Use it or lose it

Factors affecting the fitness and dominance shifts of dreissenid mussels

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Factors affecting the fitness and dominance shifts of dreissenid mussels

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Chapter 1

General introduction

General introduction

The bivalves *Dreissena polymorpha* or zebra mussel (Pallas 1771) and *D. bugensis* or quagga mussel (Andrusov 1897) originate from river estuaries north of the Ponto-Caspian basins. They spread out of their native area along rivers, attached to boat hulls and in ballast water invading hard freshwater surfaces in large parts of the northern hemisphere (Leuven et al. 2009). Both in their native and introduced areas ongoing dominance shifts are observed going from the first introduced *D. polymorpha* to *D. bugensis* as the dominant species (Karatayev et al. 2011, Matthews et al. 2014, D'Hont et al. 2018). However, the drivers of these dominance shifts are still largely unknown. During the present study, we tried to identify species traits and external factors leading to higher fitness and competitive benefits for either one of these species.

Physiology of the Black Sea and Caspian Sea

The Ponto-Caspian region harbours a system of three basins (Black Sea, Sea of Azov and Caspian Sea) that are remnants of the East Paratethys Sea (Figure 1), (Yanina 2014). They each have their unique paleogeographical history and environmental features. These basins are geologically and biologically young formations and still have natural changes taking place, in which their ecosystems are subjected to maturation processes (Ojaveer et al. 2002). Both the Black Sea and Caspian Sea are isolated from the ocean by physical and ecological barriers and can be considered as “brackish-water islands” (Ojaveer et al. 2002, Leppäkoski et al. 2009, Shiganova et al. 2014).

The Black Sea is a semi-enclosed, non-tidal waterbody with relatively low salinity (12 to 22 PSU) (Shiganova et al. 2014). It has a maximum depth of 2200 m and is bound by the Federation of Russia, Georgia, Republic of Bulgaria, Republic of Turkey, Romania and Ukraine (Baltas et al. 2017). This waterbody only has a thin (60 to 200 m) oxygenated surface layer overlying anoxic waters, making this ecosystem vulnerable to environmental changes (Paavola et al. 2005, Shiganova et al. 2014). Marine water enters the Black Sea from the Mediterranean

Sea via the Bosphorus Strait while rivers discharge freshwater (Paavola et al. 2005). The main rivers flowing into the Black Sea are the Danube, Dnieper, Dniester, Don and Kuban (Secrieru and Secrieru 2002, Mülayim and Balkis 2015, Baltas et al. 2017). Black Sea surface water temperatures range from 0 – 8 °C to 24 – 26 °C in winter and summer, respectively (Leppäkoski et al. 2009).

Figure 1. Map of our study sites in Western Europe and the Ponto-Caspian region. The Ponto-Caspian region consists of the Sea of Azov, Black Sea and Caspian Sea basins. The main rivers flowing into these basins are the Danube, Dnieper, Don and Volga. The main rivers in Western Europe are the Rhine and the Meuse. The Rhine-Meuse-Danube canal, the Dnieper–Bug canal and the Volga-Don canal connecting the waterbodies are indicated with dark dotted lines. The creation of these canals facilitated the invasion of Eurasia by dreissenids. In Chapters 2 to 4, we studied the Ponto-Caspian dreissenid mussels in the Rhine-Meuse delta at location A. In Chapter 5 we assessed the microplastic concentrations in sediment in the Black Sea and Caspian Sea basins at locations B and C.

The Caspian Sea is isolated and the world’s largest inland lake (Aladin et al. 2002, Paavola et al. 2005). It is surrounded by the five states of Azerbaijan, Federation of Russia, Islamic Republic of Iran, Kazakhstan and Turkmenistan and is located in two continents, Europe and Asia

(Aladin et al. 2002). The sea level in the Caspian Sea is about 25 – 29 m lower than the global mean sea level (Aladin et al. 2002). There are three distinct basins: The shallow northern part (average 5 m and maximum 20 m depth), the central basin (maximum depth of 788 m) and the southern basin (maximum depth of 1025 m) (De Mora et al. 2004). The average salinity of the northern part of the Caspian Sea is about 5 – 10 PSU, of the middle part 12.7 PSU and of the southern part 13 PSU (Aladin et al. 2002). About 130 rivers flow into the Caspian Sea (De Mora et al. 2004). The largest river is the Volga in the north, followed by the Kura and Ural rivers (De Mora et al. 2004, Paavola et al. 2005). The water temperature has a minimum of -0.5 °C and 13 °C in the north and the south of the Caspian Sea during winter, while in summer, the average temperature is about 24 °C and 25 – 30 °C in the north and south basins, respectively (Aladin et al. 2002).

Anthropogenic pressures on the Ponto-Caspian environment

Figure 2. Plastic debris and shells of the highly invasive species *Rapana venosa* on the Black Sea shoreline in Romania.

Unique Ponto-Caspian biota evolved in saline water bodies in the Caspian Sea - Black Sea - Aral Sea region and surrounding rivers in the past few million years (Wesselingh et al. 2019). However, during the last decades the abundances of several of these species in the Caspian Sea and Black Sea basins have severely declined, as the Ponto-Caspian ecosystems have been heavily impacted by environmental and anthropogenic pressures (Lattuada et al. 2019, Wesselingh et al. 2019). Examples of environmental pressures are water-level fluctuations and salinity changes. The anthropogenic pressures threatening the Ponto-Caspian ecosystem can be identified as increased urbanisation, chemical pollution and eutrophication due to heavy industries, intense agriculture, aquaculture and oil extraction, overfishing, poaching, introduction of invasive species, river flow regulation, high sea traffic and port activities (Figure 2)(Ojaveer et al. 2002, Paavola et al. 2005, Shiganova et al. 2014, Lattuada et al. 2019, Lattuada et al. 2020). Riverine sources are the main contributors to the pollution of the Ponto-Caspian seas (Secrieru and Secrieru 2002, Shiganova et al. 2014, Baltas et al. 2017). These anthropogenic pressures turned the Ponto-Caspian basins into some of the most polluted and mismanaged waterbodies worldwide (Shiganova et al. 2014). Consequently, the ecosystems experienced deterioration. Many taxa decreased in abundance, had a reduction of their habitat or range and several endemic species even became regionally or globally extinct over the last decades (Lattuada et al. 2019, Lattuada et al. 2020). This caused a dominance shift in species present in the Ponto-Caspian region. The main anthropogenic pressures known to affect the Ponto-Caspian biota are invasive species, chemical pollution and poaching (Lattuada et al. 2019). Within this thesis, we, therefore, focused on invasive species and pollutants (i.e. microplastic) from and within the Ponto-Caspian region.

Invasive alien species

Invasive alien and native species

It is important to distinguish between an alien species and an invasive species. An alien (or exotic, non-native, non-indigenous or introduced) species has a human introduction, accidentally or intentionally, in an area where they are not naturally present outside of their natural geographic range, after which they establish themselves into their new habitat and spread (IUCN 2013). Many alien species are non-invasive and cause no harm to their new environment (Beck et al. 2006, Skandrani et al. 2014). Some alien species lack natural predators in their new environment, allowing them to reproduce quickly and spread without limits to eventually dominate their new environment (IUCN 2013). Such alien species become invasive and are likely to cause economic loss or environmental harm or risks to human health (Beck et al. 2006). However, invasive species are not necessarily of alien origin. Native species can have similarly devastating effects and become invasive due to changes in their environment (Skandrani et al. 2014). These would be called invasive native species. Invasive species are one of the most important concerns in the context of the current biodiversity crisis because they are a potential threat to native species through predation, grazing, competition, parasitism, disease, hybridisation, altering the food chain or habitat alteration (IUCN 2013, Skandrani et al. 2014). The impact of invasive alien species on the biodiversity in their introduced range is both acute (short-term) and chronic (long-term) (Aladin et al. 2002). The acute impact can usually be seen during the first years after the introduction of an invasive alien species, after which the effect fades and becomes chronic. There are over 12 000 alien species in Europe, 15% of which are invasive (IUCN 2013). European threatened species experience invasive alien species as their third most severe threat. The European Union suffers 12 billion euros in annual damages from the impact of invasive alien species on human health, damaged infrastructure, and agricultural losses (IUCN 2013).

Ponto-Caspian species becoming invasive in other waterbodies

The Ponto-Caspian basins are impacted by invasive alien species, however, several Ponto-Caspian species have been recorded as invasive alien species elsewhere. In Western Europe in the lowland rivers and canals discharging into the North Sea, 20 – 30% of the alien species are of Ponto-Caspian origin having both biotic and abiotic impacts on their new environment (Ojaveer et al. 2002). Examples of such Ponto-Caspian invaders are the hydroid polyp *Cordylophora caspia* and the Arthropoda *Chelicorophium curvispinum*, *Pontogammarus robustoides* and *Dikerogammarus villosus* (Jazdzewski and Konopacka 2002, Slynko et al. 2002, Paavola et al. 2005, Leuven et al. 2009). However, the most famous invaders originating from the Ponto-Caspian basins are the bivalves *Dreissena polymorpha* and *D. bugensis* (Figure 3) (Ojaveer et al. 2002, Skolka and Preda 2010, Matthews et al. 2014).

Figure 3. Two invasive mussel species originating from the Ponto-Caspian region.

Dreissena polymorpha is notorious for dominating hard freshwater substrates throughout most of the northern hemisphere (Karatayev et al. 2007). *Dreissena polymorpha* was widely spread across Europe before the last glaciation, but because of this last glaciation cycle, its distribution became limited to the Black Sea, Caspian Sea, Azov Sea and Aral lake and the rivers flowing in these waterbodies (Stanczykowska 1977). This remained their geographical distribution until the beginning of the 19th century when shipping canals were created for transportation and trade connecting the Black Sea basin with the Baltic Sea and Western Europe (Karatayev et al. 1997, Pollux et al. 2010). These kinds of human activities mediated the rapid spread of *D. polymorpha* back across the freshwaters of Eurasia. The first record in the Rhine-Meuse delta dates back to 1826 and it became abundant and widespread over the next few decades (Kerney and Morton 1970). Ever since then, *D. polymorpha* became a worldwide problem, as the species spread into North America in 1986 and countries belonging to the former Soviet Union (McMahon 1996, Karatayev et al. 1998). However, during the last decade, there have been remarkable and widespread observations of the replacement of *D. polymorpha* by *D. bugensis* in both their native and introduced regions (Stoeckmann 2003, Therriault et al. 2005). The latter Ponto-Caspian dreissenid species has shown a more recent range expansion. *Dreissena bugensis* had been restricted to the Dnieper and Bug rivers and their respective estuaries in Ukraine, northern Black Sea, until the middle of the 20th century (Therriault et al. 2005, Matthews et al. 2014). By the end of the 20th century, *D. bugensis* started expanding out of its native area, moving to neighbouring Black Sea estuaries, canals and reservoirs (Mills et al. 1996). Shortly after, *D. bugensis* found its way to the Volga and Danube river systems from where it started colonising Russia and Western Europe (Molloy et al. 2007). This expansion was largely facilitated by man-made canals connecting large waterways and rivers, like the Volga-Don canal and the Rhine-Meuse-Danube canal (Figure 1)(Zhulidov et al. 2005, Bij de Vaate and Beisel 2011, Marescaux et al. 2012). Furthermore, *D. bugensis* invaded the great lakes in North America together with *D. polymorpha* (Hebert et al. 1989, May and Marsden 1992). Both *Dreissena* species cause extensive ecological (Grigorovich and Shevtsova 1995) and economic damage in their native regions (Popa and Murariu 2009) and introduced areas (Pimentel et al. 2005, Binimelis et al. 2008, Fahnenstiel et al. 2010). Ecological changes caused by dreissenids include a dramatic increase in water filtration causing a reduction in food and oxygen availability and shifts in water chemistry and species composition, relative abundances and biomass of functional groups within the aquatic ecosystem (Ojaveer et al. 2002, Binimelis et al. 2008, Fahnenstiel et al. 2010, Karatayev et al. 1997). They cause shifts in several trophic levels as they selectively remove large bacteria (< 0.9 µm); dramatic declines (30–90%) in phytoplankton biomass; declines in zooplankton due to ingestion by dreissenids themselves and by the depletion of the zooplanktons food source (phytoplankton); rapid decline of the diversity of native (unionid) mussel communities due to fouling of the dreissenids on native mussels but also due to food competition (Ojaveer et al. 2002). Additionally, the shell deposits of dreissenids have transformed soft bottoms into shell gravel, creating a local hard substrate for sessile species to settle onto (Ojaveer et al. 2002). Moreover, these species can withstand, and thus accumulate high pollutant levels (Hendriks et al. 1998, Carrasco et al. 2008). As a primary food source for some waterfowl (e.g. common coot, ducks, gulls) and fish (e.g. whitefish, eel, carp, bream, sturgeon, flounder), dreissenids transfer heavy metals and organic contaminants causing higher bioaccumulation of those contaminants in their predators and causing an increased exposure for humans (Hendriks et al. 1998). Economic damages caused by the zebra and quagga mussels include an increase in maintenance costs of overgrown man-made structures like hydraulic infrastructures and pipes in cooling water circuits (Binimelis et al. 2008, Schonenberg and Gittenberger 2008).

The invasion by these Ponto-Caspian species did not necessarily cause a local decrease in biodiversity (alpha diversity), but rather a dominance shift between several species and trophic levels. However, ultimately these dominance shifts led to a decrease in variation in species composition between regions (beta diversity) (Ojaveer et al. 2002).

Dominance shift between Dreissena polymorpha and D. bugensis

Next to the general dominance shift in species composition caused by the invasion of Ponto-Caspian species, there have been widespread observations of a dominance shift between the Ponto-Caspian mussels *D. polymorpha* and *D. bugensis* both in their introduced and native regions (Stoeckmann 2003, Therriault et al. 2005, Matthews et al. 2014). Ever since its more recent introduction, *D. bugensis* seems to be outcompeting the already present *D. polymorpha*. However, mechanisms driving this dominance shift are still not completely understood.

Microplastic pollution in the Black Sea and Caspian Sea

Humans have been experimenting with natural polymers, waxes and rubber ever since approximately 1600 BC until the modern natural and synthetic thermoplastics were developed in the 19th century (Andrady and Neal 2009). Modern-day synthetic plastics consist out of long chains of polymeric molecules created from organic and inorganic raw materials, like carbon, silicon, hydrogen, oxygen and chloride (Do Sul and Costa 2014). These raw materials are derived from oil, coal and natural gas. Modern plastics can be used in a wide range of temperatures, are chemical- and light-resistant, are very strong, and can easily be shaped as hot melt. These properties, together with the low cost, turn plastic into an important component of modern-day life. Examples of daily used products (partially) made out of plastic are clothing, shoes, public and private transportation. Additionally, the use of plastic improves public health by being able to bottle clean drinking water or provide packaging for food reducing food wastage, wrapping medicine and by creating medical devices (Andrady and Neal 2009).

Global waste production has continuously grown throughout the last decades (Löder and Gerdts 2015). Annually, global plastic production almost reached 370 million tons (Plastics Europe 2020). Wind, run-off, wastewater disposal, currents and tides can transport plastic from their land-based sources to the aquatic and marine environment. About 80% of the marine litter is assumed to have a terrestrial origin (Jambeck et al. 2015). In 1972, Carpenter and Smith were the first to raise awareness of the increased production of plastic and its accumulation in the marine environment (Do Sul and Costa 2014). Anthropogenic waste can be found in all parts of global waterbodies, ranging from the shoreline to the deep-sea (Van Cauwenberghe et al. 2013, Do Sul and Costa 2014, Ryan 2015). This is due to its high mobility and transportation by wind and currents. Plastic aggregates even created several floating plastic islands or garbage patches like the “Great Pacific Garbage Patch” (Galgani et al. 2015, Ryan 2015). The plastic particles in marine environments are exposed to ultraviolet (UV) radiation, heat, physical action by wind and waves, oxidation, bacterial decay and chemical degradation processes causing the debris to degrade (Reisser et al. 2013, Andrady 2015, Galgani et al. 2015, Ryan 2015). Due to this process, a distinction has been made based on the size of plastic debris. Plastic waste has been divided into four different size classes ranging from mega-particles (>100 mm), macro-particles (>20 mm) and meso-particles (5-20 mm) to micro-particles (<5 mm) (Barnes et al. 2009).

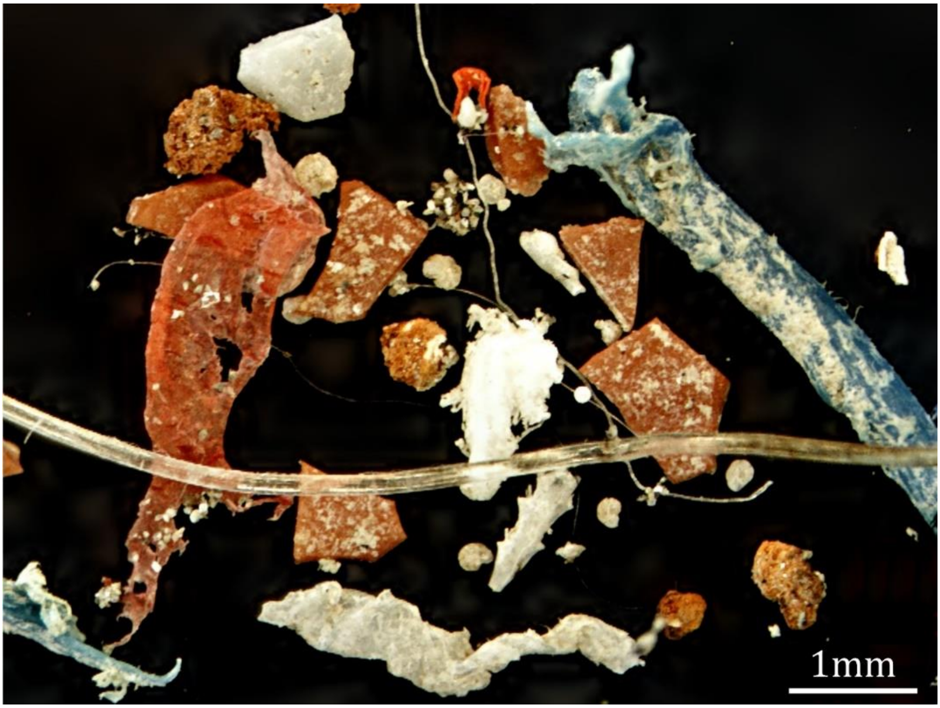

Figure 4. Plastic particles smaller than 5 mm are called microplastic particles.

Marine litter has a wide range of negative environmental, economic, social, public health and safety impacts (Newman et al. 2015). The accumulation of plastic is harmful to marine biota due to entanglement, ingestion and dispersal of alien species, especially for larger (mega- to meso-sized) plastic particles (Hidalgo-Ruz and Thiel 2015). Plastics have been demonstrated to harm at least 250 marine vertebrate species (Wright et al. 2013). This impact reduces ecosystem services as well, causing an economic impact of plastic waste. This economic impact is due to litter removal action and adverse effects on aquaculture and fisheries (Newman et al. 2015). For example, in the Asia-Pacific region alone the economic impact of marine plastic waste has been estimated at 1 billion euros per year (McIlgorm et al. 2011). Another example of the economic impact of plastic waste is the total cost of beach litter removal in Belgium and The Netherlands. This cost has been estimated at 10.4 million euros per year, which is a cleaning cost of on average about 34 000 euros per kilometre per year (Newman et al. 2015).

Most of the plastic present in the aquatic environment is represented by the smallest size class, the microplastic particles (Figure 4)(Browne et al. 2010). Two types of microplastic particles can be identified: primary and secondary microplastic. Primary microplastic particles are directly released in micro-particle size due to industrial spillage or discharge in sewage water and are produced as abrasive in cosmetic products, industrial shot-blasting abrasive or plastic pellets or powders used as feedstock for the production of larger items (Thompson 2015). Secondary microplastic is formed by the fragmentation of larger pieces of plastic debris due to ultraviolet light, heat, physical action by wind and waves and biological processes (Reisser et al. 2013, Andrady 2015). In contrast to larger plastic debris particles, knowledge of the impact of microplastic on biota and the environment is limited (Wagner et al. 2014). Microplastic ends up in the food chain, as they are available for ingestion by filter feeders at the bottom of the food chain (e.g. *D. polymorpha* and *D. bugensis*) due to their small size (Murray and Cowie 2011). Some of the effects assumed due to (micro)plastic ingestion are related to the occupation of stomach volume by debris, which may limit optimal food intake (Kühn et al. 2015, Eerkes-Medrano and Thompson 2018). This may lead to a false feeling of satiation and reduces the feeling of hunger. The reported effects of microplastic uptake include histological changes, inflammatory response, lysosomal membrane destabilisation, reduced filtering activity, neurotoxic effects, alterations of the antioxidant system, increase in haemocyte mortality, dysplasia, genotoxicity and transcriptional responses (Li et al. 2019a). Eventually, these factors may lead to a deterioration of the body condition of animals. Additionally, the chemical substances added to the plastic particles during manufacturing or adsorbed to plastics at sea are an additional source of concern (Kühn et al. 2015, Brennecke et al. 2016, Akhbarizadeh et al. 2017). Moreover, biofouling of plastic particles may lead to transportation of biota and/or invasive species (Eerkes-Medrano and Thompson 2018).

Even though the first records of microplastic pollution date back over 40 years, no thorough estimation exists of the distribution, amount and origin of microplastic in aquatic environments (Jambeck et al. 2015). After the first plastic detection in the aquatic ecosystem, it took 30 more years before microplastic particles were detected in aquatic sediments (Thompson et al. 2004). Sediment is thought to be a microplastic sink, especially for particles with a density higher than the present waterbody (Law et al. 2010, Cózar et al. 2014, Van Cauwenberghe et al. 2015). Low-density particles tend to float on or stay suspended in the overlying waterbody if their density is lower than seawater (Reisser et al. 2013, Zettler et al. 2013). Microplastic particles usually have a density ranging from 0.8 to 1.56 g/cm^3, while sediment usually has a density of about 2.65 g/cm^3 (Hidalgo-Ruz et al. 2012, Claessens et al. 2013). However, due to density modifications by biofouling, these low-density particles can sink and become trapped in between sediment grains as well (Andrady 2011). Therefore, assessing microplastic in sediment is an important part of microplastic research. Unfortunately, there is no standardised protocol yet for the extraction of microplastic from sediment. However, several techniques have been developed throughout the last two decades (Van

Cauwenberghe et al. 2015). In all cases, these extraction techniques, at least partly, depend on density separation, as the microplastic density is most likely lower than the sediment density (Hidalgo-Ruz et al. 2012).

Studies assessing the microplastic concentration and distribution in sediment of the Black Sea and Caspian Sea basins are scarce. The main pollution sources in these basins are assumed to be the rivers flowing into the region (Secrieru and Secrieru 2002, Baltas et al. 2017). Europe’s largest (Volga) and second-largest (Danube) rivers flow into the Caspian Sea and Black Sea, respectively. Therefore, both waterbodies have an unusual high river discharge into relatively small (semi-)enclosed seas, causing an increased risk of plastic pollution (Aytan et al. 2016). The Danube is estimated to discharge about 4.2 tons of plastic into the Black Sea each day (Lechner et al. 2014). This would suggest that the Black Sea might be a hotspot for microplastic pollution and retention (Figure 2) (Aytan et al. 2016). Such estimations do not yet exist for the Volga river. So far, only a couple of studies assessed microplastic pollution in the sediment of the Caspian Sea (Figure 5) (Mataji et al. 2020, Mehdinia et al. 2020). Therefore, assessments of the distribution, sources, transportation, sinks and effects on marine life of Ponto-Caspian microplastic are needed.

Figure 5. Plastic debris on the Caspian Sea shoreline near Baku, Azerbaijan.

Aim and research questions

Since its recent introduction, *D. bugensis* seems to be outcompeting the already present *D. polymorpha*. However, mechanisms driving this dominance shift are still not completely understood. This thesis aimed at unravelling some of the drivers of this observed dominance shift between *D. polymorpha* and *D. bugensis.* Therefore, comparative analyses were conducted between both species to identify species traits and external factors leading to higher fitness and competitive benefits for either one of these species.

To obtain this goal, the following research questions should be answered.

Species traits differentially leading to higher fitness and competitive benefits

- Which life-history traits provide a competitive benefit over the other dreissenid species (Chapter 2)?
- Do differential attachment and detachment traits provide a competitive benefit for either one of the species (Chapter 3)?
- Do dreissenid species exhibit differences in mobility traits (Chapter 4)?

External factors differentially affecting dreissenid fitness

- Do external factors (temperature, salinity, light) differentially affect species traits (Chapter 2, 3)?
- What are the composition, concentration and distribution of microplastic in sediments of the dreissenids natural area, the Ponto-Caspian region (Chapter 5)?
- Do microplastics and plastic adsorbed pollutants influence dreissenids fitness (Chapter 6)?

Outline of this thesis

In Chapter 2 we assessed differences in life-history traits focussing on the settlement, growth, population density and mortality of dreissenids. Additionally, we evaluated the impact of salinity and temperature on dreissenids' settlement and growth and the effect of light intensity on their settlement and population density. Settling earlier and in higher numbers, growing faster and larger, having higher population densities and lower mortality rates could lead to competitive benefits for dreissenids. Especially, when they are better at withstanding a larger range of salinity, temperature and light regimes.

In Chapter 3 we assessed differential attachment and detachment traits between both species under controlled laboratory conditions and during a field experiment. For this study, we focussed on the attachment strength, attachment rate, byssal thread morphology and the mode of byssal failure of dreissenids. A higher attachment rate or stronger attachment might be advantageous when it comes to withstanding predation and environmental disturbances such as rapid changes in water level and flow velocity (e.g. by upstream rainfall or by ship-induced currents). On the other hand, being able to detach easier and faster might facilitate relocation to more suitable habitats in case of sudden unfavourable environmental changes (e.g. salinity, temperature, light). This way, a mussel has a higher chance of reaching its ideal environment where it can thrive. The attachment traits of dreissenids can thus to some degree be one of the drivers of the observed dominance shift, especially in rapidly changing environments.

In Chapter 4 we analysed differences between dreissenids' mobility traits. To do so, we focussed on the number of individuals moving, the duration of movement, movement speed, the distance moved by individuals and the relationship between movement and the shell size of both species. Having higher overall mobility could give an individual the capacity to actively move away from unfavourable environmental conditions (e.g. poor water quality, changes in orientation, presence of conspecifics, low oxygen content and desiccation). Being able to move to a better environment where they can thrive, could count as a competitive benefit.

Rivers are one of the main transport pathways for pollutants transporting them from their sources to their sinks. According to this hypothesis, dreissenids usually live in highly polluted environments. One of those, still largely unknown, pollutants is represented by microplastic. During the last couple of years, several studies focussed on the possible impact of microplastic on filter feeders like dreissenids. However, as not much is known about microplastic concentrations in their native Ponto-Caspian area, we should first determine whether microplastic could potentially reach harmful concentrations in the dreissenids' habitats. In Chapter 5, we made a comparison between the composition and concentration of microplastic in sediments of the Black Sea and Caspian Sea basins. Additionally, we assessed whether there might be a link between the microplastic concentration in Ponto-Caspian sediment and possible microplastic sources (i.e. distance to rivers, shore or city) or sinks (sediment characteristics like grain size or water depth). By assessing microplastic pollution in this region, we aim at unravelling whether this type of pollutant might impact the filter-feeding dreissenid mussels. Hence, the synthesis reviews the contemporary body of knowledge on the impact of microplastic on filter feeders, dreissenids especially, linking the microplastic story to the dreissenids' fitness (Chapter 6).

