experimentations had highlighted aquatic invertebrate and particularly mollusc able to accumulate *C. parvum* and *G. duodenalis* cysts in their tissue (Tables 2 and 3). In fact, infective *C. parvum* oocysts were detected in mussels and cockles from a shellfish-producing region in Spain. Authors found 5×10^3 oocysts shellfish intensity (Gomez-Bautista et al. 2000). In Canada watercourses, *C. parvum* intensity on zebra mussel was 4.4×10^2 oocysts (Graczyk et al. 2001). *G. duodenalis* has been highlighted in clams and mussels in Ireland and US streams (Table 2). Nevertheless, threat research still focused on commercial organisms such as oysters or consumable mussels which can be eaten raw and transmit waterborne disease directly (Graczyk et al. 2006). In fact, Graczyk et al. had highlighted the clam ability to retain an average of 3.68×10^6 waterborne oocysts only by hemocyte internalization (Graczyk et al. 1997). Furthermore, laboratory experiments also demonstrated that shellfish have the capability to not only remove and concentrate a large number of (oo)cysts present in contaminated water but also retain (oo)cysts' infectivity after their transit in the organism (Table 3). For example, experimental contamination of oysters and clams followed by a 31-day depuration period highlighted a decrease of 70 % of *C. parvum* oocysts viability in oyster and clam tissue during the first 96 h. However, viable oocysts are still detected at the end of depuration time since half of the mice inoculated by shellfish homogenate were still infected 31 days postinfection (Freire-Santos et al. 2002; Graczyk et al. 2006). Mussels spiked by 2×10^5 *T. gondii* oocysts are infectious to mice 3 days postinfection, and oocysts are still detected in mussel hemolymph 21 days postexposure (Arkush et al. 2003). Some studies indicate that food chain could be contaminated by protozoa because aquatic mammals consume aquatic infected invertebrates (Cole et al. 2000; Miller et al. 2002; Conrad et al. 2005). More interestingly, Miller et al. (2008) report the same *T. gondii* type in terrestrial carnivores, wild mussel, and in southern sea otters occupying the same region. This study highlights the link between *T. gondii* oocyst release by wild felids, environment, and water contamination and the role of wild mussel for aquatic mammal through the food chain contamination. Indeed, various studies underscored infected mussels consumption as a threat for aquatic mammals and as a public health issue (Graczyk et al. 2003; Mead et al. 1999; Tamburrini and Pozio 1999). However, it is necessary to keep in mind that another organism could act as a vector to protozoa indirectly by food chain infection. Some aquatic organisms which have not commercially interest could internalize oocysts on their tissue and could play an important role in environmental (oo)cyst dissemination (Tables 2 and 3). By filtration, organism like filter feeders could concentrate directly in their tissues particles present in water. Moreover, (oo)cysts could survive in sea and freshwater for several months which is beneficial for aquatic invertebrates could filter and concentrate waterborne (oo)cysts in their tissues. Thus, aquatic invertebrates could be useful to highlight water bioinfection instead of water analysis since it was time consuming and expensive. In fact, parasite detection methods could be accessed by immunofluorescence or PCR presently after dissection. However, a large choice of analytical techniques made the comparison between studies problematic (Tables 1, 2, and 3). In fact, Miller et al. (2005) had tested four methods to detect low numbers of *C. parvum* oocyst in clam: direct fluorescent antibody (DFA), immunomagnetic separation–DFA (IMS-DFA), and two techniques of PCR which amplify different *C. parvum* DNA segment and use dissimilar amplification conditions. They found that IMS-DFA and first PCR are sensitive to detect a single oocyst spiked into digestive gland samples, but only PCR methods are too sensitive to detect a single oocyst spiked into clam hemolymph than other techniques (Table 3). Sotiriadou and Karanis (2008) underscored dissimilar results on *T. gondii* detection with different methods. In fact, in a 52-water sample study, authors found 48 % of *T. gondii* DNA-positive sample by the loop-mediated isothermal amplification method, whereas nested PCR products were present in only 13.5 % of samples and all were negative by immunofluorescence method. Thus, a lot of steps were needed to reach an acceptable quality of sample, and

