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RESEARCH ARTICLE

Bioaccumulation of pathogenic bacteria and amoeba by zebra mussels and their presence in watercourses

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Abstract *Dreissena polymorpha* (the zebra mussel) has been invading freshwater bodies in Europe since the beginning of the nineteenth century. Filter-feeding organisms can accumulate and concentrate both chemical and biological contaminants in their tissues. Therefore, zebra mussels are recognized as indicators of freshwater quality. In this work, the capacity of the zebra mussel to accumulate human pathogenic bacteria and protozoa has been evaluated and the sanitary risk associated with their presence in surface water has also been assessed. The results show a good correlation between the pathogenic bacteria concentration in zebra mussels and in watercourses. Zebra mussels could therefore be used as an indicator of biological contamination. The bacteria (*Escherichia coli*, *Enterococcus* spp., *Pseudomonas* spp., and *Salmonella* spp.) and parasites (*Cryptosporidium* oocysts and free-living amoebae) detected in these mussels reflect a potential sanitary risk in water.

Keywords Zebra mussel · Bioaccumulation · Pathogenic bacteria · Amoeba

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Introduction

The zebra mussel (*Dreissena polymorpha*) is a freshwater bivalve native to the drainage basins of the Black Sea and the Caspian Sea. Its colonization in southern European countries is relatively recent. The first detections in the Ribaroja and Flix reservoirs in the Ebro River occurred in 2001 (Lalaguna and Anadón 2008). From these reservoirs, zebra mussels have spread to the upper course of the Ebro River, as well as to many of its tributaries.

Organisms such as filter feeders can directly accumulate and concentrate large quantities of contaminants in their tissues. Ecotoxicological studies have already used aquatic invertebrates such as the zebra mussel as sentinel species to assess the water quality of freshwater ecosystems. In fact, zebra mussels are easy to collect in large numbers and to maintain in the laboratory (Palos Ladeiro et al. 2014).

Several studies have been reported concerning the use of the zebra mussel as an indicator of inorganic contamination (Anzano et al. 2011; Camusso et al. 2001; Magni et al. 2015; Rutzke et al. 2000) and organic pollution (Bervoets et al. 2004; Binelli et al. 2014; Parolini and Binelli 2014).

Zebra mussels may also reflect biological contamination levels using both fecal contamination indicators (*Escherichia coli*, *Enterococcus* spp., *Salmonella* spp., and *Pseudomonas* spp.) and pathogenic protozoa (*Cryptosporidium* spp., *Giardia duodenalis*, and *Acanthamoeba* spp.). Data about protozoa bioaccumulation by zebra mussels have been reported (Graczyk et al. 2001, 2004; Palos Ladeiro et al. 2014), but as far as we know, the accumulation of other bacteria apart from *E. coli* has not been studied (Selegean et al. 2001).

Escherichia coli is the most widely accepted fecal indicator since this bacterium is present in large quantities in the human digestive tract and it is not usually found in other environments. The presence of this bacterium in water indicates recent fecal
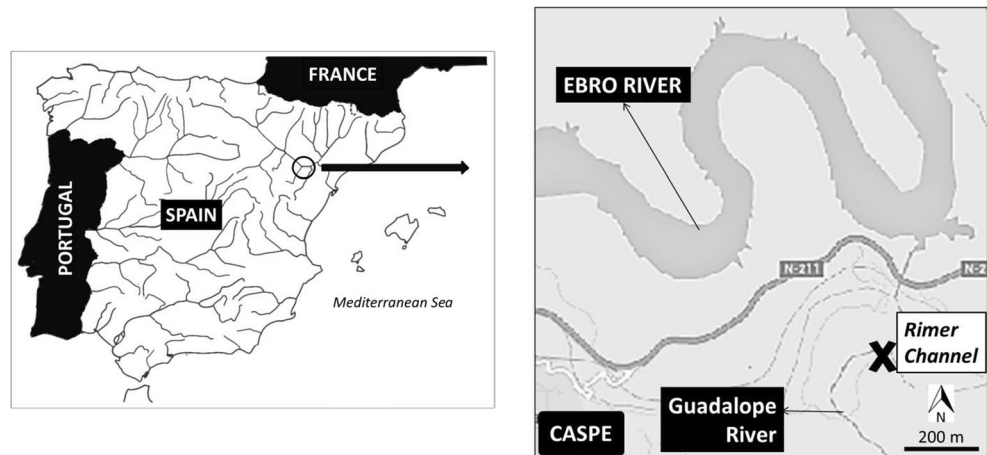
contamination and the possible existence of other pathogens (Molleda et al. 2008). The use of *Enterococcus* as an indicator bacteria is frequently suggested as an alternative to *E. coli*. Their main advantage lies in their greater resistance and their ability to grow in any environment, such as soil, water, and others. Despite the importance of *Salmonella* spp. as one of the major causes of food-borne infections worldwide, data regarding the presence of these organisms in the environment is limited (Shannon et al. 2007; Levantesi et al. 2010).

