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# Assessing microbial contamination and antibiotic resistant bacteria using zebra mussels (*Dreissena polymorpha*)

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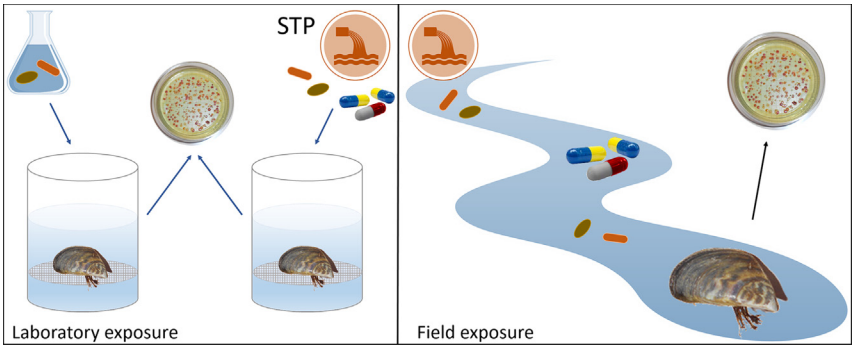
## HIGHLIGHTS

- Faecal contamination of water causes severe health effects worldwide.
- We studied the uptake of faecal indicator bacteria by zebra mussels.
- The concentration of bacteria in mussels was 132 times higher than in water.
- Antibiotic resistance was more prevalent among bacteria from mussels, than water.
- Zebra mussels are suitable for monitoring pathogens and detecting antibiotic resistance.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Aquatic pollution with faecal bacteria and subsequent consumption of contaminated water or food is a worldwide issue that causes severe health effects (e.g. meningitis, salmonellosis, dysentery). In addition, the excessive use of antibiotics in animal husbandry and human medicine has enhanced the selective pressure on pathogenic bacteria, further increasing human health risks and detrimental effects on natural microbial communities. This urges the need to monitor faecal contamination using a time-integrated approach, as grab water samples can miss pathogen peaks. We tested the ability of zebra mussels (*Dreissena polymorpha*) to take up and depurate faecal indicator bacteria such as *Escherichia coli* and intestinal enterococci. Furthermore, we quantified the frequency of antibiotic resistant bacteria in water and mussels both in controlled laboratory tests and under in situ conditions downstream of a sewage treatment plant (STP). Laboratory results show that bacterial indicators in mussels were 132 times higher than their concentration in water, and that mussels retained bacteria up to 2 days after pulse exposure. Field results show decreasing bacterial concentrations in both water and mussels downstream the STP, with maximum *E. coli* concentrations ranging 173–9 cfu $mL^{-1}$ in water and 2970–330 cfu $g^{-1}$ in mussels. Similarly, enterococci ranged 59–4 cfu $mL^{-1}$ and 1450–240 cfu $g^{-1}$ in water and mussels, respectively. High proportions of antibiotic resistant *E. coli* were found in mussels (72%) and water (65%), and slightly lower proportion of resistant enterococci was found in mussels (47%) and in water (34%). Moreover, 33% of the bacteria isolated from mussels were resistant to multiple antibiotics, which emphasizes that resistance is a common feature in surface waters and highlights the need for safe water management. Our results show that zebra mussels provide an

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efficient, time-integrating tool for quantifying faecal indicators, including resistant and multidrug resistant bacteria.



## 1. Introduction

Faecal contamination is a global problem, resulting in unsafe drinking water (WHO, 2002) and water- and foodborne diseases such as meningitis, gastroenteritis, salmonellosis, hepatitis and dysentery (WHO, 2015a). For example, diarrheal diseases are estimated to account for 1.4 million deaths each year worldwide, thus being one of the major causes of human mortality and morbidity (GBD, 2017). More than 100 types of faecal pathogens have been identified, including bacteria, viruses and parasites (Ottoson et al., 2005) which are associated with and dispersed by human and animal excrements. The abundance of faecal pathogens in water can have large and rapid fluctuations and contribute to a widespread human exposure to contaminated water. Contaminated food may cause severe health effects, high economical losses, and shortages of safe food supplies. Therefore, knowledge of the source water quality, in particular the concentrations of reference pathogens or indicators, is essential for designing water safety plans and for reaching public health protection targets (WHO, 2005). As pathogens originating from different sources can be transmitted faecally-orally from water (Pandey et al., 2014), faecal pollution is best monitored by concentrations of indicator bacteria. *E. coli* is the most commonly used indicator, but as it is more sensitive to environmental stress than several enteric viruses and protozoan cysts (Yates, 2007), additional indicators such as intestinal enterococci are also commonly used (e.g. European Union Bathing Water Directive, 2006/7/EC).

