Removal of enteric viruses and *Escherichia coli* from municipal treated effluent by zebra mussels

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ABSTRACT

Dreissena polymorpha is a widespread filter-feeder species, resistant to a broad range of environmental conditions and different types of pollutants, which has recently colonized Italian freshwaters. Although widely used to monitor pollution in freshwater environments, this species is also an important food source for some fish and water birds. It can also be used to concentrate or remove particulate organic matter to interrupt avian-to-human transmission of pollutants and control health risks for animals and humans. In this study, the accumulation/inactivation in *Dreissena polymorpha* of human health-related spiked enteric viruses was described. The removal of endogenous *Escherichia coli*, the classical indicator of faecal contamination, was tested as well. Our preliminary lab-scale results demonstrate that zebra mussels can reduce significantly poliovirus titer after 24 h and rotavirus titer after 8 h. *Escherichia coli* counts were also reduced in the presence of zebra mussels by about 1.5 log after 4 h and nearly completely after 24 h. The fate of the two enteric viruses after concentration by zebra mussels was also investigated after mechanical disruption of the tissues. To our knowledge, the accumulation from water and inactivation of human health-related enteric viruses by zebra mussels has never been reported.

1. Introduction

The zebra mussel *Dreissena polymorpha* (*D. polymorpha)* is a Ponto-Caspian zebra mussel bivalve species, that has invaded and colonized Italian freshwaters during the late 1980s (Schloesser and Schmuckal, 2013). Zebra mussels are small, sessile organisms, widespread filter feeders, resistant to a broad range of environmental conditions (Claudi and Mackie, 1993) and to different types of pollutants (Bervoets et al., 2005). They can concentrate particulate organic matter and indigestible components from water with a clearance rate ranging between 5 and 400 mL/individual/h (Ackerman, 1999; Baldwin et al., 2002). *D. polymorpha* have their most suitable habitat in stable riverbeds under high flows, a velocity below 1.2 m/s, and a depth of less than 5 m under regular flows (Sanz-Ronda et al., 2014). They have been extensively used to monitor pollution in freshwaters, especially in bio-accumulation studies, by determining the level of pollutants in their soft tissues (Voets et al., 2006). The impact of river colonization by these mussels has been considered one of the most important ecological changes in freshwater systems, both for the drop in biodiversity and for the socio-economic problems they can cause (Sanz-Ronda et al., 2014; Stankovic and Jovic, 2013). The ability of zebra mussels to attach and foul structures has also caused problems in the withdrawal of drinking water and electric-power plants (Schloesser and Nalepa, 1994) and their ability to colonize the surfaces of all solid structures in the water have caused nuisances to fishermen (Schloesser and Nalepa, 1994). On the other hand, they have also modified the aquatic environment, making the habitat more suitable for themselves and other organisms (Stankovic and Jovic, 2013). When studying their influence on physical and chemical characteristics of the Zhrebchevo water reservoir (Bulgaria) during the

periods before (1977-1980) and after (2009-2011) their invasion, water quality improved with a statistically significant effect on turbidity, pH, concentrations of dissolved oxygen, and NH_4-N, NO_2-N, NO_3-N (Kalchev et al., 2013). In particular, two different studies showed zebra mussels efficiency in nutrient (Piesik, 1983) and algae (Richter, 1986) removal.

Many works have also been published on the ability of zebra mussel to remove and accumulate chemical pollutants even when present at very low concentrations (De Jonge et al., 2012; Peck et al., 2007; Voets et al., 2006). Very recently, it was also demonstrated the role played by zebra mussel biofiltration in decreasing the concentration of many pharmaceuticals and trace metals from wastewaters (Binelli et al., 2014; Magni et al., 2015).