Pseudomonas spp. is a ubiquitous environmental bacterium and is therefore found naturally in water. Also, *Pseudomonas aeruginosa* is the species most frequently associated with disease in humans, where it acts as an opportunistic pathogen with the potential to cause infections in almost any organ or tissue, especially in patients compromised by underlying disease, age, or immune deficiency. The capacity of *P. aeruginosa* to cause disease is enhanced by both intrinsic and acquired resistance to many antimicrobials and disinfectants, virulence factors, and the ability to adapt to a wide range of environments (Loveday et al. 2014).

However, fecal contamination indicators have a limited predictive value for various pathogens, especially human viruses and protozoa. These microorganisms are now recognized as being more resistant to natural inactivation and to water treatment processes than the current bacterial indicators of water quality (Abreu-Acosta and Vera 2011).

Human pathogenic protozoa, which are not well represented by fecal contamination indicators, are present in water in resistant forms (cysts and oocysts) that protect them from environmental stress. *Cryptosporidium* and *Giardia* are genera of protozoan parasites potentially found in water and other media. *Cryptosporidium* spp. and *G. duodenalis* are major causes of diarrheal disease in humans and animals worldwide and of protozoan waterborne diseases (Moulin et al. 2010). In addition, the detection of these protozoa is complex and costly (Moss et al. 2014).

Other prevalent protozoa in the environment are free-living amoebae (FLA). These are ubiquitous microorganisms

present in soils and water. The most extensively studied FLA is *Acanthamoeba* spp. because of its abundance and its medical significance as an agent of human infections such as amoebic keratitis and granulomatous amoebic encephalitis. Another area of concern regarding FLA in water is their relationship with waterborne pathogenic bacteria, including *Legionellaceae*, *Mycobacteriaceae*, *Enterobacteriaceae*, *Vibrionaceae* and many others (Mosteo et al. 2013).

The aim of this research work is to determine the capacity of the zebra mussel to accumulate potentially pathogen bacteria (*E. coli*, *Enterococcus* spp., *Pseudomonas* spp., and *Salmonella* spp.) and protozoa (*G. duodenalis*, *Cryptosporidium* spp. oocysts, and free-living amoebae) and to compare the findings with the concentration of these microorganisms in watercourses. Moreover, the capacity of the zebra mussel to pollute clean water with these potentially pathogen bacteria is evaluated.

Materials and methods

Sample collections

Zebra mussels and water samples were collected in 2013 from the Rimer irrigation channel (N 41° 13′ 44″, E 0° 00′ 32″) in Caspe (Zaragoza, Spain) coming from the Guadalope river, a tributary of the Ebro river (Fig. 1). Sampling was carried out in

Table 1 Methods for the analysis of the physico-chemical parameters

Parameter	Method	Reference
Dissolved oxygen	Standard method 4500-O G	Cleresci et al. 2005
Temperature	Standard method 2550 B	
pH	Standard method 4500-H$^+$ B	
Conductivity	Standard method 2510 B	
Total hardness	Standard method 2340 C	
Calcium hardness	Standard method 3500 Ca B	

Table 2 Physico-chemical parameters of the water from the aquarium

Parameter	Values
pH	7.4–8.4
Conductivity (μS cm^{-1})	1715–2380
Temperature (°C)	18.5–23.8
Hardness (mg CaCO$_3$L^{-1})	760–1010
Dissolved oxygen (mg O$_2$L^{-1})	7.2–8.3

autumn and spring. The sampling protocol was developed by the Ebro Hydrographic Confederation (CHE 2006). The collected water samples zebra mussels were either used for microbiological analysis or they were kept in an aquarium for further experiments.