Also the presence of antibiotics in the environment, including source waters and their potential risks to human and environmental health (Costanzo et al., 2005), is a major concern for many governments, drinking-water regulators, water suppliers, and the public. For example, 12.7 million kg of antibiotics were consumed in the EU in 2014, with 70% applied in animal husbandry (ECDC et al., 2017). Similarly, 17.2 million kg antibiotics were sold in the US in 2013, while the estimated use in China in 2013 was almost tenfold higher (Wang et al., 2015). Between 30% and 90% of used antibiotics are excreted unchanged or as active metabolites into the environment via urine and faeces (Jjemba, 2006; Lienert et al., 2007). Despite some reduction during wastewater treatment, antibiotics are still entering surface waters (Xu et al., 2007), and can create a selective pressure for resistance among pathogens (Gullberg et al., 2011; Levy and Marshall, 2004). Subsequently, this can have detrimental effects on human health as it hampers the efficient treatment of many infections (Levy and Marshall, 2004; White et al., 2002). WHO's Global action plan on antimicrobial resistance underlined the need for an effective “one health” approach involving coordination among numerous international sectors and actors (WHO, 2015b). Besides the human health risk, antibiotics affect microbial biodiversity by selecting the resistant species and consequently contaminating the environment by dissemination of antibiotic resistant genes (Martinez, 2009).

In order to improve the detection of pathogenic bacteria and antibiotic resistance, time-integrated methods are needed, as water grab samples provide a poor chance of detection (Moles and Hale, 2003). Here we evaluated the suitability of zebra mussels (*Dreissena polymorpha*) as bioindicators of pathogenic and antibiotic-resistant bacteria in freshwater recipients. We did so by quantifying the uptake and retention of faecal indicator bacteria in mussels under laboratory and field conditions. We also assessed antibiotic resistance among bacteria isolated from sewage effluent water and mussels. We hypothesized (1) that both the concentration of bacterial indicators and the frequency of antibiotic resistant bacteria would be higher in mussels than in the water and (2) that the mussels would retain the bacteria a few days after a pulse exposure. Moreover, (3) we expected a decline in bacterial concentration and frequency of antibiotic resistance downstream from the STP.

## 2. Materials and methods

### 2.1. Test organisms

Zebra mussels are freshwater bivalves native to the Black and Caspian Seas (Ludyanskiy et al., 1993), and are widely distributed across Europe and North America (Strayer, 1991). They are epifaunal and have a life span of about 3–5 years (Strayer and Malcom, 2006). The females are highly fecund, and release up to 90,000 eggs $ind^{-1}$ $y^{-1}$ (Schneider, 1992). Zebra mussels feed on suspended particles in the range of 0.7–200 μm (Sprung and Rose, 1988; Winkel and Davids, 1982) at high filtration rates, 46 mL $mg^{-1}$ $h^{-1}$ (Fanslow et al., 1995). Hence, zebra mussels take up large amounts of suspended particles and deposit faeces and/or pseudofaeces on the bottom (Mackie, 1991). Hence, through their filter-feeding behaviour, zebra mussels are readily exposed to both particle-associated and dissolved contaminants. They are good for biomonitoring contaminants (Cope et al., 1999), as they are sessile, highly abundant, widely spread, easy to collect and have a low susceptibility to serious diseases (Karatayev et al., 2002).

We used two bacterial indicators, *E. coli* and *Enterococcus* spp. that both live in the intestinal tract of warm-blooded animals and therefore, their presence in water bodies is usually associated with faecal contamination. *E. coli* are aerobic Gram-negative, rod-shaped bacteria (Ø 0.5 μm) (Britannica). Enterococci are facultative-anaerobic, Gram-positive bacteria with ovoid shape (Ø 0.5–1 μm) (Zhou and Li, 2015).

### 2.2. Uptake and elimination of bacteria by mussels in laboratory experiments

Zebra mussels (shell length 15.8 ± 4.2 mm) were collected in winter from Lake Erken, Sweden (59°50′15.6″N 18°38′06.1″E) and transported back to the laboratory in cold lake water (approximately 5 °C). The mussels were acclimatized to experimental temperatures for 21 days, during which the temperature was increased successively from 5 (in situ) to 18 °C, and mussels were fed every 4th day with ground Tetraphyll®. During the acclimation period lake water was aerated and gradually replaced by artificial lake water, i.e. M4-medium prepared by adding nutrients to Milli-Q water (OECD Guideline 202), the medium used in laboratory experiments.

All aquaria, beakers, nets, and aeration hoses were autoclaved prior to each experiment. Laboratory experiments were done in triplicates at 17.5 ± 0.5 °C (mean ± standard deviation) with a photoperiod of 16:8 h and constant aeration throughout the experiments. Mussels were incubated in 25-L glass aquaria, placed on stainless-steel nets that separated them from accumulating (pseudo)faeces underneath the net, thus preventing re-ingestion of faecal material. Only mussels that showed an active filtering behaviour were sampled at each time point.

We studied the mussels' uptake and elimination of faecal indicator bacteria using pure cultures, as well as a cocktail of bacteria (sewage effluent) in order to better understand the filtering behaviour of the zebra mussels, and thus assess their suitability as biomonitoring organisms. Hence, we gradually increased the complexity and relevance of the exposure scenarios, from laboratory experiments, to the field experiment. The overview of the 4 laboratory experiments is given in Table 1.