Table 2 Protozoa prevalence in aquatic invertebrates, in situ experimentations

References	Country	Protozoa	Medium	Results	Techniques
Gomez-Bautista et al. (2000)	Spain	*C. parvum*	Mussels (*M. galloprovincialis*) Cockles (*C. edule)*	5.8×10^3 oocysts/mussel 5×10^3 oocysts/cockle	IFAT, mice inoculation, PCR
			Tank water	None oocyst detected after 72 h	
Graczyk et al. (2001)	Canada	*C. parvum*	Mussels (*D. polymorpha*)	4.4×10^2 oocysts/mussel	$CsCl_2$ gradient, IF
Gomez-Couso et al. (2003)	Spain Italy		Mussels (*M. galloprovincialis*)	50 % of positive sample even after 14 depuration days	Diethyl ether, IFAT, IP
	UK Ireland		Cockles (*C. edule*)/oysters (*O. edulis*)/clams (*R. decussatus*/*R. philippinarium*/*V. pulastra*/*D. exoleta*)	Contamination degree=oysters (54.8 %) >mussels (32.7 %) >clams (29.4 %) >cockles (20.8 %)	
	Portugal			Oocysts viability=clams (60 %) >oysters, mussels (51.4 %) >cockles (47.8 %)	
Graczyk et al. (2004)	Ireland	*C. parvum* *G. duodenalis*	Mussels (*D. polymorpha*)	4–16 oocysts/mussel 5–9 cysts/mussel	$CsCl_2$ gradient, IFAT + FISH
Miller et al. (2005)	USA	*C. parvum* *G. duodenalis*	Clams (*C. fluminea*)	1–7 oocysts/pool of clams 1–26 cysts/pool of clams	IF, IMS + IF, PCR
Lucy et al. (2008)	Ireland	*C. parvum* *G. duodenalis*	Mussels (*D. polymorpha*/*M. edulis*/*A. anatina*)	0–6 oocysts/g 0–13 cysts/g	FISH, IF
Giangaspero et al. (2009)	Italy	*C. parvum*	Mussels (*M. galloprovincialis*) Clams (*R. decussatus*)	All samples negatives	IF, RT-PCR
		C. parvum	In situ water	31 % *C. parvum* positive 54.8 % *G. duodenalis* positive	

Table 3 Protozoa prevalence in aquatic invertebrates, laboratory experimentations (DD=depuration days, PI=postinoculation)