Physico-chemical parameters, human pathogenic bacteria, and parasites were analyzed in the surface water samples. Human pathogenic bacteria and parasites were analyzed in five zebra mussels collected in surface water.

Analytical methodology in water samples

Physico-chemical analysis

Physico-chemical parameters such as the pH, conductivity, dissolved oxygen, temperature, and hardness were measured in situ in the Rimer irrigation channel and periodically in the aquarium using standard methods (Cleresci et al. 2005). Table 1 shows the methodology used for the analysis of each physico-chemical parameter.

Bacteriological and parasite analysis

The analysis of *E. coli*, *Enterococcus* spp., *Pseudomonas* spp., and *Salmonella* spp. was performed by the membrane filtration method (using a cellulose nitrate filter of 0.45-μm pore size, Millipore®) or by the spread plate standard method 9215C in triplicate. The culture and enumeration of *E. coli* was carried out following the procedure ISO 9308–1, using MacConkey agar (Scharlau®). The culture and enumeration of *Enterococcus* spp. and *Pseudomonas* spp. were carried out

in accordance with the procedure ISO 7899–2:2000 using Slanetz and Barntley agar (Scharlau®) and procedure EN ISO 12780 using Cetrimide agar (Scharlau®), respectively. The culture and enumeration of *Salmonella* spp. were performed using *Salmonella-Shigella* agar (Scharlau®).

The enumeration of colonies was carried out in terms of colony-forming units (CFUs) per 100 mL of sample in each contact time. A range of 30–300 colonies grew in each plate, so that the technique error was less than one order of magnitude. These concentrations were transformed to log$_{10}$ for statistical and kinetic studies.

To detect *G. duodenalis*, a direct microscopic examination of the sample was performed. To detect *Cryptosporidium* spp. oocysts, modified Zielh-Neelsen staining of the sample was required.

At least four preparations of each sample were checked in a Nikon Eclipse 80i microscope. A previous observation was performed with the ×10 objective examining all the possible fields in a smear of 22×22, later moving to ×40 and ×100. Each sample was sequentially performed by two skilled and experienced microscopists. A sample was classified as positive for a parasite or protozoa when it was detected in at least one preparation. Cultures for free-living amoebae were performed in non-nutrient agar for all the water samples, as previously described (García et al. 2011).

Zebra mussels' maintenance

A laboratory scale aquarium (volume: 100 l) was used to maintain the zebra mussels alive in order to carry out in vitro experiments related to their accumulation and infection capacity.

Both Zebra mussels and water samples from the Rimer irrigation channel were placed inside the aquarium using the most favorable environmental conditions for the maintenance of zebra mussels, taking into account previous studies (Claudi and Mackie 1994; O'Neil 1996). Table 2 shows the results of physico-chemical parameters measured in aquarium.

Leftover zebra mussel specimens were destroyed in accordance with the protocol developed by the Ebro Hydrographic Confederation (CHE 2002) with a high dosage of NaClO to

Table 3 Bacteriological analysis in zebra mussels and natural surface waters

Bacteria	Spring sampling		Autumn sampling	
	Surface natural water (CFU 100 mL^{-1})	Zebra mussels (CFU g^{-1} mussel)	Surface natural water (CFU 100 mL^{-1})	Zebra mussels (CFU g^{-1} mussel)
Escherichia coli	$2.6\pm0.4\cdot10^4$	$7.1\pm0.4\cdot10^4$	$2.4\pm0.4\cdot10^3$	$6.9\pm0.3\cdot10^3$
Enterococcus spp.	$9.1\pm0.1\cdot10^1$	$3.4\pm0.2\cdot10^2$	$9.3\pm0.2\cdot10^1$	$9.3\pm0.2\cdot10^1$
Salmonella spp.	$8.0\pm0.3\cdot10^1$	$2.9\pm0.3\cdot10^2$	$9.1\pm0.4\cdot10^1$	$1.6\pm0.3\cdot10^2$
Pseudomonas aeruginosa	$5.8\pm0.1\cdot10^4$	$6.0\pm0.0\cdot10^4$	$2.1\pm0.1\cdot10^4$	$9.0\pm0.1\cdot10^3$

Table 4 Physico-chemical parameters of water from Acequia Rimer

Parameter	Units	Value (spring sampling)	Value (autumn sampling)
pH	–	8.0	8.0
Conductivity	$\mu S\ cm^{-1}$	2420	1170
Temperature	°C	16.2	12.4
Hardness	$mg\ CaCO_3 L^{-1}$	1320	775
Dissolved oxygen	$mg\ O_2 L^{-1}$	9.1	8.2

avoid their later reproduction. The research has legal permission for the handling and storage of zebra mussel specimens.

