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Accumulation of human waterborne parasites by zebra mussels (*Dreissena polymorpha*) and Asian freshwater clams (*Corbicula fluminea*)

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Abstract Zebra mussels (*Dreissena polymorpha*) and Asian freshwater clams (*Corbicula fluminea*) are nonindigenous invasive bivalves present in North American fresh waters that are frequently contaminated with human enteric parasites, *Cryptosporidium parvum* and *Giardia lamblia*. Six-week laboratory exposure of *D. polymorpha* and *Corbicula fluminea* to both parasites seeded daily at concentrations reported from surface waters demonstrated efficient removal of *Cryptosporidium parvum* oocysts and *G. lamblia* cysts by both bivalve species. The number of parasites in mollusk tissue progressively increased in relation to the concentration of waterborne contamination, and decreased after cessation of the contamination. Oocysts outnumbered cysts in the tissue of both bivalves, and more parasites were identified in *D. polymorpha* than in *Corbicula fluminea*; overall 35.0% and 16.3% of the parasites seeded, respectively. Because *C. fluminea* and *D. polymorpha* can accumulate human waterborne parasites in proportion to ambient concentrations, these species of bivalves can be effective bioindicators of contamination of freshwater habitats with *Cryptosporidium* and *Giardia*.

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Introduction

Zebra mussels (*Dreissena polymorpha*) and Asian freshwater clams (*Corbicula fluminea*) are nonindigenous invasive bivalve species in North American fresh waters (McMahon 1991). Zebra mussels have become invasive throughout the St. Lawrence River, Great Lakes, and the eastern half of North America (McMahon 1991). *Corbicula fluminea* clams are well adapted for unstable and unpredictable habitats and are highly successful in agricultural drainage systems (McMahon 1991).

Cryptosporidium parvum and *Giardia lamblia* (syn. *G. intestinalis*, *G. duodenalis*) are intestinal protozoan parasites (Wolfe 1992; Fayer et al. 1997a). *C. parvum* significantly contributes to the mortality of people with impaired immune systems as there is no approved effective therapy (Fayer et al. 1997a). *G. lamblia* causes prolonged diarrheal illness in adults and children worldwide but the infections usually respond well to pharmacologic treatment (Wolfe 1992). *C. parvum* and *G. lamblia* are zoonotic parasites commonly infecting cattle (Wolfe 1992; Fayer et al. 1997a). *Cryptosporidium* and *Giardia* produce a long-lasting, environmentally resistant infectious stage, i.e., oocyst and cyst, respectively, which are transmitted via drinking and recreational waters as a result of contamination of surface water by agricultural and urban runoff (Wolfe 1992; Fayer et al. 1997a). The diameter of *C. parvum* oocysts does not exceed 6 μm, and *G. lamblia* cysts are oval and no longer than 15 μm (Wolfe 1992; Fayer et al. 1997a).

Bivalves can harbor environmentally-derived human pathogenic bacteria and viruses as a result of concentrating, i.e, accumulating, the recovered pathogens from the surrounding water, and therefore can be used as indicators of water pollution (Ayres et al. 1978; Trollope 1984). Bacterial and viral accumulation, i.e.,

bioaccumulation, by bivalve mollusks, understood as concentration of particles over a period of time, is well documented (Ayres et al. 1978; Trollope 1984; Wittman and Flick 1995; Selegan et al. 2001). *Cryptosporidium* and *Giardia* have been identified worldwide in bivalves (including zebra mussels) from natural waters, supporting the concept that they can serve as biological indicators for water contamination with these parasites (Chalmers et al. 1997; Fayer et al. 1998, 1999; Graczyk et al. 1999a, b, c, 2000, 2001; Freire-Santos et al. 2000; Gomez-Bautista et al. 2000; Lowery et al. 2001). However, neither studies on feral bivalves mentioned above nor experimental approaches (Fayer et al. 1997b; see Graczyk et al. 1998 for review, Tamburrini and Pozio 1999; Frischer et al. 1999; Freire-Santos et al. 2001) documented that shellfish are able to concentrate the oocysts or cysts, particles which are considerably larger than bacteria (maximum size; 2 μm). This significantly hampers any conclusions that can be drawn on the temporal occurrence and the level of the water contamination if these pathogens are identified in shellfish tissue.

The purpose of the present study was to determine if freshwater bivalve mollusks such as *D. polymorpha* and *C. fluminea* can accumulate in their tissue *C. parvum* oocysts and *G. lamblia* cysts recovered from water.