However, in spite of the numerous data on the accumulation of chemical pollutants, only a few studies are available on the ability of *D. polymorpha* to remove microorganisms. Frischer et al. (Frischer et al., 2000) demonstrated that zebra mussels use bacteria as a food source and a recent study (Winters et al., 2011) analyzed the composition of the bacterial community in zebra mussels from three locations of the Great Lakes basin waters (Michigan, USA). Bacteria, potentially pathogenic for aquatic and terrestrial animals, such as *Aeromonas* spp., *Flavobacterium* spp., *Pseudomonas fluorescens*, *Shewanella putrefaciens* and *Shigella* spp., were also detected after disrupting mussel tissues. *Cryptosporidium parvum, Giardia duodenalis* and *Toxoplasma gondii* oocysts were also found in tissues of zebra mussels exposed for one week to different protozoan concentrations, showing that their bioaccumulation was proportional to the environment contamination (Palos Ladeiro et al., 2014). *E. coli* removal by oysters and hard shell clams (Love et al., 2010) as well as by common mussel (de Mesquita et al., 1991) has also been studied, and oysters or hard shell clams were also used for Norovirus (Flannery et al., 2013), poliovirus, hepatitis A (Love et al., 2010), or Norvalk virus removal (Schwab et al., 1998). However, to our knowledge, the accumulation of human health-related enteric viruses by zebra mussels from water was never described, the only one case referring to avian influenza virus (Faust et al., 2009).

The aim of this study was to evaluate whether zebra mussels could remove the residual load of faecal bacteria and enteric viruses from treated effluents of a municipal wastewater treatment plant (WWTP) to decrease public health risks, especially in effluent dominated streams. Therefore, a series of lab-scale experiments have been carried out to verify the ability of *D. polymorpha* to remove endogenous *E. coli* and two enteric viruses (poliovirus and rotavirus) experimentally spiked into the samples. The fate of the two enteric viruses after concentration by zebra mussels was also investigated.

2. Materials and methods

2.1. Water sampling

Random samples were collected after the secondary settling of the Nosedo (Milan, Italy) wastewater treatment plant (WWTP). The Nosedo WWTP receives wastewater from 1,250,000 IE, with negligible industrial contribution. It performs a conventional mechanical-biological treatment sequence that includes pre-treatments, primary settling, biological treatment by activated sludge, secondary settling, and filtration. This treatment sequence is followed by disinfection with peracetic acid. Sampling was performed after the secondary settling of the effluent, to better mimic the conditions of many WWTPs not provided with filtration and disinfection, and discharging suspended solids that can feed zebra mussels, thus creating favorable conditions to their growth. Five samplings were performed and samples were refrigerated at 4 °C until the microbiological analyses were carried out, within 24 h.

2.2. *D. polymorpha sampling*

Zebra mussels were collected during Spring 2013 by scuba divers from Lake Lugano, located at the Italy-Switzerland border. Acclimation of mussels was performed by keeping the bivalves in a large-mesh nylon net immersed in a beaker containing 300-mL of the effluent sample, and slowly stirred. Ten mussels (around 2-cm long) were used for each experiment. The net was laid over a stainless-steel grid at around 2 cm from the bottom (Fig. 1) and the beakers were kept at room temperature (around 23 °C).

Figure 1. Beaker containing *D. polymorpha*. During the experiment, zebra mussels were slowly stirred at room temperature in a large-mesh nylon net.

2.3. *Cells*

Green monkey kidney cells (Vero) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated newborn calf serum (CS; Gibco Life Technologies, Grand Island, NY, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (P/S). *Macaca mulatta* foetal kidney cells (MA104) were grown in the same medium except that foetal calf serum (FCS; Gibco Life Technologies) was used.

2.4. Viruses

The poliovirus type I (attenuated Sabin strain from our lab) and the simian SA11 rotavirus were used. Every sample of the poliovirus- or the rotavirus-inoculated water was filtered through a 0.22-μm membrane before titration. Titration was performed in triplicate on Vero and MA104 cells, respectively, to detect alive viruses. Negative controls were carried out using unspiked water.

For poliovirus titration, cell monolayers were infected with serial dilutions of the samples for 1 h at 37 °C before adding the complete medium with 5% CS and 0.7% LE agarose (SeaKem, FMC BioProducts, Rockland, ME) as already described (La Colla et al., 1970). Briefly, after a further incubation at 37 °C for 2 days, lysis plaques were revealed after 16-18 h with 1.5% neutral red. The percent reduction of the poliovirus titer was calculated by comparison with the control, where zebra mussels were not present.