Analytical methodology for zebra mussels

Extract preparation of zebra mussels

The mussels were opened, the byssus and valves removed, the soft tissue extracted, and the total wet weight of the flesh measured. The flesh was homogenized with an equal volume g to mL of sterile phosphate buffered saline (PBS) (Graczyk et al. 2004). The obtained extract was divided in aliquots for analysis of the bacteria and parasites.

Bacteriological and parasite analysis

The methodology used to measure bacteria and parasites in the tissues of the zebra mussel extracts was the same as that used for the water analysis.

Accumulation and pollution experiments procedure

The pathogenic bacteria accumulation capacity of zebra mussels was evaluated exposing them to *E.coli*, *Enterococcus*, *Pseudomonas* and *Salmonella*, independently. In each experiment, the water volume was taken from the aquarium. The selected volume was 1 l to ensure the mussels filtered the water volume in 24 h (the time of the experiment). Firstly, the water sample and the glass bottle were sterilized (15 min; autoclave 121 °C, 1 bar). After that, the pathogenic bacterium selected was added to the water sample using two different concentrations: a high concentration (10^8–10^7 CFU 100 mL^{-1}) and a low concentration (10^6–10^5 CFU 100 mL^{-1}). Finally, some zebra mussels were superficially disinfected with NaClO and placed in the glass bottle.

The possible initial presence of each selected pathogenic bacteria in the zebra mussel was measured in two specimens taken from the aquarium. A control experiment for each pathogenic bacterium was carried out without the presence of zebra mussels.

The analysis of bacteria in the water sample, both in the accumulation and control experiments, was done at different times (initial time, 2, 5, 10 and 24 h). The analysis of bacteria in the zebra mussels was done at the beginning and at the end of the experiments (24 h). The experiments were done in duplicate.

The pollution experiments were carried out with the rest of the zebra mussels not analyzed at the end of the accumulation experiments. The zebra mussels were placed in a glass bottle (1 liter volume) containing water from the aquarium, previously sterilized. Contact was maintained for 24 h at room temperature. Analysis of the bacteria in the water was done at different times (2, 5, 10 and 24 h) and the analysis in the zebra mussels was done at the end of the experiments (24 h). The experiments were done in duplicate.

Results and discussion

Presence of pathogen bacteria and parasites in zebra mussels

The results of the pathogenic bacteria analysis in zebra mussels and surface water from the Rimer irrigation channel are shown in Table 3. It can be seen that *E.coli* and *Pseudomonas aeruginosa* were present in higher concentrations than *Enterococcus* spp. and *Salmonella* spp. both in the water and the zebra mussels. These results are consistent with the different origin of the bacteria, because while *Pseudomonas aeruginosa* is of environmental origin, *Enterococcus* spp. and *Salmonella* spp. are fecal bacteria.