Chapter 2

Drivers of dominance shifts between invasive Ponto-Caspian dreissenids *Dreissena polymorpha* (Pallas, 1771) and *Dreissena rostriformis bugensis* (Andrusov, 1897)

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Abstract

Two of the most invasive freshwater bivalve species in temperate regions worldwide are the Ponto-Caspian dreissenids *Dreissena polymorpha* and *Dreissena rostriformis bugensis*. Throughout their range, observations have been made of a dominance shift favouring *D. r. bugensis* where the two species co-occur. Although both dreissenids have been widely studied, the mechanisms driving this dominance shift are not completely understood. Our long-term and short-term field experiments assessed a selection of species traits related to growth and settlement, which may be linked to a competitive benefit for either of both species. We assessed relative population densities in time and space, mortality, and inter- and intraspecific interactions in relation to environmental factors like temperature, salinity, and light intensity, using 14 × 14 cm PVC settlement plates. Dreissenids were identified, counted and measured over 11 years in the waterbody where *D. r. bugensis* was first discovered in Western Europe*. Dreissena polymorpha* appeared to have a competitive benefit over *D. r. bugensis* by being able to settle earlier, i.e. in spring, while other studies indicate the opposite. As salinities in the study region were relatively high in spring, this discrepancy may be explained by a higher salinity tolerance of *D. polymorpha*, as is known from the literature. In addition, Common Coots predated *D. r. bugensis* clusters*. Dreissena polymorpha* were not usually found in such clusters. Regardless of these competitive benefits for *D. polymorpha*, *D. r. bugensis* is the dominant species in the region. One possible explanation could be the fact that *D. r. bugensis* was found in similar densities on both light exposed and shaded fouling plates. *Dreissena polymorpha* on the other hand, was practically absent on light-exposed plates after 12 months. Therefore, a wider range of habitats seems to be suitable for the settlement of *D. r. bugensis*. Another driver of the dominance shift can be linked to it having faster growth than *D. polymorpha*, especially during spring. More generally, *D. r. bugensis* occurred in higher population densities on plates with *D. polymorpha* than on plates with only *D. r. bugensis*. *Dreissena polymorpha* individuals may thus induce the settlement of additional individuals of *D. r. bugensis*. A final reason

for the dominance of *D. r. bugensis* concerns its low winter mortality. While the number of *D. polymorpha* individuals more than halved over the winter 2016–2017, no significant decrease in numbers of *D. r. bugensis* was recorded.

Introduction

Both the zebra mussel, *Dreissena polymorpha* (Pallas, 1771), and the quagga mussel, *Dreissena rostriformis bugensis* (Andrusov, 1897), are notorious for dominating hard freshwater substrates throughout most of the Northern hemisphere. Both species cause extensive ecological and economic damage in their native area (Grigorovich and Shevtsova 1995) and introduced regions (Binimelis et al. 2007, Fahnenstiel et al. 2010). *Dreissena polymorpha* started spreading out of its native area (the Black Sea, Caspian Sea, Azov Sea and Aral lake) to Europe and to countries of the former Soviet Union at the beginning of the 19th century (Van der Velde et al. 2010, Sousa et al. 2011). *Dreissena rostriformis bugensis* was restricted to Ukrainian river estuaries in the Northern Black Sea area until the mid-20th century (Therriault et al. 2005). Within about 20 years, *D. r. bugensis* colonized Europe and Russia and both *D. r. bugensis* and *D. polymorpha* simultaneously colonized Northern America (Molloy et al. 2007, Van der Velde and Platvoet 2007, Matthews et al. 2014). The first population of *D. r. bugensis* in Western Europe was discovered in 2006 in the estuary Haringvliet – Hollands Diep in the Netherlands (Figure 1: Location C) (Molloy et al. 2007, Schonenberg and Gittenberger 2008). The Haringvliet is a part of the Rhine-Meuse river delta which since 1970 has been separated from the North Sea by sluices. As a result, this waterbody became a freshwater system with minimal saltwater influences.

During the last decade, there have been observations of a dominance shift from *D. polymorpha* to *D. r. bugensis,* both in their introduced regions (Europe, Eurasia i.e. Russia and North America) and in their native area (the Ponto-Caspian region) (Stoeckmann 2003, Therriault et al. 2005, Matthews et al. 2014). However, the mechanisms driving this dominance shift are still not completely known. Therefore, research was initiated to disentangle drivers causing competitive benefits for either of the dreissenid species. Some of these drivers have already been assessed during short-term studies. For example, *D. polymorpha* is known to have a higher salinity tolerance and a wider salinity range than *D. r. bugensis* (Mills et al. 1996), possibly leading to a higher prevalence of this species in more saline systems. Additionally, *D. polymorpha* is able to produce more gametes during reproduction (Stoeckmann 2003). Nevertheless, *D. r. bugensis* is the dominant species in most habitats. *Dreissena rostriformis bugensis* is known to be able to persist at greater depths and in lower nutrition environments (Ram et al. 2012). Moreover, *D. r. bugensis* has a higher temperature tolerance and a wider temperature range, permitting *D. r. bugensis* to reproduce and settle earlier than *D. polymorpha* (Claxton and Mackie 1998, Gerstenberger et al. 2011). This allows for a faster colonization time of *D. r. bugensis* and potentially leads to a priority effect (Young et al. 2001). *Dreissena rostriformis bugensis* is also known to have a faster growth rate and reach a larger size than *D. polymorpha* (Neumann et al. 1993, Stoeckmann 2003). Moreover, *D. r. bugensis* is known to have a higher assimilation efficiency, a higher activity of certain enzymes (e.g. thiaminase), higher filtration rates and lower respiration rates giving it additional advantages over *D. polymorpha* (Ram et al. 2012). Despite these findings, the current body of knowledge on potential drivers of the dreissenid dominance shift has mostly been based on short-term studies and laboratory experiments. The present study assessed the impact of some of these drivers in a long-term field experiment, leading to a more complete understanding of the observed patterns. SETtLement (SETL) monitoring in the river estuary Haringvliet started in 2006 just before *D. r. bugensis* was first recorded. This was also the first record of this species in Western Europe (Bij de Vaate 2008, Schonenberg and Gittenberger 2008). Consequently, the SETL monitoring project enables the documentation of the introduction, population establishment and succession of the *D. r. bugensis* population just after its initial introduction in Western Europe. In addition, this study gives more insight into the interspecific competition of this species with *D. polymorpha* which already occurred commonly in the Haringvliet.

The present study uses the data derived from the SETL project to assess 1) the long-term effects of species traits (settlement period and growth rate), environmental factors (temperature, salinity and light intensity), and 2) processes underlying competitive benefits (population density, interspecific competition, winter mortality and predation mortality) to estimate their importance for the dreissenid dominance shift (Figure 2).

Of the known drivers, we have assessed the impact of salinity and temperature on species traits like settlement and growth. Both dreissenid species are known to favour shaded sites for settlement (Kobak 2001, Kobak and Nowacki 2007). This was assessed during the present study. Additionally, we hypothesised that light intensity may also have an impact on population density later on in the succession. The latter appears to be unknown in the literature. Other unknown drivers that may give a competitive benefit for one of the two dreissenid species concern differences in winter mortality and potential interspecific competition. Marescaux et al. (2015) hypothesize that *D. r. bugensis* may have a higher growth rate, causing it to have a larger shell size and body mass, which may help them survive winter stress better. Finally, it was tested to what degree the presence of one species may have an impact on the settlement of the other species. Interspecific competition is known to give a competitive benefit to some invasive species, for example, the invasive tunicate *Botrylloides violaceus* over the native *Botryllus schlosseri* (Gittenberger and Moons 2011).

Material and methods

Study sites

Panmictic populations of *D. polymorpha* and *D. r. bugensis* were studied in Western Europe, in the Netherlands, in Tiengemeten (Figure 1: Location A) and Numansdorp (Figure 1: Location B) in the freshwater Haringvliet. Within the Tiengemeten ferry harbour, SETL fouling plates were attached to a large floating dock near the Haringvliet connection (51°45.250′N; 4°19.050′E) and a smaller floating dock close to the control lock (51°45.261′N; 4°19.046′E). In Numansdorp the SETL fouling plates were deployed onto one large floating dock at the entrance of the village harbour (51°43.037′N; 4°26.211′E). At both locations, there was a subdivision between fouling plates deployed on the southern edge of the floating dock (fully exposed to sunlight), the long western edge (alternately exposed to light and shade) and the northern edge (completely shaded). At these locations, two field experiments were performed: 1) an ongoing long-term experiment which ran from 2006 until the present day of which the data from December 2006 to December 2017 were used, and 2) a short-term experiment running from June 2016 until December 2017 (Figure 2).

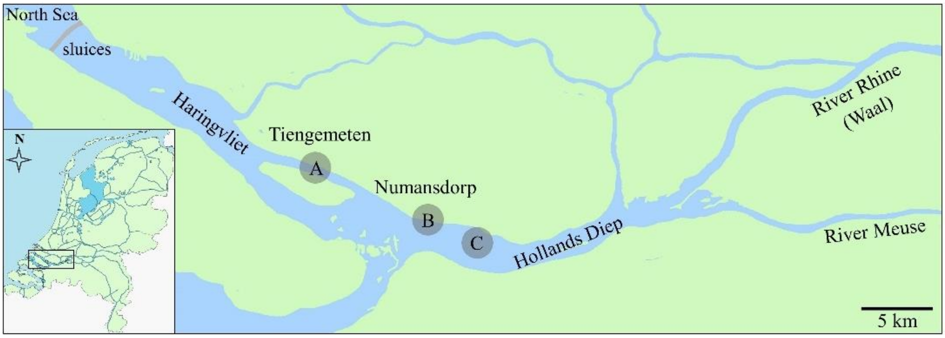

Figure 1. Locations of field experiments in the Rhine-Meuse river estuary in the Netherlands. (A) Tiengemeten ferry dock, (B) Numansdorp, (C) the first record location of *Dreissena rostriformis bugensis* in Western Europe (Molloy et al. 2007) (modified after Figure 1 in Schonenberg and Gittenberger 2008).

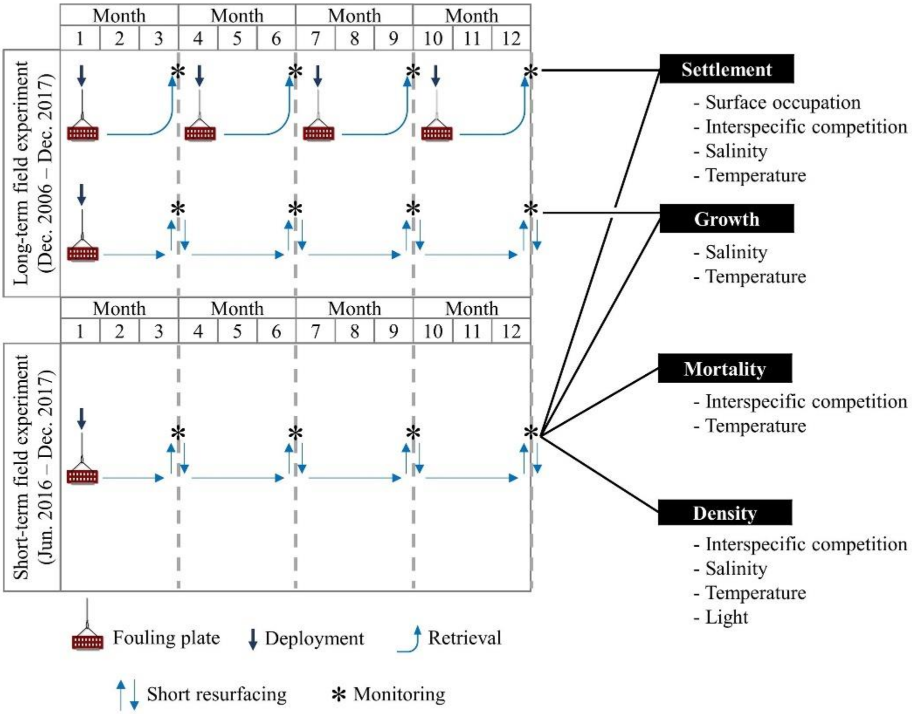

Figure 2. Summary of SETL fouling plate experiment setup concerning the long-term (11years) and short-term (1.5 years) field experiment. The bricks represent the deployment of a new plate. The * represent the monitoring of the SETL plates, which happened every 3 months. During the long-term field experiment, some fouling plates were deployed for only 3 months and replaced by a new set. Other fouling plates were deployed continuously. During the short-term field experiment, all fouling plates were deployed continuously throughout the experiment. These “continuous” plates got shortly lifted out of the water for monitoring and redeployed in the same place. The assessed parameters were represented in the black boxes. Further details on the setup of the experiment can be found in the material and method section.

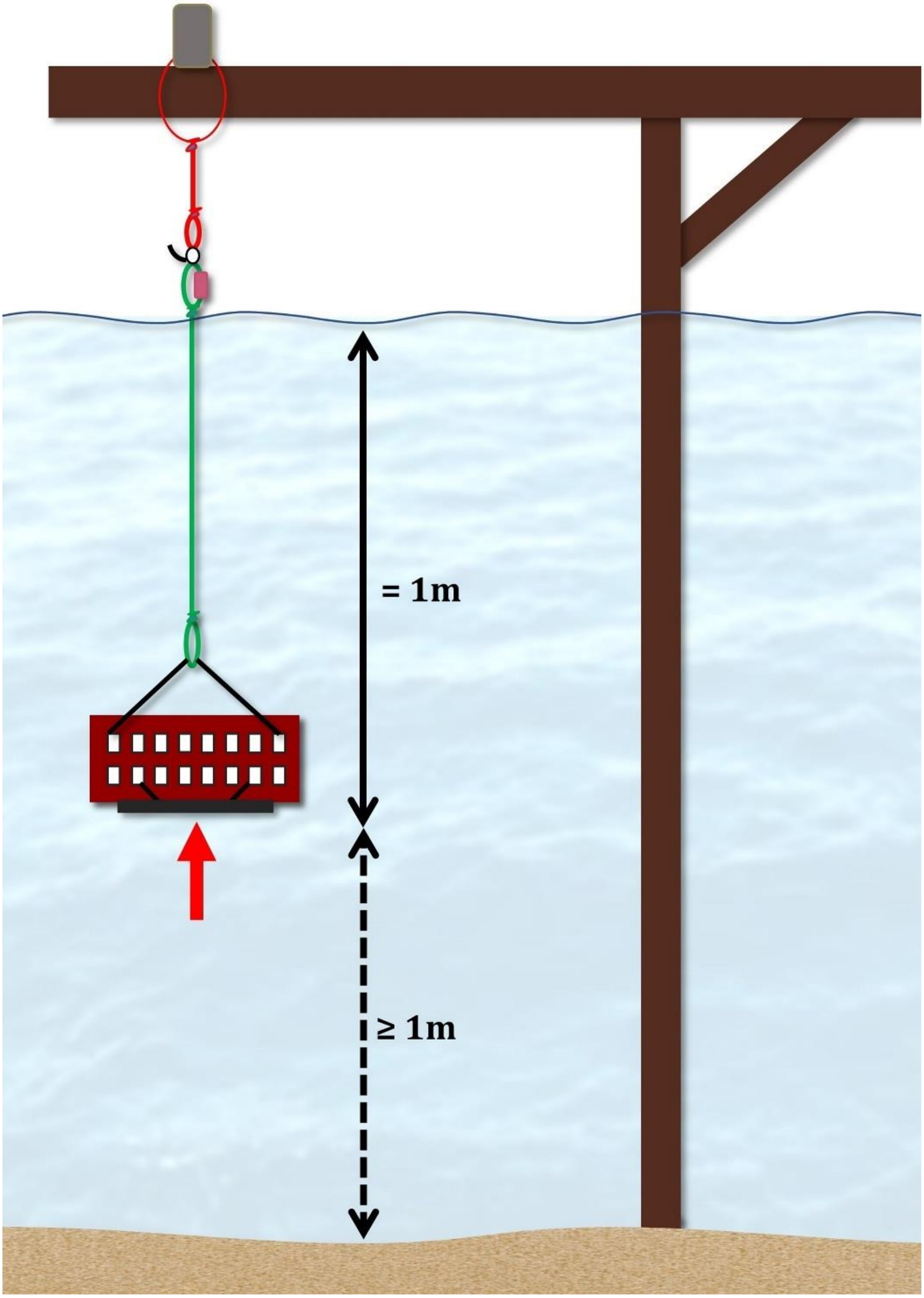

Figure 3. Schematic overview of the construction and deployment of a SETL fouling plate. Sanded 14 by 14 cm PVC fouling plates (indicated with the red arrow) are weighted down by a brick stone. The fouling plate is deployed 1 m below the water surface and preferably at least 1 m above the bottom.

Field experiments

SETL

Each SETL plate consists of a 14 by 14 cm sanded PVC plate and a brick stone attached to a rope and deployed 1 meter below the water surface (Figure 3). This setup follows the standardized method developed by the Smithsonian Environmental Research Center (SERC) (Hines and Ruiz 2001). A total of 98 SETL fouling plate structures were deployed for the Haringvliet field experiment (Table 1). Of these, 18 were already deployed during the long-term SETL project in the area and 80 were deployed for the short-term field experiment.

From the start of the short-term field experiment in June 2016, all fouling plates were monitored every 3 months: intervals here referred to as “summer” (July, August, September), “autumn” (October, November, December), “winter” (January, February, March), and “spring” (April, May, June) as they approximately correspond with the 4 seasons. Each monitoring period, the fouling plates were lifted out of the water and placed in a bucket filled with water (Figure 2). Each plate was photographed by taking one overview picture of the whole plate and about 25 detailed pictures of the fouling plate surface.

Dreissenid identification

The fouling plates were lifted out of the water for circa 15 minutes to identify and count all *D. polymorpha* and *D. r. bugensis* individuals on the plate. Both species were identified based on characteristics described in the literature (Domm et al. 1993, Pathy and Mackie 1993, Ram et al. 2012). Both living and dead individuals were recorded.

When measuring *D. polymorpha* and *D. r. bugensis*, all individuals were categorised into one of 9 size classes based on their length, each class representing 0.5 cm (A = 0–0.5 cm, B = 0.5–1 cm, C = 1–1.5 cm, D = 1.5-2 cm, E = 2–2.5 cm, F = 2.5–3 cm, G = 3–3.5 cm, H = 3.5–4 cm, I = 4–4.5 cm). Class I corresponds to the maximal size of *D. r. bugensis* based on the size range stated in the literature (Bij de Vaate 2008, Marescaux et al. 2012).

Data sources and tested parameters

Settlement period

During the long-term field experiment on settlement timing of both species, new sets, each with 3 fouling plates, were deployed at the end of autumn (December), winter (March), spring (June) and summer (September). These plates were retrieved after 3 months. The presence of young individuals (size class A–B) of *D. polymorpha* and *D. r. bugensis* on these plates were recorded to determine and compare their settlement timing and period.

Growth rate

Over the experiment, the length of dreissenids on 54 fouling plates was measured for one year. The shell length of dreissenids on plates deployed at the end of winter and the end of spring was measured every three months during the following year. In 2009–2010, three and four plates deployed in Numansdorp at the end of winter and the end of spring, respectively, were checked. In 2012–2013, dreissenids growing on three plates in Numansdorp deployed both at the end of winter and the end of spring were measured. In 2016–2017 during the short-term field experiment, 41 plates deployed at the end of spring were assessed of which 18 plates were from Tiengemeten and 23 plates from Numansdorp. These years were selected based on the availability of pictures taken during the long-term experiment involving the same plates throughout the year. In 2011–2012 *D. r. bugensis* gradually became dominant over *D. polymorpha* in the Haringvliet (*unpublished data*). Therefore, the selection of the assessed years for dreissenid growth rate revolved around this “year of shift” 2011–2012. Dreissenids measured in 2009–2010 predate the dominance shift from *D. polymorpha* to *D. r. bugensis*, while dreissenids in 2012–2013 were measured after this shift. The measurements from 2016–2017 were also included in the size curve, as these data represent the short-term extensive experiment. A correction was made for the measurements from fouling plates deployed at the end of winter 2009 for *D. polymorpha* by setting the size to 0 at the beginning of summer.

This was needed as there had already been settlement and growth of *D. polymorpha* in spring. To test whether the assigned categorical sizes were comparable to real dreissenid size evolution, the categorical sizes were subtracted by the average deviation of the categorical size from the real size during the first measurement period (= x – 0.176 cm). Individuals known to originate from external sources were eliminated from the growth experiment. We thereby ensured that the size evolution curve (Figure 4) represented the growth rate of a similar cohort. Mussels originating from external sources were too large to have settled and grown on the fouling plate itself. They were identified by comparing the maximum size of a certain dreissenid age group known from the literature, to the size of the individuals found in the field (Allen et al. 1999, Orlova and Panov 2004, Pollux et al. 2010, Wong et al. 2011). The external dreissenids exceeding the maximum size had settled on surrounding structures before fouling plate deployment in 2009, 2012 or 2016. Throughout both the long- and short-term field experiment, 6 new empty fouling plates were deployed during each control period. Therefore, we could check for settling individuals, identify them, and exclude them from the growth curve as well.

Relative dreissenid densities

To assess relative densities, only data from the large dock in Tiengemeten was considered, as for the last measurements in summer and autumn 2017 only data from this dock were available. However, population density patterns of both locations were compared to take location effects into account. The mean density and relative abundance on fouling plates populated by both species were assessed on 15, 15, 13, 8, 11 and 8 plates from summer 2016, autumn 2016, winter 2017, spring 2017, summer 2017 and autumn 2017, respectively. The density of both species was expressed in individuals per square meter to facilitate comparisons with existing literature (Stewart et al. 1998). As the bivalves were counted on 14 × 14 cm (= 196 cm^2) fouling plates, all values were thus multiplied by 51.

Table 1. The number and date of SETL fouling plates deployed at the two study sites for the long-term and short-term field experiment. The total number indicates the total amount of fouling plates deployed in the long-term experiment (18), the short-term experiment (80) in Tiengemeten (56) and Numansdorp (42).

Experiment	Deployment date	Tiengemeten	Numansdorp
Long-term			
18	Summer 2012	3	1
	Autumn 2012	2	3
	Winter 2012	1	0
	Summer 2014	2	3
	Summer 2015	3	0
Short-term			
80	Summer 2016	33	23
	Autumn 2016	3	3
	Winter 2016	3	3
	Spring 2017	3	3
	Summer 2017	3	3
Total		**56**	**42**

Light, temperature and salinity impacts

The effect of light intensity was studied by monitoring dreissenid settlement and density on 10 plates deployed from the large dock of Tiengemeten during the first year after settlement. Of these, 6 were deployed on the southern edge of the dock and exposed to sunlight (addressed as “half-shaded”, since settle plates hang upside down in the water (Figure 3)). The other 4 plates were deployed on the northern side of the dock in a shaded area. These 10 plates were the only plates strictly exposed to sunlight or shade and were therefore the only plates used for assessing light impact on settlement and density.

Data on temperature and salinity of the Haringvliet were acquired from the Dutch Ministry of Infrastructure and Water Management (Rijkswaterstaat 2018) (Figure 5). Both parameters were measured at mid-month in surface water near the Haringvliet sluice during both the long- and short-term field experiments between 2006 and 2017. Due to the unavailability of salinity data in 2017 at the time of analyses, salinity values were derived from chloride concentrations (salinity (‰) = $0.0018066 * [Cl^-]$ ($mg*L^{-1}$)) measured mid-month in surface water near the Haringvliet sluice by the Dutch Ministry of Infrastructure and Water Management (Rijkswaterstaat 2018).

Mortality

During the short-term field experiment, we kept track of empty shells on the fouling plates indicating the mortality of dreissenids. The relative number of empty shells for each period was compared to the total amount of dreissenids found during that time. Data was gathered from autumn 2016 until autumn 2017.

Interspecific competition

To assess whether the presence of *D. polymorpha* affected the density of *D. r. bugensis*, the densities of the latter on fouling plates with syntopic populations (both *D. polymorpha* and *D. r. bugensis*) and allotopic populations (only *D. r. bugensis*) were compared. The term “syntopic populations” is used to describe two species that occur together, while “allotopic populations” is used to indicate a species that occurs by itself, without the second species (Gittenberger and Moons 2011). A similar analysis for *D. polymorpha* was not possible as there were no fouling plates with allotopic *D. polymorpha* populations.

Impact of one-year-old mussels on settlement

The impact of one-year-old mussels on dreissenid settlement was assessed using a subset of the short-term experiment. The relative abundance and density of settling individuals were compared between 15-month-old fouling plates (June 2016–September 2017; containing

± one-year-old mussels) and 3-month-old empty fouling plates (June–September 2017).

Statistical analyses

A one-tailed Chi-square (χ^2) test was performed to test for differences in winter mortalities based on the absolute numbers of individuals in the winter 2017. Additionally, a one-tailed Chi-square (χ^2) test was used for assessing differences in the relative and absolute abundances of recently settled individuals of both species on 3-month-old and 15-month-old fouling plates.

A two-tailed Mann-Whitney U test was used to assess whether the abundances of *D. r. bugensis* individuals on the plates in Tiengemeten and Numansdorp were linked to the presence of *D. polymorpha* individuals on those plates. In addition, this test was used to assess whether densities of both species differed significantly for the two levels of shading, i.e. “shade” and “half shade”, during the four seasons from June 2016 to June 2017.

A linear mixed model regression was performed to determine the main factors related to variation in “shell size” between *D. polymorpha* and *D. r. bugensis* and between different years and seasons for each species. This linear mixed model regression was performed using “shell size” as independent variable, and “mussel species”, “year” and “season” as fixed response variables and “SETL plate ID” as a random effect variable. The analysis was performed using the software R (version 1.0.153, package nlme) with a statistical significance level of $P < 0.05$ (Pinheiro et al. 2017). As the H_0 for this test, we assumed there was no difference in “shell size” for the fixed variables (“mussel species”, “years” and “seasons”). Subsequently, pairwise post-hoc comparisons were carried out using Tukey HSD tests to assess within the response variables, which “mussel species”, “years” and/or “seasons” corresponded with significant differences in “shell sizes”. To validate the assumptions of normality and homogeneity of variance within the models, a histogram of the residuals was plotted, and the residuals were assessed against the fitted values (Supplementary 1).

Results

Settlement period

During the long-term field experiment over 2006–2017 both species were found to settle during summer (Table 2). *Dreissena polymorpha* showed earlier settlement starting in spring between 2006 and 2010. This changed in 2011–2012 when the settlement of *D. polymorpha* was delayed until summer. *Dreissena rostriformis bugensis* only started settling in the summer, with an exception of the early spring settlement in 2017, when both species settled in spring and summer. In conclusion, both species could settle in spring, however *D. polymorpha* did so more frequently. In the winter 2012, a one-time settlement event of dreissenids was recorded, however, the observed individuals were too small for species identification.

Growth rate

Size differed significantly between seasons, with continuous growth throughout the year (linear mixed model: F = 91.12, d.f. = 3, 138, N = 264, P < 0.0001) (Figure 4). The largest increases in size (0.25 and 0.26 cm per month) were measured during summer, while the lowest increase in size (0.03 and 0.05 cm per month) was found during winter for *D. polymorpha* and *D. r. bugensis*, respectively. The increase in *D. polymorpha* size (Figure 4A) showed no significant difference over the years. On the other hand, *D. r. bugensis* showed a significantly different size evolution over time (Figure 4B) (linear mixed model regression: F = 19.98, d.f. = 2, 27, P < 0.0001). The size evolution curves of *D. r. bugensis* on plates deployed in the same year clustered together. For *D. r. bugensis* the increase in size was significantly smaller in 2016–2017 compared to 2012–2013 (Tukey HSD: t = 3.472, d.f. = 27, P = 0.005) and to 2009–2010 (Tukey HSD: t = 3.292, d.f. = 27, P = 0.008). There was no significant difference in size between 2009–2010 and 2012–2013. These differences were usually due to a larger or smaller size increase during autumn of the respective years. When comparing the size evolution of both species with each other, *D. r. bugensis* grew faster than *D. polymorpha*, however, the difference was only significant during spring (Tukey HSD: t = 3.71, d.f. = 138, P = 0.007). These results were based on categorical size measurements, which were not significantly different from the real size evolution of dreissenids (Tukey HSD: summer t = 0.353, d.f. = 38, P = 0.726, autumn t = 0.699, d.f. = 32, P = 0.490; winter t = 0.001, d.f. = 38, P = 0.999, spring t = 0.438, d.f. = 60, P = 0.876). Therefore, we used the categorical sizes as a representation of real dreissenid size evolution.