For rotavirus, titration was performed as already described (Ruggeri and Greenberg, 1991). Briefly, cells were plated on 96-well plates overnight at 37 °C and washed with serum-free DMEM before infection. After activation with 5 μg/mL acetylated trypsin (Sigma, St Louis, MO, USA) for 30 min at 37 °C, cells were infected with serially diluted samples, and incubated for 16-20 h at 37 °C. The cells were then washed with Ca^{++}- and Mg^{++}-free phosphate-buffered saline (PBS^-) before treating with PBS^-/MetOH 1:1, and fixing with 100% MetOH at -20 °C for 20-30 min. After air-drying, the cells were washed twice with PBS^- at room temperature, before incubation for 2.5 h a 37 °C with the primary 1:1500-diluited rabbit anti-rotavirus antibody. After two washes in PBS^-, the goat anti-rabbit horseradish peroxidase (HRP)-conjugated 1:200-diluted antibody (Sigma) was added for 1 h at 37 °C. Two further washes in PBS^- were performed before adding 100 mM NaAcetate pH 5.5 for 10 min at room temperature. Foci were viewed at the optical microscope after adding a solution of 3-amino-9 ethylcarbazole (AEC, Sigma) for 15 to 40 min in the dark, until a specific color was developed and the percent reduction of the rotavirus titer was calculated as described for poliovirus. The experiments were repeated three/four times which were requested to set up the poliovirus and rotavirus concentrations to be spiked according to the different number of *D. polymorpha* and volume of water used. Only the most reliable experiment is shown, which was performed in triplicate.

2.5. E. coli counting

E. coli counting was performed by membrane filtration method (APHA/AWWA/WEF) (APHA, 1998), using 0.45 μm pore size cellulose nitrate membranes (Sartorius Stedim Biotech, GmbH, Goettingen, Germany) and chromogenic agar growth medium (EC X-GLUC agar, Biolife Italiana SrL, Milan, Italy). Inoculated plates were incubated at 44 °C for 24 h and *E. coli* concentration was expressed as colony-forming units (cfu)/100 mL sample. The percent of *E. coli* removal was calculated with reference to the blank, with no mussels, at the same sampling times. Five replicates were performed.

2.6. Determination of poliovirus and rotavirus removal by D. polymorpha

Two test beakers were prepared per each virus, one with *D. polymorpha* and the other in the absence of the bivalves. The beakers were filled with 300 mL of the effluent water and then spiked to reach a final titer of 5 x 10^4 plaque-forming units (pfu)/mL for poliovirus and 3.7 x 10^3 focus-forming units (ffu)/mL for rotavirus. The titers were experimentally determined previously to give the adequate concentration both for the amount of water and the number of *D. polymorpha* to make the virus detectable after being in contact with the bivalve. The virus-spiked effluent was slowly stirred for 15 min, then the mussels were transferred from the acclimation to the test beakers. Spiked-effluents with no mussels were used as a control. Samples were collected from the test beakers after 15 min (T_0), 4, 6, 8 and 24 h (T_4, T_6, T_8 and T_{24}, respectively) for poliovirus and after 4, 8, 12, and 24 h (T_4, T_8, T_{12} and T_{24}, respectively) for rotavirus. Poliovirus and rotavirus samples were kept at -80 °C and at 4 °C, respectively, till they were analyzed, after filtration through 0.22 µm membranes. The percentage of virus removal by *D. polymorpha* from the water phase was calculated at the same sampling times with reference to the control.

Viral presence was also determined in the pseudofaecal or faecal discharge material for the three days after the experiment, during which the mussels were kept in tap water and fed with *Spirulina* spp. algae. Briefly, after two washes with 100 mL of maintenance medium to remove the residual inoculum, further water was added for 1 day (200-mL) to obtain faeces and pseudofaeces. An aliquot of water was collected, filtered and analyzed before adding 100 mL of water containing *Spirulina* spp. Two days later, the water was centrifuged at 2,000 *x g* to separate faeces, which were then resuspended in 3 mL of Earle BSS, filtered through 0.45 µm membrane, and titrated.

The search for residual virus was extended to the soft-tissue homogenates of zebra mussels. Briefly, 150 or 30 mL of the effluent were spiked with either 5 x 10^4 pfu/mL of poliovirus or with 3.7 x 10^3 ffu/mL of rotavirus and left with the bivalves for 24 h. The zebra mussels were then removed from the effluent, and, after 48 h in tap water within a beaker with extensive stirring, the tissues were mechanically desegregated with scissors, as already performed for primary cell culture preparations from chick embryos (Temin and Rubin, 1958). Two mL of serum-free medium was then added to the homogenate, which was centrifuged at 2,100 *x g* for 15 min at 4 °C. The supernatant was then frozen at -80 °C until titration was performed.