The characteristics of the water from the Rimer irrigation channel are shown in Table 4. The analysis of the physicochemical parameters shows that every parameter, except the temperature, were between the high and optimal tolerance range for the survival of zebra mussels (Claudi and Mackie 1994). These results indicate that the zebra mussels had a
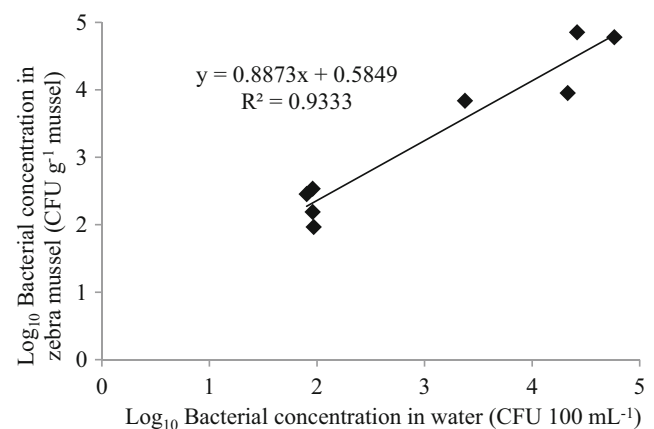
Fig. 2 Correlation between bacterial concentration in water and in zebra mussel. (Bacterial concentration is in log units)
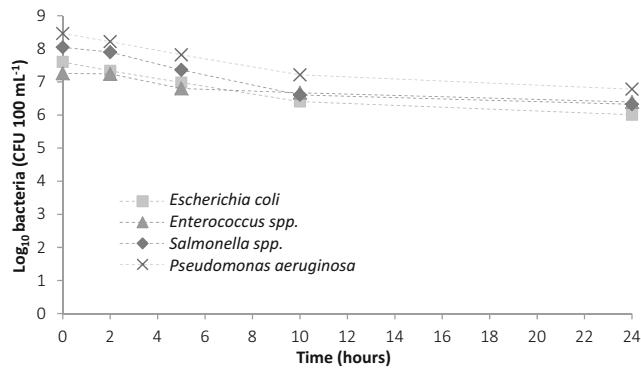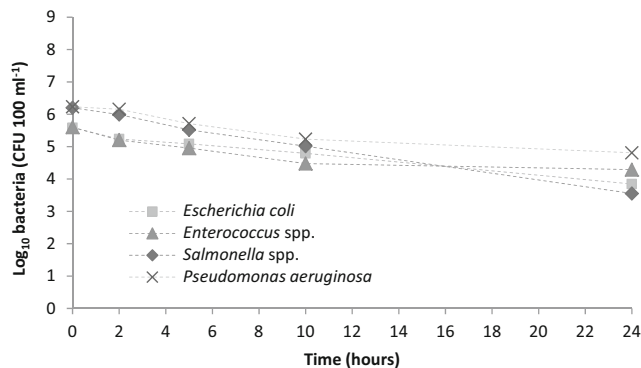
Fig. 3 Reduction of the bacterial concentration in water in the presence of zebra mussel. Accumulation experiment with high concentration of bacteria (10^8–10^7 CFU 100 mL^{-1})

similar behavior in both spring and autumn, so no influence of the water variables is observed.

A linear correlation of the bacteriological pollution in water and in zebra mussels, without differentiating the bacterial genera, is shown in Fig. 2. As can be observed, there is a good correlation between the pathogenic bacteria concentration in the zebra mussels and in the water, suggesting that zebra mussels could be used as indicators of the biological quality of water. This is of considerable interest since although some studies have been published about the presence of parasites inside the zebra mussel and its use as an indicator of biological contamination (Graczyk et al. 2001, 2004; Minguez et al. 2011; Palos Ladeiro et al. 2014), as far as we know the accumulation of other bacteria apart from *E. coli* has not been studied (Selegean et al. 2001).

To control the microbiological pollution of waters, fecal contamination indicators are usually employed since their detection and quantification is easy and cheap, and this determination provides information relating to the presence and behavior of the principal human pathogens present in waters (Abreu-Acosta and Vera 2011). Therefore, it is important to investigate the validity of using bacteria as contamination indicators of zebra mussels.
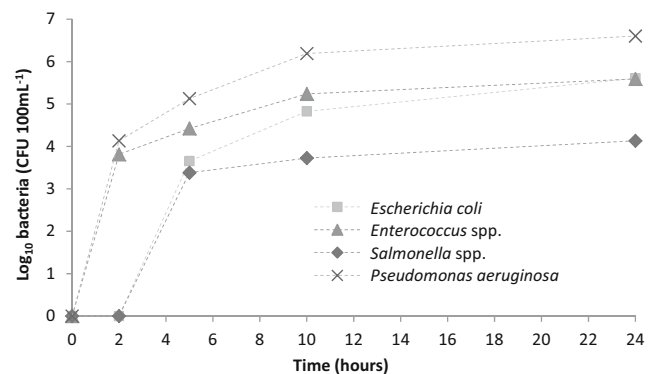
**Table 5** Accumulation results after 24 h of experiments