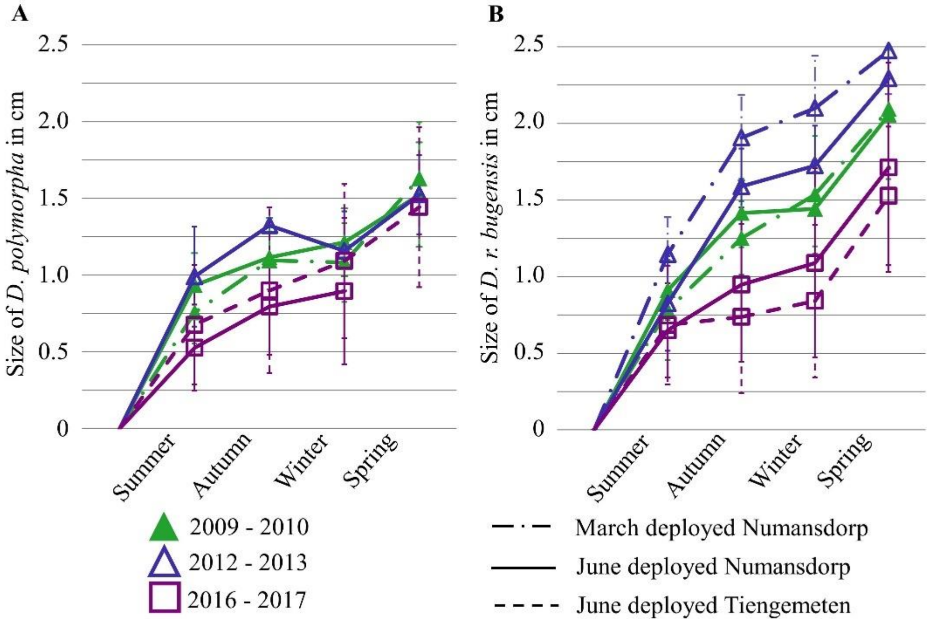

Figure 4. Mean categorical size of (A) *Dreissena polymorpha* and (B) *Dreissena rostriformis bugensis* on fouling plates deployed at the end of winter and/or the end of spring of 2009, 2012 and 2016. The size was measured at four occasions throughout the course of one year at the end of summer (September), autumn (December), winter (March) and spring (June). A correction was made for the measurements of the March deployed fouling plates in 2009 for *D. polymorpha* to set the size to 0 at the beginning of summer, as there had already been settlement and growth in spring.

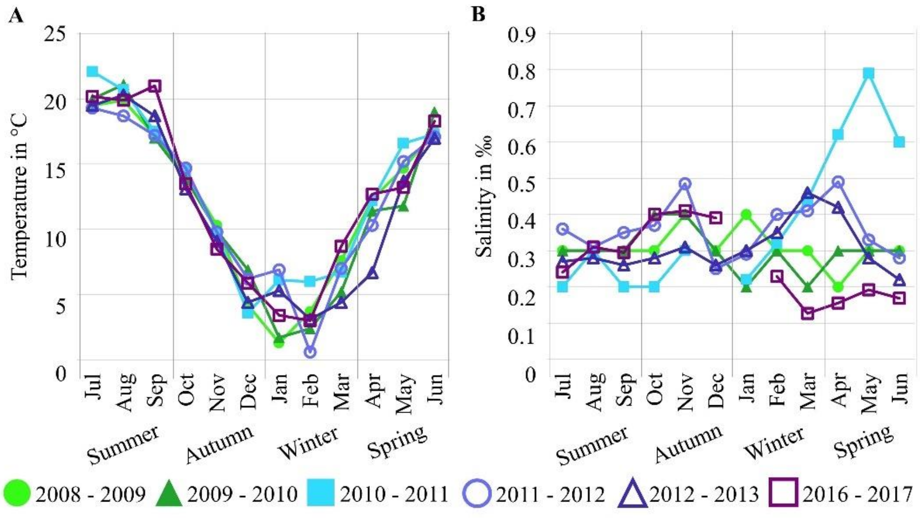

Figure 5. (A) Water temperature and (B) salinity in the Haringvliet measured mid-month in surface water near the Haringvliet sluice before (2008–2010), during (2011–2012) and after (2013) the dominance shift from *Dreissena polymorpha* to *Dreissena rostriformis bugensis*. Additionally, measurements during the short-term field experiment between June 2016 and May 2017 have been plotted. Gaps in the graph are due to lack of data (Rijkswaterstaat 2018).

Relative dreissenid densities

During the short-term field experiment in summer 2016, right after settlement, we observed the highest relative *D. polymorpha* abundance throughout the whole first year with a 40%–60% ratio for *D. polymorpha* and *D. r. bugensis* (Figure 6). During other periods of this experiment, there was a relative increase in *D. r. bugensis*. The highest relative abundance of *D. r. bugensis* was found in spring 2017 with a 5%–95% *D. polymorpha* : *D. r. bugensis* ratio. The new settlement period had already started in spring 2017, however, the *D. polymorpha* proportion did not increase. *Dreissena polymorpha* showed later settlement as the relative amount of this species increased to about 25% of total dreissenids in summer 2017. In autumn 2017 the relative amount of *D. polymorpha* increased even further to 29%.

Between summer and autumn 2017 there was a drop in the density of dreissenids when nearly three-quarters of the individuals disappeared and the number of individuals reduced from 10464 to 2749 per m². This decrease was stronger for *D. r. bugensis* than for *D. polymorpha* (Figure 6).

Table 2. Settlement period of *Dreissena rostriformis bugensis* and *Dreissena polymorpha* with a size up to 1 cm recorded over the period 2006–2017. P: *D. polymorpha* present; B: *D. r. bugensis* present; –: No dreissenids present; V: Dreissenids present, but too small for species identification; ?: No data.

Year	Winter	Spring	Summer	Autumn
2006	?	P	?	?
2007	–	P	P&B	?
2008	?	P	P&B	–
2009	–	P	P&B	–
2010	–	P	?	?
2011	?	?	P&B	?
2012	V	–	P&B	–
2013	–	–	P&B	–
2014	–	–	?	?
2015	?	–	P&B	–
2016	–	–	P&B	–
2017	–	P&B	P&B	–

Light effect on density

Figure 7 depicts the density of dreissenids per square meter on a subset of the short-term field experiment representing shaded and half-shaded plates. Both species had a higher settlement rate during summer 2016 on fouling plates deployed in shaded areas (Mann-Whitney U test: *D. polymorpha* W = 24, d.f. = 8, P = 0.01; *D. r. bugensis* W = 24, d.f. = 8, P = 0.01). However, the differences in densities on plates deployed in shaded and half-shaded areas minimized over time. When comparing the density in relation to light intensity throughout the year, both species showed the same pattern except for spring 2017. During this period *D. polymorpha* almost completely disappeared on half-shaded plates but remained present in low numbers on shaded plates. *Dreissena rostriformis bugensis*, on the other hand, had an equal density on both shaded and half-shaded plates.

Mortality

During the long-term SETL project, predation of dreissenids by Common Coots (*Fulica atra*) was recorded for the first time in our study area at the end of autumn 2017. The Common Coots were seen diving and resurfacing carrying clusters of dreissenids. Thereafter, they shook the clusters in the water or banged them on a rock to break up the clusters and swallow the shells. Additionally, a high number of remnants of byssal threads were present, up to a 10:8 ratio of remnants of byssal threads to living dreissenids during autumn 2017 (D'Hont, *pers. obs.*).

The relative contribution of empty, dead shells to the total number of dreissenids on all fouling plates was 3%, 9%, 1%, 2%, 0% for autumn 2016, winter 2017, spring 2017, summer 2017 and autumn 2017, respectively. Natural mortality appeared to be highest during winter (Supplementary 2). Additionally, the observed winter mortality was mostly due to the death of *D. polymorpha* as it significantly halved in number going from 629 individuals per m^2 in autumn 2016 to 294 individuals per m^2 in winter 2017 (Chi-square test: $\chi^2 = 78.724$, d.f. = 12, $P < 0.001$). The number of *D. r. bugensis* remained more or less stable going from 1473 individuals per m^2 in autumn 2016, to 1432 individuals per m^2 in winter 2017 (Chi-square test: $\chi^2 = 0.865$, d.f. = 12, $P = 0.352$) (Figure 6).

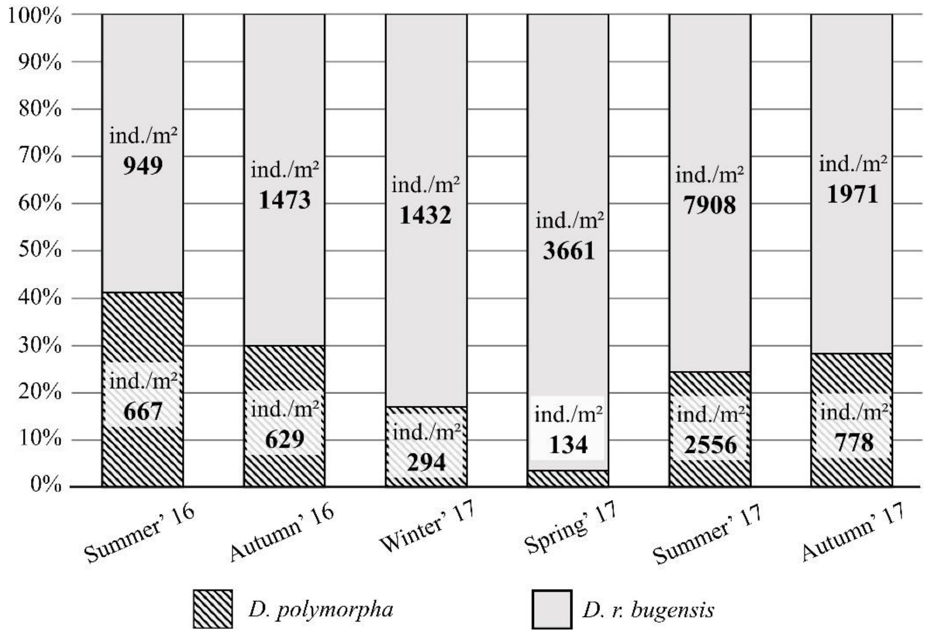

Figure 6. Percentage of *Dreissena rostriformis bugensis* and *Dreissena polymorpha* on fouling plates between summer 2016 and autumn 2017 measured on the large dock in Tiengemeten. The mean density of both species per square meter is indicated in the columns.

Interspecific competition

Dreissena rostriformis bugensis was found to have higher abundance in syntopic populations on fouling plates than in allotopic populations (Supplementary 3). This trend was recorded for both the small and large dock in Tiengemeten and the dock in Numansdorp. We found a significant difference for autumn 2016 in Numansdorp (Mann-Whitney U test: W = 9, d.f. = 4, P = 0.014).

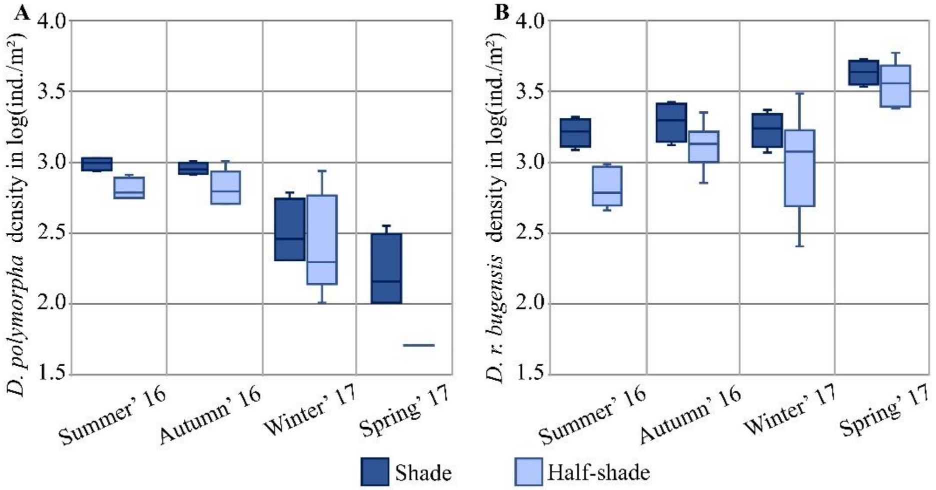

Figure 7. Density (log-transformed) of dreissenids (individuals per square meter, ind./m^2) of (A) *Dreissena polymorpha* and (B) *Dreissena rostriformis bugensis* on fouling plates shortly after settlement (summer; July–September 2016), after 6 months (autumn; October–December 2016), after 9 months (winter; January–March 2017) and one year after settlement (spring; April–June 2017). Six fouling plates were exposed to half-shade and four fouling plates were deployed in shade. The box-and-whisker plots represent the minimum, first quartile, median, third quartile and maximum density per sampling date.

Impact of one-year-old mussels on settlement

When comparing the young dreissenid ratio on 3-month-old fouling plates (22–78%; *D. polymorpha* – *D. r. bugensis*) with the young dreissenid ratio on 15-month-old fouling plates already containing one-year-old *D. polymorpha* and *D. r. bugensis* (11–89%; *D. polymorpha* – *D. r. bugensis*), we found a significant difference between both ratios (Chi-square test: χ^2 = 80.909, d.f. = 4, P = 0.0027). However, the absolute number of young individuals settling per plate was for both species still higher on the 3-month-old plates (Chi-square test: χ^2 = 6.579, d.f. = 4, P = 0.01) (Supplementary 4).

Discussion

Settlement period

Studies suggest that temperature is the main factor in determining the timing of settlement of both mussel species (Gerstenberger et al. 2011, Ram et al. 2012) (Table 2) which seems to correspond with our data. In winter 2012, the water temperature was close to 7 °C in January, cooled down to ± 0 °C in February and warmed up to 7 °C again in March (Figure 5A). This was the most extreme shift in winter temperature observed over the duration of the experiment since 2006. The extreme temperature shift was probably the reason for the settlement of dreissenids during that winter (Table 2). Settlement was only recorded during spring and summer in all other years. This agrees with Ram et al. (1996) who stated that changes in absolute temperature can trigger settlement. Due to their small size, it was impossible to provide certain identification based on pictures. If these were *D. polymorpha*, this temperature shift may explain the dominance shift to *D. r. bugensis* in the region recorded after 2011–2012 (*unpublished data*). In this case, *D. polymorpha* individuals who settled during winter may not have survived (see “Relative dreissenid densities and mortality” below).

In the American Great Lakes *D. r. bugensis* is able to settle at greater depths, and thus in cooler habitats, than *D. polymorpha* (Mills et al. 1993). Other studies also show earlier settlement onset for *D. r. bugensis* compared to *D. polymorpha* (Claxton and Mackie 1998, Gerstenberger et al. 2011, Ram et al. 2012). However, we found the opposite (Table 2). This earlier settlement onset for *D. polymorpha* may be related to salinity levels in the Haringvliet which were clearly higher in spring 2010–2013 than in summer (Figure 5B): *D. r. bugensis* has a lower salinity tolerance (Mills et al. 1996). In 2017, when salinities were relatively low, *D. r. bugensis* settled in spring for the first time (together with *D. polymorpha*). Such settlement patterns, possibly linked to salinities varying over the years, can only be explained based on long-term studies.

Growth rate

The highest growth rate of *D. polymorpha* was measured during summer (Figure 4) in agreement with Pollux et al. (2010), but in contrast to Allen et al. (1999) who recorded the highest growth rate during spring. Based on our study it is clear that growth rates can significantly differ between years, which may explain why a relatively fast growth rate was measured in spring over a study lasting just one year (Allen et al. 1999).

We observed *D. polymorpha* having a constant growth rate pattern over several years, in contrast to *D. r. bugensis*. For *D. r. bugensis* the growth rate can be almost twice as high in the same season of different years (Figure 4B). These yearly differences for *D. r. bugensis* may be explained by variations in temperature and salinity as these parameters showed strong annual variation in the Haringvliet (Figure 5). The observed variation in growth (Figure 4B) for *D. r. bugensis* did not seem to coincide with the differences in seasonal water temperatures in those years (Figure 5A), but was connected with salinity (Figure 5B). *Dreissena rostriformis bugensis* seems to adjust its growth rate to salinity concentrations measured in the Haringvliet, growing faster when salinity is lower: salinity in autumn 2012 was low while *D. r. bugensis* showed a remarkably high growth rate (Figure 5B). Additionally, salinity at the end of the autumn in December 2017 was high compared to the average salinity during that season. This might explain the low growth during autumn 2017. During winter, *D. r. bugensis* growth rate is generally low, so this likely explains why the correlation between growth rate and salinity was not very clear during winter. Dreissenids are known to have a decreased growth rate with increasing water salinity (Wright et al. 1996). This effect may be stronger for *D. r. bugensis*, as it is known that this species has a lower salinity tolerance than *D. polymorpha* (Mills et al. 1996). This lower salinity tolerance and the slightly brackish water (on average 0.3–0.4‰) may explain why *D. polymorpha* is still commonly present in the Haringvliet. *Dreissena rostriformis bugensis* is known to dominate at salinities between 0.00–0.02‰ (Mills et al. 1996).

Relative dreissenid densities and mortality

The relative dreissenid densities indicated that *D. r. bugensis* is the dominant species in the Haringvliet. Throughout the experiment, 60% to 95% of all dreissenids were *D. r. bugensis* (Figure 6). This may partly be explained by winter mortalities. For example, *D. polymorpha* density more than halved over winter 2016–2017, while densities of *D. r. bugensis* remained stable (Figure 6). This may be explained by higher growth rates for *D. r. bugensis*, permitting a large shell size and body mass by winter, which probably gives this species a better chance at surviving the winter (Marescaux et al. 2015).

During the secondary settlement event one year after deployment, in spring and summer 2017, *D. polymorpha* started to increase again in relative and absolute number (Figure 6). In autumn 2017 there was an even further increase in relative abundance of *D. polymorpha* giving an unexpectedly high relative abundance of *D. polymorpha* compared to *D. r. bugensis*, deviating from previous years. Based on observations in 2016, we would expect the relative *D. polymorpha* abundance to decrease again after the secondary settlement (Figure 6). This event co-occurred with a strong drop in the density of dreissenids in general on the fouling plates, losing almost three-quarters of the individuals, and a high number of empty byssal threads. This sudden unexpected drop in dreissenid densities may be related to predation by Common Coots which is well known in the literature (Cavé et al. 1989, Hamilton and Ankney 1994, Werner et al. 2005). For the first time in our study area, one Common Coot was observed feeding on dreissenids, which it picked from the fouling plates, in autumn 2017. The predation by Common Coots might even be selective for *D. r. bugensis*, as it preferably predated on clusters. *Dreissena polymorpha* individuals are usually not found in such clusters. Another potential explanation for the recorded sudden drop of *D. r. bugensis* may be that *D. r. bugensis* individuals died because of a change in the environment, for example in salinity. If that had happened, we would have expected to record empty dreissenid shells on the plates after winter. As such empty shells were not found, predation by Common Coots remains the most likely explanation.

In the Haringvliet, both species preferred shaded habitats as their initial density was higher on those fouling plates (Figure 7), which corresponds to results of other studies (Kobak 2001, Kobak and Nowacki 2007, Marsden and Lansky 2000). The shading preference was higher in settling individuals than at later stages during succession (Figure 7), in contrast to the findings of Kobak and Nowacki (2007). This might be due to spatial competition during later stages of succession causing individuals to migrate to, or settle on, half-shaded plates with a higher light intensity. One year after settlement the densities of *D. r. bugensis* on half-shaded fouling plates were equal to that on shaded fouling plates (Figure 7). Most likely *D. r. bugensis* is better at coping with higher light intensities giving it a competitive advantage over *D. polymorpha* in our study area. This suggests that *D. r. bugensis* can settle in a wider range of habitats (Mills et al. 1993).

Interspecific competition and the impact of one-year-old mussels on settlement

Dreissena rostriformis bugensis was more abundant in syntopic populations than in allotopic populations for settling individuals, 6 and 12-month-old individuals (Supplementary 3) thus showing a greater increase in abundance in syntopic populations compared to *D. polymorpha*. The capacity of an invasive species to increase its abundance in the presence of competitors is a known trait for some invasive species, such as the invasive tunicate *Botrylloides violaceus* and the native *Botryllus schlosseri* (Gittenberger and Moons 2011). It may be that the presence of *D. polymorpha* causes *D. r. bugensis* to increase in abundance by somehow promoting settlement of the latter. A similar phenomenon was observed when comparing 3-month-old fouling plates to 15-month-old plates: *D. r. bugensis* showed higher relative abundance on 15-month-old plates where one-year-old *D. polymorpha* were already present (Supplementary 4). This interspecific relationship provides an advantage for *D. r. bugensis* in the competition for space against *D. polymorpha* (Connell and Slatyer 1977, Jackson 1979).

Conclusions

Dreissena polymorpha appeared to have a competitive benefit over *D. r. bugensis* through earlier settlement onset, settling in spring in 2006–2010. *Dreissena rostriformis bugensis* on the other hand was only found to settle in summer except for 2017. This may be linked to relatively high Haringvliet salinities in the spring of those years as *Dreissena polymorpha* has a higher salinity tolerance. Our results support this hypothesis as in 2017, when salinities were relatively low, settlement of *D. r. bugensis* was also recorded in spring. In the Haringvliet the Common Coot preferred to predate *D. r. bugensis* clusters over *D. polymorpha* individuals, as the latter are usually not found in clusters. Despite these competitive advantages for *D. polymorpha*, *D. r. bugensis* is the dominant species in the region. One explanation is that *D. r. bugensis* is more light tolerant while *D. polymorpha* was practically absent on light-exposed fouling plates. This suggests that *D. r. bugensis* can settle in a wider range of habitats than *D. polymorpha.* Higher growth rate, especially during spring, may also contribute to the dominance of *D. r. bugensis*, despite the negative impact of salinity on growth rate. Generally, *D. r. bugensis* occurred in higher densities on fouling plates than *D. polymorpha*. A striking new finding is that *D. polymorpha* may induce the settlement of *D. r. bugensis* individuals: the density of *D. r. bugensis* was significantly higher in syntopic populations in comparison to allotopic populations. A final potential reason for the dominance of *D. r. bugensis* concerns winter mortalities. While the absolute number of *D. polymorpha* individuals on the plates more than halved over the winter 2016–2017, no significant decrease in numbers of mussels was recorded that winter for *D. r. bugensis*.

In summary, although *D. polymorpha* is able to settle earlier, is more salinity tolerant, and is predated less than *D. r. bugensis*, *D. r. bugensis* remains the dominant species in the region. This may be explained by being able to settle in a wider range of habitats, adjusting growth and settlement better to seasonally and annually varying salinities and temperatures, having lower winter mortality, and settling in higher numbers when *D. polymorpha* individuals are already present on the substrate.

This study shows the importance of long-term data to assess competitive benefits like settlement, growth and interactions between *D. polymorpha* and *D. r. bugensis*. The impact of drivers such as movement speed and duration, grouping and clustering behaviour, small-scale location preferences, interactions with other species and fouling plate coverage on this dominance shift is still not completely understood, especially related to long-term data.

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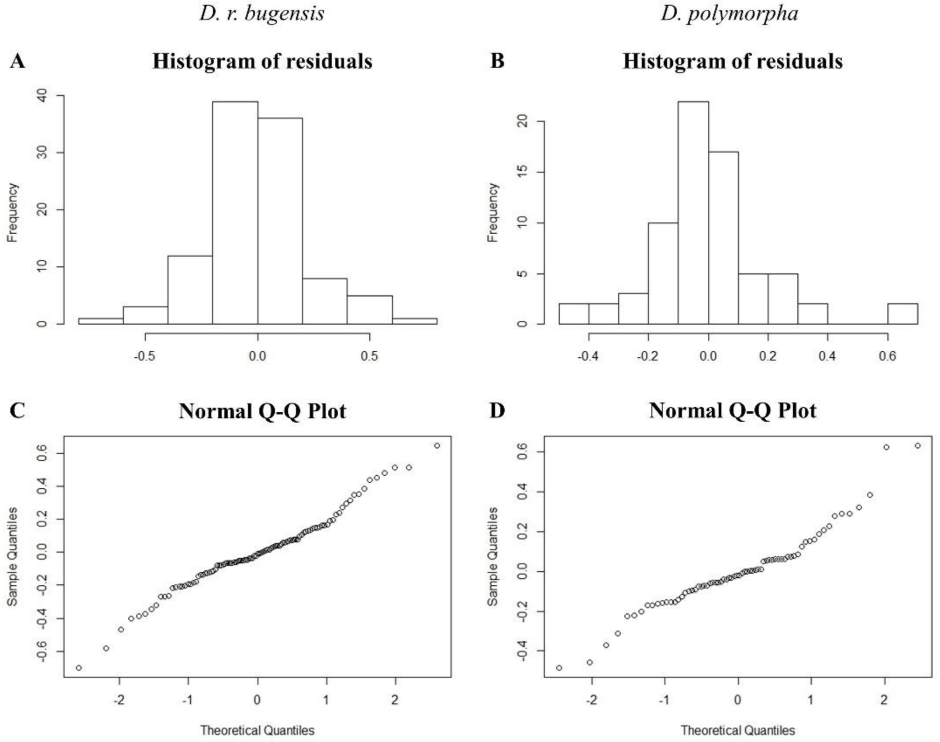
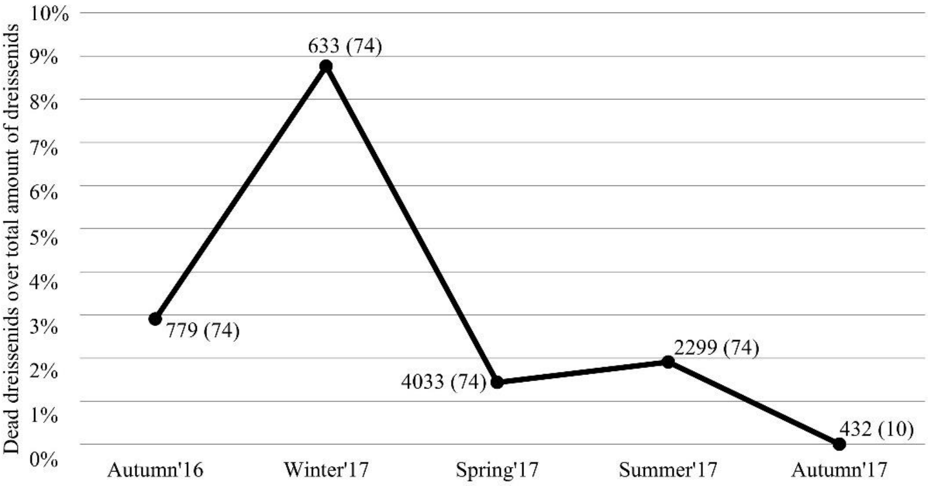
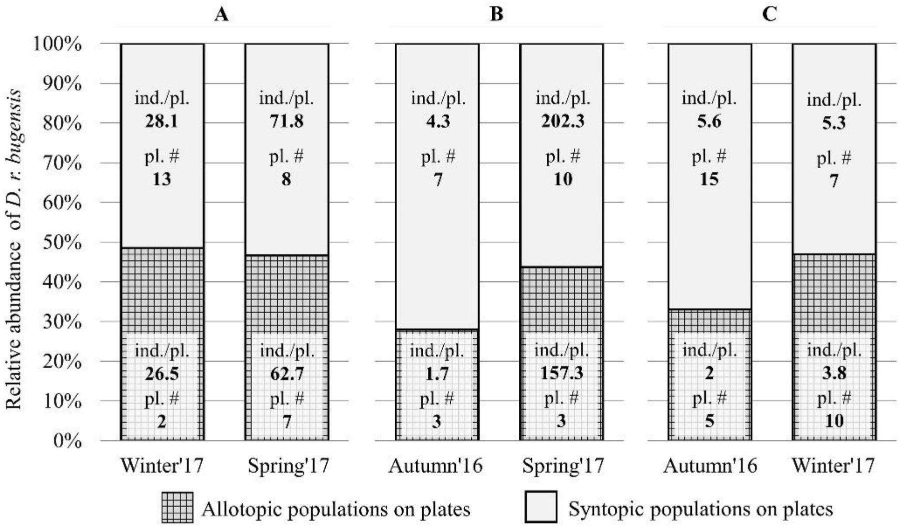


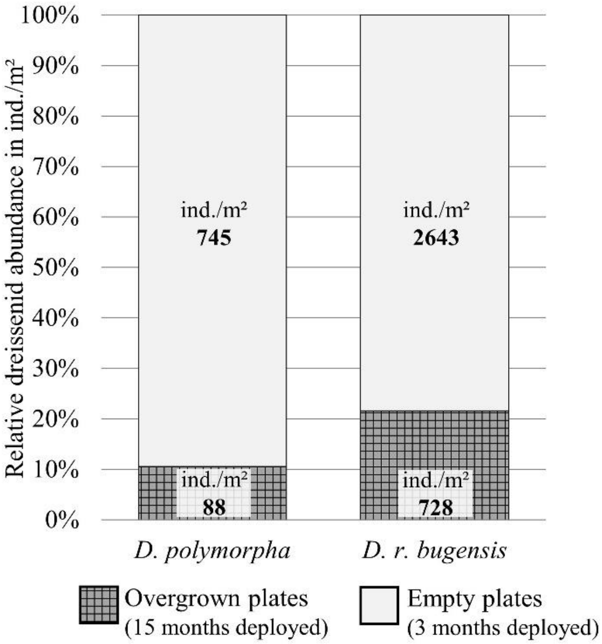
Supplementary 1. To validate the assumptions of normality and homogeneity of variance within the statistical models, a histogram of the residuals was plotted, and the residuals were assessed against the fitted values. (A) and (B) represent the histogram of residuals of *Dreissena rostriformis bugensis* and *Dreissena polymorpha,* respectively. (C) and (D) represent the qq plots of *Dreissena rostriformis bugensis* and *Dreissena polymorpha,* respectively.