2.7. Determination of E. coli removal by D. polymorpha

After acclimation, the net containing *D. polymorpha* was transferred into 300-mL beakers containing the effluent samples. The effluent sample with no mussels was used as a blank. *E. coli* were counted after 4, 24 and 48 h contact time (T_4, T_{24} and T_{48}, respectively).

2.8. Statistical analyses

Statistical analyses were performed using the one-way ANOVA parametric test with the Bonferroni post-hoc analysis of variance, and the GraphPad Prism 5 software. The significance was set as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

3. Results

3.1. Zebra mussels can reduce poliovirus titer from wastewater

To verify the ability of zebra mussels to decrease the amount of poliovirus, the effluent water experimentally spiked with the virus in the presence or absence of *D. polymorpha* was analyzed at different times post inoculation (p.i.) (Fig. 2A). Preliminary experiments were performed to determine the poliovirus titer to be spiked to detect a decrease by ebra mussels in experimentally-tested volumes (data not shown). The final poliovirus concentration was thus established as 5×10^4 pfu/mL. A constant and significant 33.3% reduction of the virus titer was found 4 h (T_4, $p < 0.05$) and 6 h (T_6, $p < 0.01$) p.i., that further increased at 8 h (T_8, 49.7%, $p < 0.001$) and 24 h p.i. (T_{24}, 75.7%) (Figs. 2A and B). No virus was detectable when looking for endogenous live poliovirus in the effluent water before virus spiking. Negative results were also obtained from *D. polymorpha* faeces and pseudofaeces, whereas 3.4×10^4 pfu/mL of poliovirus were found in the 2-mL total mussel homogenate supernatant, corresponding to 0.9% of the spiked virus, 48 h after washes in clear water (Table 1). Controls using unspiked wastewater were always negative, as expected.
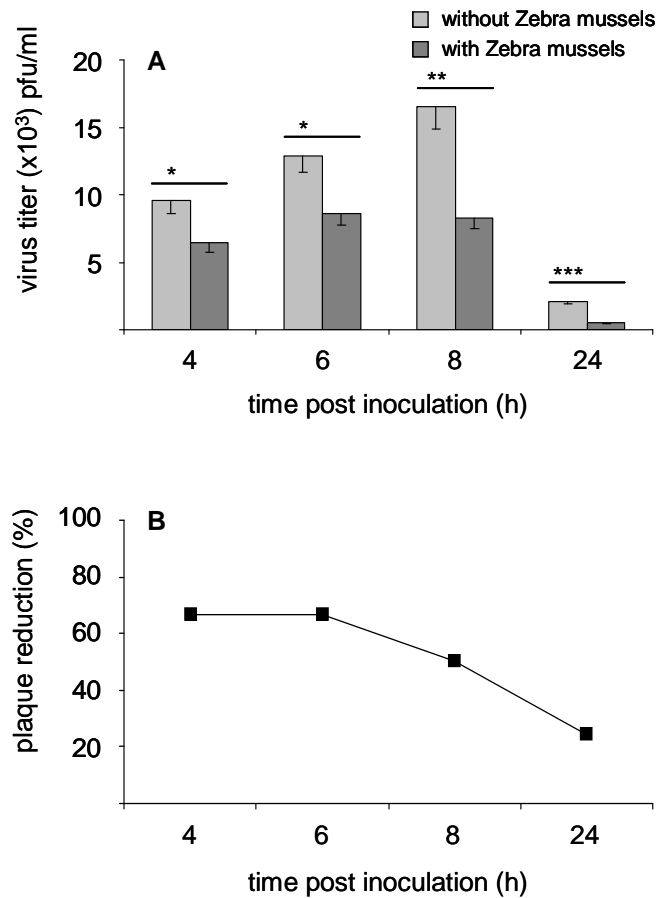
Figure 2. Reduction of poliovirus titer by *D. polymorpha*. The effluent was spiked with poliovirus in the presence or absence of *D. polymorpha* and analyzed at T_4, T_6, T_8, and T_{24} (panel A). A significant reduction of the viral titer can be observed over time in the effluent containing the bivalves (panel B) compared to the titer found in the effluent without *D. polymorpha*. The average of three replicates is shown.

