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Zebra mussels (*Dreissena polymorpha)* for assessing microbial contamination and antibiotic resistant bacteria in freshwaters

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Abstract

The suitability of zebra mussels (*Dreissena polymorpha*) as biomonitoring organisms for faecal contamination of surface waters was examined in both laboratory and field experiments. In addition, the presence of antibiotic resistant bacteria was investigated. In the first two laboratory experiments, the mussels were first exposed to a known concentration of *Escherichia coli* and *Enterococcus facecalis* and then depurated. In the other two experiments, the mussels were exposed to a tenfold dilution of sewage treatment plant effluent and then depurated. Zebra mussels showed a maximum uptake and elimination rate during the first hours after exposure to contaminated, and respectively, clean water. The field experiment was conducted at 6 sites located upstream and downstream from the sewage treatment plant outlet in the Fyris river (Uppsala, Sweden). During the 19 days of exposing caged mussels, the presence of faecal indicators, as well as *Salmonella* spp. was investigated. All the experiments demonstrated the ability of the zebra mussels to accumulate much higher concentrations of bacteria than in the surrounding water. This is in accordance with the work of previous studies and sustains the usefulness of zebra mussels in detecting bacterial peaks. *Salmonella* spp. was found on three occasions at the outlet, in both mussels and water, and on two occasions in the water downstream. All the *E. coli* and 41.3 % of the *Enterococcus* spp. isolates from both water and mussels were resistant to at least one antibiotic. A large percentage (48.5 %) of those found in mussels was multi drug resistant, thus providing evidence for this emerging problem that needs to be controlled.

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1. Introduction

The contamination of waters with faeces is an emerging problem worldwide, being more critical in developing countries which lack proper sanitation (UN Water). Human and animal faeces are carriers for the primary agents of many severe waterborne and consequently, food-borne diseases such as gastroenteritis, salmonellosis, typhoid and paratyphoid fever, meningitis, cholera, hepatitis, encephalitis or dysentery (WHO, 2011a). Besides the health effects, these food-borne outbreaks may cause shortages of food supplies and high economical losses. Therefore, it is crucial to have a good control of the microbiological quality of water.

The impracticality of synchronizing the water sampling events with pathogen outbreaks (Moles and Hale, 2004) has lead to the need of finding other methods for detecting peaks of bacteria in water bodies. This master's thesis aims at studying the uptake and elimination rate of faecal indicators by zebra mussels, in order to assess their efficiency as biomonitoring organisms in freshwaters receiving sewage effluents. By determining for how long time can the mussels retain bacteria, useful information can be provided for designing monitoring programs and for assuring food safety through proper depuration of mussels. Furthermore, this project aims at studying antibiotic resistance among the bacteria isolated from sewage effluent water, thus contributing to the on-going research about this expanding issue.

1.1. Hypothesis

This master's thesis tests the validity of the hypothesis: there is a significantly higher concentration of bacteria in the mussels than in the surrounding water, which shows the ability of zebra mussels to accumulate bacteria and thus, their suitability as biomonitoring organisms.

2. Literature review

2.1. Description of the test organism

2.1.1. Geographical range

Zebra mussels (*Dreissena polymorpha,* below referred to simply as *Dreissena*) were first identified in 1769 by Pallas in Russia (Ural River) and in the Caspian Sea (USGS a). However, they were spread from their native areas to freshwaters in Western Europe and North America by means of commercial and recreational ships, through attachment of adult mussels to ships or the transport of larvae in ballast water. In the 1920's they appeared in Sweden and are now distributed in the eastern basins of Lake Mälaren, Lake Hjälmaren and several other lakes in central eastern part of Sweden (Hallstan et al., 2010), including the Fyris river (Berglund et al., 2005), which is the location for the present field experiment. In 1988 zebra mussels were first discovered in Lake St. Clair (Garton and Haag, 1991) and have rapidly invaded the Great Lakes (DAISIE, 2006).

2.1.2. Morphology

Dreissena polymorpha is a bivalve with triangular shell having an acute hinge end called umbone (ISSG). The ventral surface is flat and presents an opening - pedal dape - through which the byssal threads are extended, making possible the attachment on hard surfaces (Mackie, 1991; US Army

Corps of Engineers® a). When the two valves are opened, the siphons are visible: the larger one is the inhalant siphon, which usually presents 80-100 small tentacles that help the filtration process. The exhalant siphon is located dorsal to the inhalant one and it lacks the tentacles (US Army Corps of Engineers® a).

The shell is composed of three layers: the periostracum, the prismatic layer, and the nacre (US Army Corps of Engineers® b). The periostracum is smooth and has a dark stripe pattern from which the common name of "zebra mussel" was derived (ISSG). Hinge teeth are not present (Mackie, 1991).

Figure 1. *Dreissena polymorpha* showing its byssal threads

The maximum length of the shell is usually 2-3 cm (Mackie, 1991), although 5 cm long mussels have also been identified (NPS, 2007; USGS b). The growth rate of adults is approximately 1.5 - 2 cm per year (Mackie, 1991) and the life span is 2-3 years in general (Chase and Bailey, 1999), although in exceptional cases they might live more than 4 years (Sprung, 1995; AIS, 2005).

2.1.3. Reproduction

Dreissena polymorpha is a dioecious species with external fertilization (Mackie, 1991). Gametogenesis occurs when water temperatures are above 12 °C (Sprung, 1987). There are two periods of reproduction each year: one in the spring (May-June) and the other one in the fall (September-November) (Mackie, 1991). The eggs, ranging in size between 30-50 µm are expelled through the exhalant siphons of the females. A *Dreissena* female can release up to 1 million eggs each year and a male up to nearly 10 billion sperm (US Army Corps of Engineers® a).

It usually takes four weeks to complete the larval life cycle, during which three different stages are identified (Fig. 2): a veliger stage, a post-veliger stage and a settling stage (Mackie, 1991). This planktonic larval stage (the veliger) is a unique feature of *Dreissena* among freshwater mussels (Garton and Haag, 1991). After attaching to a substrate, the mussel enters the benthic state and lives an epifaunal life until it dies (Mackie, 1991), a feature unusual for freshwater bivalves (USGS a). After one year (~ 9 mm shell length) the mussels are reproductively mature and thus can start contributing to the veliger population (Mackie, 1991).

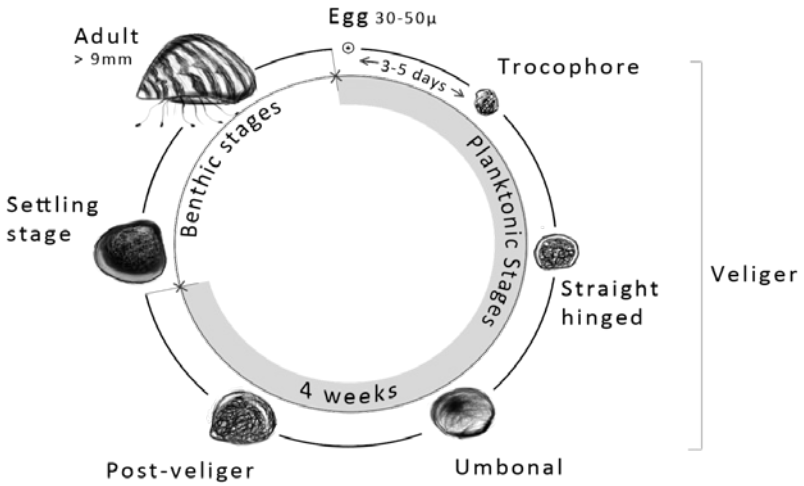

Figure 2. Larval cycle of zebra mussel (after Mackie 1991, US Army Corps of Engineers® and DFW)

2.1.4. Filter-feeding mechanism

Dreissena is a filter-feeder and the gills are the major food collecting organs. These are covered by cilia which create a current of water that aids the collection and transport of particulate material towards the inhalant siphon. The particles are bound in mucus and transported by the frontal cilia to the marginal grooves and from there, via the palps, they reach the mouth (Winter, 1978).

Zebra mussels have a selective filtration mechanism, dependent on particle size. However, the optimal size range varies significantly between different authors: 0.7-1.2 µm (Sprung and Rose, 1988), 15-40 µm (Winkel and Davids, 1982) or 15-200 µm (Gossiaux et al., 1998). Even at low food concentrations, the smaller mussels are able to sustain very high filtration rates, compared to the larger ones (Winter, 1978; Reeders and Bij de Vaate, 1990), which might be explained by a degenerative state of the oldest (and largest) mussels (Reeders and Bij de Vaate, 1990).

In general, *Dreissena*'s filtration rate varies between 5-400 mL/(individual*h) (Ackerman, 1999). This high variability may be due to different factors that affect the filtration efficiency, such as temperature, viscosity, pH, turbidity (Morton, 1971), water velocity, particle type, size and concentration (Lei et al., 1996), resting periods (Elliot, 2008), or particle surface charge (negative charge might antagonize uptake) (Hernroth, 2000).

The rejected particles are agglutinated with mucus and eliminated as pseudofaeces through the inhalant siphon (Reeders et al., 1989; Naddafi et al., 2007; US Army Corps of Engineers® a). Over 90 % of all *Dreissena*'s excrements may consist of pseudofaeces (Reeders and Bij de Vaate, 1992). These will deposit on the bottom of the water body, thus increasing the organic content of the sediments (Burlakova et al., 2011), or they can contain certain rejected but still viable algal species which can be resuspended in the water column and thus return to the phytoplankton population (Naddafi et al., 2007).

2.1.5. Habitat requirements

Dreissena are most abundantly found at depths between 4 - 12 m (Wu et al., 2010) and can tolerate a wide range of temperatures, from -2 to 40 °C. However, the optimal temperature is around 18-20 °C (DAISIE, 2006), with 15-20 °C being the preferred range for byssus thread formation (Rajagopal et al., 1996). Zebra mussels prefer mesotrophic water bodies (DAISIE, 2006) and require alkaline waters, with pH values between 7.2 and 9 (AIS, 2005).