References	Protozoa	Medium	Experimental conditions	Results	Techniques
Fayer et al. (1997)	*C. parvum*	Oysters (*C. virginica*)	Single dose of 5×10^5 oocysts/oyster and 30 DD	Oocysts in gills and hemocytes 3 h PI In stomach lumens and intestine 24,168, and 720 h PI	IF, immunohistology
Graczyk et al. (1998)	*C. parvum*	Clams (*C. fluminea*)	19×10^4 oocysts/clam during 24 h and 14 DD	Oocysts in gills and gastrointestinal tract 24 and 48 h PI, in feces until 3 days, in hemolymph until 7 days	Acid fast stain, IF
		Tank water	10^6 oocysts/L and 3 DD	No oocyst detected after 24 h PI	
Tamburrini and Pozio (1999)	*C. parvum*	Mussels (*M. galloprovincialis*)	8×10^6 oocysts/mussel during 24 h and 14 DD	Oocysts in gills 3 days PI, in hemolymph 7 days PI, and in gut 14 days PI	Immunohistology, mice inoculation
		Tank water	13×10^6 oocysts/L	Infective oocysts detected after 14 days PI	
Freire-Santos et al. (2001)	*C. parvum*	Clams (*R. philippinarium*)	10^6 oocysts/clam during 48 h	Infective oocysts in gill and gastrointestinal tract	IF, IFAT
Lindsay et al. (2001)	*T. gondii*	Oysters (*C. virginica*)	10^6 oocysts during 24 h and 6 DD	Infective oocysts after 6 days PI	Mice inoculation
Freire-Santos et al. (2002)	*C. parvum*	Clams (*T. decussatus*)	Single dose of 5×10^5 oocysts/organism and 31 DD	Decrease of 70 % oocyst viability after 96 h	Diethyl ether, IP, IFAT
		Oysters (*O. edulis*)		Infective oocysts detected after 31 days PI	
		Tank water	10^6 oocysts/L	From 10^6 to 25×10^3 oocysts/L after 24 h	
Arkush et al. (2003)	*T. gondii*	Mussels (*M. galloprovincialis*)	2×10^5 oocysts/mussel and 21 DD	24 h PI; 50 % in hemolymph; 3 days PI: 25 % in digestive gland; 7 days PI: 17 % in gill; 21 days PI: 33 % in hemolymph	Taqman PCR, mice bioassay
		Tank water	6×10^5 oocysts/L	740-fold reduction in 6 h	
Graczyk et al. (2003)	*C. parvum*	Mussels (*D. polymorpha*)	0.5 oocysts/organism and 1.4 cysts/organism daily during 5 weeks and 14 DD	70±25.8/pool of 30 mussels	$CsCl_2$ gradient + IF
	G. duodenalis	Clams (*C. fluminea*)		48±24.9/pool of 30 clams Oocysts are still detected until 2 depuration weeks	
Izumi et al. 2004	*C. parvum*	Clams (*C. japonica*)	6.73×10^4 oocysts/clam and 14 DD	2 h PI: 90 % in gastrointestinal tract, 5 % in mantle, 0.1 % in gills; 48 h PI: 85 % in feces still infective; 14 days PI: total recovery	Sucrose gradient, IFAT
		Tank water	7.29×10^5 oocysts/L	No oocysts detected 2 h PI	
Lindsay et al. 2004	*T. gondii*	Oysters (*C. virginica*)	6.67×10^4 oocysts/oyster and 85 DD	Oocysts are still infective in oyster tissues until 85 days PI	Mice bioassay
Gomez-Couso et al. 2005	*C. parvum*	Clams (*T. decussatus*)	Single dose of 3.3×10^5 oocysts/clam and 10 DD (without remove water)	24 h: 2.85×10^5 oocysts/clam	Diethyl ether + IFAT, immunohistology
Miller et al. 2005	*C. parvum*	Clams (*C. fluminea*)	Exposition during 6 h of: 2.5 oocysts/clam, 25 oocysts/clam, 250 oocysts/clam, and 21 DD	3 h PI: 1 oocyst/clam; 3 h PI: 8 oocysts/clam; 3 h PI: 40 oocysts/clam	IF, IMS + IF, PCR
		Tank water	20/200/2 000 oocysts/L and 21 DD	6 h PI: decrease of 55 %	
Graczyk et al. 2006	*C. parvum* *G. duodenalis*	Oysters (*C. ariakensis*)	2.6×10^3 (oo)cysts/oyster and 33 DD	Viable oocysts were identified up to 33 days PI Viable cysts are detected until 14 days PI	IFAT, FISH

brought by a strong loss of parasites, the results were not constant between experimentations and even between samples.