3.2. Rotaviruses are almost completely removed from wastewater by zebra mussels

As rotaviruses might be present in the effluent water, the ability of *D. polymorpha* to reduce their contamination was verified after experimental spiking (Fig. 3A). Preliminary experiments were also performed to determine the rotavirus titer to be spiked and detect its decrease by zebra mussels in tested volumes (data not shown), before using the final concentration of 3.7×10^3 focus-forming units (ffu)/mL. An initial reduction was observed 4 h p.i. (T_4, 74.5%, $p < 0.001$), that increased 8 h (T_8, 88.6 %, $p < 0.001$), 12 h (T_{12}, 99.0%, $p < 0.001$), and 24 h p.i. (T_{24}, 96%, $p < 0.01$) (Figs. 3A and B). No endogenous rotavirus was found

in the effluent before virus spiking nor when rotavirus was searched in the mussel homogenate supernatant (Table 1). Controls using unspiked wastewater were always negative, as expected.
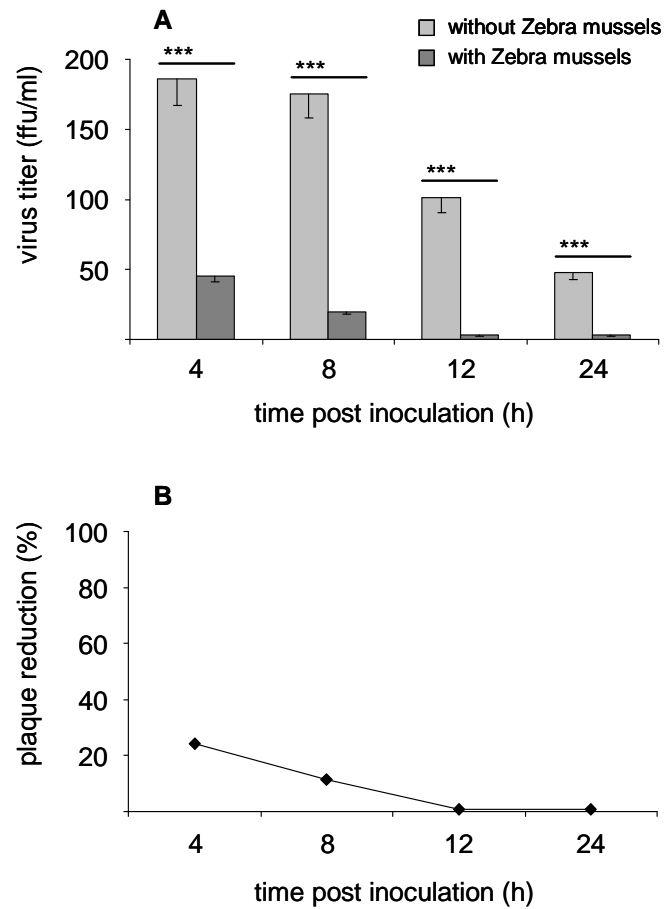

Figure 3. Reduction of rotavirus titer by *D. polymorpha*. The effluent was spiked with rotavirus in the presence or absence of *D. polymorpha* and analyzed at T_4, T_8, T_{12}, and T_{24} (panel A). A dramatic reduction of the viral titer can be already observed in the effluent containing the bivalves at T_4 that was more evident at T_8, and almost reached 100% at T_{12} and T_{24} (panels A and B). The average of three replicates is shown.