Dreissena is therefore most prolific in hard waters, but can also occur in waters with a Ca^{2+} concentration around 12 mg/L (ISSG). Although they are predominantly found in freshwaters, they can tolerate brackish waters also, as long as the salinity is not above 0.7 % (DAISIE, 2006). However, their feeding rates are an order of magnitude higher in freshwaters than in brackish waters (Lauringson et al., 2007). Another particular feature of *Dreissena* is its incapacity of surviving in Mg^{2+} deficient waters or in deionized water (Dietz et al., 1994).

According to some sources (Alexander and McMahon, 2004), *D. polymorpha* is poorly adapted to hypoxic conditions, while according to others (Reeders and Bij de Vaate, 1992), *Dreissena* is well adapted to low oxygen contents, showing a significant decline in respiration only below 36 % O_2 saturation (DAISIE, 2006). However, if they are acclimated at low temperatures, their oxygen regulation ability is increased. This might be a form of adaptation to the poorly oxygenated conditions created when ice cover is present over their habitat (Alexander and McMahon, 2004).

Although they are not social animals, zebra mussels attach to one another forming clusters called druses (Burlakova et al., 2011). They might even be found in densities greater than $10^5/m^3$ (Horohov et al., 1992). After dying, the empty shells will be accumulated on the bottom creating hard-substratum habitats which can provide shelter for many other species, such as *Gammaridae* or *Trichoptera*. These formations offer efficient protection against predators, strong waves or currents (Burlakova et al., 2011).

2.1.6. Ecological impact

Severe detrimental effects have been observed since *Dreissena*'s invasion to new water environments. Being very prolific animals, they are found in significantly high numbers clustering on the native bivalves (Gu and Mitchell, 2002). This impedes the colonized bivalves (usually Unionids) to fully open in order to perform several metabolic functions such as feeding, reproduction, respiration and excretion or impedes the closing of the valves, which leads to the vulnerability of the Unionids against predators and parasites or bad water quality. Moreover, the high biomass of the zebra mussel disturbs the normal locomotion and burrowing activity of the native bivalves (Mackie, 1991).

Other negative consequences of zebra mussel invasion result from competition for food and space with native bivalves or with other filter-feeding organisms (DAISIE, 2006). By feeding on large amounts of phytoplankton, zebra mussels disturb the trophic chain leading to detrimental impacts on animals situated at higher trophic levels, such as fish (AIS, 2005).

By selectively rejecting *Microcystis* in their pseudofaeces, zebra mussels can promote blooms of these toxic cyanobacteria which lead to severe ecological problems in freshwater bodies subjected to eutrophication (Juhel et al., 2006; AIS, 2005). Contrary to their ability to increase water clarity, zebra

mussels can also impair the quality of water by excreting nutrients and by increasing sediment BOD (James et al., 2000).

Dreissena is responsible for altering the algal species dominance in phytoplankton communities (Juhel et al., 2006; Naddafi, 2007; Wojtal-Frankiewicz and Frankiewicz, 2011) and for causing shifts in lakes' energy balance, from pelagic production to benthic production (Naddafi, 2007; Mackie, 1991).

2.1.7. Economical impact

Dreissena polymorpha is regarded as an important pest in areas highly invaded, being nominated amongst the 'World's Worst' 100 invaders (ISSG). These mussels can cause a lot of problems to power plants (Quinn et al., 2004) or water treatment plants which had to spend, in the recent years, about $30,000 annually to remove them (Connelly et al., 2007). Industrial as well as domestic pipelines can present high infestations with zebra mussels, leading to a significant decrease in their functionality, sometimes even interrupting the flow of drinking water for entire communities (Gu and Mitchell, 2002; NPS, 2007). Moreover, the mussels attach to the hulls of ships and boats, impairing their sailing efficiency (Mackie, 1991), or they damage docks and breakwaters (NPS, 2007).

Despite these, there are also several economical benefits of the zebra mussels, in places where their presence is intended. For instance, their efficiency in removing nutrients (Goedkoop et al., 2011) and suspended matter (Noordhuis et al., 1992) from the water column can be utilized to improve the quality of water. Moreover, zebra mussel shells can be crushed and used as a fertilizer or poultry feed (Birnbaum, 2011).

2.1.8. Environmental monitoring and assessment

Mussels have been used in monitoring programs since the mid 1970's, when the 'Mussel Watch' program was established for assessing the trends in chemical pollution in coastal and estuarine areas (Smolders et al., 2003).

The zebra mussel is particularly easy to use in biomonitoring programs because it is easy to sample, it is highly abundant and widely spread (Noordhuis et al., 1992; Lucy et al., 2008; Minguez et al., 2011a) and due to their moderate sensitivity to anthropogenic pollution, they are able to sustain large populations even in urban areas (Contardo-Jara and Wiegand, 2008). Some studies claim that the mussel filtration bio-assay may be more sensitive than the more widely used acute daphnid test (Kraak et al., 1994), i.e. test no. 202 - OECD Guidelines for the Testing of Chemicals. Their sedentary life provides useful insight of site specific pollution (Voets et al., 2006).

The quality of water can be evaluated by studying the physiological responses of the mussels to pollution. These responses are reflected in the animals' condition status, described by measuring growth, reproduction, survival, condition indices or attachment capability (exposure to pollutants can negatively affect byssogenesis) (Smolders et al., 2002; Moles and Hale, 2004). Acute changes in water quality can also be identified by observing the valve movement of the mussels (Kraak et al., 1994). Dreissena has also been successfully used in freshwaters as bioindicator of endocrine disruption (Quinn et al., 2004) and Sr^{90} contamination (Kinney et al., 1994).

Moreover, due to their tolerance and capacity of accumulating metals and organic pollutants in their tissues, zebra mussels are considered efficient tools for measuring the bioavailability of these substances in the environment (Camusso et al., 1994, Kraak et al., 1994; Bervoets et al., 2005; Riva et

al., 2010). They have also been successfully used in experiments for removing PAH and hexachlorobiphenyl from the water column and decreasing their bioavailability by depositing them as pseudofaeces on the bottom of the water body (Gossiaux et al., 1998).

In addition to its role in detecting chemical pollution, *Dreissena* can also be used for sanitary assessment of water quality, due to its ability to concentrate waterborne pathogens (Lucy et al., 2008) and retain them for several days (Selegean et al., 2001). This feature in particular is the focus of this report.

Besides their utility as biomonitoring organisms, zebra mussels have proven to be important contributors to improving water quality or to sewage sludge treatment (Mackie and Wright, 1994). For instance, they can make on-site water treatment processes much easier and less expensive, if used as biofilters (e.g. for removing chlorophyll) (Elliot, 2008).

Moreover, zebra mussels can be used for improving the quality of water in eutrophied lakes. They have been shown to be able to remove over 90 % of organic matter from the water (Elliot, 2008) and thus, they can decrease water turbidity and, as a consequence, the growth of aquatic macrophytes is stimulated (Reeders and Bij de Vaate, 1990; Mackie and Wright, 1994; Birnbaum, 2011).

2.2. Microbial water monitoring

2.2.1. Water quality

The quality of surface waters as well as groundwater is regulated in Europe by the Water Framework Directive (WFD, 2000/60/EC). While this directive presents well-established criteria for determining the chemical and ecological status of water bodies (Annex V), it lacks however any criteria for assessing the microbiological status of water. Yet, water represents the most important common source of infectious diseases and therefore, providing a good sanitation and control of water microbial quality is a crucial measure for assuring public health (Madigan and Martinko, 2006).

Whether they are washed off from land by rainfall or come from sewage treatment plants (STPs), microbes can have very rapid fluctuations in the receiving waters and even short-term peaks of pathogens can initiate outbreaks of waterborne diseases. An important aspect of these peaks is that, by the time they are actually detected, a significant amount of people may have been exposed to the noxious water (WHO, 2011a).

2.2.2. Faecal pathogens

So far, more than 100 types of faecal pathogens have been identified, which consist of pathogenic bacteria, viruses and parasites (e.g. helminthes or protozoa). These are the main concern in faecally-contaminated environments, such as surface waters receiving effluents from STPs or runoff water from agricultural lands. These pathogenic organisms can cause a wide range of diseases, such as meningitis, gastroenteritis, salmonellosis, hepatitis or dysentery (WHO, 2011a). Diarrheal diseases in particular account for 2.4 million deaths each year and add over 73 million Disability - Adjusted Life

Years (DALY)[1]. On a global level, this makes diarrheal diseases the sixth cause of mortality and the third cause of morbidity (WHO, 2005b).

Faecal pathogens are easily transmitted via the faecal-oral route, mainly due to contaminated drinking water, contaminated food (e.g. bad irrigation practices of vegetables using wastewater or shellfish harvested from contaminated water), recreational activities such as swimming or simply through poor hand hygiene. Thus, knowing the source water quality, in particular the concentration of reference pathogens or indicators, is mandatory for designing water safety plans and for meeting the targets for public health protection (WHO, 2005b).

Salmonella spp.

The *Salmonella* genus is part of the *Enterobacteriaceae* family and comprises 2500 identified serovars, or subspecies (WHO, 2005a). All serovars are pathogenic, with few manifesting a limited host-spectrum. They are gram negative, motile and mesophilic organisms distributed all over the world, usually inhabiting the intestinal tracts of animals. The most common sources of *Salmonella* spp. are poultry eggs, beef, raw milk and water. Besides typhoid and paratyphoid fever, they can cause a wide-spread food-borne disease – salmonellosis - which determines millions of infections each year and even thousands of deaths, posing at the same time a high cost for supporting this public health burden (FAO, 2002; WHO, 2005a; Prescott et al., 2005, Alcamo, 1987).