A problem appeared when results did not indicate presence of parasites in a sample: was it effective because no parasites were in the sample or may be because nothing was detected? Therefore, to facilitate protozoa detection, it is conceivable to use only a few organs instead of whole organisms to detect protozoa since the final matrix could be too complex for a detection technique. Fayer et al. demonstrated that *C. parvum* could be accumulated by eastern oyster, *Crassostrea virginica*, and oocysts would be preferentially accumulated in gills and hemocytes (Fayer et al. 1997). In fact, gills are directly in contact with contaminated water and provide parasites with an access to the organism and, *in fine*, in hemolymph. Although filtration activity was a complex phenomenon depending on water chemistry, water parameters, and particle concentration (Bourgeault et al. 2011), invertebrate could filter water, keep nutrients, and concentrate small particles. Protozoa such as *Cryptosporidium* is a small-size organism measuring 4–5 μm (Carey et al. 2004) and *Giardia* and *Toxoplasma*, 10–15 μm (Adam 2001; Jones and Dubey 2010). Thus, filter feeders could retain protozoa during feeding and respiration (Gomez-Couso et al. 2003). Indeed, some studies found a high level of infective parasite in hemolymph, gills, gastrointestinal tract, and feces (Fayer et al. 1997; Graczyk et al. 1998; Tamburrini and Pozio 1999; Freire-Santos et al. 2001). Gomez-Couso et al. attempted to bring out the transit of *C. parvum* oocysts in clam (*Tapes decussatus*). They found that oocysts could be found in siphons, stomach, intestine, digestive diverticula, branchial mucus, and gills. Moreover, they found the highest number of oocysts in the intestine (Gomez-Couso et al. 2003). Another study indicated that, after a single exposition of 7.29×10^5 *C. parvum* oocysts per liter, almost all oocysts were concentrated by clam. Moreover, authors pointed out that 90 % of oocysts are present in the gastrointestinal tract, 5 % are in the mantle, and 0.1 % in the gill only 2 h after exposure; 85 % of oocysts were excreted via feces between 4 and 8 h postexposure (Izumi et al. 2004). Clam exposition to *C. parvum* indicated that 81.6 % of the oocysts could be phagocytosed by 93 % of the clam hemocytes (Graczyk et al. 1997), whereas Gomez-Couso et al. did not highlight oocyst internalization in clam cells (Gomez-Couso et al. 2003). Concerning *T. gondii*, an experimental study highlighted *Toxoplasma* RNA most often in digestive gland than in hemolymph or in gill sample (Arkush et al. 2003). Moreover, some researches underscored the potential infectivity of oocysts after their passage in the organism and found that oocysts were still infective (Fayer et al. 1997; Lindsay et al. 2001). These conclusions highlight some biological interrogations since viable oocysts consequences on invertebrate homeostasis were poorly studied in these experimentations. From now, most studies deal with specific parasite since parasitism could be an important confounding factor in ecotoxicological investigations (Neves et al. 2000; Sures 2004; Coors et al. 2008; Sures 2008; Marcogliese et al. 2009; Morley 2010). In fact, various studies had investigated macroparasites such as trematodes, nematodes, or acanthocephalan that are specific parasites for their host. These parasites often produce negative effects on their host or on their offspring (Taskinen 1998; Hasu et al. 2006; Gangloff et al. 2008; Minguez et al. 2009; Ben-Ami et al. 2011). For example, Robledo et al. found an inhibiting gonadal development and a decrease in condition index of mussels affected by a copepod parasite (Robledo et al. 1994). Another study had highlighted a lower lipid level in gravid *Gammarus pulex* female infected by a macroparasite (Plaistow et al. 2001). In the same way, Hasu et al. had underscored a smaller offspring in female infected by an acanthocephalan than noninfected female because infected female has a less resource for offspring care (Hasu et al. 2006). Regarding their hosts, parasite could reduce tolerance to chemical contamination: infected snails by trematodes are less tolerant to zinc exposure (Guth et al. 1977), infected cockle have a less tolerance to hypoxia than noninfected one (Wegeberg and Jensen 1999), and increased trematode densities predict reduced mussel reproductive output and physiological condition (Gangloff et al. 2008). According to biomarkers' responses, SOD activity was significantly reduced in infected shrimp by isopods (Neves et al. 2000), infected zebra mussels by ciliates and bacteria displayed a more developed lysosomal system revealed by a larger number of lysosomes (Minguez et al. 2012), and metallothionein concentrations increased in healthy cockles whereas decreased significantly in trematode-parasited ones (Baudrimont et al. 2006). Nevertheless, some studies had highlighted the synergistic or antagonistic effects of biological and chemical stressors in aquatic organisms (Khan 1990; Lafferty 1997; Sures 2006, 2008; Morley 2009).