Materials and methods

Bivalves

Dreissena polymorpha, 2.0–3.5 cm shell length, collected on the north shore of the St. Lawrence River, Quebec, Canada, and *Corbicula fluminea*, 2.0–2.5 cm shell length, from Lake Cheston, Franklin County, Tenn., USA, were shipped overnight on ice to the laboratory. Mussels and clams were depurated for 3 weeks as described previously (Graczyk et al. 1998), and after depuration 30 randomly selected clams and mussels were individually tested for *Cryptosporidium* and *Giardia* (Graczyk et al. 1998). Three, 38-l aquaria (approximately 10 gallons), i.e., aquarium A, B, and C, were filled with dechlorinated drinking water filtered by the Filterite 10-μm-pore yarn-wound cartridge (Memtec America Corp., Baltimore, Md.). Each aquarium was equipped with a Fluval filter (model 403) (Askoll, Italy) and two air-stones. Two hundred-twenty specimens of *Corbicula fluminea* or *D. polymorpha* were placed separately in aquarium A and B, respectively, and 110 of each bivalve species were placed in aquarium C. Shellfish in aquaria were maintained as described previously (Graczyk et al. 1998).

Inoculum

Cryptosporidium parvum oocysts and *G. lamblia* cysts originated from experimental infection of a calf and were purified by $CsCl_2$ gradient centrifugation (Kilani and Sekla 1987). Oocysts and cysts were enumerated by flow cytometry (Bennett et al. 1999). Water in each aquarium was spiked daily in the early morning with 106 oocysts and 304 cysts for 31 consecutive days. The inoculum size was calculated to produce the concentration of oocysts and cysts reported from surface water, i.e., 28 oocysts/10 l, and 80 cysts/10 l (Atherholt et al. 1998).

Sampling and processing

Thirty bivalves were sampled 7 times at weekly intervals in the late afternoon with the first sampling timepoint, i.e., week 1, on three days after the first water contamination event (Fig. 1). The fifth sampling timepoint, i.e., week 5, occurred the day of the last water contamination timepoint (Fig. 1). Each time the sampled bivalves included 30 clams (aquarium A), 30 mussels (aquarium B), and 15 of each mollusk species (aquarium C) (Fig. 1). The bivalves were opened (Graczyk et al. 1998), the soft tissue and hemolymph from 30 shellfish was pooled, homogenized with a doubled volume (w/v) of phosphate-buffered saline (pH 7.4), and the homogenate was sieved, sedimented (Graczyk et al. 1999b), and purified over $CsCl_2$ gradient (Kilani and Sekla 1987). The oocyst- and cyst-containing fraction of $CsCl_2$ was centrifuged (1,000 *g*; 3 min; 4°C), and the pellet resuspended in 4 ml deionized water. Approximately 500 μl of resuspension was placed in each of eight wells on an 8-well-chamber tissue culture glass slide (Nalge Nunc International, Naperville, Ill., USA). After 3 h incubation at 20°C, the fluid was aspirated from each well, the plastic dividers were removed, and the slide was air-dried. *Cryptosporidium parvum* oocysts and *G. lamblia*