Supplementary 2. The percentages of all dreissenids scored in various seasons which were found to be dead (empty shells). The total number of dreissenids recorded (alive and dead) is indicated for each season. In between brackets, the number of plates is indicated on which the dreissenids were scored. The SETL plates were deployed at the end of spring 2016 and checked after each season (at three-month intervals). After the first three months (Summer 2016 check) 721 dreissenids were scored. As these were too small (< 0.5 cm) to differentiate between alive and dead specimens, the graph starts with presenting the results of Autumn 2016.



Supplementary 3. The relative abundance of *Dreissena rostriformis bugensis* in syntopic populations on plates (both *Dreissena polymorpha* and *D. r. bugensis* were present) and allotopic populations on plates (only *D. r. bugensis* was present). Allotopic populations were only found for *D. r. bugensis*. The plates were deployed off docks A (large dock) and B (small dock) in Tiengemeten and dock C in Numansdorp. Only seasons where both syntopic and allotopic populations on fouling plates were present were included. The average number of specimens per plates (ind./pl.) and the number of plates (pl. #) on which these were scored are indicated in the column.



Supplementary 4. The relative abundance of newly settled *Dreissena polymorpha* and *Dreissena rostriformis bugensis* (< 1 cm). They were assessed on 15-month-old fouling plates containing about one-year-old dreissenids (*D. polymorpha* and *D. r. bugensis*) and empty three-month-old fouling plates. The average density per square meter (ind./m²) is indicated in the columns.

Chapter 3

Dreissenids' breaking loose: Differential attachment as a possible driver of the dominance shift between two invasive mussel species

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Key words: Attachment strength, byssus threads, detachment location, Quagga mussel, shell size, Zebra mussel

Abstract

Ponto-Caspian dreissenids are notorious freshwater invaders. Recently, widespread observations show a dominance shift from the early invader, *Dreissena polymorpha*, to its successor, *Dreissena bugensis*. These observations likely reflect congeneric species differences in physiological and behavioural traits. Here, we assessed the mussel attachment strength, attachment rate, and the mode of byssal failure as trait differences that could potentially contribute to dominance shifts. The attachment traits were measured in field and laboratory experiments. Fouling plates were deployed in the Rhine-Meuse river delta and dreissenids were collected and acclimatised in 60 litre non-aerated freshwater tanks. Attachment strength was positively correlated with shell size. The attachment strength of *D. bugensis* was significantly greater compared to slower growing *D. polymorpha* individuals of a dreissenid field assemblage. This corresponded to the superior byssal thread morphology of *D. bugensis* (i.e. higher number and two times wider byssal threads). Moreover, our results indicated that byssal threads of *D. bugensis* are stronger than those of *D. polymorpha*, as the latter ruptured more often. Additionally, *D. bugensis* had a significantly lower attachment rate than *D. polymorpha*. Having a greater attachment strength gives *D. bugensis* an advantage when it comes to withstanding currents and predators. On the other hand, not being attached allows an individual to actively move around. This would allow them to move away from fast changing unfavourable environmental conditions. These attachment traits indicate competitive benefits for *D. bugensis* over *D. polymorpha*, therefore possibly contributing to the dominance shifts.

Introduction

Both the zebra mussel *Dreissena polymorpha* (Pallas 1771) and the quagga mussel *Dreissena bugensis* (also described as Dreissena rostriformis bugensis auct., Andrusov 1897, Wesselingh et al. 2019) have successfully invaded hard, freshwater substrates in large parts of Europe and North America (Karatayev et al. 2011, Matthews et al. 2014, Collas et al. 2018). *Dreissena polymorpha* was introduced in Western Europe at the beginning of the 19th century (Van der Velde et al. 2010, Sousa et al. 2011). *Dreissena bugensis* spread in the same area during the mid-20th century (Therriault et al. 2005). This led to the co-occurrence of both species in their native (Zhulidov et al. 2004) and introduced areas (Grutters et al. 2012). During the last decades, there have been widespread observations of a shift going from the first introduced *D. polymorpha* to the recent invader *D. bugensis* as the dominant species (Matthews et al. 2014, Marescaux et al. 2015, Ginn et al. 2018). These shifts were for example observed in the 1970s in their native area in Ukraine (Wesselingh et al. 2019) and in the 1990s in introduced regions in the Great Lakes in North America (Ricciardi and Whoriskey 2004). Only a few studies have assessed mechanisms driving this dominance shift (D'Hont et al. 2018). These studies so far found that although *D. polymorpha* is able to settle earlier in the season, is more salinity tolerant, and predators seem to target this species less than *D. bugensis*, *D. bugensis* usually remains dominant where both species co-occur (Karatayev et al. 2014, Naddafi and Rudstam 2014, D'Hont et al. 2018). *Dreissena bugensis* is able to settle in a wider range of habitats, is found at a greater depth range, grows faster and larger and adjusts its growth and settlement better to seasonally and annually varying salinities and temperatures than *D. polymorpha* (Claxton and Mackie 1998, Orlova et al. 2005, Gerstenberger et al. 2011, Metz et al. 2018). *Dreissena bugensis* is known to have a higher assimilation efficiency, a higher activity of certain enzymes, higher filtration rates and lower respiration rates (Stoeckmann 2003, Ram et al. 2012, Karatayev et al. 2014). Moreover, this species has lower winter mortality, has a higher resistance to cold temperature and settles in higher numbers when *D. polymorpha* individuals are already present on the substrate (Orlova et al. 2005, D'Hont et al. 2018). These characteristics proved a competitive benefit for *D. bugensis* over *D. polymorpha*. However, several other plausible mechanisms have not been studied extensively, such as differences between both species in mobility, attachment strength and detachment ability (Peyer at al. 2009, Balogh et al. 2019).

Both dreissenid species attach to hard substratum using byssal threads secreted by the byssus gland at the base of the foot of the mussel (Kobak et al. 2009). Biofouling of hard substrates such as rock, wood, ship hulls, hydraulic engineering structures and water intake facilities causes severe environmental problems and high economic costs (Pimentel et al. 2005, Grutters et al. 2012). Moreover, other species e.g. crayfish or native freshwater mussels can be smothered to death by heavy fouling of dreissenids, blocking their siphons and competing for resources (Ricciardi et al. 1996, Sousa et al. 2011, Gonçalves et al. 2013, Bódis et al. 2014).

Comparative studies on the attachment traits of both dreissenids are relatively scarce as most studies have so far focused on *D. polymorpha* (Ackerman et al. 1996, Dormon et al. 1997, Kobak et al. 2009, Czarnołęski et al. 2010). Moreover, in situ field data is lacking making it difficult to interpret attachment traits in natural communities (Kobak 2001). Studies assessing the attachment strength and byssal thread synthesis rate in laboratory setups found higher values for *D. polymorpha* compared to *D. bugensis* (Peyer et al. 2009, Grutters et al. 2012, Collas et al. 2018). Balogh et al. (2019) unravelled a more complex pattern as the attachment strength increased with shell size. For larger individuals the previously observed pattern reversed as larger (> 1.2 cm) *D. bugensis* individuals had a higher attachment strength than *D. polymorpha* individuals of the same size (Balogh et al. 2019). Next to the shell size, the number and thickness of byssal threads have a positive impact on the attachment strength of dreissenids as well (Bell and Gosline 1997, Kobak 2006). Additionally, the comparison between the natural detachment mechanisms of both dreissenids is lacking. The common blue mussel (*Mytilus edulis*) is known to voluntary detach its entire byssal mass (Eckroat et al. 1993). Voluntary detachment is also known for *D. polymorpha* individuals (Kobak et al. 2009, Dzierżyńska-Białończyk et al. 2018). However, the mechanism behind this voluntary detachment is not well understood

(Eckroat et al. 1993, Kobak et al. 2009). Most dreissenid attachment studies focus on the forced detachment of the Ponto-Caspian mussels. The mode of byssal failure or detachment location of dreissenids indicates the part of the attachment structure (adhesive plaques, byssal threads or stem detachment) that ruptures during forced detachment of an individual (Dormon et al. 1997). Adhesive plaque detachment is mostly due to the detachment of the glue from the substrate. The byssal threads themselves can rupture as well. The rupture of the stem (where all byssal threads are joined together) from the byssal gland causes the entire byssal mass to be separated from the mussel. To our knowledge, our study was the first comparative assessment of the detachment location of both species. Dormon et al. (1997) studied the detachment location of *D. polymorpha* individuals. They found that the force required to detach the mussels was directly proportional to the percentage of broken byssal threads and to the detachment location.

It has been proven that the presence of other species, might affect dreissenid behaviour. This effect of species assemblage has been assessed by comparing syntopic populations (both *D. polymorpha* and *D. bugensis* together) and allotopic populations (either *D. polymorpha* or *D. bugensis*) to each other. D'Hont et al. (2018) found that a higher number of *D. bugensis* settled when *D. polymorpha* individuals were already present on the substrate. However, the effect of the presence of *D. bugensis* on the *D. polymorpha* attachment rate (whether an individual attached to the substrate or not) and vice versa is not yet known. The effect of the light regime on the attachment rate of both species has not been thoroughly studied either. Generally, *D. polymorpha* individuals prefer dark environments as they have higher attachment rates in dark environments (Kobak 2013, Kobak et al. 2009, D'Hont et al. 2018). However, no such preference for dark environments was found for *D. bugensis* individuals on field deployed fouling plates (D'Hont et al. 2018).

This study aimed at unravelling whether differences in attachment traits of both dreissenid species might affect the outcome of interspecific competition. A stronger attachment or higher attachment rate might cause a competitive benefit when it comes to withstanding predation and environmental disturbances such as rapid changes in water level and flow velocity of rivers and canals, caused for example by upstream rainfall or by ship-induced currents (Koopman et al. 2018). On the other hand, being able to detach faster and easier might help mussels to relocate to a more suitable habitat in case of sudden unfavourable environmental changes (e.g. light, temperature, salinity). This way, an individual has a higher chance of reaching its ideal environment where it can thrive. Consequently, the attachment traits of dreissenids can to some degree be one of the drivers of the observed dominance shift between both species, especially in rapidly changing environments. Therefore, we conducted a comparative study focused on these attachment traits. Balogh et al. (2019) unravelled a more complex pattern in the attachment strength of both dreissenid species when comparing different sized individuals to each other. During our study, we wondered whether a comparison between the frequently selected shell size range (1-2 cm) in laboratory experiments and the shell size range found in the field would result in a similar pattern. For individuals of the same size, we expected a higher attachment strength for *D. polymorpha*, while for individuals of the same age, we would expect *D. bugensis* to have a higher attachment strength. This hypothesis is based on the fact that the attachment strength increases with shell size and that *D. bugensis* individuals grow faster and larger than *D. polymorpha* individuals do (D'Hont et al. 2018, Balogh et al. 2019). Additionally, the link between the morphology of the byssal threads (number and thickness) and the attachment strength was assessed. Based on previous studies, we expect a positive relationship between these two attachment traits. The differential attachment rate and whether or not the attachment rate is affected by the species assemblage and light regime was studied as well. Finally, the detachment location for both species and a possible link between the detachment location and the individual's attachment strength was assessed. We would expect that the detachment location is related to the attachment strength of an individual and to the strength of its byssal threads. For example, having a lower number of byssal thread failures, combined with a greater attachment strength, could indicate stronger byssal threads for this species. By testing these hypotheses, we aimed at elucidating whether differential attachment and detachment of mussel species can be one of the driving forces behind the observed dominance shift between *D. polymorpha* and *D. bugensis*.

Material and methods

Sampling sites

The sampling sites of dreissenids for this study were located in the Haringvliet and Hollands Diep, which are a part of the Rhine-Meuse delta in the Netherlands. Individuals of both dreissenid species were collected in front of the sluice in the ferry harbour of the island Tiengemeten (N 51°45.261'; E 4°19.046') and about 8 km upstream in the same river system at the harbour entrance of Numansdorp (N 51°43.037'; E 4°26.211'). *Dreissena polymorpha* was already present in this area when *D. bugensis* was observed here for the first time in Western Europe in 2006, after which these species co-existed (Molloy et al. 2007, Schonenberg and Gittenberger 2008). These mussels were monitored ever since using fouling plates of the SETtLement (SETL) project (Gittenberger et al. 2017). Dreissenids were collected at Numansdorp and Tiengemeten in four separate batches on the 19th of December 2017 (batch 1), 6th of March 2018 (batch 2), 12th of June 2018 (batch 3), 5th of December 2018 (batch 4; Figure 1). These batches consisted of 80 (40 *D. bugensis* and 40 *D. polymorpha*, batch 1-3) and 200 (100 *D. bugensis* and 100 *D. polymorpha*, batch 4) individuals, respectively. In addition to the collection of dreissenids, 21 extra fouling plates were deployed from the floating dock in the ferry harbour of Tiengemeten in June of 2016. Structures in the SETL project consisted of 14x14 cm sanded PVC plates weighed down by a brick stone and deployed one meter below the water surface (Hines and Ruiz 2001, Schonenberg and Gittenberger 2008, D'Hont et al. 2018). The dreissenids on these SETL plates were considered to be batch 5 measured on the 6th of March 2018 (Figure 1). Batch 5 consisted of 169 individuals (79 *D. polymorpha* and 90 *D. bugensis*).

Field experiment

SETL plates were deployed in front of the sluice in the ferry harbour of the island Tiengemeten for 21 months before retrieval for attachment measurements on the 6th of March 2018 (batch 5; Figure 1). The attachment strength of 169 dreissenids on field-deployed fouling plates was measured. This number of dreissenids represented all individuals of each species present on the fouling plates, which we will further address as the whole dreissenid field assemblage. For *D. polymorpha* and *D. bugensis*, this corresponded to a shell size of 0.4-2.0 cm and 0.4-3.2 cm, respectively.

Laboratory experiment

The laboratory experiments were conducted with dreissenids from batch 1-4 who all had a shell size between 1-2 cm and were all collected from both Numansdorp and Tiengemeten. After collection in the field, the mussels were acclimatised to room temperature (±20 °C) for 24 to 48 hours after which all byssal threads were cut off using small scissors. Each dreissenid batch was kept in four polyethene tanks each containing 60 litres of non-aerated standing fresh tap water. The tap water had an initial oxygen level of 6.0-8.0 mg/l and a pH of 7.84, temperature of 16.9 °C and salinity of 0.28 PSU as measured on the second day of the experiment. Within the Haringvliet the pH normally ranges between 7.7 and 8.6, the salinity between 0.2 and 0.8 PSU and the temperature between 0 and 28 °C (Rijkswaterstaat 2020). All tanks were uniformly illuminated with daylight coming through a window at the north side of the laboratory, with a natural photoperiod (batch 1 and 4: 8L:16D, batch 2: 11L:13D, batch 3: 16L:8D).

Attachment traits

Attachment rate

The attachment rate of 435 dreissenids from batches 1, 2, 3 and 4 to the hard substrate was assessed. Mussels from each batch were placed in four polyethene tanks (20 individuals per tank for batches 1-3, 50 individuals per tank for batch 4) (Figure 1). During the experiment with batch 4 five individuals died, explaining the odd number of individuals tested. The experiments to assess the attachment rate of batches 1-4 ran for eleven, four, seven and seven days, respectively. These experiments differed in duration because they were originally designed as a movement experiment by D'Hont et al. (2021). However, as can be seen in our results, the difference in experiment duration had no effect on the number of individuals attached.

Next to the species-specific attachment rate, the effect of the presence of the other species on the attachment rate was assessed. The laboratory setup consisted of two tanks containing a syntopic population (two tanks: 10 *D. polymorpha* and 10 *D. bugensis*; 40 individuals in total) and two tanks containing an allotopic population (one tank: 20 *D. polymorpha*; and one tank: 20 *D. bugensis*; 40 individuals in total). This setup was replicated for batches 1 and 2 for in total 80 individuals for both species.

Batch 3 was used for assessing the effect of light regime on the attachment rate of dreissenids. In total 40 individuals of each species were divided amongst the four tanks, two tanks for each species. One of these two tanks was exposed to a light regime while the other one was kept in the dark at all times (20 *D. polymorpha* exposed to light and 20 to darkness, 20 *D. bugensis* exposed to light and 20 to darkness). The tanks with light treatment were illuminated by daylight with a natural photoperiod (16L:8D). The dark treatments were achieved by covering the tanks with a lid. This exposed the dreissenids to complete darkness, as the tanks were not translucent.

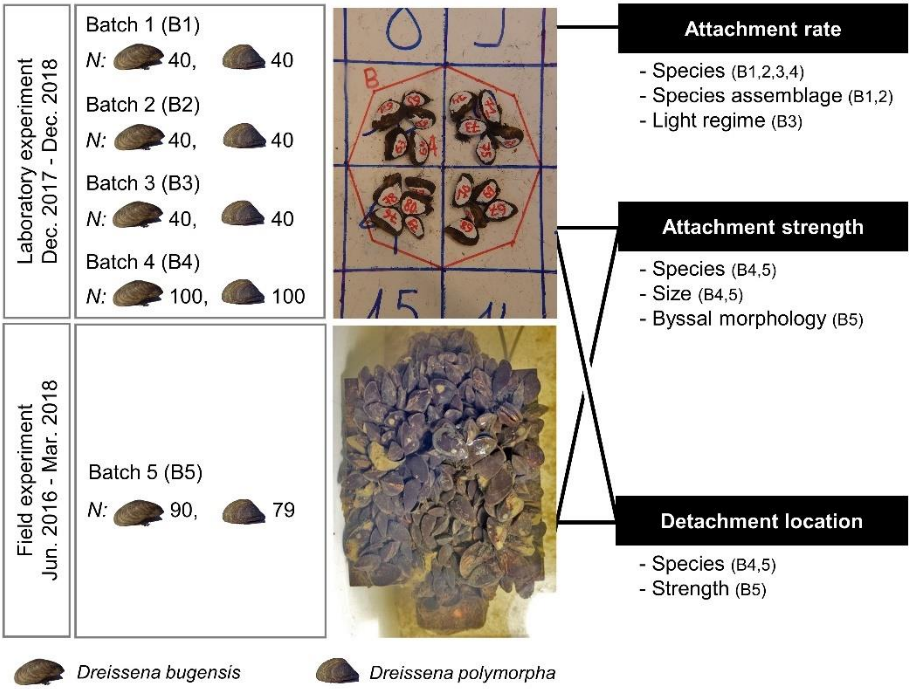


Figure 1. The setup of the field and laboratory experiments. The number of individuals is indicated by N. The right part of the figure shows the research topics (between brackets: batch(es) of dreissenids used for analyses). For assessing the attachment strength and detachment location, mussels from field and laboratory experiments were used. The attachment rate was derived from laboratory experiments.

Attachment strength

The attachment strength was measured for individuals that attached themselves under laboratory circumstances (batch 4), as well as for individuals that attached themselves in the field (batch 5, Figure 1). This way the attachment strength of mussels in an artificial environment was compared with that of mussels on plates in natural conditions. This comparison allowed for an assessment of the effect of the environment, substrate and attachment duration on the attachment strength of individuals. For the laboratory experiment the attachment strength of 200 mussels (100 *D. polymorpha* and 100 *D. bugensis*) was measured. The individuals were left to attach for seven days in the four tanks (four times 50 individuals). A duration of seven days was chosen as the attachment strength pattern of *D. polymorpha* and *D. bugensis* is supposed to remain equal after seven days, as was found by Balogh et al. (2019) for same sized (1-2 cm) individuals in the laboratory.

The attachment strength of both dreissenid species was measured using a Sauter FK10 digital force gauge (capacity: 10 N, resolution: 0.005 N) (Collas et al. 2018). Individuals exceeding the 10 N limit of the Sauter FK10 digital force gauge were detached using an analogue G&G 20N spring balance. A harness of fishing wire was used to get hold of the mussel on each of both sides of the byssal threads (Figure 2). Mussels were attached to the force gauge and detached from the substrate by gently pulling up the force gauge vertically and perpendicular to the substrate. Individuals of the same size (1-2 cm, batch 4 and 5) and of the whole dreissenid field assemblage (batch 5) were compared to each other. The same sized individuals did not differ significantly in size (Mann-Whitney U test: U = 1224.00, N1 = 53, N2 = 54, P = 0.190).

The relationship between the attachment strength and the byssal morphology was assessed by measuring the width and number of byssal threads for individuals of the whole dreissenid field assemblage. The byssal threads were collected by carefully cutting them from the individual as close as possible to the byssal gland and/or by picking the remaining ones from the substrate and were stored on 96% ethanol. The width of the collected byssal threads was measured using a Dino-Light AM7013MZT digital microscope and the program DinoCapture 2.0 version 1.5.27.B. The width in the middle of each byssal thread was measured in μm at a magnification of 250x. On average 25 byssal threads were measured per individual and these values were used to calculate the average width of their byssal threads. Furthermore, the number of byssal threads was counted for field experiment mussels, which detached at the byssal gland in the mussels' foot, as this represented the only detachment type where all byssal threads could certainly be accounted for.

Detachment location

The detachment location of the byssal threads was determined by distinguishing between three categories: 1) plaques detachment: mostly due to the detachment of adhesive plaques or glue from the substrate, 2) byssal thread detachment: mostly due to the rupture of the byssal threads, 3) stem detachment: the rupture of the stem from the byssal gland, causing the entire byssal mass to be separated from the mussel. The difference between detachment locations was assessed between both species for batch 4 and 5. Within each species we additionally assessed whether there was a relationship between the detachment location and their attachment strength (batch 5).

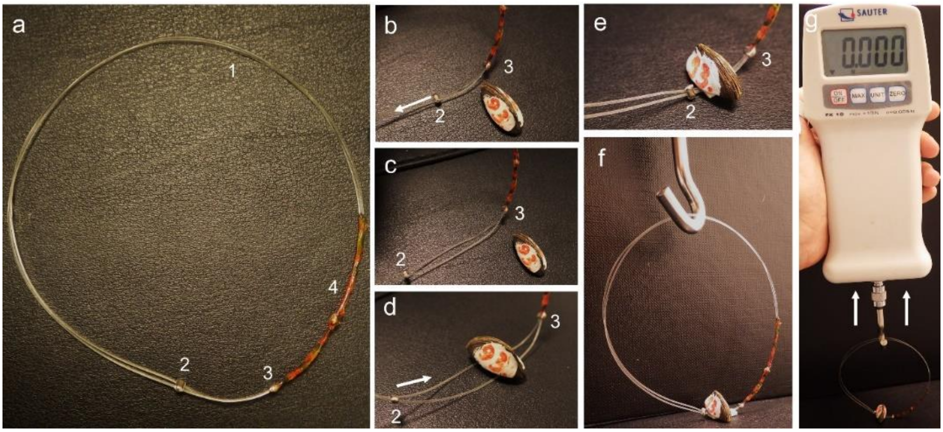


Figure 2. The harness of fishing wire and Sauter FK10 digital force gauge used to measure the attachment strength of dreissenids. Panel a gives an overview of the harness with 1) two fishing wire loops, 2) a movable aluminium crimp, 3) a fixed aluminium crimp, and 4) the red and black area where both loops are glued together. The movable aluminium crimp (2) can be slid back and forward (panel b-e) to secure the mussel while detaching it from the substrate (panel f-g).

Statistical analyses

To assess the effects of byssal morphology traits on the attachment strength, a general linear model was constructed by backwards stepwise selection. The dependent variable "attachment strength" and explanatory variables “species”, “shell length”, “byssus width”, “byssus count” and all their first order interactions were included in the initial model. Backwards stepwise model selection led to the exclusion of all non-significant first order interaction effects and the byssal thread counts. We tested whether adding tank as a random factor would improve model fit by comparing model fit parameters. However, the models with this random factor did not lead to a significantly better model fit. Therefore, we chose to use the least complex model without random factors. To assess the linear interdependence of both byssal thread traits (width and count), a Pearson correlation test was performed. A Spearman rank correlation test was performed to check for a linear correlation between the shell size, byssal thread width and count for both species separately.

Zooming in on the interaction of the shell length and attachment strength, a general linear model was assessed with shell length as a continuous variable. To assess whether the frequently selected shell size range (1-2 cm) in laboratory experiments had the same outcome regarding the species displaying the strongest attachment, a comparison was made with the complete dreissenid shell size range found in the field. We compared three types of attachment strength experiments: 1) same sized individuals in the laboratory (1-2 cm), 2) same sized individuals in the field (1-2 cm), 3) the whole dreissenid field assemblage. A general linear model with dependent variable “attachment strength” and explaining factors “species”, “experiment type” and their first order interaction term was constructed. All assumptions for the use of a general linear model were met. Additionally, a Tukey HSD test was conducted as a post-hoc test for this general linear model.

A one-tailed Chi-square (χ^2) test was used to test whether the attachment rate was affected by species, species assemblage and light regime. This one-tailed Chi-square test was also used to assess whether there was a difference in detachment location of the byssal threads for both species. Finally, a Kruskal-Wallis rank-sum test was performed to check whether there was a link between the detachment location and the measured attachment strength. All analyses were performed using the software R (version 3.6.2) with a statistical significance level of $\alpha < 0.05$.

Results

Attachment rate

The statistical test used, the test statistics, degrees of freedom and the P-values are represented in table 1 and 2. Combining the attachment rate for all batches (1-4), *D. polymorpha* individuals (85%) attached significantly more often than *D. bugensis* individuals (76%) to the substrate (P= 0.013; Table 1a, Figure 3). We found this pattern in three out of four measured batches (1-3). Additionally, we conducted a separate attachment analysis of batch 4, as this batch had an opposite result with significantly more *D. bugensis* attaching to the tank wall (Chi-square test: $\chi^2 = 5.76$, d.f. = 1, P = 0.016; Figure 3).

When comparing syntopic and allotopic populations there was no significant difference recorded in attachment rate either for *D. polymorpha* (P = 0.608) nor for *D. bugensis* (P = 0.058; Table 1b).