The incubation time for salmonellosis is between 8 and 72h. After entering the host, bacteria multiply and occupy the intestinal mucosa where they produce an enterotoxin and cytotoxin that damages the epithelial cells. The major symptoms are abdominal pain, diarrhea, nausea, vomiting and fever, which can last up to several weeks (WHO, 2005a). In the acute phase of the disease, the infected humans can excrete up to 1 billion bacterial cells per gram of faeces (Prescott et al., 2005).

In many parts of the world, contamination of shellfish with Salmonella is a problem that originates mainly in the lack of proper sanitation of the water used for aquaculture (Feldhusen, 2000; WHO, 2005a). Thus, considering the fact that bivalves are usually eaten raw or just slightly cooked (Oliveira et al., 2011), the risk of infection is much higher and therefore, systematic microbial surveillance is mandatory for assuring food safety.

2.2.3. Faecal indicators

Indicator organisms present several characteristics that allow them to be reliable options for evaluating the quality of water, despite the fact that no ideal indicator has been determined so far. For instance, indicator bacteria are found in higher numbers than the pathogens and thus are easier to detect, persist in the environment for a longer time, are more resistant to disinfectants and are not pathogenic. Moreover, because it is too expensive and laborious to analyze all pathogens, quantifying indicators gives sufficient information to get an idea about the health risk (FAO, 1994). Furthermore, a frequent examination of water quality using this simple indicator method is more valuable than a more rarely performed examination using a complex test. (WHO, 2011a)

[1] One DALY represents one lost year of "healthy" life (i.e. reference level for the risk of infection). The sum of these DALYs across a population gives the burden of disease (WHO, 2011a)

The coliforms represent the most widely used group of bacteria for identifying microbes of faecal origin. It is considered to be a strong relationship between the number of indicators found and the number of bacterial pathogens existing in the environment. However, weaker correlations can be established between viruses, parasites and these indicators. To this category belongs the most studied and utilized bacterial indicator – *Escherichia coli* (FAO, 1994).

Escherichia coli

The *Escherichia* genus is also part of the *Enterobacteriacee* family and it comprises facultative anaerobic species which are almost exclusively found in the intestines of humans and vertebrates (Von Baum and Marre, 2005), where they might have a nutritional function by synthesizing vitamins, particularly vitamin K (Madigan and Martinko, 2006).

They are rod-shaped, motile or non-motile Gram negative bacteria with a length of about 2 µm and a diameter of 0,5 µm (Singleton, 1997). Some pathogenic strains of *E. coli* are EIEC (enteroinvasive), EHEC/VTEC (enterohaemorrhagic), ETEC (enterotoxigenic), EPEC (enteropathogenic), EAggEC/EAEC (enteroaggregative), which cause severe food-borne diseases (Singleton, 1997; WHO, 2011c).

Due to the high specificity of its habitat, *E. coli* represents the most commonly used indicator organism for detecting faecal contamination (Alcamo, 1987). However, the efficiency of this monitoring method is questionable because not detecting *E. coli* in the studied environment does not assure the absence of enteric pathogens (FAO, 1994; Oliveira et al., 2011).

Furthermore, *E.coli* has a low resistance to adverse physical or chemical conditions, which leads to the necessity of using additional indicators for monitoring water or frozen products (FAO, 1994). One of these indicators, which is able to thrive in poor conditions is *Enterococcus* spp., which is described below.

Enterococci

Enterococci are non-sporulating, non-motile, Gram-positive bacteria with ovoidal shape, having a diameter of about 0.5 µm. They are facultatively anaerobic and live in commensalism in the intestinal tract of humans and other mammals. However, in the last decades they have been increasingly isolated from nosocomial (hospital-acquired) and other infections (Ludwig et al., 2009).

Their ability to adhere to the host's cells and to invade the tissues is a result of the activity of several aggregation substances such as surface carbohydrates, cytolisins, hyaluronase or lipase (Ludwig et al., 2009). Enterococci have the ability to secrete bacteriocins ("enterocins") which are effective against other bacterial groups, as well as other enterococci. *Enterococcus faecalis* presents some of the most well studied enterocins: AS-48, cytolysins $CylL_L$ and $CylL_S$.

The most commonly used as indicators are *Enterococcus faecalis* and *Enterococcus faecium* and their presence in water bodies is generally interpreted as a sign of faecal contamination. This, together with their ability to withstand low water temperatures makes *enterococci* good indicators for water quality (Ludwig et al., 2009). Moreover, in situations where it is important to distinguish between human or animal induced faecal pollution, *Enterococcus* spp. might a useful tool (although not very discriminative – Blanch et al., 2006), as it has been shown that this bacteria prevails in the faeces of warm-blooded animals, whereas faecal coliforms are found more abundantly in human faeces (Baudišová, 2009). The ability of *Enterococcus* spp. to tolerate the conditions inside the mussels'

digestive tract makes it a suitable indicator for detecting faecal pollution (De Mesquita et al. 1990) and therefore it has been chosen for this study.

2.3. Antibiotics in water bodies and their implications

Antibiotics represent molecules that have an antagonistic effect upon bacteria and fungi, either by stopping their growth or by killing them. While most of the antibiotics used for treating infectious diseases are natural products (produced either by bacteria or fungi), synthetic chemicals have also been developed and used since the 1930s (the sulfa drugs), 1960s (the quinolones) and most recently (2000), oxazolidinone (Walsh, 2003). Globally, an estimated 172.4 million kg of antibiotics were produced in 2006, with approximately 68 million kg used in animal husbandry (Rysz, 2007).

The presence of antibiotics in water, even at very low concentrations, has raised concerns among drinking-water regulators, governments, water suppliers and the public, regarding the potential risks to human health (WHO, 2011b) and the environment.

While some subsets of bacteria have been producing antibiotics for hundreds of millions of years, the ''attacked'' bacteria were under the evolutionary pressure of developing resistance mechanisms in order to survive. Because bacterial populations contain large numbers of cells and their generation times are short, the development of mutants is easily encouraged (Walsh, 2003).

Bacteria can acquire resistant genes through *de novo* mutations, vertical gene transfer – when daughter cells inherit the parent genetic material (Rysz, 2007) or through horizontal gene transfer. The latter occurs via several paths: cell-to-cell conjugation, transduction mediated by phages (viruses) or transformation by free DNA which is released by dead cells (Rysz, 2007; Andersson and Hughes, 2010). This spread of resistant genes is influenced by several environmental factors such as spatial separation between bacteria, their capacity to adsorb to particles, temperature, pH, nutrient availability and the presence of other stressors such as heavy metals (Rysz, 2007; Merlin et al., 2011).

The molecular mechanisms of antibiotic resistance in bacteria involve the inactivation of the drug or its decreased accumulation in the cell, the modification of the target site or the overproduction of competitive metabolites (Karlsson, 2001; Andersson and Hughes, 2010). However, a detailed description of these mechanisms is not part of this thesis' topic.

Regardless of their use for treating infections in humans, as food additives in farming or in agriculture, antibiotics end up in the water (Costanzo et al., 2005) and it is a matter of months, or sometimes years until the pathogens develop resistance mechanisms. Thus, the more widely used the antibiotic, the more probable the resistance is (Walsh, 2003). In addition, the unregulated use of cheap, low quality and easily available antibiotics or their misuse by health professionals are social factors which enhance the incidence of resistant genes in developing countries (Okeke et al., 1999; Ojo et al., 2008). This has severe consequences on human health, in particular, because it limits the efficiency of the treatments required for many infections (White, 2002; Merlin et al., 2011). Moreover, even if so far it has received less attention, antibiotics impact biodiversity to a certain extent, by altering the microsphere and by representing the cause of antibiotic resistant gene pollution (Martinez, 2009).

3. Materials and Methods

3.1. Laboratory experiments

3.1.1. Zebra mussel collection and acclimatisation

Some 600 zebra mussels (16.2 ± 0.47 mm shell length) were collected from Lake Erken, Sweden, in December 2011 and transported to Uppsala in cooling boxes, together with 60 L of lake water for supplying the aquarium. The zebra mussels were acclimatized for 21 days. During the first week they were kept at 5 °C and then the temperature was gradually increased with one degree per day. Along the whole acclimatization period the mussels' aquarium was permanently aerated and the lake water was changed every 4th day during the first week and every 3rd day in the last two. The mussels were fed with pulverized Tetraphyll™ (Tetra) every 4th day and not fed at all during the week before the start of the experiments. During the last 5 days of acclimatization the aquarium water was gradually replaced with artificial lake water (M4): first 50%, after 48 h 75 % and after another 48 h, 100 % M4 was used. This standardized artificial lake water was prepared according to OEDC Guideline 202.

All acclimatization water was treated with 3 % citric acid before being discarded, in order to avoid the spread of potential veligers in the Fyris river. The same concentration of citric acid was used to disinfect all aquarium water containing bacteria, before discarding it.

All experiments were performed in triplicates. At each sampling occasion, only mussels that showed active filtering were taken out of the aquaria for analysis. All the aquaria, beakers, nets and aeration hoses were sterilized prior to the start of each experiment (i.e. the beakers and hoses were autoclaved at 121 °C and the aquaria and the stainless-steel nets were dry-sterilized at 120 °C for 2 h). The temperature (17.5 ± 0.5 °C) and photoperiod (16:8) were kept constant throughout all the experiments.

3.1.2. Experiment 1: Bacteria uptake

25 L glass aquaria were filled with 10 L of artificial lake water. Stainless steel nets were placed in each of the three aquariums in order to avoid the exposure of zebra mussels to their (pseudo)faeces (Fig. 3a,b). Approximately 32 g mussels (35-38 individuals with a shell length of 16.38 ± 2.1 mm) were rinsed with deionized water and placed in each aquarium. Aeration was provided during the whole experiment (Fig. 3b).