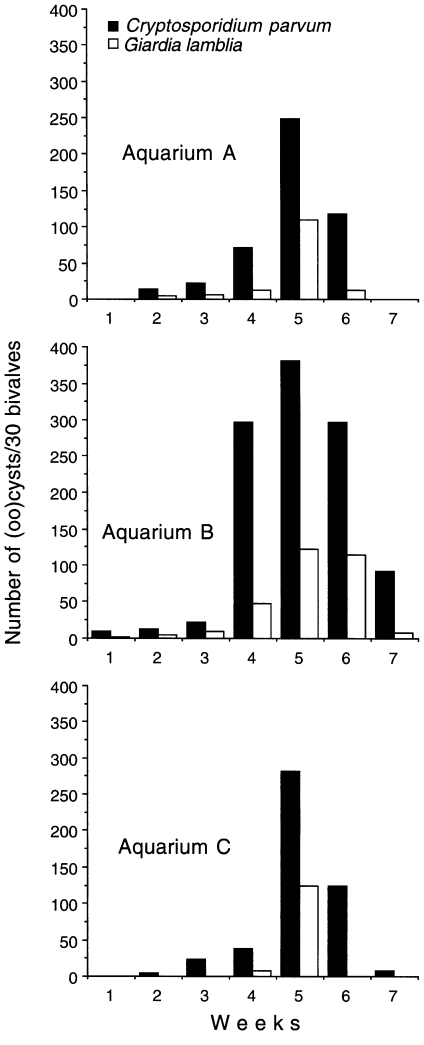

Fig. 1 Identification of *Cryptosporidium parvum* oocysts and *Giardia lambia* cysts recovered from artificially contaminated water by freshwater bivalve mollusks, *Corbicula fluminea* (aquarium A), *Dreissena polymorpha* (aquarium B); aquarium C contained equal numbers of both bivalve species which were sampled equally. Water in each 38-l aquarium seeded daily for 31 consecutive days, i.e., up to week 5, with 106 oocysts and 304 cysts. Aquarium C; *Cryptosporidium parvum* and *G. lamblia* identified in *D. polymorpha* tissue only. Oocysts and cysts identified by immunofluorescent antibody

cysts were visualized by immunofluorescent antibody of the MERIFLUOR test kit (Meridian Diagnostic, Cincinnati, Ohio) and enumerated (Graczyk et al. 1999b). The overall numbers of oocysts and cysts were adjusted for the method recovery efficiency, i.e., 51.1%, as described previously (Graczyk et al. 1999b).

Sediments from all aquaria were tested for *Cryptosporidium* and *Giardia* as described previously (Graczyk et al. 1998) every time the bivalves were sampled. Efforts were made to collect all sediments.

All water from all aquaria was filtered by the cellulose acetate membrane disk, 393-mm diameter, 3.0-μm pore size (Millipore, Bedford, Mass.) (Fayer et al. 1999) every time the bivalves were sampled. After total aquarium drainage the filtered water was re-circulated back to the aquarium. The membranes were processed to detect *C. parvum* and *G. lamblia* as described previously (Rodgers et al. 1999a, b). To confirm the recovery efficiency of this method five 38-l water samples were processed as described above except that each sample was spiked with 106 *C. parvum* oocysts and 304 *G. lablia* cysts.

Statistical analysis

Statistical analysis was carried out with Statistix 4.1 (Analytical Software, St. Paul, Minnesota). The variables were examined by the Runs test to determine conformity to a normal distribution. The degree of linear association between variables was evaluated using Pearson's correlation coefficient (R), two-sample t-test was used to assess the significance of differences between mean values, and fractions were compared using the G-heterogeneity test. Mean values were associated with standard deviation (SD). Statistical significance was considered to be $P < 0.05$.

Results

Neither *Cryptosporidium parvum* oocysts nor *Giardia lamblia* cysts were identified in water and sediment samples, and no parasites were found in shellfish after depuration. The numbers of *C. parvum* oocysts and *G. lamblia* cysts identified in shellfish tissue increased progressively through week 5, and both parasites were identified for the first time, i.e., on week 1, in *Dreissena polymorpha* tissue (Fig. 1). There was a significant correlation observed in all three experiments (Fig. 1) between the cumulative numbers of *C. parvum* oocysts seeded to the water (as presented in Fig. 2 upper panel) and identified in bivalve tissue (Pearson correlation; $P = 0.94$, $P < 0.02$). This was also the case for *G. lamblia* in two experimental options, i.e., aquarium A and B (Pearson correlation; $P = 0.96$, $P < 0.01$). The parasite levels decreased at week 6 after cessation of water contamination, but *C. parvum* and *G. lamblia* were still detected in *D. polymorpha*, i.e., aquarium B and C, 2 weeks after the last water contamination event (Fig. 1).

In general, more cystic stages of both parasites were identified in the tissues of *D. polymorpha* (aquarium B) than *Corbicula fluminea* (aquarium A) (Fig. 1). In aquarium C, in which equal numbers of each bivalve species were kept (and sampled), most parasites were identified in the *D. polymorpha* tissue (Fig. 1). Based on the data from all three 7-week-long experiments, on average 48 ± 24.9 pathogen cystic stages (both *Cryptosporidium parvum* and *G. lamblia*) were identified in the tissue of 30 *C. fluminea* clams, and 70 ± 25.8 in 30 *D. polymorpha* mussels. Analysis of these results by two-sample t-test demonstrated that significantly higher numbers of parasites were identified in *D. polymorpha* than in *Corbicula fluminea* ($t = 3.03$, $P < 0.05$).