The light regime had no significant effect on either *D. polymorpha* and *D. bugensis* (P = 1.000, P = 0.320, respectively; Table 1c, Supplementary 1).

Attachment strength

The three attachment strength experiments (Same sized individuals in the laboratory, same sized individuals in the field and the whole dreissenid field assemblage) did not have the same outcome regarding the species displaying the strongest attachment (P = 0.002, Table 2a). Where it concerns the 1-2 cm size class in controlled laboratory conditions, there was no significant difference in attachment strength between both species (P = 0.972; Table 2b). Additionally, no significant difference was found for individuals of the 1-2 cm size class during the field experiment (P = 1.000; Table 2c). When assessing the whole dreissenid field assemblage with individuals of all sizes (*D. bugensis* 0.4-3.2 cm, *D. polymorpha* 0.4-2.0 cm), the attachment strength of *D. bugensis* mussels was significantly higher than the attachment strength of *D. polymorpha* mussels ($P < 0.001$; Table 2d). For both species an approximately four times greater attachment strength was found for individuals on fouling plates in the field, in comparison to individuals that attached themselves to the polyethene tanks within the laboratory setup (Mann-Whitney U test: *D. bugensis*: U= 3323.00, d.f. = 44, $P < 0.001$; *D. polymorpha*: U = 3404.00, d.f. = 49, $P < 0.001$).

The attachment strength of dreissenids in general was positively related to their shell size (Figure 4). Larger dreissenids attached significantly stronger to the substrate (P = 0.001; Table 2f). This relation between the shell size and attachment strength was stronger for *D. polymorpha* as the increase in attachment strength with shell size was stronger for this species (Figure 4).

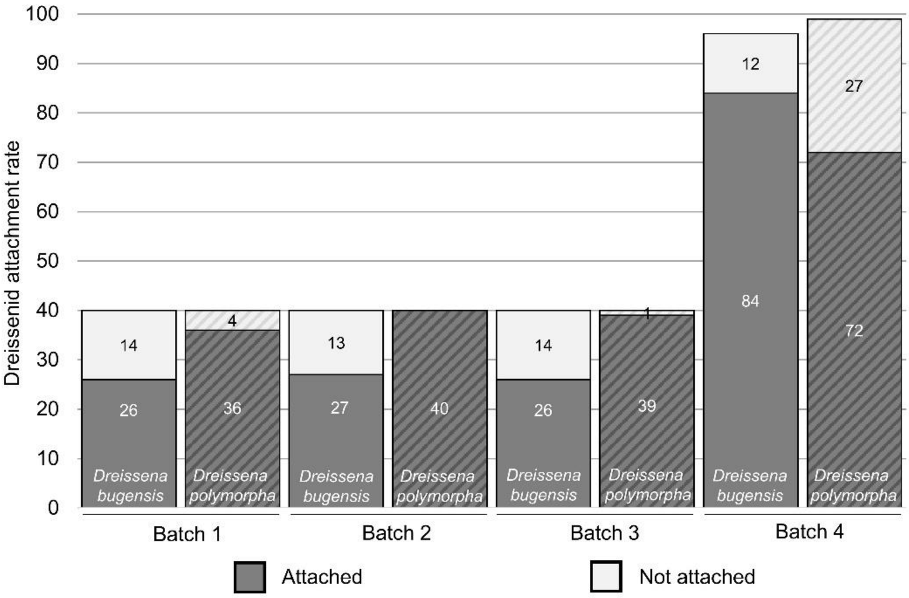


Figure 3. The attachment rate and numbers of (un)attached dreissenids in the laboratory experiment for *Dreissena bugensis* and *D. polymorpha* of each batch.

Byssal threads

The byssal thread width of *D. bugensis* was found to be significantly different and nearly double the width of *D. polymorpha* during the field experiment (P < 0.001; Table 2h). The byssal thread width and count were positively correlated to each other (P = 0.024; Table 2g). Therefore, the effect of byssal thread morphology (width and count) should not be analysed separately. The byssal thread count had a low sample size (N = 4 for *D. polymorpha*). To obtain results for the byssal thread count we rely on the correlated byssal thread width results. The positive correlation between the byssal thread width and count indicate a similar result for both byssal morphology traits. Assessing the average byssal counts we found about twice as many byssal treads for *D. bugensis* in comparison to *D. polymorpha* (Table 2i). The byssal thread width was positively correlated to the shell size for *D. bugensis*, while there was no correlation found for *D. polymorpha* (P = 0.027, P = 0.553, respectively; Table 2h). The number of byssal threads was positively correlated to the shell size for *D. bugensis*, while there was no correlation found for *D. polymorpha* (P < 0.001, P = 0.301, respectively; Table 2i).

Detachment location

Both species detached at the plaques, stem or by rupture of the byssal threads when they were pulled away from the SETL plate (Table 1d; Supplementary 2). At the end of the field experiment, *D. bugensis* had no significant difference between the detachments at the adhesive plaques (38% of the detachments) and by the rupture of byssal threads (43%)(Chi-square test: χ^2 = 0.20, d.f. = 1, P = 0.642). The stem detachment happened significantly less often than the adhesive plaque or byssal thread rupture (Chi-square test: χ^2 = 7.31, d.f. = 1, P = 0.007 and χ^2 = 10.91, d.f. = 1, P = 0.001, respectively). *Dreissena polymorpha* detached in most cases due to the rupture of the byssal threads (61% of the detachments). The byssal thread rupture happened significantly more often than the adhesive plaque or stem detachment (Chi-square test: χ^2 = 10.70, d.f. = 1, P = 0.001). *Dreissena polymorpha* detached significantly more often because of the rupture of byssal threads than *D. bugensis* did (P = 0.031; Table 1d). *Dreissena bugensis*, on the other hand, detached about four times more often because of the rupture of the stem than *D. polymorpha* did (P = 0.021). For *D. polymorpha* there was no significant difference between detachment locations found for individuals originating from the field experiment and controlled laboratory conditions (Table 1f). However, *D. bugensis* individuals in controlled laboratory conditions detached about 6 times more often by rupture of the byssal threads (P < 0.001), about four times less by rupture of the stem (P = 0.035) and about two times less by the detachment of the adhesive plaques (P = 0.003) than those who settled during the field experiment (Table 1e).

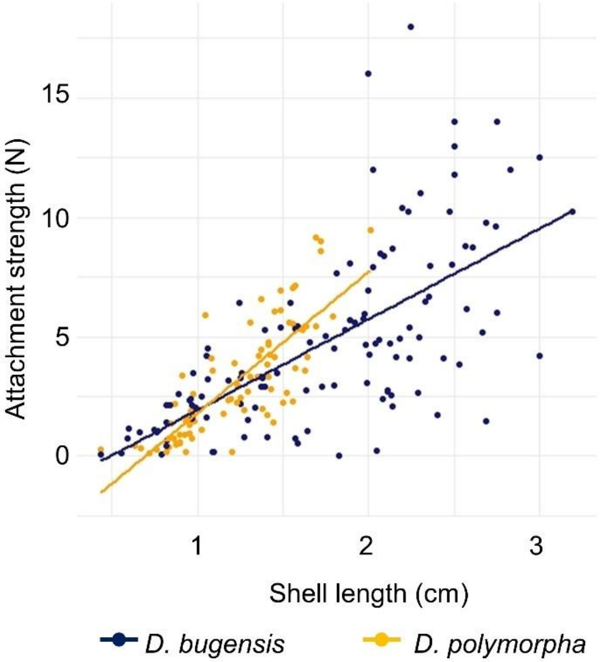


Figure 4. The attachment strength in Newtons (N) for field deployed dreissenids in relation to their shell sizes (cm). The attachment strength is positively related to their shell size. This increase is stronger for *Dreissena polymorpha* in comparison to *D. bugensis*.

It took nearly double the amount of force to detach *D. bugensis* individuals experiencing stem failure from the substrate compared to individuals detaching at the adhesive plaques or the byssal threads (P = 0.002; Table 1g, Supplementary 3). There was no significant difference in attachment strength between individuals detaching at the adhesive plaques or by rupture of the byssal threads. For *D. polymorpha* no significant differences in attachment strength were found for the different detachment locations (P = 0.855; Table 1h, Supplementary 3). During the course of the experiment, entire byssal masses were found free floating in the tanks for both species.

Table 1. The relative number or mean with standard deviation, minimum and maximum values for the attachment rate and detachment location of *Dreissena bugensis* and *D. polymorpha*. The relative numbers were indicated in % and attachment strength values were indicated in Newtons (N). The results of various experiments were statistically tested with a Kruskal-Wallis rank-sum test (K) or Chi-squared test (χ^2). Significant results were indicated with an asterisk symbol (*).

Experiment		Relative number or Mean (±SD) – Minimum – Maximum	Statistical test
Attachment rate			
(a) Species			
	D. bugensis	76%	$\chi^2 = 6.20$, d.f.= 1, $P = 0.013$*
	D. polymorpha	85%	
(b) Syntopic vs allotopic populations			
	D. bugensis	Syntopic = 55%, Allotopic = 78%	$\chi^2 = 3.58$, d.f.= 1, $P = 0.058$
	D. polymorpha	Syntopic = 98%, Allotopic = 93%	$\chi^2 = 0.26$, d.f.= 1, $P = 0.608$
(c) Light regime			
	D. bugensis	Light = 75%, Dark = 55%	$\chi^2 = 0.99$, d.f.= 1, $P = 0.320$
	D. polymorpha	Light = 95%, Dark = 100%	$\chi^2 < 0.001$, d.f.= 1, $P = 1.000$
Detachment location			
(d) Field experiment for both species			
Byssal threads	*D. bugensis*	43%	$\chi^2 = 4.70$, d.f.= 1, $P = 0.031$*
	D. polymorpha	61%	
Adhesive plaques	*D. bugensis*	38%	$\chi^2 = 0.30$, d.f.= 1, $P = 0.617$
	D. polymorpha	33%	
Stem	*D. bugensis*	19%	$\chi^2 = 5.30$, d.f.= 1, $P = 0.021$*
	D. polymorpha	5%	
(e) Laboratory vs field experiment for D. bugensis			
	Byssal threads	Laboratory = 7%, Field = 43%	$\chi^2 = 18.50$, d.f.= 1, $P < 0.001$*
	Adhesive plaques	Laboratory = 17%, Field = 38%	$\chi^2 = 8.90$, d.f.= 1, $P = 0.003$*
	Stem	Laboratory = 76%, Field = 19%	$\chi^2 = 4.50$, d.f.= 1, $P = 0.035$*
(f) Laboratory vs field experiment for D. polymorpha			
	Byssal threads	Laboratory = 64%, Field = 61%	$\chi^2 = 0.03$, d.f.= 1, $P = 0.866$
	Adhesive plaques	Laboratory = 33%, Field = 33%	$\chi^2 < 0.001$, d.f.= 1, $P = 1.000$
	Stem	Laboratory = 3%, Field = 5%	$\chi^2 = 0.17$, d.f.= 1, $P = 0.677$
Detachment location and strength			
(g) Species - D. bugensis			
	Byssal threads	5.01 (±2.37) - 0.01- 10.00N	$K = 12.40$, d.f.= 2, $P = 0.002$*
	Adhesive plaques	4.88 (±3.57) - 0.72 - 14.00N	
	Stem	8.95 (±4.41) -2.49 - 18.00N	
(h) Species - D. polymorpha			
	Byssal threads	3.22 (±2.39) - 0.12 - 9.15N	$K = 0.70$, d.f.= 2, $P = 0.855$
	Adhesive plaques	3.18 (±2.26) - 0.17 - 8.99N	
	Stem	1.99 (±1.45) - 0.93 - 4.08N	

Table 2. The mean with standard deviation, minimum and maximum values for the attachment strength and byssal thread morphology of *Dreissena bugensis* and *D. polymorpha*. The attachment strength values were indicated in Newtons (N). The attachment strength in relation to size indicates the shell size of each species (in mm). The byssal thread morphology includes the byssal thread width (in µm) and count. The results of various experiments were statistically tested with a Linear model (Lm(t)), Tukey's HSD post-hoc test (THSD), Pearson correlation test (r) or a Spearman rank correlation test (S). Significant results were indicated with an asterisk symbol (*).

Experiment	Mean (±SD) – Minimum – Maximum	Statistical test
Attachment strength		
(a) Difference between three setups		
Lab same size vs field same size vs field whole assemblage		*Lm(t)*= 33.55, d.f.= 5, 397, P = 0.002* *Adjusted* R^2 = 0.288
(b) Laboratory experiment (same size: 1-2 cm) – linear model df(5, 397)		
D. bugensis	0.81 (± 0.58) - 0.05 - 2.05N	T_{HSD}: P = 0.972
D. polymorpha	1.12 (± 0.68) - 0.10 - 2.94N	
(c) Field experiment (same size: 1-2 cm) – linear model df(5, 397)		
D. bugensis	3.67 (±2.26) - 0.01 - 10.00N	T_{HSD}: P = 1.000
D. polymorpha	4.19 (±2.07) - 0.17 - 9.15N	
(d) Field experiment – linear model df(5, 397) *(whole dreissenid assemblage; D. bugensis 0.4-3.2 cm, D. polymorpha 0.4-2.0 cm)*		
D. bugensis	4.73 (±3.80) - 0.01 - 18.00N	T_{HSD}: P < 0.001*
D. polymorpha	3.13 (±2.40) - 0.12 - 9.46N	
Attachment strength in relation to shell size		
(e) Dreissenid shell size (whole dreissenid field assemblage)		
D. bugensis	17.57 (±6.55) - 4.54 - 31.94mm	*NA*
D. polymorpha	12.70 (±3.32) - 4.37 - 20.64mm	*NA*
(f) Attachment strength in relation to shell size (whole dreissenid field assemblage)		
D. bugensis	4.73 (±3.80) - 0.01 - 18.00mm	*Lm(t)*= 11.14, d.f.= 1, 196, P = 0.001* *Adjusted* R^2 = 0.049
D. polymorpha	3.13 (±2.40) - 0.12 - 9.46mm	
Byssal thread morphology		
(g) Byssal thread morphology correlation		
Byssal thread width	NA	r= 0.52, d.f.= 17, P = 0.024*
Byssal thread count	NA	
(h) Byssal thread width – linear model df(3, 36)		
D. bugensis	56.77 (±13.33) - 33.89 - 86.35µm	*Lm(t)*= 15.93, d.f.= 1, P < 0.001* *Adjusted* R^2 = 0.466
D. polymorpha	35.13 (±4.60) - 26.25 - 43.33µm	
Correlation size and byssal width D. bugensis	NA	S= 3327.00, N= 32, P = 0.027* rho = 0.390
Correlation size and byssal width D. polymorpha	NA	S= 2568.10, N= 26, P = 0.553 rho = 0.122
(i) Byssal thread count		
D. bugensis	235 (±136) - 32 -570	*Sample size too small*
D. polymorpha	116 (±72) - 40 - 290	
Correlation size and byssal count D. bugensis	NA	S= 339.13, N= 19, P < 0.001* rho= 0.703
Correlation size and byssal count D. polymorpha	NA	S= 1219.10, N= 18, P = 0.301 rho= -0.258

Discussion

Attachment strength and byssal thread morphology

This study presents a first time comparison between the attachment strength of two invasive freshwater mussel species in laboratory and field experiments. The comparisons of individuals of the same size in both the laboratory and field experiment did not result in significant differences in attachment strength between both species, contrary to our hypothesis. Based on previous studies, we would expect *D. polymorpha* to have a greater attachment strength in this situation (Peyer et al. 2009, Grutters et al. 2012, Collas et al. 2018). However, judging from the results obtained by Balogh et al. (2019), laboratory kept individuals with a size between 1-2 cm and an attachment duration of about one week, would not have a clear difference in attachment strength for both species. In accordance with our hypothesis, attachment strength was found to be positively related to mussel size (Table 2; Figure 4) as also found by Kobak (2006) and Balogh et al. (2019). The curve in figure 4 clearly indicates why the attachment strength differed significantly between both species when assessing individuals of the whole size range, and why no significant difference was found when assessing individuals with a shell size between 1 and 2 cm. *Dreissena polymorpha* individuals displayed a stronger increase of attachment strength per unit length (though starting from a lower initial attachment strength for smaller individuals), resulting in a stronger adhesion for larger specimens of the same size as *D. bugensis*. However, *D. bugensis* grows faster and becomes larger than *D. polymorpha* individuals do (D'Hont et al. 2018). Therefore, the large *D. bugensis* individuals end up with a greater attachment strength than *D. polymorpha* individuals of the same age. Balogh et al. (2019) found a similar, however, reversed pattern, as *D. bugensis* individuals displayed a stronger increase of attachment strength per unit length. Since dreissenids attach to hard substratum using byssal threads, factors affecting their byssogenesis might impact their attachment strength. The byssogenesis is influenced by multiple environmental cues, such as temperature, salinity, dissolved oxygen, light, hydrodynamics, adhesion surface and season (Grutters et al. 2012, Balogh et al. 2019). Our results were obtained from field deployed fouling plates and individuals who had 9 to 21 months to attach themselves to the fouling plates. We know the possible duration of attachment as the two settlement events during this study happened respectively 9 and 21 months prior to our measurements. These plates were exposed to various seasons, temperatures and currents. Moreover, Dutch river deltas and upstream reaches of the rivers Rhine and Meuse are intensively used for navigation, causing ship-induced changes in flow velocity (Koopman et al. 2018). Adaptation of collected dreissenids to harsh environmental conditions could cause discrepancy with results of Balogh et al. (2019), as they tested randomly collected mussels kept under constant laboratory conditions for a maximum of one month.

Considering shell size affected the attachment strength, the size of individuals was taken into account when comparing results of our attachment experiments. Individuals of both species of in dreissenid field assemblage differ in size as *D. bugensis* (0.4-3.2 cm) grows faster and larger than *D. polymorpha* (0.4-2.0 cm) (D’Hont et al. 2018). The attachment strength of the larger *D. bugensis* was significantly greater than that of smaller *D. polymorpha* individuals. Larger individuals are probably more affected by currents and are more visible for predators (Hunt and Scheibling 2001, Balogh et al. 2019). The individuals involved in the field experiment were all collected in front of the sluice of the ferry harbour to the island Tiengemeten, so they are regularly exposed to strong ship-induced currents. Moreover, *D. bugensis* individuals have a strong clustering behaviour causing the larger and older individuals to carry conspecifics on their shell (D’Hont et al. 2018, Jermacz et al. 2021). We can hypothesise that the larger *D. bugensis* individuals had to compensate for these circumstances by investing in a higher attachment strength. These larger mussels might secrete mechanically superior and thicker byssal threads compared to smaller individuals, as has already been found in *Mytilus galloprovincialis* (Babarro et al. 2008, Babarro and Carrington 2013). In accordance with our hypothesis, our data indicate that the byssal threads of *D. bugensis* were nearly twice as thick as those of *D. polymorpha*. Likewise, the number of byssal threads was twice as high for *D. bugensis*. Additionally, we found a positive correlation between the byssal thread width and count and the shell size of *D. bugensis*. This relates to the significantly greater attachment strength observed for the larger *D. bugensis*. It is known that the number and thickness of byssal threads have a positive impact on the attachment strength (Bell and Gosline 1997). Another possible explanation for the higher attachment strength for *D. bugensis* during the field experiment might be a difference in physical condition between both species (cf. Balogh et al. 2019). If *D. bugensis* has a better physical condition due to favourable environmental parameters (e.g. light, temperature, salinity) for this species over *D. polymorpha*, they might be able to spend more energy on their attachment to the substrate. Nevertheless, the significantly greater attachment strength makes *D. bugensis* a stronger competitor during disturbances than *D. polymorpha* individuals of the same age.

Comparing the mean attachment strength during the laboratory experiment for *D. polymorpha* (1.12 N) and *D. bugensis* (0.81 N) to the results from Peyer et al. (2009) for respectively *D. polymorpha* (1.13 N) and *D. bugensis* (0.97 N), we found that these measurements do not differ from one another. The experiment of Peyer et al. (2009) was run for two months while ours lasted for seven days. This would thus suggest that the attachment strength of both species does not increase that much after seven days. Ackerman et al. (1996) conducted an attachment experiment in the field for *D. polymorpha* individuals and found an attachment strength between 0.35-1.60 N depending on the substrate type. During our field experiment, we found a mean attachment strength of 4.16 N for *D. polymorpha* and 3.67 N for *D. bugensis* for individuals with a similar size (1-2 cm) as reported by Ackerman et al. (1996). The attachment strength obtained in the present study was thus more than two times higher. This might be due to the experimental setup. Ackerman et al. (1996) kept their substrate containing the mussels in a laboratory setup for one month after retrieval from the field before conducting the measurements. These artificial environmental conditions might have caused the dreissenids to start looking for a new environment by voluntary detaching and reattaching themselves within one-month time. The present study measured the attachment strength of the dreissenids immediately after recovery from the field assuring their attachment strength did not change.

The attachment strength for both dreissenids was about four times higher during the field experiment in comparison to the laboratory experiment. In the field experiment, we used polyvinylchloride (PVC) plates especially sanded to increase the attachment, while the laboratory experiment used smooth polyethene tanks. The difference in roughness of the substrate most likely had an effect on the attachment strength of both species (Ackerman et al. 1996). An alternative explanation may be the duration of the attachment period, as dreissenids in the field experiment may have had a longer attachment period. Balogh et al. (2019) indicated that attachment strength still slightly increases after seven days for both *D. bugensis* and *D. polymorpha*. Moreover, unlike the laboratory experiment, individuals in the field experiment were potentially exposed to currents due to their location in front of the sluice. The correlation between attachment strength and currents has already been proven for blue mussels (*Mytilus edulis*) and Mediterranean mussels (*Mytilus galloprovincialis*) (Hunt and Scheibling 2001, Lachance et al. 2008, Balogh et al. 2019).

Attachment rate

In total 76% of *D. bugensis* and 85% of *D. polymorpha* attached to the substrate during the laboratory experiment (Table 1). Thus, *D. polymorpha* attached significantly more often to the hard substrate in controlled laboratory conditions than *D. bugensis*, which corresponds to previous findings (Collas et al. 2018). This was the case for three out of four experiments (Figure 3). Batch 4, however, had an opposite result with more *D. bugensis* attaching to the substrate. The number of individuals in each tank for this batch was more than two times higher (Figure 1). This might be one of the reasons causing the observed difference for batch 4. The attachment rate of a species might be related to their search for an optimal settlement environment. D'Hont et al. (2021) found that more *D. bugensis* individuals than *D. polymorpha* individuals moved around the tanks during the laboratory experiment, instead of settling down. Dreissenids usually move for a short period, i.e. a few minutes up to maximum of three hours (D'Hont et al. 2021). Furthermore, changes in the environment due to, for example, intense rainfall will generally take place within a short timeframe as well (minutes to a few hours). In these extreme cases it may be important that an individual can quickly escape the resulting poor environmental conditions. As the laboratory setup likely resembled suboptimal environmental conditions, this would indicate that *D. bugensis* individuals were actively seeking a better environment. This trait would give *D. bugensis* a benefit over *D. polymorpha* possibly explaining the dominance shift between these species.

The co-occurrence of both dreissenid species is known to affect their densities, as a higher number of *D. bugensis* individuals settled when *D. polymorpha* individuals were already present on the substrate (D'Hont et al. 2018). The presence of individuals of the same species positively affects the attachment rate, while injured conspecifics reduced the byssal attachment as a reaction to the presence of potential predators (Kobak 2001, Czarnołęski et al. 2010). In our study, however, the presence or absence of the other dreissenid species had no significant effect on the dreissenids' attachment rate (Table 1).

The presence or absence of light in the present study had no significant effect on the attachment of *D. polymorpha* or *D. bugensis* individuals, which corresponds to the results of Grutters et al. (2012). *Dreissena polymorpha* is known to have a preference for settlement in dark environments and mussels in illuminated environments changed their initial sites less often than those kept in darkness (Kobak 2006, Kobak and Nowacki 2007, Kobak et al. 2009). The unfavourable environmental conditions created during our laboratory setup may be strong enough to counteract the effect of light on dreissenids' settlement. This might also have been the case in the study of Grutters et al. (2012). The sample size and replication rate for this experiment were relatively low as well (N = 20 for each treatment, light and dark). This low sample size might have caused possible significant trends to get lost. Supplementary 1 indicates a trend in which light exposed *D. bugensis* individuals had a higher attachment rate than individuals kept in a dark environment. The in depth assessment of this interaction would be recommended for future studies.

Detachment location

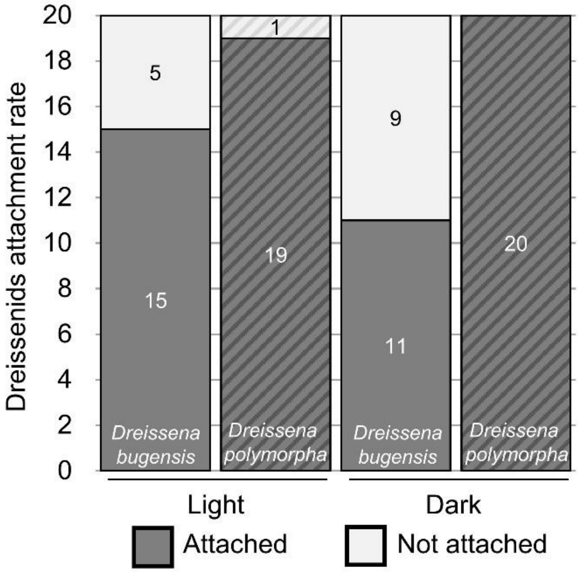
Dreissena polymorpha individuals detached 20% more often because of the rupture of their byssal threads than *D. bugensis* individuals did (Table 1; Supplementary 2). This would mean that the *D. bugensis* byssal threads are stronger than the byssal threads of *D. polymorpha*, which corresponds to the attachment strength related to a detachment by the rupture of the byssal threads for both species (Table 1; Supplementary 3). *Dreissena bugensis*, on the other hand, displayed four times more often stem or gland detachment than *D. polymorpha*. This type of detachment is relatively uncommon for *D. polymorpha* (Dormon et al. 1997). In our study, stem detachments of *D. bugensis* were associated with relatively high attachment strengths and the presence of relatively high byssal thread counts. Only few *D. polymorpha* individuals showed stem ruptures, however, these cases were not associated with a higher attachment strength. Information on the detachment location of either species under natural conditions is scarce. A remarkable observation were the free floating byssal masses for both species. The common blue mussel (*Mytilus edulis*) is known to detach its entire byssal mass, however, the underlying mechanisms are not yet well understood (Eckroat et al. 1993). Voluntary detachment is also known for *D. polymorpha* individuals (Kobak et al. 2009, Dzierżyńska-Białończyk et al. 2018). Apart from that, not much is known on the voluntarily detachment of dreissenids, as most studies only assessed forced detachment of both species (Eckroat et al. 1993, Kobak et al. 2009). Especially a comparison between the voluntarily detachment of both species is missing. This would therefore be recommended for future studies. Overall, our results support the hypothesis that differential attachment-detachment traits might to some degree be one of the contributing drivers of the ongoing dominance shift between *D. polymorpha* and *D. bugensis*.