Bacteria	Estimated accumulated bacteria after 24 h according to zebra mussel analysis (CFU g^{-1} mussel)	Estimated accumulated bacteria after 24 h according to water analysis (CFU g^{-1} mussel)
High initial concentration of bacteria		
Escherichia coli	$2.1\pm0.2\cdot10^5$	$1.1\pm0.4\cdot10^8$
Enterococcus spp.	$7.5\pm0.5\cdot10^5$	$4.2\pm0.2\cdot10^7$
Salmonella spp.	$7.4\pm0.4\cdot10^4$	$2.9\pm0.3\cdot10^8$
Pseudomonas aeruginosa	$2.2\pm0.2\cdot10^7$	$1.1\pm0.2\cdot10^9$
Low initial concentration of bacteria		
Escherichia coli	$2.3\pm0.3\cdot10^4$	$8.9\pm0.0\cdot10^5$
Enterococcus spp.	$9.7\pm0.2\cdot10^4$	$8.6\pm0.5\cdot10^5$
Salmonella spp.	$2.2\pm0.2\cdot10^2$	$5.2\pm0.2\cdot10^5$
Pseudomonas aeruginosa	$1.7\pm0.7\cdot10^6$	$7.0\pm0.1\cdot10^6$

Direct microscopic examination of the mussel extract shows the presence of *Cryptosporidium* oocysts (modified Zielh-Neelsen staining) and free-living amoebae. However, cysts or trophozoites of *Giardia* are not observed in the mussel extract. To determine pathogen protozoa in water, such as *Cryptosporidium* and *Giardia*, it is necessary to filter a high volume of the water. Natural filtration by zebra mussels allows the detection of accumulated protozoa inside them, the origin of which is always the surrounding water. However, the presence of bacteria in zebra mussels can be indicative of their presence in the watercourse. Moreover, high amounts of these bacteria inside the mollusk suggest its continuous presence in the water or the occurrence of an occasional wastewater discharge.

Furthermore, the detection of pathogen bacteria and parasites in zebra mussels reflects the sanitary risk associated with the presence of zebra mussels in surface waters in Aragón.

Fig. 4 Reduction of the bacterial concentration in water in the presence of zebra mussel. Accumulation experiment with low concentration of bacteria (106–105 CFU 100 mL^{-1})

Fig. 5 Pollution of bacteria from zebra mussel in sterilized water. Experiments with high concentration of bacteria
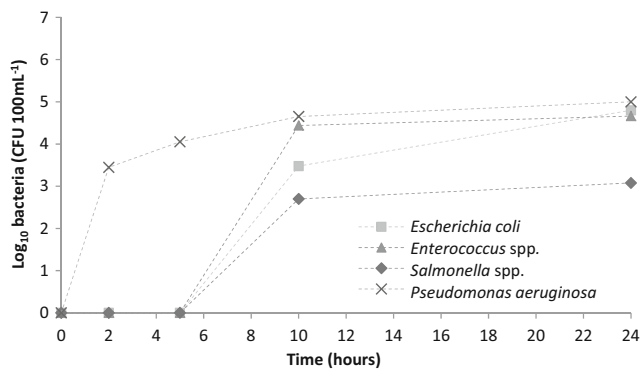
Fig. 6 Pollution of bacteria from zebra mussel in sterilized water. Experiments with low concentration of bacteria

Zebra mussels can concentrate these pathogenic bacteria and parasites and expel them when the bivalve organism dies or when some environmental event favors this process. This can lead to a significant increase in pathogens in waters, mainly during drought periods in which the water flow decreases considerably.

Accumulation capacity of zebra mussels

The analysis of pathogen bacteria in zebra mussels taken from the aquarium shows that the initial concentration of bacteria is low enough to be considered negligible (*Enterococcus* spp. and *Salmonella*=0 CFU g^{-1}; *E. coli*=6.13 CFU g^{-1} mussel; *P. aeruginosa*=4.72 CFU g^{-1} mussel).

The results for the reduction of the bacterial concentration in water during accumulation assays carried out with high and low concentrations of bacteria are reflected in Figs. 3 and 4. The main reduction takes place during the first 10 h, achieving more than 99 % reduction (1–1.5 log inactivation) for each bacterium without any influence of the initial concentration of the bacteria. Moreover, the results do not reflect any

differences in the accumulation capacity dependent on the bacteria shape.