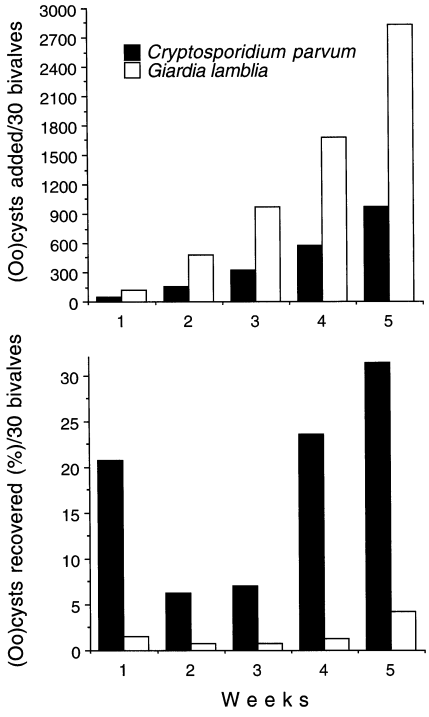

Fig. 2 *Upper panel* The theoretical cumulative numbers of *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts seeded to the water in three 38-l aquaria with freshwater mollusks, *Corbicula fluminea* clams and *Dreissena polymorpha* mussels (aquarium A, B, and C, as described in Fig. 1). *Lower panel* The overall mean percentage of oocysts and cysts identified in the tissue of bivalves maintained in aquaria with *Cryptosporidium parvum* and *G. lamblia*-seeded water

Figure 2 (upper panel) presents the theoretical cumulative numbers of pathogen cystic stages (calculated per 30 mollusks) in each of the three aquaria with respect to their constant levels seeded daily to the water and progressively decreasing number of bivalves related to sampling, i.e., 30 specimens/week. These cumulative numbers were used to calculate the mean percentage of parasites identified in bivalve tissue (lower panel). On average, from 7% to 32% (mean, 17.8%) of all *Cryptosporidium parvum* oocysts added to the water could be identified in the bivalve tissue for the 31-day duration of water contamination (Fig. 2). This level was significantly higher than the level of *G. lamblia* cysts (range: 1–5%; mean, 1.7%) (two-sample t-test; $t = 59.2$, $P < 0.01$). Overall, for all three 7-week-long experiments 35.0% and 16.3% of the parasite cystic stages seeded into the water were identified in *D. polymorpha* and *Corbicula fluminea*, respectively (G-heterogeneity test: $G = 6.8$, $P < 0.01$).

Discussion

Although multiple experimental studies demonstrated that bivalves can filter out and temporarily trap, i.e.,

retain, *Cryptosporidium* and *Giardia* (Fayer et al. 1997b; see Graczyk et al. 1998 for review; Frischer et al. 1999; Tamburrini and Pozio 1999; Freire-Santos et al. 2001), the question of whether molluscan shellfish can actually accumulate these parasites has not been answered. The present study demonstrated that freshwater bivalve mollusks, i.e., *Corbicula fluminea* and *Dreissena polymorpha*, are able to accumulate waterborne parasites recovered from contaminated water in proportion to ambient concentrations.

Surface waters are frequently polluted by both *Cryptosporidium parvum* oocysts and *G. lamblia* cysts and their levels in waters impacted by agricultural and urban runoff can be much higher (Rose et al. 1997) than used in the present study. Concentration of oocysts and cysts in the Delaware River, New Jersey, USA (drawn by drinking water plants) was 14 and 40 per 5 l, respectively (Atherholt et al. 1998), and 260 and 2,100 per 100 l, respectively, in water supplies of San Pedro Sula, Honduras (Solo-Gabriele et al. 1998). The present study demonstrated that zebra mussels and *Corbicula* clams performed very well as indicators for waterborne contamination under the pollution levels reported from surface drinking water supplies (Rose et al. 1997; Atherholt et al. 1998; Solo-Gabriele et al. 1998). This conclusion is reinforced by the negative results of water testing by the method recommended by the U.S. Environmental Protection Agency (Rodgers et al. 1999a, b). Thus, *D. polymorpha* and *Corbicula fluminea* can prove water contamination even when water testing produces negative results.