Conclusions

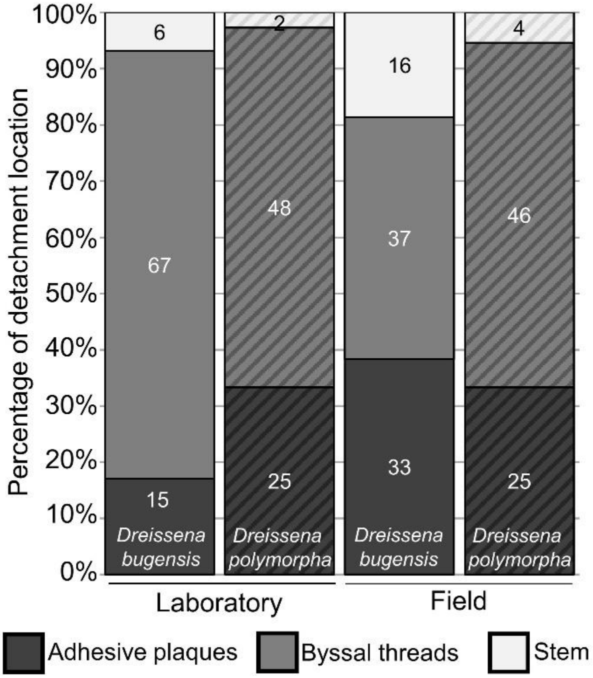
The goal of this study was to assess whether the attachment of dreissenids explained, at least for some degree, the ongoing dominance shift between *D. bugensis* and *D. polymorpha*. Shell size and attachment strength were positively correlated in both species. *Dreissena polymorpha* individuals showed a faster increment of attachment strength per unit length, resulting in a stronger adhesion for larger specimens of the same size as *D. bugensis*. However, *D. bugensis* grows faster and becomes larger than *D. polymorpha*. Therefore, for field deployed individuals of the whole available size range, the attachment strength in *D. bugensis* was significantly higher in comparison to *D. polymorpha*. This higher attachment strength can at least partially be explained by the byssal thread morphology as these byssal threads were about two times thicker for *D. bugensis*, which also correlated to a higher number of byssal threads. Additionally, *D. polymorpha* individuals detached significantly more often because of the rupture of their byssal threads than *D. bugensis* individuals did. This indicates that the byssal threads of *D. bugensis* are stronger than the byssal threads of *D. polymorpha*. These attachment traits likely give *D. bugensis* mussels an advantage over *D. polymorpha* mussels within a dreissenid field assemblage when it comes to withstanding currents and predators. Additionally, *D. bugensis* had a significantly lower attachment rate. This may be explained by a higher number of *D. bugensis* individuals searching for the optimal habitat, as the laboratory setup likely resembled suboptimal environmental conditions. The results support our hypothesis that the differences in attachment-detachment traits might to some degree be one of the contributing drivers of the ongoing dominance shift between both species.

Acknowledgements

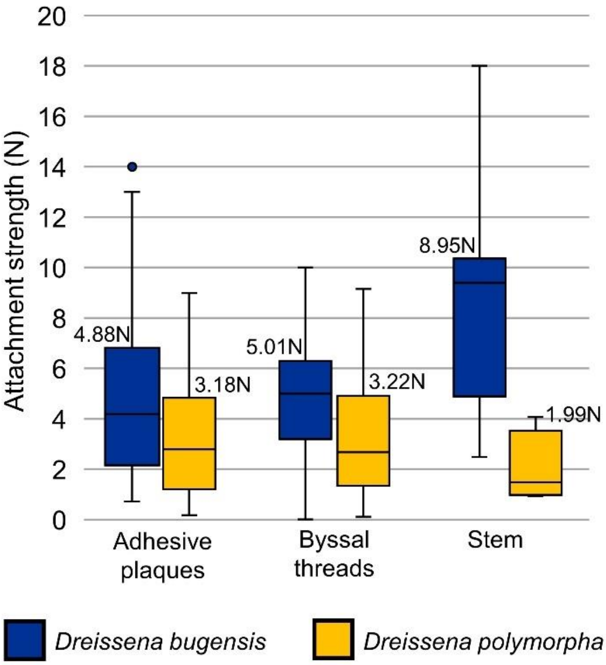
The authors would like to thank the European Union's Horizon 2020 research and innovation programme and the Innovative Training Network 2015–2019 Drivers of Pontocaspian Biodiversity Rise and Demise (PRIDE) under the Marie Sklodowska-Curie Grant Agreement No 642973 of the European Commission for funding and supporting this research. We thank Naveen Parthiban and two anonymous reviewers for their critical comments and suggestions to improve this paper. We thank the GiMaRIS team for their assistance during the practical part of this research. We acknowledge Mr Veldhoen, Mr Kruithof and ferry service 'de Hoeksche Vaart' for facilitating fieldwork in Numansdorp and Tiengemeten. Finally, special thanks go to Martijn Van Roie for his support throughout this research.



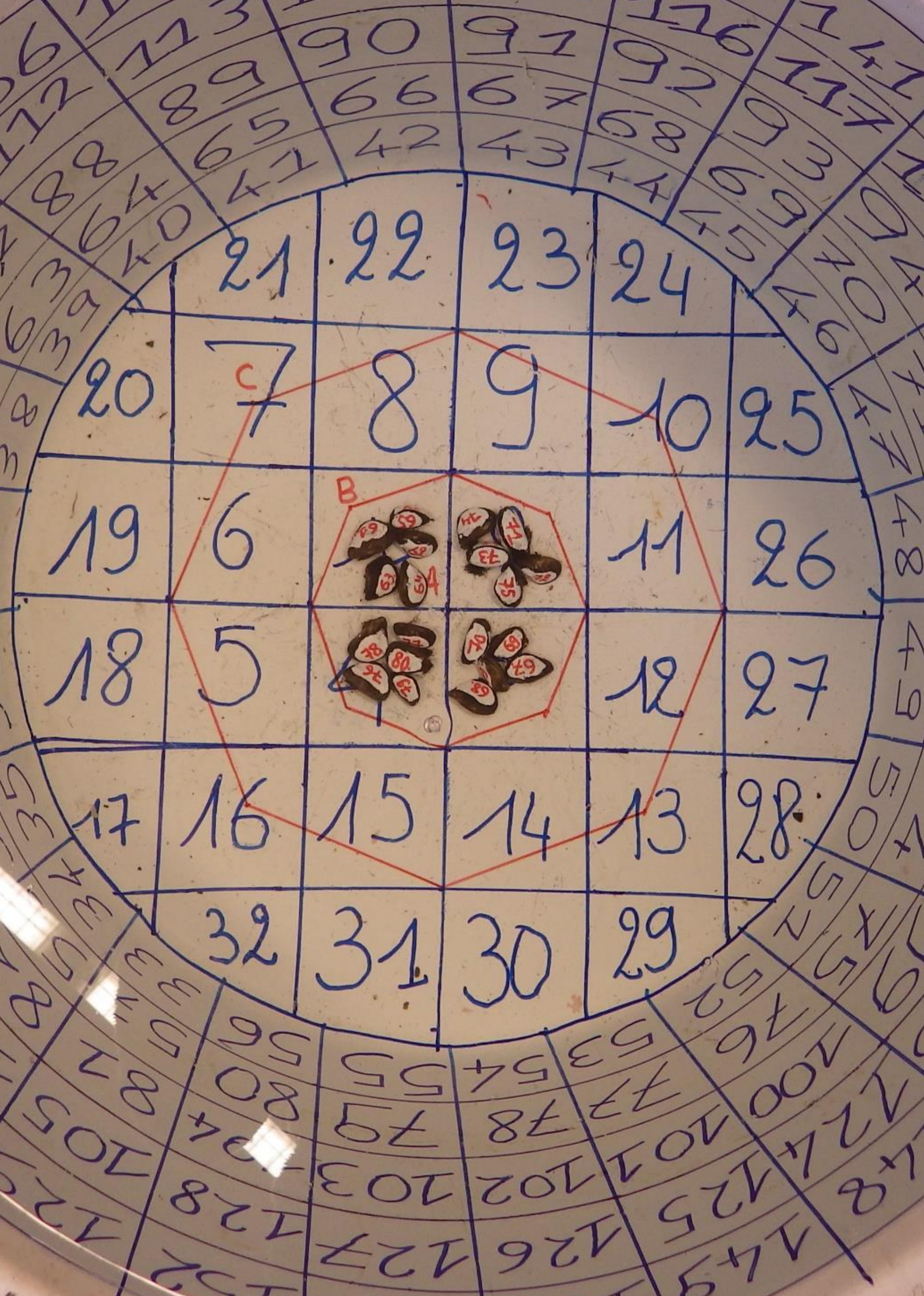
Supplementary 1. The attachments rate and numbers of (un)attached dreissenids in controlled laboratory experiments under light and dark conditions.



Supplementary 2. The location of the detachment of both dreissenids from laboratory and field-deployed plates. The values indicate the number of individuals for each detachment type (byssal threads: rupture of the byssal threads; adhesive plaques: detachment of the plaques; stem: rupture of the stem from the byssal gland).



Supplementary 3. The attachment strength in Newtons (N) of the two dreissenid species concerning various detachment locations (byssal threads, adhesive plaques and stem) of mussels deployed during the field experiment. The box-and-whisker plots represent the minimum, first quartile, median, third quartile and maximum attachment strength. The values represent the mean attachment strength for each detachment location category.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Chapter 4

Dreissenids' need for speed: Mobility as a driver of the dominance shift between two invasive Ponto-Caspian mussel species

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Key words: distance, *Dreissena bugensis*, *Dreissena polymorpha*, duration, motility, movement, size

Abstract

Both the quagga mussel (*Dreissena bugensis*) and the zebra mussel (*Dreissena polymorpha*) are notorious for dominating hard substrates in freshwater ecosystems throughout most of the Northern hemisphere. Despite widespread observations of a dominance shift favouring *D. bugensis*, where both Ponto-Caspian dreissenids co-occur, mechanisms driving this shift are still largely unknown. This study assessed whether movement behaviour differs between these two mussel species. That way we aimed at assessing whether mobility might be a contributing driver to the observed dominance shift. The mobility of dreissenids was assessed in an experimental set-up consisting of polyethene tanks marked with squares and concentric circles facilitating location tracking of the dreissenids by time-lapse photography. Specimens were collected at the Haringvliet and Hollands Diep in the Rhine-Meuse river delta. The experiments mimicked unfavourable habitat conditions by drying, cleaning, tagging and placing mussels in a new environment. After these disturbances, the movement rate, duration, distance, pattern and speed of 299 individuals were monitored. For both species, most individuals moved in more or less circular patterns, causing their actual movement distance to be twice as high as their displacement distance. The average movement duration within 24 hours after the start of each experiment was 65 min, with an average speed of 28 cm/h and an average distance of 29 cm. Hereby no significant differences were found between *D. polymorpha* and *D. bugensis*. However, a higher top speed was observed for *D. bugensis* than for *D. polymorpha*. The fastest individuals of these two species moved at 90 cm/h and 60 cm/h, respectively. Moreover, about twice as many *D. bugensis* individuals moved during the experiments in comparison to *D. polymorpha* individuals. Hereby it was recorded that any point in time close to 10% more *D. bugensis* specimens were moving around. The results support our hypothesis that *D. bugensis* could have a competitive benefit over *D. polymorpha* by having a higher top speed and a significantly higher number of individuals moving after a disturbance of their population. Detachment and mobility of sessile

mussel species are supposed to be avoidance mechanisms during unfavourable environmental conditions. Therefore, mobility might be one of the contributing drivers of the observed dominance shift between both species.

Introduction

The zebra mussel (*Dreissena polymorpha* Pallas, 1771) and the quagga mussel (*Dreissena bugensis*, also described as *Dreissena rostriformis bugensis* auct. Andrusov, 1897) are notorious freshwater invaders. Both dreissenid species cause extensive ecological effects (Grigorovich and Shevtsova 1995) and economic damage in their native area (Popa and Murariu 2009) as well as introduced regions (Pimentel et al. 2005, Binimelis et al. 2008, Fahnenstiel et al. 2010). Ecological changes caused by dreissenids include a dramatic increase in water filtration causing a reduction in food and oxygen availability and shifts in species composition, relative abundance and biomass of functional groups within aquatic ecosystems (Binimelis et al. 2008, Fahnenstiel et al. 2010). Economic damages include an increase in maintenance costs of overgrown man-made structures like hydraulic infrastructures, water corridors, boat hulls and pipes in cooling water circuits (Binimelis et al. 2008, Schonenberg and Gittenberger 2008). They originate from the Ponto-Caspian region and have spread to many parts of the Northern hemisphere (Europe, Eurasia and North America) (Therriault et al. 2005, Molloy et al. 2007, Van der Velde et al. 2010, Sousa et al. 2011). *Dreissena polymorpha* started to spread out of its native area at the beginning of the 19th century (Van der Velde et al. 2010, Sousa et al. 2011). *Dreissena bugensis* followed in its footsteps at the end of the 20th century (Therriault et al. 2005). Recently, there have been widespread observations of a shift from *D. polymorpha* to *D. bugensis* as the dominant species in their introduced range as well as in their native area (Karatayev et al. 2011, Matthews et al. 2014, D'Hont et al. 2018). Although *D. polymorpha* is able to settle earlier in the season, is more salinity tolerant, and is predated less upon than *D. bugensis*, *D. bugensis* usually remains the dominant species where both species co-occur (D'Hont et al. 2018). *Dreissena bugensis* is able to settle in a wider range of habitats and adjust its growth and settlement better to seasonally and annually varying salinities and temperatures (Claxton and Mackie 1998, Gerstenberger et al. 2011, Marescaux et al. 2015, D'Hont et al. 2018, Balogh et al. 2019). *Dreissena bugensis* is known to have a higher assimilation efficiency, a higher activity of certain enzymes, higher filtration rates and lower respiration rates (Ram et al. 2012). Moreover, this species has lower winter mortality and settles in higher numbers when *D. polymorpha* individuals are already present on the substrate (D'Hont et al. 2018). Additionally, a higher attachment strength is found for *D. bugensis* compared to *D. polymorpha* (D'Hont et al. 2021). This may give *D. bugensis* an advantage over *D. polymorpha* when it comes to withstanding predators and disturbances such as currents (Hunt and Scheibling 2001, Kobak 2001, Czarnołęski et al. 2010) and navigation-induced changes in flow velocity (Koopman et al. 2018).

Dreissenids are generally considered sessile and sedentary animals (Kobak and Kakareko 2009). However, dispersal and migration are common for a dreissenids' larval life stage. The larval settlement is a critical dispersal period in its life cycle (Wilson et al. 1999). Additionally, settled mussels can relocate by detaching their byssus from the substrate and actively moving around using their foot (Toomey et al. 2002). They do so in reaction to changes in environmental factors, like water quality, orientation, presence or absence of conspecifics, oxygen and desiccation during water level drawdown (Kobak 2001, Kobak and Nowacki 2007, Collas et al. 2018). Higher movement ability may thus promote the avoidance of unfavourable environmental conditions.

Most knowledge of the mobility of dreissenids is based on studies with *D. polymorpha*. Light, temperature and water hardness are found to have no effect on the distance travelled by this species (Toomey et al. 2002, Coons et al. 2004). However, the presence of crushed individuals can cause a significant decrease in movement in the remaining individuals (Toomey et al. 2002). The urge to move and the distance moved by *D. polymorpha* individuals is inversely proportional to shell size, as small individuals move more than large ones (Toomey et al. 2002).

We conducted a comparative analysis of the rate, duration, speed and distance moved and the relationship between the movement and shell size of *D. polymorpha* and *D. bugensis*. To assess whether mobility may contribute to the dominance shift from *D. polymorpha* to *D. bugensis*, we studied whether any of these characteristics differed between both species.

Material and methods

Study sites

Individuals of *D. bugensis* and *D. polymorpha* were collected in the Haringvliet and Hollands Diep, which are a part of the Rhine-Meuse delta in the Netherlands. They were collected in the ferry harbour of the island Tiengemeten (51°45.261′N; 4°19.046′E) and about 8 km upstream in the same river system in the harbour entrance of Numansdorp (51°43.037′N; 4°26.211′E). *Dreissena bugensis* was observed for the first time in Western Europe in 2006 in this part of the Rhine-Meuse delta (Molloy et al. 2007, Schonenberg and Gittenberger 2008). Dreissenids were collected from bricks deployed for about 1.5 years from the local docks at a depth of 1 m. Individuals of both species were collected at both locations to be able to collect enough specimens. There were no indications for differences in population structure of both species between both locations.

Experimental setup

In total 299 dreissenids were collected in three separate batches on 19 December 2017 (water temperature 5.2 °C), 6 March 2018 (1.4 °C) and 9 December 2019 (7.2 °C) (batch 1, 2 and 3, respectively; Figure 1) (Rijkswaterstaat 2020). Batch 1 and 2 consisted each of 40 *D. polymorpha* individuals and 40 *D. bugensis* individuals with shell lengths between 1 and 2 cm. To test the potential impact of shell size on the movement rate, duration, speed and distance of dreissenids, batch 3 was collected. This batch was collected from bricks which were deployed for about 1.5 years at a depth of 1 m. Batch 3 consisted of 54 collected individuals with shell lengths between 0.86-2.27 cm of *D. polymorpha*, and 68 individuals with shell lengths between 1.02-3.04 cm of *D. bugensis*. As *D. bugensis* grows faster and larger, the collected individuals of *D. bugensis* were slightly larger (D'Hont et al. 2018). By specifically searching for the largest individuals present, additionally, eight about 3 cm long *D. polymorpha* and nine about 4 cm long *D. bugensis* individuals were found and included in the experiments. Unfortunately, these were the only maximum-sized individuals to be found at that time on the sampled bricks.

Movement experiments were conducted to assess the speed (cm/h), distance (cm), duration (min) and movement rate (%) of both dreissenid species. After collection in the field, the dreissenids were gradually acclimatized to room temperature (± 20 °C) for 24 to 48 hours. This was done by slowly increasing the water temperature from the ambient temperature at the sampling sites (1.4–7.2 °C) to room temperature (± 20 °C). Acclimation of 4–48 hours is common practice in experimental studies with dreissenids (Vanderploeg et al. 2001, Toomey et al. 2002, Juhel et al. 2006a, b, White et al. 2015, Whitten et al. 2018). Subsequently, the mussels were dried, cleaned and tagged by applying a number to both sides of the shell using Tipp-Ex correction fluid and permanent marker. Finally, the byssal threads were cut off using small scissors before placing them in the tanks. The mortality rate throughout this experiment was low (< 1%). The deceased individuals were excluded from the analyses. The experimental setup consisted of four polyethene containers with 60-litre non-aerated fresh tap water. The surface area of these containers was subdivided into 152 grid cells (6 cm^2 squares) and eight concentric circles, to indicate the location of dreissenids (Figure 1). The tap water had a pH of 7.84, and salinity of 0.28 PSU. Within the Haringvliet the pH normally ranges between 7.7 and 8.6, the salinity between 0.2 and 0.8 PSU and the temperature between approximately 0 and 28 °C (Rijkswaterstaat 2020). Dreissenids were added to a tank with tap water of room temperature (± 20 °C). The four containers were stocked with 20 individuals each. All tanks were illuminated with daylight coming in through windows of the laboratory, with a natural photoperiod (batch 1 and 3: 8L : 16D, batch 2: 11L : 13D; Light : Dark).

In total six repeated runs of a movement experiment were carried out (batch 1: run 1 – 3; batch 2: run 4 – 6, Figure 1). The movement of the same individuals was tracked for three consecutive runs because of the limited collection capacity of dreissenids from the bricks at our study sites. Dreissenids were allowed to move within the tanks for 4 to 10 days. The initially selected duration of the experiments was 10 days. However, after noticing that most individuals stopped moving after 24–48 hours, the following experiments were shortened. This way we were able to run more experiments in a shorter amount of time. In between each run, the mussels were allowed to recover for 3 days in aerated and acclimatized Haringvliet water. The particulate organic matter present in this Haringvliet water served as their only food source. An additional experiment was conducted with differentially sized mussels collected in batch 3 to assess whether the size of an individual affected its movement.

Movement rate, duration, speed and distance

To assess the movement rate, data from all runs (1–6) were analysed. The movement rate was measured as the number of moving individuals over the total number of individuals (%) for both dreissenid species during each of the days of the 6 runs. The total number of each dreissenid species used differed between the days as the duration of the experiments varied between 4 and 10 days. The maximum and average movement duration, speed and distance were assessed during runs 4 to 6 with a focus on the first 24 hours of the experiment, as most of the dreissenids moved within this timeframe. These parameters were measured using 2-minute interval time-lapse photography. Once an individual started moving, we recorded this as an “active period” for this specific individual. We defined an active period as the moment at which an individual started to move at a speed higher than 15 cm/h for at least 2 consecutive minutes. The active period came to an end at the moment the individuals remained at the same location in two consecutive photos, which corresponds to 2 minutes. One individual might display multiple active periods throughout the experiment, therefore, the number of active periods was registered for each individual. The maximum and average movement duration of an individual was registered as the duration of all active periods of that individual within the first 24 hours and as the duration of the first active period. The maximum and average speed was measured as the distance travelled by an individual in between two consecutive photos. This distance was noted as the number of centimetres covered in 2 minutes and recalculated to centimetres per hour. The distance moved could be subdivided into two different types: the displacement distance and the actual distance (Figure 2). The displacement distance is the shortest distance between the dreissenids' location at the beginning of the experiment and its location after 24 hours. The actual distance moved by the dreissenids was calculated as the sum of distances measured between consecutive time-lapse photos. The actual distance was estimated for the first 24 hours and the first active period of each individual.

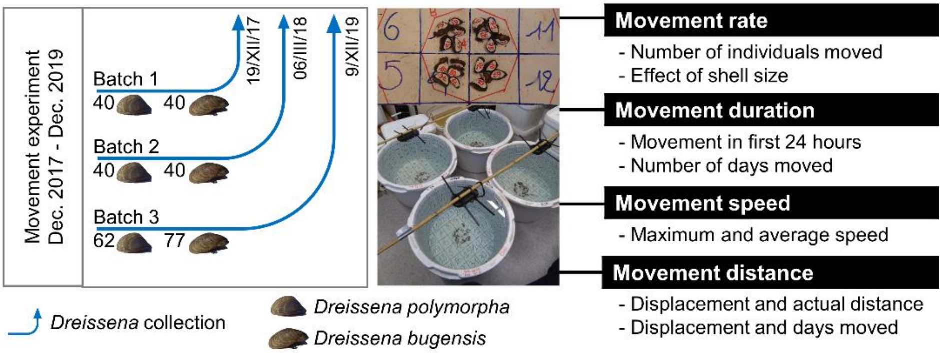

Figure 1. The setup of the movement experiments. The dreissenids were collected in three separate batches consisting of 40 *D. bugensis* and 40 *D. polymorpha* (batch 1-2) and 77 *D. bugensis* and 62 *D. polymorpha* (batch 3). The right part of the figure indicates the research topics. Photo by Anouk D'Hont.

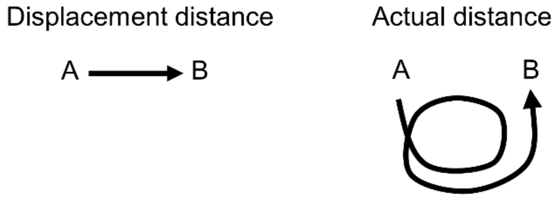

Figure 2. The difference between displacement distance and the actual distance travelled by dreissenids (A: location at the beginning of the movement experiment; B: location after 24 hours).

Statistical analyses

A generalized linear mixed model was performed to determine whether the number of individuals moving for several days, the total number of individuals moving throughout all of the movement experiments and the number of individuals moving each separate day of the experiments, differed between both species. This test was chosen because the data contained repeated measurements, as the movement of each mussel was tracked for three consecutive runs. We used three separate binomial generalized linear mixed models to test for differences between species in the following independent test variables: (i) individuals moved for multiple days ("yes" or "no"), (ii) individuals moved at some point during the experiment runs ("yes" or "no"), (iii) individuals moved each day ("yes" or "no"). The mussel "species" was the fixed response variable, while the "run" number and individual "mussel" number were the random effect variables. The analysis was performed using the package nlme (Pinheiro et al. 2017). As the H0 for this test, we assumed there was no difference in total individuals moved or individuals that moved for multiple days for the fixed variables ("species"). A one-tailed Chi-square (χ^2) test was used to assess whether the movement rate for individuals with different shell sizes differed significantly. The Mann-Whitney U test was used to analyse the statistical significance of differences in movement duration, speed and distance between both species. Additionally, the relationship between shell size and movement rate was assessed using the Mann-Whitney U test. The Kruskal-Wallis test was used to check the effect of the number of days moved on the displacement distance. All analyses were performed using the software R (version 3.6.2) with a statistical significance level of $P < 0.05$.

Results

Movement rate and shell size

Dreissena bugensis individuals with a shell size of 1–2 cm moved every day during the experiments (Figure 3). The *D. polymorpha* individuals with the same shell size were found to be moving on each day except day 9 (Figure 3). During the first 24 hours, the percentage of moving individuals of *D. bugensis* was higher than that of *D. polymorpha* (circa 50% vs 40%, respectively; Generalized linear mixed model: $P = 0.003$, Table 1). Throughout all remaining days, i.e. up to the 10th day, the number of moving individuals was also recorded to be higher for *D. bugensis* than for *D. polymorpha*. Apart from the first day, this difference was also found to be significant for day 2 (Generalized linear mixed model: $P < 0.001$, Table 1), while for days 3 to 10 the difference was not significant (Generalized linear mixed model: all $P \geq 0.613$, Table 1) (Figure 3). Throughout the whole experiment, the number of individuals that moved at any point in time was almost two times higher for *D. bugensis* (58%) compared to *D. polymorpha* (39%) (Generalized linear mixed model: $P < 0.001$, Figure 3, Table 1).

When comparing various sized individuals to each other (1.02–3.04 cm and 0.86–2.27 cm, respectively), *D. bugensis* (41%) had twice as many moving individuals in comparison to *D. polymorpha* (19%) (Chi-squared test: $P = 0.013$). No significant relationship was found between the shell size and the movement rate of individuals for either *D. polymorpha* or *D. bugensis* (Mann-Whitney U test: $P = 0.193$, $P = 0.172$, respectively, Figure 4). However, for *D. polymorpha* none of the individuals with a shell size larger than 2 cm moved, while *D. bugensis* individuals of the whole size range moved. The largest, and thus oldest collected *D. bugensis* (± 4 cm) had a tendency to move (two out of nine individuals), while none of the large *D. polymorpha* (± 3 cm) individuals moved (zero out of eight individuals).

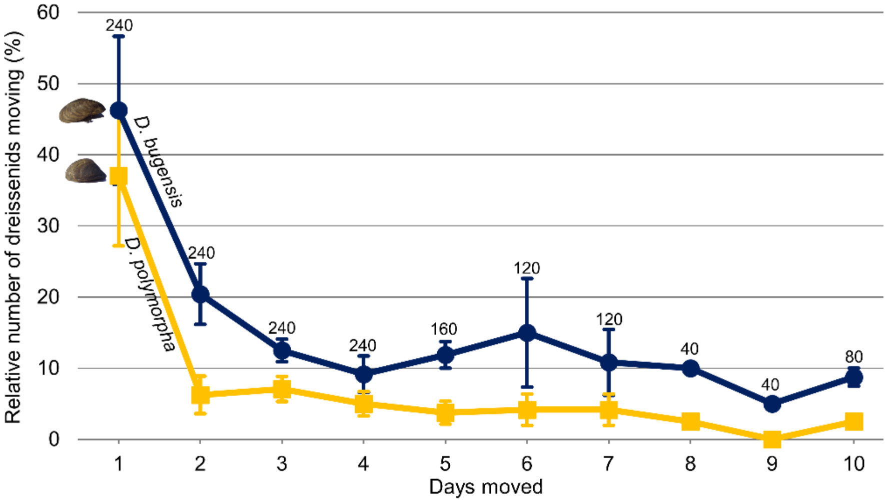

Figure 3. The percentage of individuals moving for both dreissenid species throughout the movement runs. The error bars represent the standard error. An equal number of dreissenids of each species was used to calculate the percentage for each day. The total number of each dreissenid species used (= N indicated above dots) differed between the days as the results were based on 2, 1, 1 and 2 experiments, which ran respectively for 4, 5, 7 and 10 days. During one of the experiments of 10 days, the movement was not tracked on day 8 and 9, explaining the lower N on those days.

Movement duration

The movement duration of dreissenids measured during the first 24 hours was not significantly different between the two species (Mann-Whitney U test: P = 0.511, Table 1, Figure 5). Additionally, there was no significant difference found between the duration of the first active period of each species (Mann-Whitney U test: P = 0.583, Table 1).