Figure 3a. Design of the uptake experiments, representing the three replicate aquaria

Figure 3b. View of the zebra mussels placed on the stainless-steel nets and provided with aeration stones

Escherichia coli (SLV 082) and *Enterococcus faecalis* (SLV 051) were cultured overnight at 37 °C and then diluted 10 times. One milliliter of each bacterial suspension was added to the aquaria, thus giving a concentration of 10^4 CFU (colony forming units)/mL water. The mussels were exposed to this concentration for 60 h, during which samples consisting of 2.58 ± 0.28 g mussels were taken at times: 0, 2, 4, 6, 8, 12, 24, 48 and 60 h. The samples taken at time 0 (before adding the bacteria in the aquaria) served as control. Water was sampled from the replicates at the same time points as the mussels.

Bacteria were cultured following the methods mentioned in Table 1, in all the laboratory and field experiments.

Table 1. Methods for isolating bacteria from water and mussel samples, used in all the laboratory and field experiments

Indicator	**Sample**	**Method**
E. coli	Water	ISO 9308-1
	Mussels	Cultivation on Mac Conkey agar, 44 °C, 24 h
Enterococcus spp.	Water	ISO 7899-2
	Mussels	Nordic Committee on Food Analysis No. 68, 5th ed. (2011)

The zebra mussels were entirely homogenized (i.e. including shells) and diluted 10:1 with neutralized bacteriological peptone water (SPW), produced at BVF [2]. The homogenate was further serially diluted for spreading 1 mL and 100 µL from each dilution on SLABA plates (Slanetz and Bartley, PO5018A, Oxoid, Malmö, Sweden) for enumerating *Enterococcus* and on Mac Conkey plates (CM00115, SVA, Uppsala, Sweden) for *E. coli*. All samples were incubated at 44 °C for 24 h (*E. coli*) and 48 h for *Enterococcus* spp. After the incubation period, the colonies grown were counted and expressed as CFU per gram mussel.

Water samples were serially diluted with SPW and filtered using MicroFunnel™ 0.45 µm filter funnels (PALL Corporation). The filter membranes were then placed on agar plates (SLABA CM0377 for

[2] Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, Uppsala, Sweden

Enterococcus and Lactose TTC with tergitol-7, 54232, for *E.coli,* both prepared at BFV) and incubated as previously described. After the incubation period the colonies formed were counted where possible, or otherwise estimated and expressed as cfu/mL water. Mussel samples were processed within 1.5 h after sampling. Water samples were kept at 4 °C and analyzed within 12 h.

As shown in figure 4, typical colonies of *E. coli* grown on Mac Conkey agar are red with bile precipitation and those grown on lactose TTC agar are light orange-yellow. Typical *Enterococcus* colonies are raised and have a red, brown or pink colour (in the center of the colony or throughout).

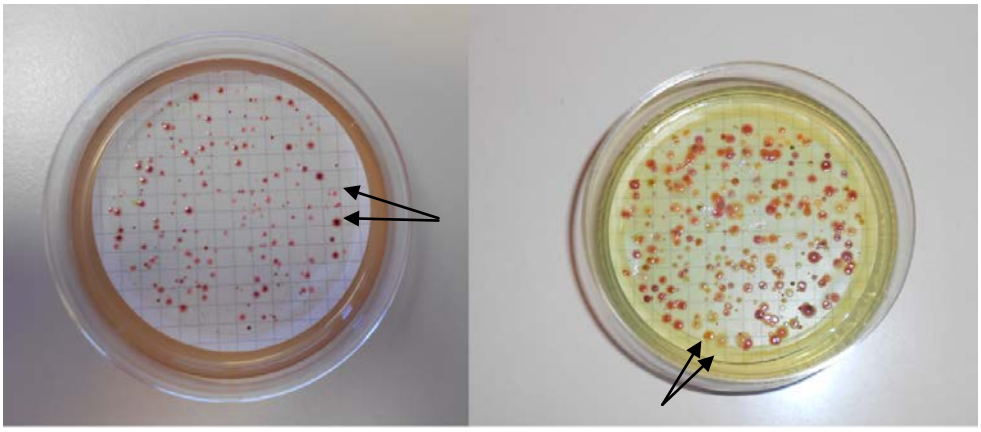

Figure 4. Plates showing bacteria isolated from water samples: *Enterococcus faecalis* (left) and *E. coli* (right); typical colonies are indicated by arrows

3.1.3. Experiment 2: Bacteria elimination

Each of the three replicates contained 32 g (between 32-39 individuals) of zebra mussels that were placed in 10 L of artificial lake water in the same type of aquaria as in the previous experiment. The mussels were exposed to 10^4 *E. coli* and *Enterococcus* per mL for 8 h, after which approximately 3 g (3.08 ± 0.88 g) of mussels from each aquarium were placed into eight[3] 1 L beakers containing 700 mL of M4 (Fig. 5). The beakers were provided with nylon nets in order to allow the mussels to be separated from their faeces and were positioned on a stirring table. The whole depuration experiment lasted 60 h, during which the mussel and water samples were analyzed every 12 h. The mussels were prepared as previously described and the water samples included the faeces this time.

Figure 5. Scheme showing the depuration beakers

[3] Only the first 5 samples were analyzed because towards the end of the experiment the mussels stopped filtering.

3.1.4. Experiment 3: Bacteria uptake from sewage treatment plant effluent

Sewage effluent water was collected one hour before the start of the experiment, from Uppsala waste water treatment plant, Kungsängsverket. The sewage effluent was diluted 10 times with oxygenated M4 in 25 L glass aquariums. Approximately 33 g mussels (37-44 individuals with an average shell length of 16.84 ± 1.54 mm) were rinsed with deionized water and placed in each of the three replicate aquaria. Water (200 mL) and mussels (3.26 ± 0.92 g) were sampled at times: 0, 2, 4, 6, 8, 12, 24, 36 and 48 h after exposure. The mussel and water samples were analyzed following the same procedure as in the first experiment. However, bacterial colonies that differed in colour, shape or texture from the typical ones needed further confirmation by cultivation on blood agar at 37 °C for 24 h and by performing biochemical tests – API 20E (Biomérieux) for *E. coli* and sorbose tests for *Enterococcus faecalis*. One independent colony from each sample was dispersed in 1.1 mL BHI (Brain Heart Infusion, BVF) with 17 % glycerol and frozen at -70 °C for antibiotic resistance testing.

The antibiotic resistance was tested using VetMic™ GN-mo and VetMic™ E-cocci microdilution panels (SVA). Bacteria from the frozen samples were cultured on blood agar at 37 °C overnight. Three to five colonies were suspened in 5 mL Mueller Hinton broth (CAMHB) and incubated for 3 hours at 37 °C, diluted 1000 times before 50 µL were placed into each of the 96 wells of the VetMic plates, which were then covered with tape and incubated at 37 °C for 16-18 h. The antibiotics against which resistance was tested in both bacteria are presented in Table 2. After the incubation period, the wells showing visible growth above a certain threshold concentration were considered to contain resistant bacteria. Isolates resistant to 3 or more antibiotics were classified as multi drug resistant (MDR).

Table 2. Antibiotics contained in the VetMic plates and their cut-off values

E. coli	**Cut-off value (µg/ml)**	***Enterococcus* spp.**	**Cut-off value (µg/ml)**	***E. coli* and *Enterococcus* spp.**	**Cut-off value (µg/ml)[4]**	
Sulfamethoxazole	>256	Bacitracin	>32 U/ml	Kanamycin	>8	>1024
Nalidixic acic	>16	Virginiamycin	>32	Streptomycin	>16	>512
Florfenicol	>16	Erythromycin	>4	Chloramphenicol	>16	>32
Trimethoprim	>2	Vancomycin	>4	Gentamicin	>2	>32
Colistin	>2	Linezolid	>4	Tetracycline	>8	>4
Ceftazidime	>0.5	Narasin	>2	Ampicillin	>8	>4
Cefotaxime	>0.25					
Ciprofloxacin	>0.06					

[4] The first column contains the values for *E. coli* and the second for enterococci.

3.1.5. Experiment 4: Bacteria elimination

Sewage effluent water was sampled 30 minutes before the start of the exposure and diluted tenfold with oxygenated M4. Between 59-79 zebra mussels (~41 g) were placed in the same glass aquariums (which have been previously sterilized) and were exposed to the diluted effluent water for 2 h. At the end of the exposure phase, the mussels from each replicate were rinsed with deionized water and divided into 7 samples placed on nets in beakers containing 700 mL clean artificial lake water. Mussels (4.6 ± 1.12 g) and water (200 mL including faeces) were sampled after 2, 4, 6, 12, 24, 36 and 48 h of depuration. The bacteria concentrations were determined according to the same method as in all the previous experiments.

3.2. Field experiment

3.2.1. Zebra mussel collection and depuration

Zebra mussels (shell length 20.82 ± 3.10 mm) were sampled on the 23rd of April from Lake Ekoln, Sweden, at a depth of approximately 0.5 m. They were washed from mud and debris and kept in filtered lake water for 48 h, after which the water was replaced with sterilized lake water. The mussels were kept in a climate room with a photoperiod of 16:8 and were fed Tetraphyll^{TM} every 3rd day, after changing the water in the aquarium. Aeration was provided throughout the depuration period. An initial analysis of 8 mussels showed no *E. coli* and 180 cfu/g *Enterococcus* spp. Thus, the mussels were suitable for the field experiment after 9 days of depuration, when no *E. coli* and no *Enterococcus* spp. were found in 7.6 g of mussels.