In the present study *Cryptosporidium parvum* oocysts and *G. lamblia* cysts were first identified in *D. polymorpha* tissue 3 days after water contamination, and were still detectable 2 weeks after the cessation of contamination. In aquarium C with equal numbers of *D. polymorpha* and *Corbicula fluminea* higher numbers of both parasites were found in the former species. Also, the overall number of parasites identified in *D. polymorpha* was significantly higher than in *C. fluminea*. Together this suggests that *D. polymorpha* may be a better indicator than *C. fluminea* for contamination with waterborne protozoan parasites, predominantly *Cryptosporidium*. However, because *D. polymorpha* occurs in cooler climates and under different environmental conditions than *C. fluminea*, they do not usually occur in the same bodies of water or in the same aquatic habitats within those bodies (McMahon 1991). Thus, the most useful species will vary with the specific area being monitored, or both species may be used together to cover a broad geographical area.

The reason why higher numbers of parasite cystic stages were detected in *D. polymorpha* is not clear. *C. fluminea* clams and *D. polymorpha* mussels are both very efficient filter feeders (McMahon 1991). The pathogen filtration rates measured previously under laboratory conditions in similar 38-l aquariua were 1.9×10^5 *Cryptosporidium parvum* oocysts/clam per 24 h (Graczyk et al. 1998), 5.0×10^2 *G. lamblia* cysts/clam per 24 h (Graczyk et al. 1999a), and 4.9×10^2 *C. parvum* oocysts/mussel per 24 h (Frischer et al. 1999). As no parasites were found in aquarium sediments and water, the possibility cannot be rejected that *Cryptosporidium parvum* and *G. lamblia* resisted digestion to a lesser extent in *D. polymorpha* than in *Corbicula fluminea* tissue. From a water-monitoring standpoint the extended presence of water-recovered human parasites observed in *D. polymorpha* tissue indicates again their high applicability for monitoring.

The levels of *G. lamblia* cysts detected in both mollusk species were significantly lower than those of *Cryptosporidium parvum* oocysts despite the fact that more cysts than oocysts were seeded into the water. This cannot be a detection artifact as the cysts are bigger than oocysts and easier to enumerate. Interestingly, studies on wild-collected oysters that utilized immunofluorescent antibody against both *Cryptosporidium* and *Giardia* never identified *Giardia*, whereas the load of *Cryptosporidium* was quite high (Fayer et al. 1998, 1999). *Cryptosporidium* and *Giardia* originate from similar sources and *Giardia* usually occurs in higher concentrations than *Cryptosporidium* in surface water (Atherholt et al. 1998; Solo-Gabriele et al. 1998). This may indicate that *Giardia* cysts do not resist digestion and disintegrate in the tissue of molluscan shellfish.

Zebra mussels and *Corbicula* clams have an important role in aquatic habitats and act by filtering suspended particles, thereby making the water clear and generally improving water quality (McMahon 1991). The potential removal of particulates by *Corbicula fluminea* is so high that this species is considered to be the major seston consumer in North American freshwater surface drainage systems (McMahon 1991). Zebra mussels collected from the St. Lawrence River near a wastewater discharge site contained on average approximately 440 *Cryptosporidium parvum* oocysts (Graczyk et al. 2001), and the *C. parvum* filtration rate was 4.9×10^2 oocysts/mussel per 24 h (Frischer et al. 1999). Based on these results and reported *D. polymorpha* densities (McMahon 1991), it has been calculated that during 24 h approximately 1.3×10^7 waterborne *C. parvum* oocysts can be removed by a square meter of a zebra mussel bed (Graczyk et al. 2001).

Zebra mussels serve as an excellent biological indicator of chemical, viral, and bacterial pollutants in the Great Lakes and the St. Lawrence River predominantly because they can bioaccumulate these pollutants in their tissue (Brieger and Hunter 1993; de Lafontaine et al. 1999; Horgan and Mills 1999; Regoli et al. 2001). Similarly, *Corbicula fluminea* clams have been used as a bioindicator of agricultural toxicants due to their ability to accumulate chemical compounds in their tissue (Tatem 1986; Leland and Scudder 1990). Demonstrated herein, the ability to accumulate waterborne protozoan pathogens by *D. polymorpha* and *Corbicula fluminea* clams proves their equally high applicability (as for chemical, viral and bacterial pollutants) for biomonitoring of freshwater habitats contaminated with human protozo-

an parasites. *Dreissena polymorpha* and *C. fluminea* are convenient for such purposes because they form dense populations, are of no economic value, are easily collected, are relatively small, and form clumps that facilitate collection of a large sample.

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