Individuals started moving within 2.5 and 12 hours after the start of the experiment runs. The number of individuals that moved for more than one day was significantly higher for *D. bugensis* (35%) during each of the six runs compared to *D. polymorpha* (13%) (Generalized linear mixed model: $P < 0.001$, Table 1). *Dreissena bugensis* specimens moved for a maximum of 5 out of 10 days. *Dreissena polymorpha* specimens moved for a maximum of 4 out of 10 days.

Movement speed

The average movement speed did not differ significantly between both dreissenid species (Mann-Whitney U test: $P = 0.273$, Table 1, Figure 5). The top speed of *D. bugensis* (90 cm/h for 2 min) was a third higher than the top speed measured for *D. polymorpha* (60 cm/h for 4 min). The maximum speed was estimated for each individual. When comparing this maximum speed of the separate individuals of each species with each other, no significant difference was found between both species (Mann-Whitney U test: $P = 0.225$, Table 1).

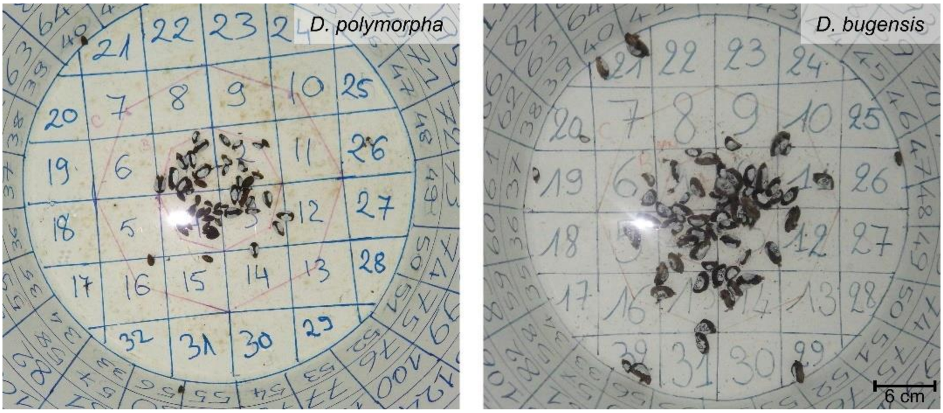

Figure 4. The effect of shell size on the movement of dreissenids after 24 hours. In general, twice as many *D. bugensis* moved in comparison to *D. polymorpha*. For *D. polymorpha* only the small (< 2 cm) individuals moved, while for *D. bugensis* individuals of all sizes moved around in the tank. Photo by Anouk D'Hont.

Movement distance

The average displacement distance within the first 24 hours did not differ significantly between both dreissenid species (Mann-Whitney U test: P = 0.848, Table 1, Figure 5). Additionally, the actual distance moved within the first 24 hours and during one active period by either of the dreissenids did not differ significantly (Mann-Whitney U test: P = 0.809, Table 1, Figure 5). There was no significant relationship between the number of days moved and the displacement distance for either *D. polymorpha* or *D. bugensis* (Kruskal-Wallis test: P = 0.245, P = 0.643, respectively).

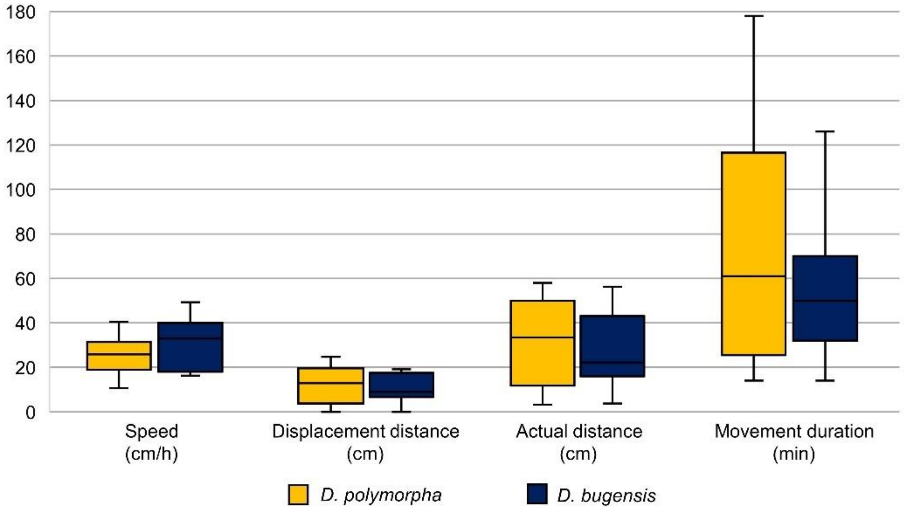

Figure 5. Average speed, average displacement distance, average actual distance and average movement duration for the two dreissenid species. For each of the parameters, 14 *D. polymorpha* and 11 *D. bugensis* individuals were assessed.

Table 1. The movement rate, duration, speed, actual distance, displacement distance and the relationship between movement and shell size for both species in the first 24 hours of the movement experiments. The standard error is represented next to the average values. Where appropriate, a Generalized linear mixed model (Z), Mann-Whitney U test (U), Kruskal-Wallis test (K) or Chi-squared test (χ^2) was performed and represented.

	Dreissena polymorpha	*Dreissena bugensis*	Statistical test
Movement rate same size (1-2 cm)			
Total number moved (%)	39	58	Z = -4.1 $N_{1,2}$= 240, **P < 0.001***
Number moved Day 1 (%)	33	46	Z = -3.0, $N_{1,2}$= 240, **P = 0.003***
Day 2 (%)	6	20	Z = -1247, $N_{1,2}$= 240, **P < 0.001***
Day 3 (%)	7	13	Z = -0.4, $N_{1,2}$= 240, P = 0.706
Day 4 (%)	5	9	Z = -0.3, $N_{1,2}$= 240, P = 0.746
Day 5 (%)	4	12	Z = -0.5, $N_{1,2}$= 160, P = 0.634
Day 6 (%)	4	15	Z = -0.5, $N_{1,2}$= 120, P = 0.613
Day 7 (%)	4	11	Z = -0.4, $N_{1,2}$= 120, P = 0.726
Day 8 (%)	3	10	Z = -0.2, $N_{1,2}$= 40, P = 0.816
Day 9 (%)	0	5	Z = -0.01, $N_{1,2}$= 40, P = 0.911
Day 10 (%)	3	9	Z = -0.3, $N_{1,2}$= 80, P = 0.772
Movement and shell size			
Movement rate (%) different sized individuals	19	41	χ^2 = 6.2, N_1= 54, N_2= 68, **P = 0.013***
Relationship between shell size and movement rate	NA	NA	*D. polymorpha:* U = 161.0, N= 54, P = 0.193 *D. bugensis:* U = 670.0, N= 68, P = 0.172
Movement duration			
Maximum duration (min)	178	126	NA
Average duration (min)	74 ± 14	57 ± 11	U = 64.5, N_1= 14, N_2= 11, P = 0.511
Average duration in one active period (min)°	55 ± 14	35 ± 7	U = 66.5, N_1= 14, N_2= 11, P = 0.583
Multiple days moved (%)	13	35	Z = -5.4, $N_{1,2}$= 240, **P < 0.001***
Speed			
Maximum speed (cm/h)	60.0 ± 3.1	90.0 ± 6.1	U = 99.0, N_1= 14, N_2= 11, P = 0.225
Average speed (cm/h)	25.4 ± 2.3	30.4 ± 3.4	U = 97.5, N_1= 14, N_2= 11, P = 0.273
Maintained top speed for	4.0 cm / 4 min	3.0 cm / 2 min	NA
Actual distance			
Maximum distance (cm)	58.0	56.2	NA
Average distance (cm)	31.0 ± 5.5	27.4 ± 5.3	U = 72.0, N_1= 14, N_2= 11, P = 0.809
Average distance per active period (cm)°	24.1 ± 5.9	17.9 ± 4.0	U = 72.0, N_1= 14, N_2= 11, P = 0.809
Displacement distance			
Maximum distance (cm)	24.7	19.2	NA
Average distance (cm)	11.6 ± 2.2	11.6 ± 1.9	U = 81.0, N_1= 14, N_2= 11, P = 0.848
Distance in relation to number of days moved	NA	NA	*D. polymorpha:* K = 4.2, df= 3, P = 0.245 *D. bugensis:* K = 2.5, df= 4, P = 0.643

° Values derived from the first active period instead of the first day

* Significant p-values

Discussion

Movement distance

The displacement and actual distance moved, did not differ significantly between both species (Figure 5). The maximum distance of 58.0 and 56.2 cm and average distance of 31.0 and 27.4 cm measured during this study for *D. polymorpha* and *D. bugensis*, respectively, are comparable to the values measured in literature (Table 1) (Toomey et al. 2002, Coons et al. 2004, Kobak and Nowacki 2007, Kobak and Kakareko 2009, Kobak 2013). In other studies, the maximum distance travelled by *D. polymorpha* was estimated to be between 20.5 and 80.3 cm with an average between 5.9 and 27.5 cm (Toomey et al. 2002, Coons et al. 2004, Kobak and Nowacki 2007, Kobak and Kakareko 2009, Kobak 2013). The maximum distance moved by both species is thus within the range reported by the previously mentioned studies. However, *D. polymorpha* moved up to 10–20 cm further in Kobak and Nowacki (2007) and Kobak and Kakareko (2009) compared to the maximum distance measured during our study. These studies assessed the effect of light and the presence of several predators on the distance moved by *D. polymorpha*, representing lesser optimal environmental conditions for this species than the ones in our study. This would support the hypothesis that suboptimal environmental conditions promote dreissenid movement.

Both dreissenids species displayed non-linear, corkscrew-like or circular movement paths resulting in a higher actual distance travelled in comparison to the displacement distance. For both species the actual distance travelled was more than two times higher than the displacement distance (Table 1). In case they would be exposed to a directional nuisance, like for example strobe lights, they might have displayed a linear and directional movement path, moving away from the nuisance source (Coons et al. 2004).

Movement duration

No significant differences were found for the movement duration within the first 24 hours (65 min) and the duration of one active period (45 min) between individuals of both species (Table 1, Figure 5). However, the number of individuals moving for more than one day (2-5 days) was more than two times higher for *D. bugensis* compared to *D. polymorpha*. Having more than one movement day might have an effect on the distance moved by an individual. Unfortunately, we do not have time-lapse photos throughout the whole experiment, to check whether this is actually true. Therefore, we were not able to assess the relationship between the actual movement distance and number of days moved by an individual. Consequently, we do not know whether having several movement days is beneficial for an individual. We could hypothesize that having several movement days gives *D. bugensis* a higher chance to avoid unfavourable conditions and find optimal environments.

All relocating individuals started moving within 2.5 and 12 hours after the start of the experiments. During this research, none of the individuals moved during the first 2.5 hours of the experiments, whereas Toomey et al. (2002) recorded movement for about half of the assessed *D. polymorpha* during their 2-hour lasting experiment. Toomey et al. (2002) exposed their *D. polymorpha* individuals to several temperature, light and water hardness treatments. These treatments might have provoked the mussels, more than our setup, to start looking for a better environment, mobilising them earlier. Additionally, we acclimatized the dreissenids for 24 to 48 hours before starting each experiment, while Toomey et al. (2002) collected their mussels only 4 hours prior to the start of their experiment. Our dreissenids might have moved during the first two hours after placement in the acclimatization tank, however, we did not monitor the dreissenids during acclimatization.

Movement speed

The observed top speed was a third higher for *D. bugensis* (90 cm/h) in comparison to *D. polymorpha* (60 cm/h). These top speeds are higher than the known top speed of 48 cm/h for *D. polymorpha* (Toomey et al. 2002, Czarnołęski et al. 2010). Both dreissenid species moved at more or less the same average speed throughout the experiments (Table 1, Figure 5). However, the average speed measured during this study (25.4 cm/h and 30.4 cm/h for *D. polymorpha* and *D. bugensis*, respectively) was more than two times higher than the average speed known from literature for *D. polymorpha* (between 6 and 12 cm/h) (Toomey et al. 2002, Czarnołęski et al. 2010). The cited articles might have underestimated the movement speed of *D. polymorpha*, as they extrapolated possible speed values from the distance moved over the 2-hour duration of the experiment (Toomey et al. 2002) or with intervals of 15 minutes over 3 hours (Czarnołęski et al. 2010). Individuals probably did not move at a constant speed throughout the experiment, as they might have stopped moving within the 2 hours or 15-minute interval, respectively.

Movement rate and shell size

The shell size and movement rate of individuals were not correlated for either *D. polymorpha* or *D. bugensis*. This result is different than generally reported in literature where, for *D. polymorpha*, the movement rate and the distance moved is inversely proportional to their shell size and thus also to their age (Kobak 2001, Toomey et al. 2002). Even though our study found no significant effect of the shell size on the movement rate of both species, we did observe that none of the individuals of *D. polymorpha* larger than 2 cm moved (Figure 4).

Movement rate

This study exposed that about twice as many *D. bugensis* individuals moved during the movement experiments in comparison to *D. polymorpha* individuals when looking at variously sized specimens (41% and 19%, respectively). This experiment mimicked a natural dreissenid community as *D. bugensis* grows faster and larger than *D. polymorpha* (D'Hont et al. 2018). When comparing individuals with an equal shell size to each other, we obtained a similar result with a significantly higher movement rate for *D. bugensis* (58% and 39%, respectively). Additionally, about 10% more *D. bugensis* specimens were moving around the polyethene tanks at any point in time during the experiments (Figure 3). This comparison has to our knowledge not been made before in literature. In another experiment by the first author, a significantly lower percentage of *D. bugensis* (69%) individuals were found to be attached to the substrate in comparison to *D. polymorpha* (85%) individuals (D'Hont et al. 2021). This lower attachment rate for *D. bugensis* could correspond to its higher movement rates, as dreissenids will most likely not attach to the containers while moving. Since we assume that our experimental circumstances (drying, cleaning and tagging of mussels and non-aerated, nutrient-poor water) mimicked unfavourable conditions for dreissenids, they were likely triggered to look for a better environment. Examples of such unfavourable environmental conditions in their natural habitat are poor water quality, changes in orientation, presence of conspecifics, low oxygen content or desiccation (Kobak 2001, Alexander and McMahon 2004, Kobak and Nowacki 2007, Collas et al. 2018). These environmental conditions are proven stimuli of detachment and/or movement in dreissenids (Kobak 2001, Kobak and Nowacki 2007, Collas et al. 2018). One could expect that a higher percentage of moving individuals gives *D. bugensis* a higher chance to find optimal environments.

The results of this research can contribute to assessing the risk of spread and establishment of both dreissenid species (Andersen et al. 2004, Verbrugge et al. 2012). Boat traffic and, to a lesser degree, birds and fish were suggested to be the main (upstream) dispersal vectors for dreissenids (Wilson et al. 1999, Karatayev et al. 2011). This type of dispersal most likely includes passage through unfavourable environmental conditions like overland transport or changes in salinity. *Dreissena polymorpha* individuals endure these types of e.g. overland transport better than *D. bugensis* does (Collas et al. 2018).

Dreissena bugensis will in such situations probably detach looking for a better environment (D'Hont et al. 2021). Consequently, the risk of introduction is higher for *D. polymorpha* in comparison to *D. bugensis* (Baldwin et al. 2002). However, the higher number of moving individuals with the ability to look for better locations allows *D. bugensis* to become a stronger invader once the population is settled and established. This might give *D. bugensis* a competitive benefit over *D. polymorpha* and possibly be a contributing driver to the observed dominance shift between both species. This will mostly be the case in highly fluctuating environments, i.e. with strong water currents, large fluctuations in water level or salinity. As our study site, the Haringvliet, has little to no currents and well-regulated constant water levels, the motivation to move to better environments will be low in this area. This might explain why both species still co-occur in the Haringvliet, 14 years after the introduction of *D. bugensis* and why *D. bugensis* was not able to fully outcompete *D. polymorpha*.

The movement of dreissenids might be influenced by environmental conditions, like e.g. temperature, salinity, light conditions, oxygen and food availability (Kobak 2001, Toomey et al. 2002, Coons et al. 2004, Kobak and Nowacki 2007, Collas et al. 2018). We chose to keep these environmental conditions constant throughout the experiment while aiming at elucidating the differences in moving behaviour of two dreissenid species. Our study was conducted with a temperature difference of about 15 °C between the temperature at the collection site and the experimental water temperature (using gradual acclimatization). Future research may be focused on unravelling differential effects of various environmental conditions and procedures for acclimatization. Therefore, we recommend future studies to include movement experiments conducted at several temperatures and other environmental conditions.

Conclusions

The top speed of *D. bugensis* was higher than that of *D. polymorpha*, moving at maximum 90 cm/h and 60 cm/h respectively, after a disturbance of environmental conditions. Additionally, about twice as many individuals of *D. bugensis* moved during the experiments in comparison to *D. polymorpha*. The same result was found when comparing individuals with various shell sizes to each other. About 10% more *D. bugensis* specimens were moving around the polyethene tanks at any point in time during the experiments. This gives them the capacity to actively move away from unfavourable environmental conditions. Examples of such unfavourable environmental conditions for dreissenids are poor water quality, changes in orientation, presence of conspecifics, low oxygen content and desiccation. The average movement duration, average speed and average distance moved did not differ significantly between *D. polymorpha* and *D. bugensis*. Most individuals moved in more or less circular patterns, causing their actual movement distance to be twice as high as their displacement distance. Consequently, the average movement duration, average speed, average distance moved and general movement pattern of these two mussel species probably do not contribute to the species displacement. However, the results support our hypothesis that *D. bugensis* could have a competitive benefit over *D. polymorpha* by having a higher top speed and a significantly higher number of individuals moving when their populations are disturbed. Therefore, mobility might be one of the contributing drivers of the observed dominance shift between both species.

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Chapter 5

Dropping the microbead: Source and sink related microplastic distribution in the Black Sea and Caspian Sea basins

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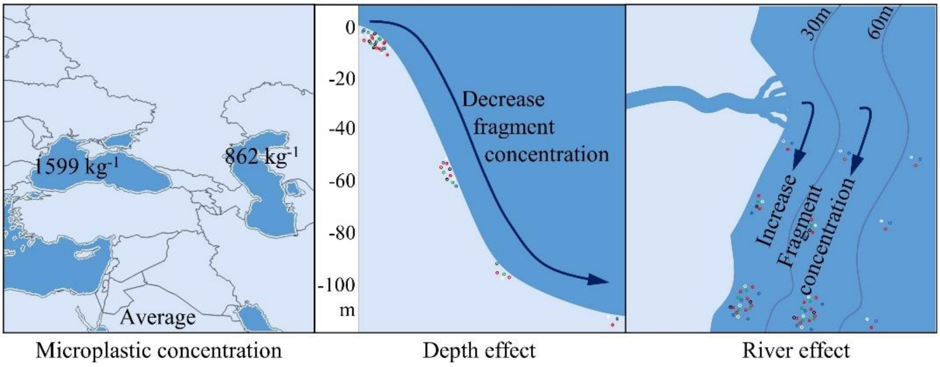
in Marine Pollution Bulletin

Key words: city, depth, grain size, Ponto-Caspian, river, shore

Graphical abstract



Abstract

Microplastic pollution is a growing, yet poorly understood problem. Here, we assessed the relationship between microplastic concentration and distance to rivers, shorelines, cities, sediment grain size or water depth in sediments of the world's largest (semi-)enclosed aquatic basins. Microplastic was extracted from sediment using density separation and elutriation. Fibers and transparent or white microplastic particles were the most abundant shape and color. The microplastic concentration in sediments of the Black Sea was about twice as high compared to that in the Caspian Sea. Fragment concentrations decreased with depth, while fiber concentrations were independent of depth. Overall, no relationship with distance to shores, rivers and cities or with grain size was observed. However, within some depth classes concentrations were related to the distance from rivers, shores and cities.

Introduction

Global waste production has grown continuously throughout the last decades (Löder and Gerdts 2015). The annual global litter production almost reached 370 million tons in 2019 (Plastics Europe 2020). About half of this amount concerns non-biodegradable materials and about 80% of marine waste has a terrestrial origin (Ioakeimidis et al. 2014, Jambeck et al. 2015). Due to its high mobility in the marine environment, transported by currents and wind, anthropogenic waste is present in all compartments of water bodies ranging from the shorelines to the remote deep-sea (Van Cauwenberghe et al. 2013, Do Sul and Costa 2014, Ryan 2015). Plastic is the most common type of anthropogenic waste representing 95% of the litter accumulated on shorelines, water and the seabed (Galgani et al. 2015). Plastic debris in the aquatic environment can be divided into four size classes ranging from mega-particles (> 100 mm), macro-particles (> 20 mm) and meso-particles (5-20 mm) to micro-particles (< 5 mm) (Barnes et al. 2009). Most of the plastic present in the aquatic environment is represented by microplastic particles (Browne et al. 2010). The microplastic particles can be divided into two sub-classes: Primary and secondary microplastic. Primary microplastic is directly released in the aquatic environment due to industrial spillage or discharge in sewage water. This type of microplastic is produced as abrasive in cosmetic products, industrial shot-blasting abrasive or as plastic pellets used as feedstock for the production of larger items (Thompson 2015). An important and often underestimated primary microplastic source is the discharge of microplastic fibers by washing machines (Browne et al. 2011, Napper and Thompson 2016). Secondary microplastic is formed by the fragmentation of larger pieces of plastic debris due to ultraviolet light, heat, biological processes and physical action by wind and waves (Reisser et al. 2013, Andrady 2015). In contrast to larger plastic debris particles, there is no rigorous knowledge on the impact of microplastic (Wagner et al. 2014). Microplastic particles can impact biota due to biofouling and ingestion (Eerkes-Medrano and Thompson 2018). The ingestion of microplastic (e.g. by bivalves, crustaceans, echinoderms, bryozoans and vertebrates) causes stress responses such as inflammation or oxidative stress at tissue and cellular levels (Von Moos et al. 2012, Eerkes-Medrano and Thompson 2018). Microplastic can enter food chains due to ingestion by filter feeders at the bottom of the food chain (Murray and Cowie 2011). The accumulation of microplastic particles in digestive tracts reduces feeding or causes false satiation affecting nutritional gain and energy reserves (Eerkes-Medrano and Thompson 2018). Microplastic particles may also be a medium to expose biota to environmental contaminants adsorbed to their surfaces (Bakir et al. 2016, Brennecke et al. 2016, Akhbarizadeh et al. 2017). Additionally, biofouling of (micro-)plastic particles may lead to transportation of biota and/or invasive species (Eerkes-Medrano and Thompson 2018).

Even though the first reports of microplastic in the aquatic environment date back to the early 1970s, 50 years later, no profound estimation exists of the distribution, amount, and origin of microplastic (Jambeck et al. 2015). Early studies focused on the water column (Carpenter et al. 1972, Carpenter and Smith 1972). It took 30 more years before the first observations of microplastic in sediment were made (Thompson et al. 2004). Sediment serves as a sink for microplastic, especially the particles with a density higher than that of the overlying water compartment (Law et al. 2010, Cózar et al. 2014, Van Cauwenberghe et al. 2015). Low-density particles tend to float on the sea surface, however, they might as well sink to the bottom due to density modifications by processes such as biofouling (Andrady 2011, Reisser et al. 2013, Zettler et al. 2013). Therefore, assessing microplastic in sediment is a relevant issue in aquatic pollution research.

Microplastic concentrations found in sediment might be influenced by source and sink characteristics. Concentrations may depend on the distance to the source. Sink related concentrations would correlate with the saturation capacity of the sediment (i.e. sediment grain size) or factors influencing the settlement of plastic particles, like water depth and hydrodynamics. Coarser sediment particles have larger gaps in between the particles allowing for microplastic particles to accumulate and to be washed out from in between the sediment particles by water currents. Additionally, low water turbulence favors the settlement of (micro)plastic particles (Pohl et al. 2020). Decreasing turbulence along the depth gradient causes deeper areas to become a sink for fine and slowly settling microplastic particles (Enders et al. 2019). Moreover, water turbulence relates to sediment grain size as well. High water energy levels correlate with coarse sediment grains, while low energy environments usually correlate with fine sediment grains. Therefore, small sediment particles and microplastic particles are both likely to be transported by water currents and to be deposited in low energy environments (Browne et al. 2010).

Rivers are important transport pathways for plastic in the aquatic environment connecting their sources and sinks (Ballent et al. 2016, Van Calcar and Van Emmerik 2019). This was confirmed as microplastic concentrations in sediment decreased with an increasing distance from river mouths (Luo et al. 2019, Falahudin et al. 2020). Most plastic sources are land-based, therefore, microplastic concentrations decreased further away from the shoreline (Vianello et al. 2013, Graca et al. 2017, Zhang et al. 2019a, Zhang et al. 2019b, Wang et al. 2020a). This shoreline effect is mainly influenced by urban activities and population density along the shore (Ballent et al. 2016, Wang et al. 2017, Waldschläger et al. 2020).

Information on microplastic pollution in the Black Sea and Caspian Sea basins is scarce. The main pollution sources of these basins are the rivers (Secrieru and Secrieru 2002, Baltas et al. 2017). The major rivers flowing into the Black Sea are the Danube, Dnieper, Dniester, Don and Kuban (Secrieru and Secrieru 2002, Mülayim and Balkis 2015). The Danube is Europe’s second-largest river and has an estimated plastic input in the Black Sea of 4.2 tons per day (Lechner et al. 2014). The relatively high microplastic input in an enclosed basin means that the Black Sea is a hotspot for accumulation of microplastic and there is an urgency to understand their distribution, origins, transportation and effects on marine life (Aytan et al. 2016). The Caspian Sea is the largest fully enclosed basin in the world. The largest fresh water contributor to this sea is the Volga, followed by the rivers Kura and Ural (De Mora et al. 2004). Microplastic concentration in the southern coastal zones were 25 to 330 items per kilogram of dry sediment, with fibers as the most abundant type (Mataji et al. 2020, Mehdinia et al. 2020). Additionally, the fact that these basins are (semi)enclosed allows us to exclude the influence of certain parameters (e.g. sea currents, tides) when assessing possible microplastic sources and sinks. The Black Sea and Caspian Sea basins are therefore ideal as model systems to investigate microplastic sources and sinks.

Despite these pressures, the composition and concentration of microplastic in sediments of the Black Sea and Caspian Sea are largely unknown (Aytan et al. 2016, Mataji et al. 2020, Mehdinia et al. 2020). The present study, therefore, aims to answer two main research questions. First, we assessed whether the microplastic composition and concentration differed between the Black Sea and Caspian Sea basins. Second, we assessed whether there is a link between the microplastic concentration in Ponto-Caspian sediment and various microplastic sources (rivers, shore or city) or characteristics of sinks (like grain size of sediment and water depth).