The results of control experiments for each pathogenic bacterium carried out without the presence of zebra mussels showed that changes in the concentrations of bacteria in waters are only due to the accumulation in zebra mussels, except for *Salmonella* spp. The control experiment for *Salmonella* spp. showed a significant decrease in the concentration, reflecting the high death rate of this bacterium in waters.

Table 5 shows the accumulation capacity of zebra mussels at the end of the experiments, taking into account the bacteria analysis in water and in the tissues of the zebra mussels. The high concentration of *Salmonella* in zebra mussels suggests that the mollusk protects the bacteria although their accumulation in all cases is less than that for the other bacteria.

The results of bacteria accumulation in the zebra mussels show important differences with respect to the estimated value calculated on the basis of the results of the disappearance of bacteria in water. This difference is due to various causes such as the extraction of zebra mussel tissue (Selegean et al. 2001) or the metabolization of bacteria by the mollusk (Frischer et al. 1996).

Pollution capacity of zebra mussels

The bacterial pollution of sterilized water by zebra mussels during 24 h as shown in the accumulation experiments is represented in Figs. 5 and 6. The pollution capacity of *E. coli*, *Enterococcus* spp., *Salmonella* spp. and *Pseudomonas* spp. differed depending on the initial concentration of the bacteria and the type of microorganism (Figs. 5 and 6). The pollution is faster when the bacteria concentration in the zebra mussel is higher because after 2 h of contact time, all the bacteria were present in water. *Pseudomonas* spp. is the only bacterium that polluted water from the beginning of the experiment

Table 6 Pollution results after 24 h of experiments	Bacteria	Bacterial pollution by zebra mussels after 24 h according to zebra mussel analysis (CFU g^{-1} mussel)	Bacterial pollution by zebra mussels after 24 h according to water analysis (CFU g^{-1} mussel)
	High initial concentration of bacteria		
	Escherichia coli	$1.3\pm0.3\cdot10^5$	$2.4\pm0.5\cdot10^6$
	Enterococcus spp.	$5.6\pm0.2\cdot10^5$	$2.5\pm0.1\cdot10^6$
	Salmonella spp.	–	$6.9\pm0.3\cdot10^4$
	Pseudomonas aeruginosa	$2.0\pm0.6\cdot10^7$	$2.4\pm0.3\cdot10^7$
	Low initial concentration of bacteria		
	Escherichia coli	$5.1\pm0.1\cdot10^3$	$2.9\pm0.3\cdot10^5$
	Enterococcus spp.	$6.8\pm0.2\cdot10^4$	$2.5\pm0.4\cdot10^5$
	Salmonella spp.	–	$5.5\pm0.1\cdot10^3$
	Pseudomonas aeruginosa	$1.5\pm0.4\cdot10^6$	$7.9\pm0.2\cdot10^5$

independently of the concentration of the bacteria in the zebra mussels.

Table 6 shows the results after 24 h of contact time for both water pollution by the bacteria and the bacteria concentration in the zebra mussel tissue. The results reflected in Table 6 show that the pollution capacity of zebra mussels is high, and very close to the accumulation capacity taking into account the results shown in Table 5. A comparison between the accumulation and pollution capacities indicate that a certain amount of bacteria is assimilated by the zebra mussels since the CFU per gram mussel is higher for the accumulation results taking into account both the water and zebra mussel values. Furthermore, the pollution values based on water analyses are higher than the CFU per gram mussel calculated using the data relating to the zebra mussel tissue. Thus, these results are consistent with those of Selegean et al. (2001) and Frischer et al. (1996).

Conclusions

This work shows that zebra mussels can be used as an indicator of the biological quality of water. The pathogen bacteria (*Escherichia coli*, *Enterococcus* spp., *Pseudomonas* spp., and *Salmonella* spp.) and parasites (Cryptosporidium oocysts and free-living amoebae) detected in these mussels reflect a potential sanitary risk in the surface waters of Aragón (Spain). Further studies of more pollutants and zebra mussels from different water samples are needed to know more precisely their potentially use as bioindicators of water quality.

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