3.2.2. Experimental design

Zebra mussels were placed in metal cages (10x10x10 cm) and positioned in the Fyris river at a depth of 0.5 m at 6 sites upstream and downstream from the source of faecal contamination, i.e. the outlet of a sewage treatment plant which treats approximately 20 million m^3 of wastewater yearly (uppsalavatten.se). Site 1 was located 120 m upstream from the STP outlet, site 2 was right at the outlet, site 3 was 200 m downstream from the outlet and sites 4, 5 and 6 were at 500, 1000 and 1,500 m downstream from the outlet (Fig 6). Each site had two replicate cages, with approximately 90 g of mussels each.

Figure 6. Map showing the location of the experimental sites along the Fyris river

Water (200 mL) and mussels (6.74 ± 1.18 g) were sampled for determining the concentrations of faecal indicators after 1, 2, 5, 7, 12 and 19 days of exposure.

Samples for analyzing *Salmonella* spp. were taken at three occasions: days 5, 12 and 19. Water samples (300 mL) were filtered (0.45 µm) and the filter membranes were immersed into 50 mL of buffered peptone water (BPW) and incubated at 37 °C overnight, according to ISO 19250.

Salmonella spp. in mussels were analyzed according to the Nordic Committee of Food Analysis No. 187 (2007). Approximately 25 g of mussels were homogenized and diluted tenfold with BPW and incubated in the same way as the water, after which 100 µL of all the samples (water and mussel homogenates) were dropped onto MSRV plates (Modified Semi-solid Rappaport Vassiliadis medium) and incubated for 24 h at 42 °C. The plates showing visible growth needed further confirmation by cultivating on brilliant green - BG and xylose lysine desoxycholate - XLD agars (SVA) for 24 h at 37 °C. A positive XLD plate shows black colonies surrounded by a pink-red zone and BG shows pink colonies (Fig. 7).

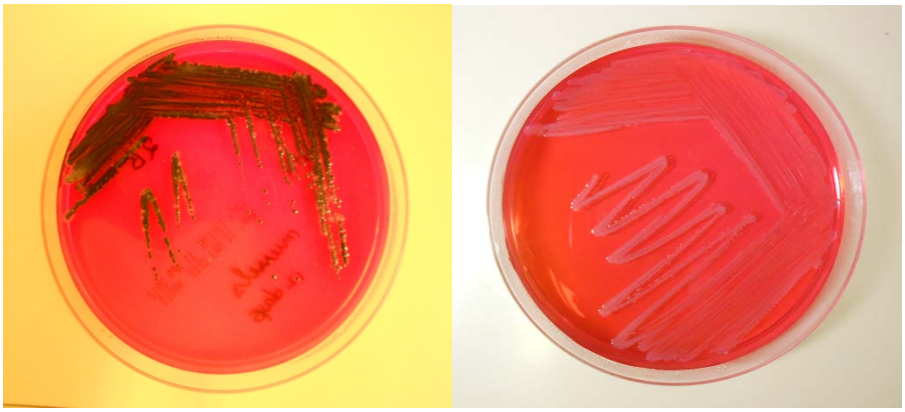

Figure 7. Positive *Salmonella,* detected in mussels: XLD agar (left) and BG agar (right).

On every sampling occasion, water temperature and pH were measured at each site. Flow data was available from the Swedish Meteorological and Hydrological Institute. Antibiotic resistance was tested for *Salmonella* spp., *E. coli* (the first four samples) and *Enterococcus* spp. (samples taken after 1 and 5 days of exposure), using the same method as in the third laboratory experiment.

3.3. Data analysis

All statistical analysis was performed using JMP software version 9 (SAS Institute Inc). One-tail paired t-tests were applied to assess if the concentration of bacteria in the mussels was significantly higher than the one in water ($\alpha = 0.025$). A one-way ANOVA was used in all occasions for testing the difference between replicates ($\alpha = 0.05$). In the field experiment, one-way ANOVA was used to test if there is a significant difference in the amount of bacteria in both mussels and water between sites and sampling occasions. A stepwise regression analysis using a p-value threshold of 0.05 was performed for identifying, at each site, the factors that influence the concentration of indicators found in mussels and water.

4. Results

No significant difference was found between the resplicates, as regards the concentration of bacteria in both mussels and water. All experiments have shown a significant ($p < 0.025$) capacity of the mussels to concentrate bacteria from the water. On several occasions the indicators were below the detection limit of 10 cfu/g or mL and thus, those values were replaced with 1.

4.1. Experiment 1: Bacteria uptake

The zebra mussels showed a high capacity of concentrating and eliminating *Enterococcus* spp. in all laboratory experiments.

The uptake experiment showed, on average, a concentration of *E. coli* about 5 times greater in the mussels than in the water, whereas the concentration of *Enterococcus faecalis* was almost 99 times greater in the mussels than in the water (Fig. 8). The zebra mussels showed a maximum uptake of *E. coli* after 6 h of exposure and of *E. faecalis* after 2 h, followed by a decline in the filtration rate towards the end of the experiment (Fig. 8).

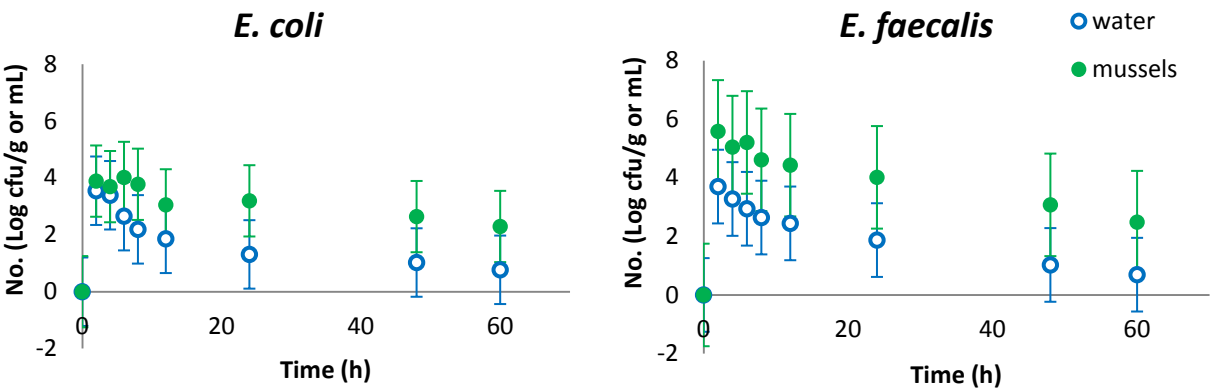

Figure 8. Uptake of *E. coli* and *Enterococcus faecalis* by zebra mussels during 60 h. Each point represents an average Between the three replicate aquaria. Error bars indicate the standard deviation

4.2. Experiment 2: Bacteria elimination

After 8 h of exposure, during which the mussels had accumulated 8.8 x 10^3 and 3 x 10^5 cfu/g *E. coli* and *E. faecalis*, respectively, the depuration phase showed a rapid clearence of the mussels during the first 12 h, when the bacteria concentrations had decreased 40 and 60 times, respectively (Fig. 9). After 60 h of depuration, 99.6 % and 99.9 % of the initial amount of *E.coli* and *Enterococcus* were eliminated from the mussels' bodies (i.e. 2.34 and 2.91 log reduction, respectively) (Fig. 9).

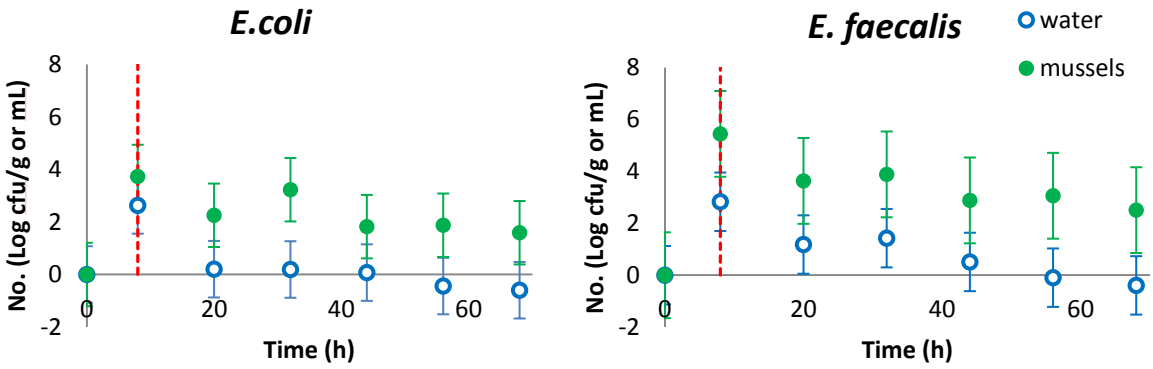

Figure 9. Depuration of zebra mussels in still water (M4), after 8 h of exposure to faecal indicators; the dotted line marks the end of exposure

4.3. Experiment 3: Bacteria uptake from sewage treatment plant effluent

When exposed to sewage effluent water, the zebra mussels concentrated *E. coli* 8 fold and *Enterococcus* spp. 37 fold, as compared to their concentrations in the surrounding water (Fig. 10). The uptake of both indicators was maximal within the first 2 h of exposure to the effluent water and decreased until a steady-state was reached for *Enterococcus* spp. and a slight increase was observed for *E.coli.*

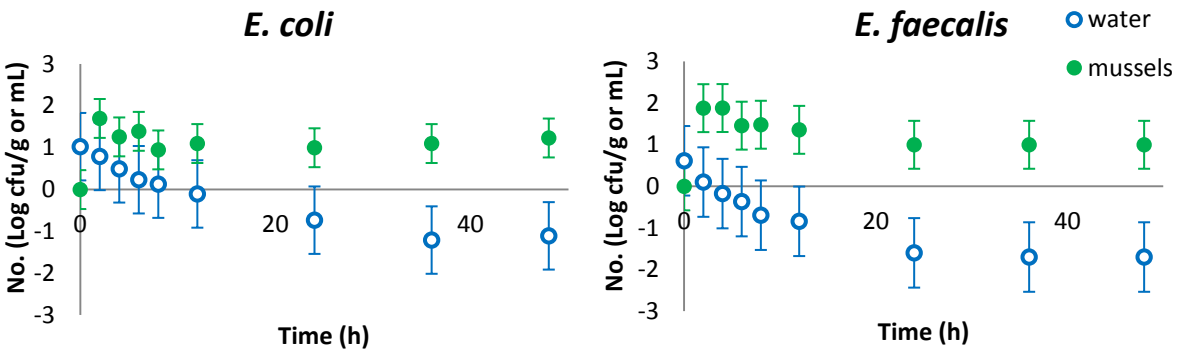

Figure 10. Zebra mussels' uptake of faecal indicators from sewage effluent

4.4. Experiment 4: Bacteria elimination

The depuration experiment lead to a 83 % decrease in the concentration of *E. coli* and 96.4 % in that of enterococci in the mussels (i.e. 0.78 and 1.45 log reduction, respectively)(Fig. 11).