3.3. Zebra mussels significantly reduce endogenous E. coli counts in the effluent

Although the number of *E. coli* colonies decrease spontaneously overtime also in the absence of zebra mussels (Fig. 4A), a significant efficiency in enterobacterial removal was found starting from 4 h after exposure (T_4, 83.1%, $p <0.001$) to *D. polymorpha*. The decrease was even higher at 24 and 48 h (T_{24} and T_{48}; 98.6 and 99.4%; $p <0.001$), when *E. coli* colonies were found in very limited numbers (8 cfu/100 mL) (Figs. 4A and B).
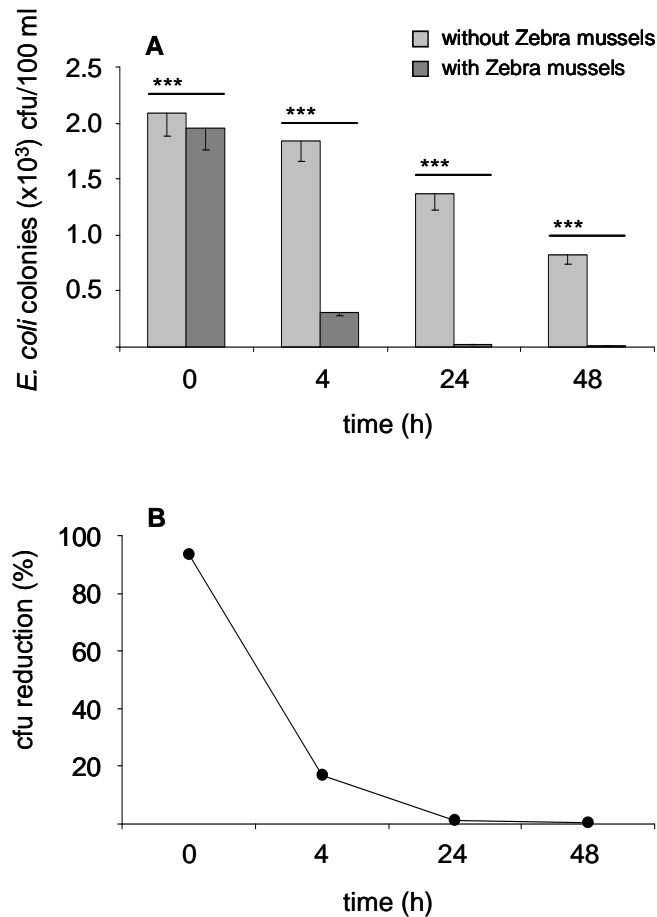
Figure 4. Reduction of endogenous *E. coli* colony numbers by *D. polymorpha*. Analyses were carried out at T_0, T_4, T_{24}, and T_{48} (panel A) in the presence or absence of *D. polymorpha*. A significant efficiency in bacterial removal was found starting from T_4 after exposure to zebra mussels, which reached almost 100% at T_{24} and T_{48} (panel B).

4. Discussion

Several studies already showed that zebra mussels can accumulate or remove pathogenic microorganisms from water bodies. However, only a few studies were related to viruses. A reduced viral infectivity was shown in wood ducks (*Aix sponsa*) inoculated intranasally with water spiked with avian influenza virus, previously filtered by *Corbicula fluminea* (Faust et al., 2009), which allowed bird survival. It was also demonstrated that zebra mussels could accumulate avian influenza virus from wastewaters for an extended period (Stumpf et al., 2010), after which the virus was still detectable inside the mussels even after transfer to freshwater.

In the present study, samples of treated effluents were used to evaluate the capability of *D. polymorpha* to remove or inactivate endogenous *E. coli* or spiked enteric viruses from wastewaters. Our results demonstrate that zebra mussels can reduce significantly: (1) poliovirus titer after 24 h; (2) rotavirus titer after 4 h; and (3) *E. coli* counts by about 1.5 log after 4 h and nearly completely after 24 h.

Differently from *E. coli*, which was already present in the effluents, the enteric viruses had to be spiked into the effluent samples. In particular, the live-attenuated poliovirus Sabin strain was utilized, as no live and titrable virus can be recovered from wastewaters, due to the use of the inactivated poliovirus Salk strain for the current vaccination protocols. Although unexpected, the increase of poliovirus titer up to 8 h after spiking, in the absence of zebra mussels, can be ascribed to the frequent presence of poliovirus pseudo-crystals (Kawanishi, 1978) that can desegregate over time, whereas the sharp decrease in viral titer at 24 h was probably caused by environmental factors, that favoured poliovirus inactivation. Conversely, after rotavirus spiking, the titer in wastewater spontaneously decreased over time, also in the absence of zebra mussels, whereas, in the presence of *D. polymorpha*, a more significant reduction was observed starting from 4 h contact time.

Few and sometimes contrasting data are available about the long-term survival of pathogens inside the bivalves and their release in the aquatic environment. Although zebra mussels may be an important reservoir for bacteria, it was demonstrated that *Aeromonas* species, potentially pathogenic for humans (*A. jandaei, A. veronii, A. salmonicida, A. hydrophila* and *A. media)*, were also lethal to this species (Gu and Mitchell, 2002; Maki et al., 1998). In our study, by analyzing the fate of the two spiked enteric viruses, a considerable amount of the inoculum was found to be removed and concentrated by mussel filtration, as demonstrated by viral detection in the mussel homogenate liquid phase.