Even if they underscored the simultaneous effect of both contaminations, they deal with intestinal parasites of fish and invertebrate infected by trematodes, nematodes, or isopods. Only a few studies took into account biological effects by parasite on biological response. Minguez et al. (2009) had suggested that specific parasitism of zebra mussel must be taken into account in ecotoxicology studies. However, as we see previously, nonspecific parasite such as protozoa could be present in strong density in watercourses and could be accumulated and concentrated by aquatic organism, particularly by filter feeders like molluscs and crustacean. Effects of nonspecific parasite on aquatic invertebrate biomarkers would be an interesting investigation because protozoa may modulate biological responses even if they are nonspecific and because aquatic invertebrate biological

responses had been largely used in biomonitoring programs. Even if aquatic invertebrates were not protozoa host specific, viable parasites could interfere with organism and with biological responses. Since invertebrate hemocytes are involved in various biological functions like wound repair; shell repair; nutrient digestion, transport, and excretion (Cheng 1981); gills ensure respiration; and digestive gland ensures nutrition, viable (oo)cysts could interfere with these organs and, in fine, damage their important functions. Moreover, these organs are already used in ecotoxicological studies as exposure indicators like antioxidant system in digestive gland (Bigot et al. 2010), energy metabolism (Palais et al. 2012), DNA damage in gills cells and hemocytes (Vincent-Hubert et al. 2011), immunological functions (Ellis et al. 2011), or host defense (Xu and Faisal 2009). When immunological functions and defense need to be increased by the presence of foreign body, other organism resources could be impacted such as survival, energy, or reproduction resource (Nisbet et al. 2000; Ren and Ross 2005; Kooijman and Troost 2007; Sousa et al. 2010). Protozoa effects on biological responses should be investigated since they were poorly accessed in literature instead of finding false positive or false negative results in experimentations.

Conclusion

T. gondii, Cryptosporidium spp., and *Giardia* spp. are human waterborne parasites. These worldwide parasites have been detected in various watercourses as recreational, surface, drinking, river, and seawater. Thus, interaction with aquatic organisms is unavoidable since they can filter a large quantity of water for feeding and respiration. Impact on the immune system of foreign body could activate and limit immunological system to the detriment of another large biological function such as survival, growth, or reproduction. Protozoa could also damage the defense system which was already used in ecotoxicological studies to discriminate pollution risk sites. As chemical contamination, consequence of protozoa could interfere with biological response. Moreover, associated chemical and biological stress could distort ecotoxicological conclusion giving false positive or false negative results.

While confounding factors had to be considered, effect of nonspecific parasitism on biological responses was poorly accessed. Protozoa could be present in strong density in watercourses and could be accumulated and concentrated by aquatic organisms, particularly by filter feeders like molluscs and crustaceans. The high filtration rate of aquatic invertebrate allowed the filtration of large amount of water and concentration of protozoa with their food. Moreover, concentration of protozoa by phyto- and zooplankton did not be neglected. Their role in aquatic food chain was important and permitted the bioconcentration and bioaccumulation in higher organisms such as aquatic invertebrates. Nonetheless, invertebrate's physiological state could be an important factor of parasite accumulation or parasite depuration. As a result, in situ experimentations had highlighted more prevalence in organism than laboratory ones. It is probably the reason why a lot of studies process the whole sample of organism before to realize the parasite detection technique. Thus, they can integrate the entire parasite amount in their results. However, realization of parasite detection organ by organ could be interesting to understand the parasite kinetics and parasite effects on organism.

Aquatic organisms could be more integrative of the biological pollution and useful than water since they already could filter and concentrate parasite in their tissues. Biological response of aquatic organism could be an alternative to water analysis technique which had a poor sensitivity. Since they are sedentary and could accumulate protozoa, they could be a new useful tool to highlight biological water contamination. However, other research studies are needed to complete the lack of data with exposition to active filter feeders at nonspecific parasite to understand the kinetic and the effects of these biological confounding factors on aquatic organisms: *ex vivo* exposure will permit to understand nonspecific parasite and cells' interaction whereas in vivo exposure will point out protozoa's impact on the whole organism. It is all the more necessary since aquatic organisms are used as sentinel or indicator organisms of watercourses, and parasitism could be a source of misinterpreted results.

Acknowledgments This PhD work was supported by grants from the "Région Champagne Ardenne" (projet INTERBIO). Financial support was provided by CNRS-INSU (Programme EC2CO, projet IPAD) and the Programme Interdisciplinaire de Recherche sur l'Environnement de la Seine (PIREN-Seine).

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