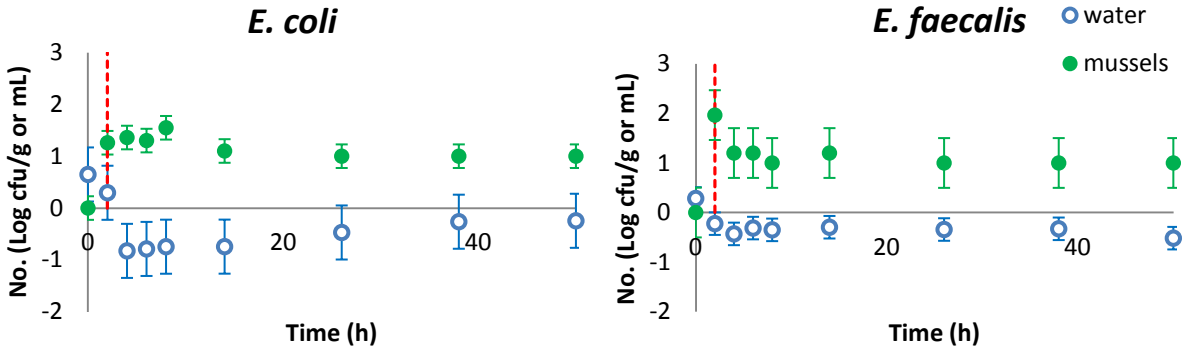

Figure 11. Depuration of zebra mussels in still water (M4), after 2 h exposure to sewage effluent; the dotted line marks the end of exposure

4.5. Field experiment

The transplanted mussels reached their maximum concentration of *E. coli* during the first 24 h of exposure to the river water and the maximum of enterococci after 5 days of exposure. The concentration of bacteria in both mussels and water decreased over time at all sites. However, *E. coli* concentrations in the mussels peaked in the second day of sampling at sites 4 and 6, in the fifth day at site 5 and in the seventh day at site 4 (Fig. 12a). Peaks of enterococci in mussels were observed at

site 4 in the first, second, seventh and twelfth sampling day and at site 6 in the fifth and seventh day (Fig. 12b).

During the six sampling occasions, water temperature varied between 13.9 and 15 °C at the outlet and 10.9 – 13.1 °C at the other sites. The water at site 2 had the lowest pH at all occasions, varying between 6.64 and 7.87, while pH at the other sites varied between 7.65 – 8.11. At two occasions (i.e. after 5 and 12 days of exposure) the water turbidity was increased and a higher number of dead mussels were found in the cages.

At all occasions, the concentration of faecal indicators was significantly higher ($p < 0.05$) at site 2 (the STP outlet). On average, zebra mussels contained twice more *E. coli* than the water and four times more enterococci; the concentrations of these two indicators found in mussels were very similar.

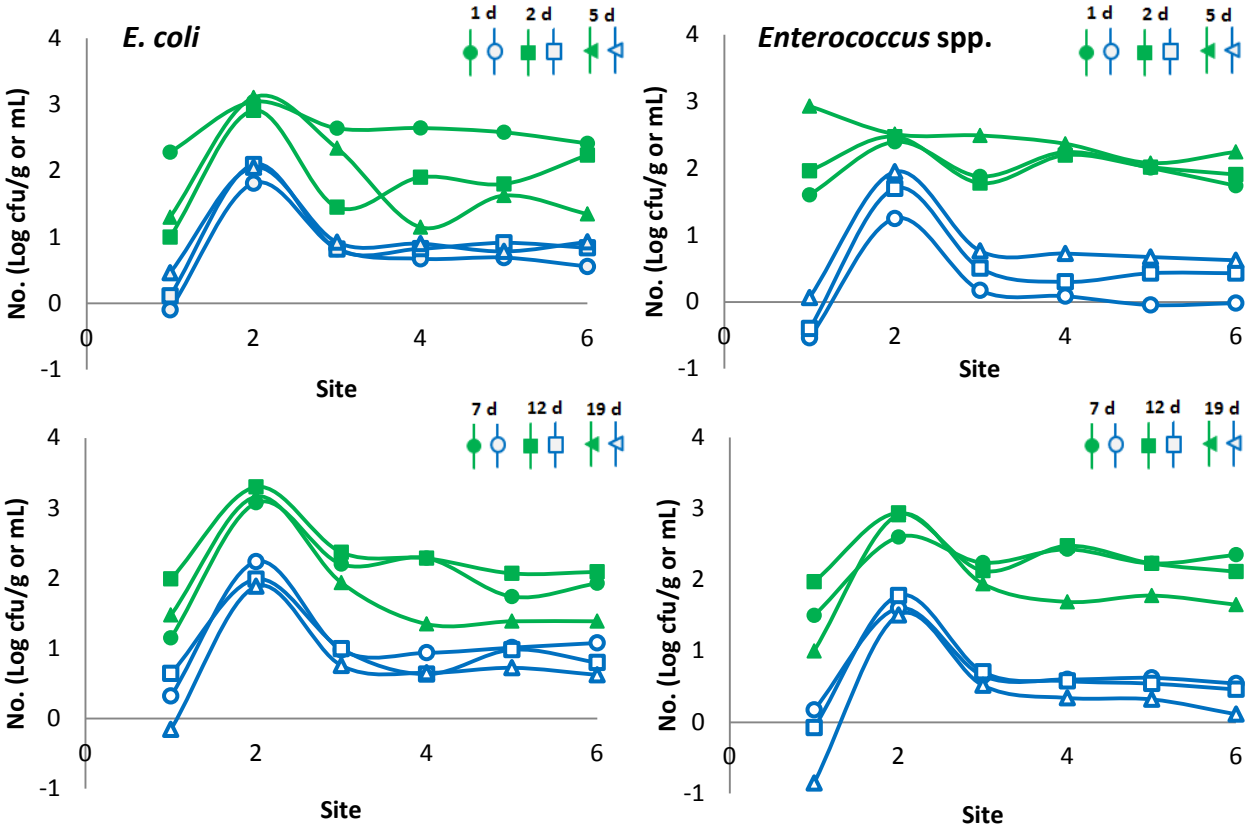

Figure 12ab. Concentrations of faecal indicators in water (empty bullets) and mussels in the Fyris river during 19 days of exposure. Site 1 is the upstream location, site 2 represents the outlet and the rest are downstream sites. Each point for mussels is an average between the two replicate cages. The lines connecting the markers are meant to make it easier for the reader to spot the peaks (i.e. the lines do not represent a model of the uptake)

The stepwise regression showed good relationships between pH, temperature and the concentrations of faecal indicators in the mussels and water (Table 3). In addition, the amount of indicators in the water was influenced by the rainfall events and, consequently, flow and sampling times.

Table 3. Parameters affecting the concentration of indicators at each of the 6 sites (Stepwise regression, α = 0.05)

	Site	1	2	3	4	5	6
Mussels	***E. coli***				temperature pH	pH	pH flow
	***Enterococcus* spp.**	temperature	time		temperature	temperature	temperature time
Water	***E. coli***	temperature pH rainfall flow time	rainfall	temperature time rainfall flow	temperature flow time	rainfall	temperature rainfall
	***Enterococcus* spp.**	temperature pH rainfall flow time		pH rainfall flow time			

Out of the 42 samples analyzed for Salmonella, 9 were positive. Salmonella was confirmed for both mussels and water collected from the outlet on all three occasions. In addition, Salmonella was detected in water samples taken from sites 4 and 3 after 12 and 19 days, respectively.

4.6. Antibiotic resistance

The antibiotic resistance analysis performed for the third laboratory experiment showed that *E. coli* colonies isolated from both mussels and water were more resistant than *Enterococcus faecalis* colonies: resistance against at least one antibiotic was found in 100 % of the *E. coli* samples and in 43.6 % of *E. faecalis* samples.

As regards *E. coli*, these were most resistant against sulfamethoxazole: 100 % of the mussel samples and 96.3 % of the water samples (Fig. 13a). Multi drug resistant (MDR) colonies were found in both mussels (73.3 %) and water (51.9 %) (Fig. 13b).