The remaining live poliovirus was irrelevant outside or inside the zebra mussels, and we hypothesize that the inoculum might have been either inactivated by the wastewater or by mussel-released virucidal factors, making the virus undetectable by the used live-virus plaque-counting assay. Live rotavirus was also undetectable in the soft-tissue homogenate liquid phase for the same reasons as for poliovirus. However, its absence might also be due to the natural high liability of rotavirus during the experimental manipulations. It is important to note that rotavirus cannot be frozen/thawed several times without a clear reduction of the viral titer and that a low titer can generally be obtained during its production on *in-vitro* cell cultures. This is also the reason for having used a lower concentration and a smaller volume of effluent water for rotavirus than for poliovirus during the experimental design.

Although in studies regarding enterobacterial removal from wastewater by *D. polymorpha* a 50% reduction was found after 24 h and almost 100% after 48 h (Selegean et al., 2001), our results showed a reduction of already 83.1% and 98.6% at T_4 and T_24, respectively, and almost 100% at T_{48}. In the control samples, where zebra mussels were absent, the overtime *E. coli* decrease was slower, and can be due to the inactivation by toxic factors present in the wastewater.

Since viruses are intracellular parasites that can be released in the environment, it is not unlikely that bivalves may concentrate viruses from the wastewater and may act as a source of contamination. Zebra mussels are an important food source for some fish and waterbirds (Tucker et al., 1996; Zimmermann et al., 1997), and viruses might be transmitted to waterbirds, which are the natural predators of *D. polymorpha*. However, our data suggest that zebra mussels cannot only retain enteric viruses, but also inactivate them, thus preventing avian-to-human transmission. In effluent-dominated streams, if the conditions provide the suitable habitat for their presence and growth, the ability of *D. polymorpha* to filter and inactivate pathogens might have positive effects to control health risks for animals and human beings.

5. Conclusions

Overall, although the number of *D. polymorpha* we used may not be representative of the zebra mussel density in watercourses, our preliminary data seem to demonstrate the positive effects of this invasive species on the hygienic condition of surface waters. Zebra mussels were able to remove two enteric viruses (the poliovirus and rotavirus), probably not in the way that bacteria are removed, given the viral size. To our knowledge, the uptake and inactivation of poliovirus and rotavirus by zebra mussels has never been reported.

This invasive species can thus be useful to inactivate *E. coli*, reduce viral concentrations and decrease the levels of some pollutants recalcitrant to the traditional treatments of WWTPs, as also shown by other studies carried out by our group in the pilot-plant built at Nosedo WWTP (Binelli et al., 2014; Magni et al., 2015). These three studies also seem to share similar results, in that better performances were obtained using treatments with longer exposure times and low water flow, so that the next step should be the large-scale use in small reservoirs with biofiltration techniques, such as the phytodepuration and lagooning systems, where the permanence time is generally a-few day long.

As zebra mussels are often considered an alien species, their use for these purposes will require the necessary measures to avoid diffusion in the surrounding water systems both of adult mussels and veliger larvae. Thus, we would not suggest the introduction of this invasive species for water quality management, but rather consider their attractive qualities in controlled systems, in areas where they are already growing, following specific precautions, such as the installation of narrow grids or sand filters in the outlet.

Native bivalves, such as the Unionidae, could be one of the best solutions, if they were not an endangered species, unsuitable for the above-mentioned purposes. Preliminary studies should therefore include the search for a rapid method to grow such species and produce a large amount of zebra mussels, necessary for waste treatment. A less-expensive method to eliminate the bivalve biomass contaminated by xenobiotics and pathogens should also be found out.

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Notes

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Figure 1

Table 1. Virus titer in wastewater and zebra mussel homogenate

	endogenous virus in effluent water	total virus in faeces and pseudofaeces	spiked virus (pfu/ml)	total spiked virus (pfu)	total virus (pfu) in mussel homogenate
poliovirus	n.d.	n.d.	5.0×10^4	$7.5 \times 10^{6\,a}$	6.8×10^4
rotavirus	n.d.	n.d.	3.7×10^3	$1.0 \times 10^{6\,b}$	n.d.

[a], 150 ml were used; [b], 30 ml were used; n.d., not detectable