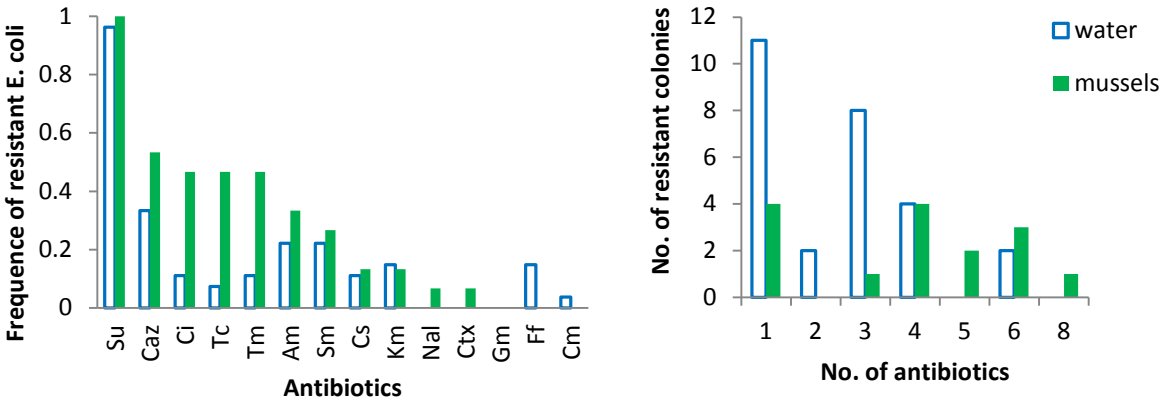

Figure 13ab. Cases of resistance and multi-drug resistance among *E. coli* colonies isolated from water and mussel samples

Enterococcus spp. showed a high sensitivity to antibiotics: 56.3 % of those isolated from mussels and 56.5 % from water. However, MDRs were also found: 18.8 % and 4.4 % from mussels and water, respectively (Fig. 14b). Overall, most of the colonies were resistant against tetracycline and erythromycin (Fig. 14a).

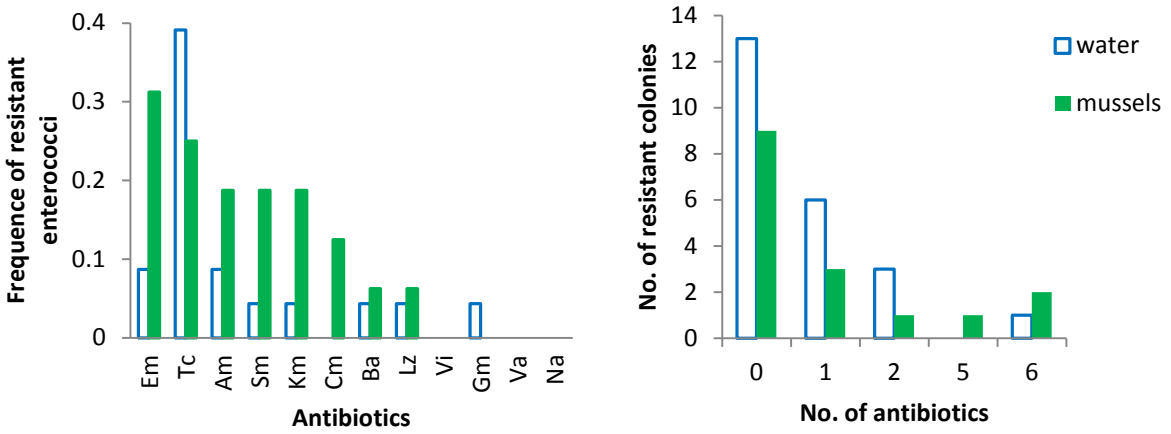

Figure 14ab. Cases of resistance and multi-drug resistance among *Enterococcus* spp. colonies isolated from water and mussel samples

In the field experiment, a very similar number of MDR colonies were found in both water and mussels, as were found in the lab experiment. As before, MDRs prevailed in the mussels samples more than in water samples: 65.2 % (mussels) and 50 % (water) for *E. coli* (Fig. 15b) and 25 % for enterococci isolated from mussels; no MDR enterococci were found in water (Fig. 16b).

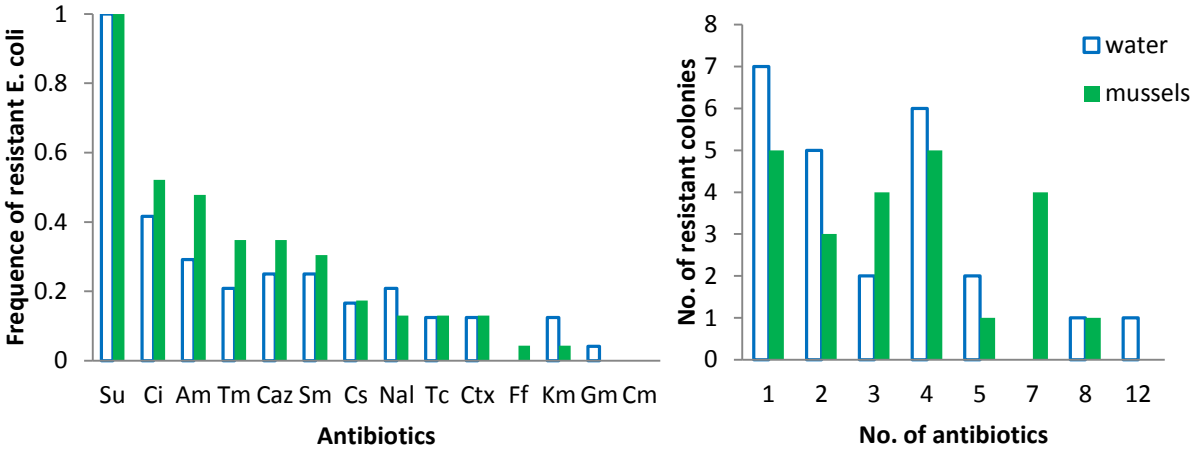

Figure 15ab. Frequence of resistant colonies and occurrence of MDR *E. coli* isolated from water and mussels

All *E. coli* colonies were resistant to at least one antibiotic (sulfamethoxazole). However, all *E. coli* were sensitive to chloramphenicol (Fig. 15a). In addition, all *E. coli* isolated from water were sensitive to florfenicol and those isolated from mussels were 100 % sensitive to gentamicin (Fig. 15a).

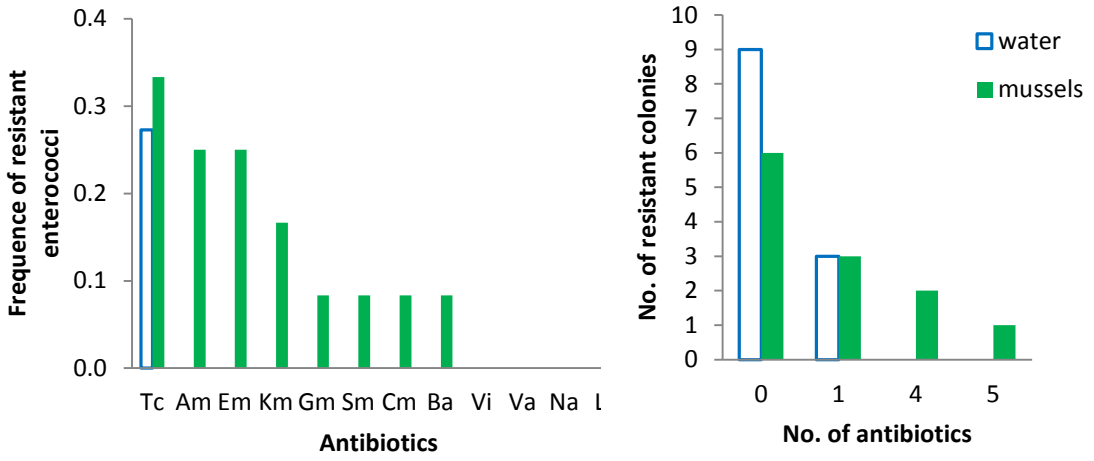

Figure 16ab. Frequence of resistant colonies and occurrence of MDR *Enterococcus* spp. isolated from water and mussels

The highest number of resistant cases for *Enterococcus* was against tetracycline: 33.3 % of the mussel samples (Fig. 16a). Enterococci isolated from water samples were resistant only to tetracycline (25 %) (Fig. 16a), whereas 50 % of those isolated from mussels were resistant to at least one antibiotic.

Overall (i.e. mussel and water samples), the upstream site contained the highest number of resistant and MDR *E. coli*, followed by sites 2 and 5. The highest prevalence of resistant *Enterococcus* spp. was at sites 2, 6 and 4.

All *Samonella* spp. found were resistant to sulfamethoxazole. In addition, one colony isolated from water collected at the outlet after 19 days exposure was resistant to gentamycin. No MDR *Salmonella* spp. were detected.

5. Discussion

The main purpose of this thesis was to study the ability of zebra mussels to accumulate bacteria from water and to observe the rate at which the gut is cleared from bacteria, particularly faecal indicators. All laboratory and field experiments have confirmed the initial hypothesis that zebra mussels are able to concentrate bacteria from water and the suitability of the mussels for biomonitoring is further on discussed.

Uptake

Zebra mussels showed a maximal uptake during the first hours of exposure to bacteria. This was followed by a decrease in the filtration rate after 48 h in experiment 1, but a rather constant rate was kept in experiment 3. This might be because the initial concentration in experiment 1 was much higher than that used in experiment 3, which might have irritated the mussels' tactile receptors, determining them to slow down their filtration activity (Morton, 1971).

During the four laboratory experiments, the uptake of *Enterococcus* by zebra mussels was between 1.4 - 34 times higher than the uptake of *E. coli*, which might be a consequence of the stronger adherence to the mussels' tissues due to several aggregation substances produced by enterococci or they outcompeted *E. coli* through secretions of bacteriocins (Ludwig et al., 2009).

In the **field experiment**, more variables contributed to the concentration of indicators found in water and in mussels, than in the laboratory experiments. Variations in water temperature, pH, rainfall events, the distance to the source of contamination and the exposure time can influence the uptake of bacteria to a certain extent. In particular, it seems that such environmental parameters affect more the concentration of *E. coli* in the *water*, than the amount taken up by *mussels*; a similar situation was found for *Enterococcus* spp.(this is according to the relationships given by the regression analysis; this data can be found in the annex). This might indicate there could be more chances of detecting a more ''accurate'' concentration of indicators by sampling the mussels, instead of the water.

The amount of indicators in the mussels seems to vary more in time, compared to the amount in water. This could be explained by the continuous discharge of STP effluent into the river, whereas zebra mussels filter only about half of the time (Morton, 1971). However, the mussels are able to detect bacterial peaks and provide evidence of faecal pollution. For instance, no sign of contamination would have been seen if only water would have been sampled at any of the five events in this study when peaks actually occurred (Fig. 12ab).

Out of the 10 peaks of bacteria that occurred in the 5 occasions mentioned, only two corresponded to both faecal indicators. This might imply that the use of only one indicator for assessing shellfish sanitary quality is not reliable enough, as was previously shown by De Mesquita et al. (1990).

The results of this study are in accordance with the work of other authors (Selegean et al., 2011) and show the ability of zebra mussels to retain the indicators even 2 days after the peaks and demonstrate the utility of *Dreissena polymorpha* as a tool for detecting these elevated concentrations of bacteria that would have been otherwise missed by conventional water analysis. Thus, sampling mussels every 2^{nd} or 3^{rd} day could provide a more cost-efficient plan for detecting faecal pollution.

Moreover, the zebra mussels were able to detect elevated concentrations of bacteria that were not necessarily related to rainfall events, which implies that other sources of contamination have to be considered and suggests the utility of the mussels for microbial source-tracking (i.e. for distinguishing between human and animal faecal pollution).

On four occassions mussels from one of the replicate cages at each site contained higher concentrations of bacteria than mussels from the other cage (although overall, these differences were not statistically significant). The cause of these large fluctuations is not known (e.g. like the situation at site 1, in the 5^{th} day of sampling, when the number of enterococci in cage B was 90 times higher than in cage A). It is possible that sample B contained one or several abnormally contaminated mussels. However, mussels from different sites might vary in their filtration efficiencies (Selegean et al., 2001), which should be considered for a strategic placement in a monitoring program.

Depuration

Similar to the uptake, the elimination of bacteria was maximal during the first hours after placement of the mussels in clean water. The first depuration experiment was not possible to continue until the mussels were completely clean because after 60 h in the same still water, noxious conditions were probably created, which determined the mussels to close their valves and stop filtering. However, such high faecal indicator bacteria concentrations as used in the first two experiments are not likely

to be found under natural conditions. Moreover, studies show that depuration of mussels should not be done for more than 48 h because this process might affect their palatability (Oliveira et al., 2011). What is important from a biomonitoring perspective is the ability of the zebra mussels to keep the bacteria in their bodies even after short exposures and especially after filtering clean water for 60 and 48 h, respectively.

Rather unexpected was to find more variation in the depuration rate of the mussels during experiment 2, which contained standardized lake water (M4) and only two bacteria species, than in experiment 4 where the mussels were exposed to a cocktail of bacteria and other substances from the sewage treatment plant effluent. However, this might be explained by the longer exposure time and higher faecal indicator concentrations used in experiment 2. After 6 h of being in clean water, the mussels in experiment 4 kept their filtration rate rather constant, whereas those in experiment 2 seemed to filter more actively every 12 h.

Antibiotic resistance

In all experiments, the prevalence of resistant and multi drug resistant bacteria was much higher in mussels than in water. Although the literature for comparison is very limited, this finding can be considered quite similar to the results obtained by Cooke (1976), who showed that the percentage of resistant faecal coliforms isolated from oysters was higher than that from the surrounding seawater. This high occurrence of antibiotic resistant bacteria in the mussels is an important discovery because it suggests that sampling mussels could be more reliable for detecting resistant microbes, than sampling water. Furthermore, because the occurrence of resistant bacteria is usually related to the presence of antibiotics in the environment, this might indicate that analyzing mussels can be used as an alternative to the expensive methods for detecting antibiotics. Moreover, it is interesting to see if there is any selective pressure in the mussels, which might determine the higher prevalence of antibiotic resistant bacteria in their bodies than in the water. Thus, further studies are needed to analyze the concentration of antibiotics in the tissues of the mussels exposed to sewage effluent and to compare them with the minimum inhibitory concentrations (MIC). However, Gullberg et al. (2011) showed that resistance can occur even at concentrations below MIC and therefore, mussels containing even traces of antibiotics might serve as reservoirs for resistance.

The main paths through which antibiotics reach surface waters are via runoff from farms, landfills and from STP effluents. Yet, in this study, no spatial gradient was observed and thus, the presence of the STP outlet did not seem to impact the prevalence of resistant bacteria, because more of these were found at other sites (e.g. sites 1 and 5 for *E. coli*).

Nevertheless, finding such high numbers of resistant bacteria in the Fyris river is a serious concern for human health and it emphasizes the necessity to prioritize this emerging problem in the context of water safety management. In addition, the higher occurrence of antibiotic resistant and MDR bacteria in the mussels is a food safety issue that requires a better control of the sanitary quality of shellfish. Besides raising awareness for the human health risk, these observations are important signals of the degree of alteration of aquatic ecosystems by anthropogenic action (Baquero et al., 2008).

Limitations

Using zebra mussels in biomonitoring programs can pose some practical limitations. For instance, they can only be used in water bodies which are already populated by zebra mussels, because transplanting them to new sites is conflicting with the activity of power plants, water treatment plants or other stakeholders. Moreover, caution must be taken for avoiding the spread of veligers in unpopulated sites when using caged mussels, as was done in this study. This aspect was considered by placing the mussels in the Fyris river under conditions that do not favour spawning, such as temperatures below 12 °C at all sites (except the outlet). It is unlikely however that veligers, if produced, have survived in that heavily polluted site. In any case, the Fyris river discharges into Lake Mälaren, which is already colonized by zebra mussels, so our field experiment did not represent a risk of contamination.

Vandalism is also an issue encountered by some authors (Selegean et al., 2001) during field experiments, which causes the damage of cages and thus, loss of data. Luckily, this kind of situation did not occur during this study.

Furthermore, like any other living organism, mussels might have unpredictable behaviour (e.g. stop filtering for no apparent reason or die), which can make the interpretation of the results more difficult. In addition, preparing the mussel samples for analysis is more laborious and time-consuming than analyzing water samples which are easy to filter for bacterial enumeration. However, other microorganisms such as viruses are more difficult to filter and thus they require a different approach; in this case sampling mussels could be an efficient option.

Conclusion

This study contributes to the existing evidence that zebra mussels are efficient accumulators of bacteria and thus can be successfully used in biomonitoring programs, despite some practical difficulties. It also emphasizes the potential use of zebra mussels for detecting antibiotic resistant microbes and suggests the need of further studies to assess whether there is a selective pressure in the mussels.

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Annex

Table 4. The relationships between the environmental parameters and the amount of each indicator isolated from mussels and water, from each site, are presented in terms of adjusted R^2 values, obtained from stepwise regression; empty fields indicate the fact that the parameters did not fulfill the condition of $p < 0.05$ for entering the model. The parameters accepted in the model are marked with '+'.

E. coli												
Site	**1**		**2**		**3**		**4**		**5**		**6**	
	mussels	**water**	**mussels**	**water**	**mussels**	**water**	**mussels**	**water**	**mussels**	**water**	**mussels**	**water**
pH		+					+		+		+	
temperature		+				+	+	+				+
rainfall		+		+		+				+		+
flow		+				+		+			+	
time		+				+		+				
R^2 adj.	-	1.000	-	0.570	-	0.999	0.701	0.880	0.356	0.358	0.614	0.675
***Enterococcus* spp.**												
pH		+				+						
temperature	+	+					+		+		+	
rainfall		+				+						
flow		+				+						
time		+	+			+					+	
R^2 adj.	0.365	1.000	0.461	-	-	0.967	0.587	-	0.373	-	0.588	-

Table 5. Correlations (r values) obtained from the multivariate analysis done for each site

E. coli												
Site	**1**		**2**		**3**		**4**		**5**		**6**	
	mussels	**water**	**mussels**	**water**	**mussels**	**water**	**mussels**	**water**	**mussels**	**water**	**mussels**	**water**
pH	-0.027	-0.767	0.458	-0.023	-0.139	0.290	-0.623	-0.342	-0.598	-0.568	-0.617	-0.382
temperature	0.016	-0.616	0.078	-0.112	-0.300	-0.670	-0.205	-0.632	-0.366	-0.378	-0.285	-0.568
rainfall	-0.340	0.159	-0.068	0.755	0.008	0.471	0.266	0.626	-0.141	0.593	0.041	0.708
flow	0.486	0.548	0.379	-0.172	0.232	0.491	0.344	-0.604	0.295	0.405	0.282	-0.137
time	-0.061	-0.030	0.369	-0.160	-0.086	-0.104	-0.353	-0.452	-0.521	-0.028	-0.527	-0.183
***Enterococcus* spp.**												
pH	-0.593	-0.831	0.547	0.353	0.513	0.778	-0.727	-0.095	-0.594	-0.047	-0.246	-0.039
temperature	-0.604	-0.861	0.395	-0.316	-0.402	-0.240	-0.766	-0.362	-0.611	-0.273	-0.579	-0.502
rainfall	-0.201	0.552	-0.070	-0.058	0.214	0.219	0.278	0.284	0.406	0.223	0.500	0.209
flow	0.001	0.122	0.449	0.162	-0.043	0.215	0.294	0.456	0.352	0.326	0.057	0.384
time	-0.400	-0.330	0.679	0.073	0.044	0.344	-0.461	0.336	-0.276	0.269	-0.180	-0.014

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