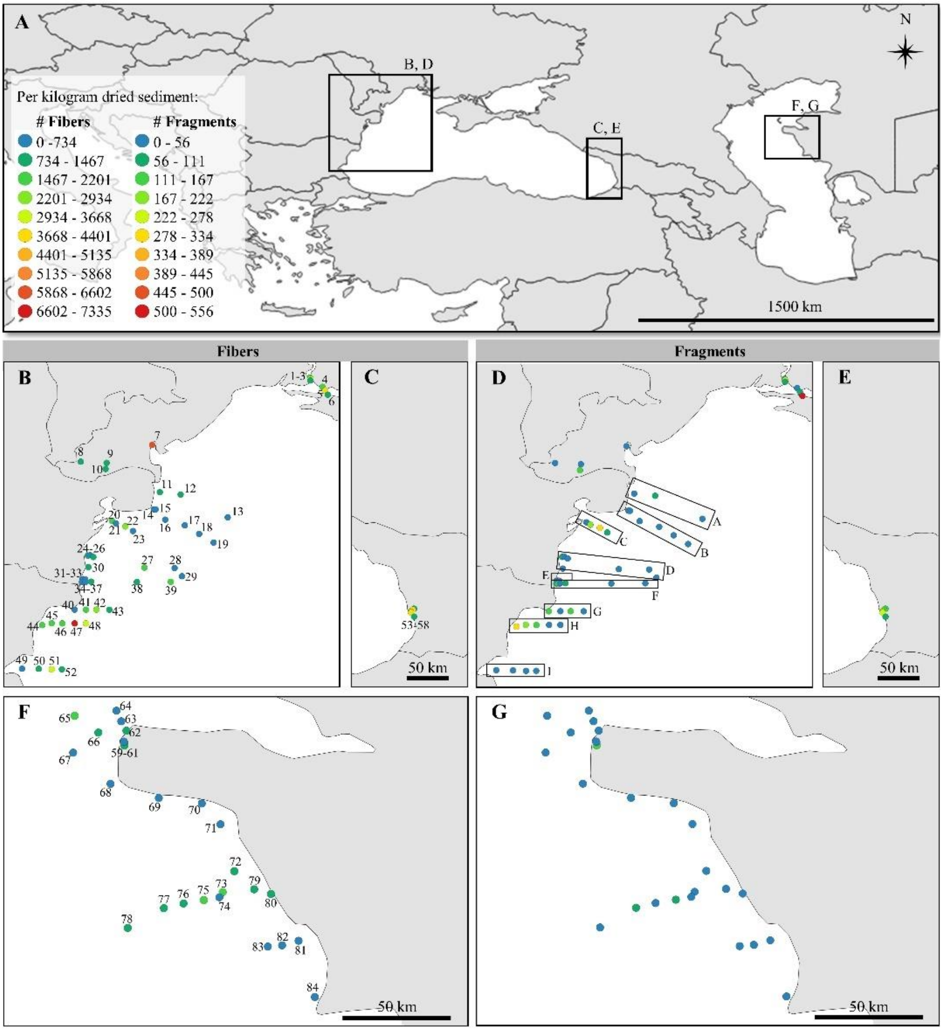


Figure 1. Microplastic fibers and fragments in the Ponto-Caspian basins. (A) Overview of the assessed research areas and all 84 samples collected in the Black Sea (B-E) and Caspian Sea (F-G). The results are represented as the concentration of the fibers (B, C, F) and fragments (D, E, G) per kilogram dried sediment. The sample identification numbers are represented (B, C, F). Panel (D) represents the transect numbers for the Black Sea (transect numbers A-I). The samples were color coded according to their concentration.

Material and methods

Study sites

The samples were collected in the Black Sea (Eastern Europe and Western Asia) and Caspian Sea (Western Asia) (Figure 1). The Black Sea has a surface area of about 423 000 km2, a maximum depth of 2200 m and is surrounded by the Federation of Russia, Georgia, Republic of Bulgaria, Republic of Turkey, Romania and Ukraine (Baltas et al. 2017). The Caspian Sea has a surface area of about 390 000 km^2 and is surrounded by Azerbaijan, the Federation of Russia, the Islamic Republic of Iran, Kazakhstan and Turkmenistan. This sea is divided into three basins (De Mora et al. 2004): 1) the shallow northern part (average 5 m and maximum 20 m depth), 2) the central basin (maximum depth of 788 m), and 3) the southern basin (maximum depth of 1025 m).

Sampling locations

In total 84 sediment samples were collected. In the Black Sea, 58 samples were taken in front of major harbors (i.e. Constanța, Mangalia, Varna and Boergas), river mouths (e.g. Danube, Dniepr, Bug and Rioni) and nature reserves (i.e. Razim lake, Balgarevo and Kolkheti national park) (Figure 1, Supplementary 1). Those samples were collected in four Black Sea countries: Ukraine, Romania, Bulgaria and Georgia. In the Caspian Sea area of Kazakhstan, 26 samples were collected in front of harbors (e.g. Aktau and Bautino) and along the shoreline (Figure 1). These samples were used to make a general comparison between both seas.

To assess the relationship between the microplastic concentration and characteristics of possible sinks (depth, grain size) and sources (river, shore, city), a subset of 42 sediment samples in open water (sample numbers 11 to 52, Figure 1B) from the Black Sea sample set was chosen as it was the largest one with several sources and sinks present. This set of samples consisted of 9 transects (transect A-I, Figure 1D). Some of these transects (E-H) were probably not optimal, as these transects

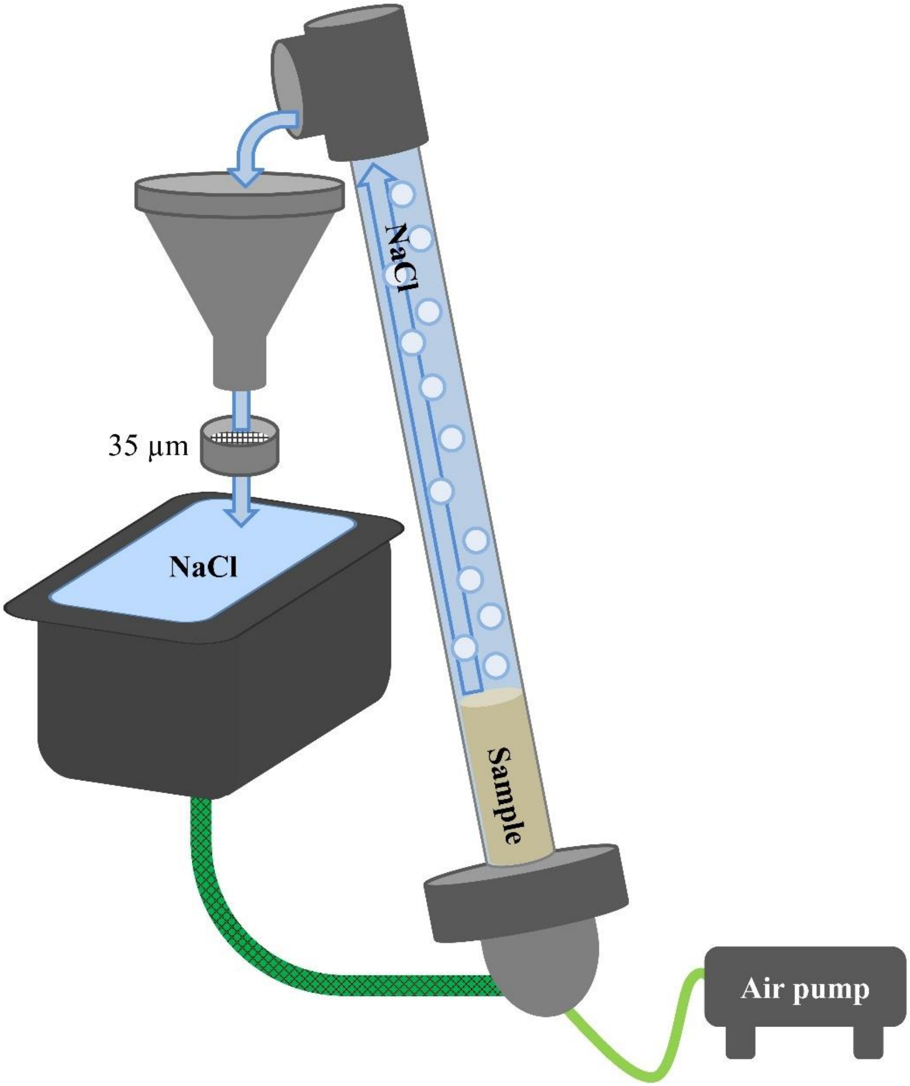


Figure 2. Density separation, elutriation column. Setup of the elutriation column for extracting microplastic from sediment. Methodology adapted from Claessens et al. (2013).

did not run perpendicular to the shoreline and the isobaths (Figure 1D). For comparison, the relationship between the microplastic concentration and their sources and sinks was assessed for all 26 Caspian Sea samples. Two sets of Caspian Sea samples were identified in the Caspian Sea: 1) a depth transect perpendicular to the coastline (samples number 73-78), and 2) a shoreline transect parallel to the coastline in a single depth category (0-30 m, sample numbers 59-72 and 79-84, Figure 1F).

Sediment collection

Sediment sampling was carried out from a vessel using a 0.11 m^2 Van Veen grab. Once on board, around 500 ml of the upper 3 cm was collected for microplastic analysis. A subsample was used for grain size analysis. All samples were stored in 500 ml aluminum containers and wrapped in aluminum foil and plastic bags to avoid contamination by airborne microplastic particles.

Water depth, distance to shore, river, city and seafloor profiles

The water depth at all sediment-sampling sites in open water zones of the Black Sea and Caspian Sea was determined using the bathymeter of the research vessel.

The shortest distance to the shore, distance to the nearest river mouth and distance to the nearest city were measured using Google Earth Pro 7.3.3.7786.

The seafloor profiles were obtained using a Black Sea bathymetric map by the EMODnet Bathymetry Consortium (2018). This data file was processed to obtain the seafloor profile where the Black Sea open water samples were collected using the terrain profile plugin tool in the program QGIS 3.6.0.

Analyses of grain size

The grain size analyses were conducted for 33 and 18 sediment samples from the open water zones in the Black Sea and Caspian Sea, respectively. These sediment samples were dried and analyzed using a Geotech Sieve Analysis Field Kit. The grain size analyses were in accordance with the OSPAR/HELCOM protocol for port monitoring (Gittenberger et al. 2014). In total five grain size classes were identified: silt (<0.06 mm), fine sand (0.25-0.06 mm), medium sand (0.5-0.25 mm), coarse sand (1.80-0.50 mm) and (fine) gravel (>1.80 mm). After the removal of shells, the dried sediment was carefully grinded to separate all sediment grains. The shells and grinded sediment were added to the Geotech Sieve and separated by size by shaking stacked sieves. Each size fraction of the sample was weighed using a scale with a sensitivity up to 0.001 g. For each grain size class, the percentage of the sediment composition was calculated. For the statistical analyses, the obtained percentages for each size class were combined with the average size of that size class to get a single grain size value for each sediment sample: % silt x 0.06 mm + % fine sand x 0.13 mm + % medium sand x 0.35 mm + % coarse sand x 1.15 mm + % gravel x 1.80 mm.

Microplastic extraction

The microplastic extraction was based on density separation in an elutriation column. This method was adapted from Claessens et al. (2013). Plastic particles between 5 cm and 35 μm were considered to be microplastic. A subsample of 200 g dried sediment was added to a 500 mL glass jar and mixed with saturated NaCl (1.18 g/mL). Samples were stored in these jars for 5 days until the whole sediment sample was dissolved and suspended in NaCl. The dissolved sample was added to an elutriation column (Figure 2, Supplementary 1) consisting of an aerated PVC tube with a length of 1 m and a diameter of 50 mm. The air bubbles created a current within the elutriation column, releasing microplastic particles from the sediment and pushing particles with a lighter density upwards. The column was left to mix for 10 minutes before creating an overflow with saturated NaCl from the column onto a 35 μm sieve collecting the particles with the lowest density. The overflow was created by opening up the tap to a pump for 3 seconds. Following this overflow, the tap to the pump and the connection to the air pump were closed off and the sediment was left to settle for 5 minutes in the column. After this 5-minute settlement step, a new 3 seconds overflow was created onto the sieves. This settlement step was repeated two more times. Finally, the 35 µm sieve containing the fraction of the sample with the lightest density, including the microplastic particles, was rinsed with fresh tap water. The tap water and leftover sediment particles smaller than 35 µm were forced through the sieve using a small paintbrush. The sample was rinsed until all particles smaller than 35 µm were flushed through the sieve. The sieve was then covered using another 35 µm sieve to avoid contamination from airborne microplastic particles, and dried in an oven at 50 °C.

Analyses of microplastic

After drying, all samples with extracted microplastic particles were inspected under a Byomic light microscope with a magnification range of 7.5 to 50x. The microplastic particles in each sample were counted and identified based on the criteria described by Hidalgo-Ruz et al. (2012). The shape and color categories identified by Hidalgo-Ruz et al. (2012) were adjusted to the most commonly found shapes and colors in our samples. The microplastic particles were subdivided into 6 different shape categories (sphere, fiber, angular, elongated, random and film) and 7 color categories (blue, red, white/transparent, black, green, yellow and other colors). Examples of each shape and color can be found in Figure 3.

Statistical analysis

When comparing the microplastic composition between the Black Sea and Caspian Sea, the data were log transformed before creating a resemblance matrix using the Bray curtis similarity index. A multidimensional scaling (MDS) plot with a Pearson correlation was created. A PERMANOVA test was performed using the factor “waterbody”. The permutation distance to the centroid (PERMDISP) was checked to be non-significant. A SIMPER analysis was performed to assess the average dissimilarity between both waterbodies and the microplastic types explaining these differences. Additionally, a

Kruskal-Wallis rank sum test was performed to assess differences in microplastic concentration between both seas.

When assessing possible relationships between microplastic sources (river, shore, city) and sinks (grain size, depth) we mainly focused on the 42 Black Sea open water samples and two main types of microplastic (fibers and fragments). Using a Spearman correlation test, we found a correlation between all of the parameters, apart from “grain size”. Therefore, we decided to analyze these parameters separately instead of including them all into one model. To assess the effect of source and sink parameters, the data were log transformed as the fiber count was on average 50 times higher than the fragment count. Next, a resemblance matrix using the Bray curtis similarity index was created and a PERMANOVA with an unrestricted permutation of raw data was conducted. The PERMANOVA tests were performed for the categorical factors “distance to river”, “distance to city”, “distance to shore”, “depth” and “grain size”. For each PERMANOVA test, the permutation distance to the centroid (PERMDISP) was checked to be non-significant.

More in-depth analyses were performed on the association between the depth of the sediment-sampling site and the concentration of fibers and fragments in those samples. When analyzing spatial data, spatial autocorrelation should be checked. We plotted the residuals of our models against their spatial position (longitude/latitude) and visually assessed for geographical patterns. No spatial autocorrelation was found. The goodness of fit was analyzed for a linear model (Lm) in comparison to a linear mixed effects model (Lme) including the “transect number” as a random factor (random intercept). Based on the AIC, BIC and Log likelihood, the linear model seemed to give the best fit to our data. Therefore, this model was used during the analysis of the concentration of fibers and fragments with continuous depth data. The function “Lme” of the package “nLme” was used to run this analysis on the log transformed data.

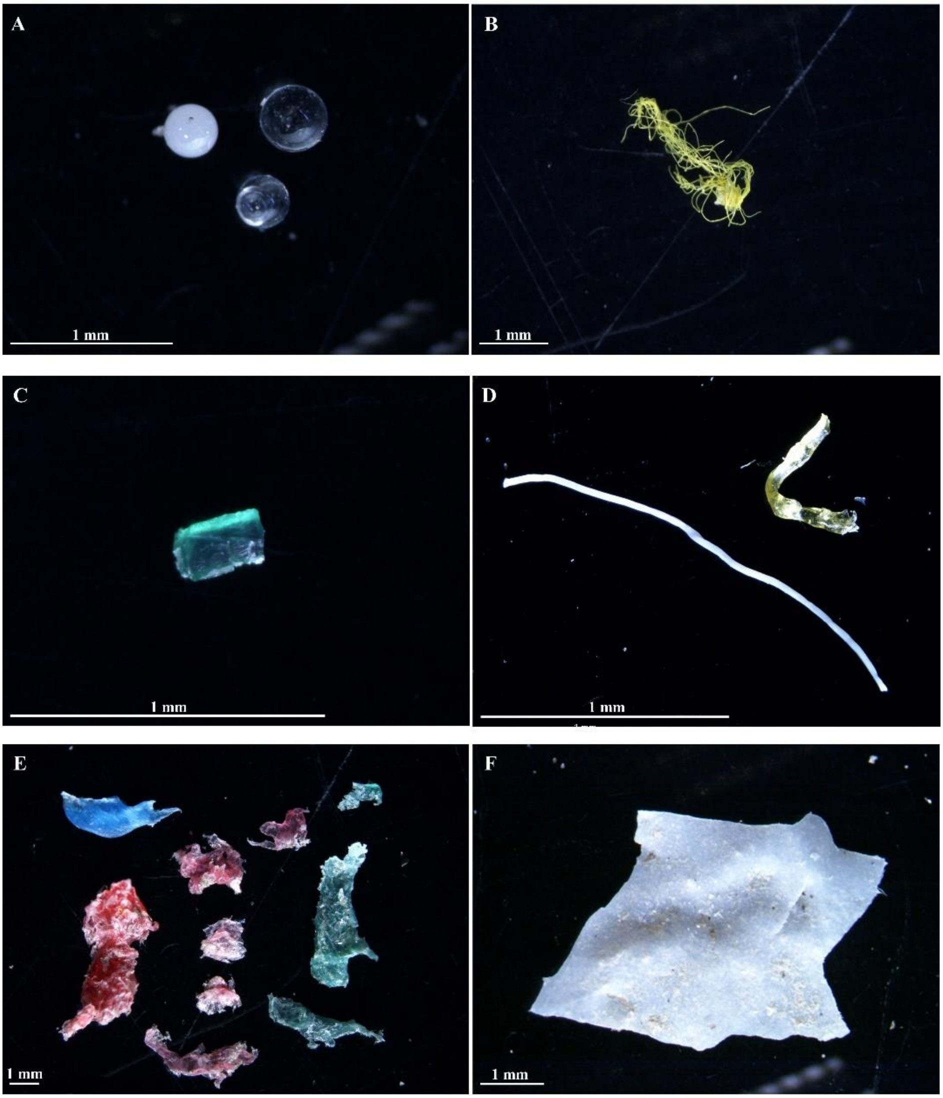


Figure 3. Microplastic types: shape and color. The different colors and shapes of microplastic identified during this study: A) White and transparent spheres, B) Yellow fibers, C) Green angular fragment, D) White and yellow elongated fragments, E) Blue, red and green irregular particles, F) White films. Types A, C - F are considered to be fragments, while B is considered to be fibers.

Additionally, PERMANOVA analyses without data transformation and using a Bray Curtis resemblance matrix were performed to assess the effect of the microplastic shape and color in relation to depth.

Finally, the data of sediment samples from the open water zones in the Black Sea were subdivided into three depth categories (0-30 m, 31-60 m and 61-100 m) representing a more or less equal division of the number of samples and depths (N = 15, N = 11, N = 16, respectively). Within these depth categories, linear models were performed to assess whether the distance to sources (river, shore or city) and a characteristic of sinks (grain size of sediment) might affect the microplastic densities. Similar tests were used on the Caspian Sea samples to assess the relationship between the concentration in fibers and fragments and the distance to sources (river, shore, city) and characteristics of sinks (water depth and grain size of sediment).

The programs R (version 3.6.2), Primer (version 6.1.13) and PERMANOVA (version 1.0.3) were used for all data analyses.

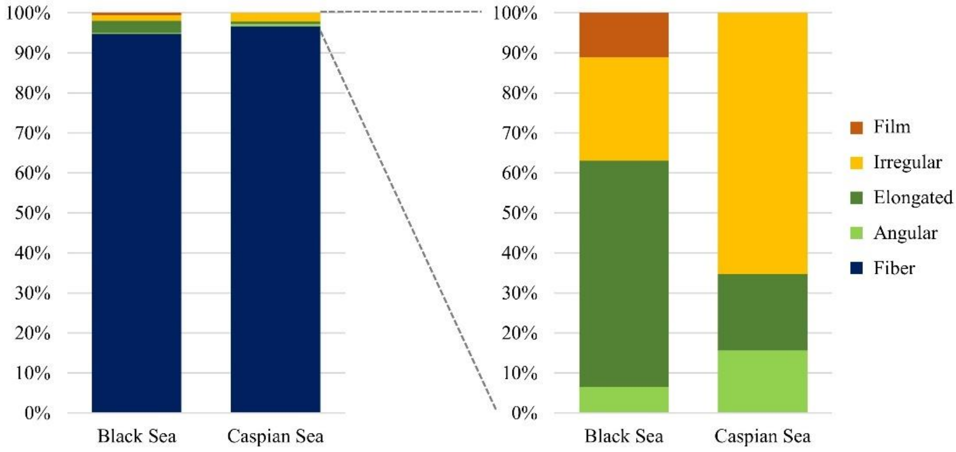


Figure 4. Microplastic shapes. The relative microplastic shape distribution in the Black Sea and Caspian Sea basins.

Results

Differences between microplastic types

The most abundant microplastic shapes were the fibers, followed by thicker, longer and more elongated particles and the random or irregular shaped particles (Figure 4; Table 1). Spheres were not found in the Black Sea and Caspian Sea. The most abundant microplastic colors were white or transparent, followed by red and black. Green was the least abundant color (Figure 5; Table 1).

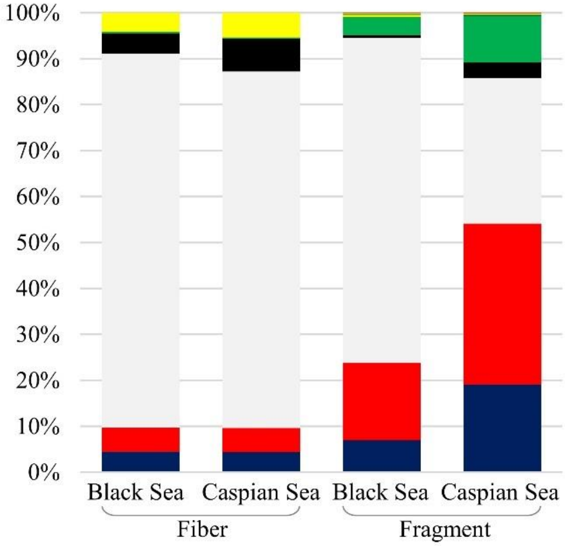


Figure 5. Microplastic colors. The relative distribution for fibers and fragments with various colors in the Black Sea and Caspian Sea basins.

Difference between Black Sea and Caspian Sea

The Black Sea and Caspian Sea differed significantly from each other in microplastic composition ($P = 0.001$, Table 2). The SIMPER analysis indicated an average dissimilarity of 41.6% between both seas, based on their microplastic composition. The elongated white fragments and red and blue fibers were mostly present in the Black Sea, while yellow and black fibers were mostly found in the Caspian basin. The microplastic concentration was about two times higher in the Black Sea in comparison to the Caspian Sea (P = 0.029, Figure 6, Table 2). This was also the case when separately assessing microplastic fibers and fragments (P = 0.003 and P < 0.001, respectively; Figure 6, Table 2). Because of these differences, the other analyses were performed on each waterbody separately. We mainly focused on the Black Sea basin to assess the possible relationship of sinks (water depth, grain size) and sources (river, shore, city) with the microplastic concentration in sediment. A subset of 42 open water samples from the Black Sea sample set was chosen as it was the largest one with several sources and sinks present.

Table 1. Total number of items per shape and color in 84 sediment samples, each per one kilogram dried sediment.

Shape - Color	Blue	Red	Transparent/White	Black	Green	Yellow	Other	Total
Spheres	0	0	0	0	0	0	0	**0**
Fiber	4 912	5 701	88 357	5 435	341	4 823	0	**109 570**
Angular	83	230	45	25	45	5	0	**433**
Elongated	25	30	2 827	5	0	0	0	**2 887**
Irregular	262	800	457	20	173	25	0	**1 736**
Film	109	20	339	0	51	0	20	**539**
Total	**5 390**	**6 781**	**92 025**	**5 485**	**611**	**4 853**	**20**	115 165

Black Sea basin

Effect of water depth

The ratio of fiber and fragment density in sediments changed significantly over depth (P = 0.029, Figure 7). The concentration of fragments decreased significantly with depth (P = 0.014, Figure 7). Fiber concentrations were not significantly related to depth (P = 0.096, Figure 7, Table 2). The shape of microplastic fragments (angular, film, irregular and elongated) did not significantly relate to the water depth at sampling sites (P = 0.108, Table 2). Similarly, there was no significant relationship between the color of the fibers or fragments and the water depth (P = 0.375, P = 0.094, respectively, Table 2).

Effect of distance to river, city, shore and sediment grain size

No significant relationship was found between the concentration of microplastic particles and the distance to the nearest upstream city (P = 0.112), distance from the nearest river (P = 0.081), distance from the shore (P = 0.143) and the sediment grain size (P = 0.828) for samples collected in the Black Sea basin (Table 2).

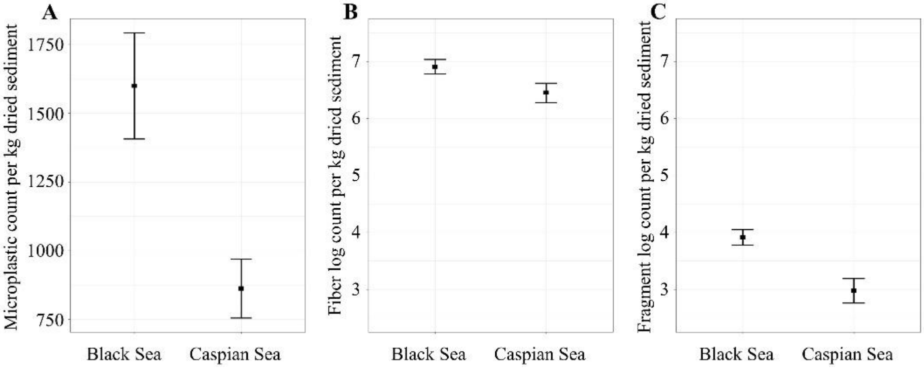


Figure 6. Microplastic concentrations per waterbody. The boxplots (with standard error) for both the microplastic concentration (A), the log of the microplastic fiber concentration (B) and the log of the microplastic fragment concentration (C) counted as the particles per kilogram dried sediment from both seas.

Effect of distance to river, city, shore and sediment grain size within a single depth category

As water depth was found to affect the composition and concentration of fragments in the sediment, we assessed whether distance to rivers, shorelines and cities as well as grain size affected the microplastic concentration within one single depth category (0-30 m, 31-60 m or 61-100 m). The microplastic fragment concentration was found to increase significantly further away from the river in both the 0-30 m and 31-60 m depth categories (P = 0.039; P = 0.005, for 0-30 m and 31-60 m respectively, Table 2). Additionally, the concentration of microplastic fragments increased significantly when moving further away from the shore in the 31-60 m depth category (P = 0.034, Table 2). In the deepest depth category (61-100 m) the fiber concentrations decreased further away from the city and the shoreline (P < 0.001; P=0.034, respectively, Table 2). No relationship between sediment grain size and fragment or fiber concentrations was found in any of the depth categories (P ≥ 0.160, Table 2).

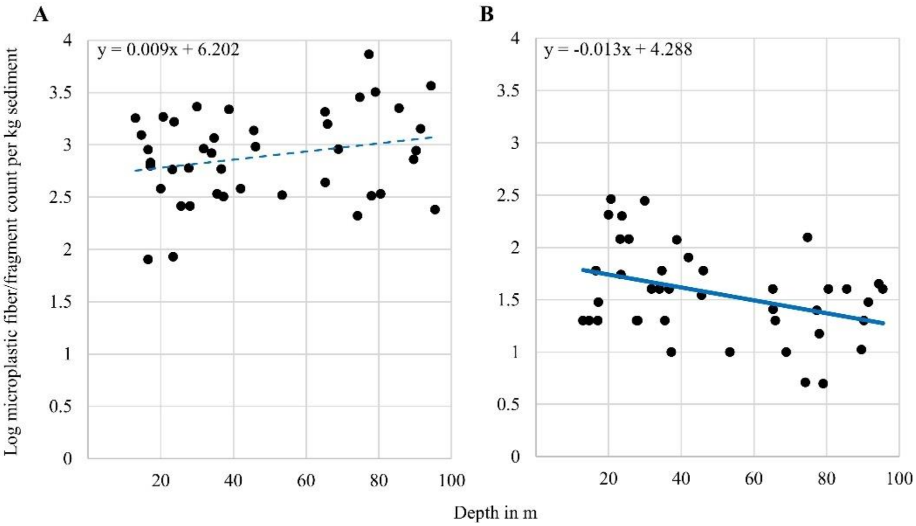


Figure 7. Microplastic concentrations with depth. The relationship between the concentration of fibers (A) and fragments (B) per kilogram dried sediment and water depth. The slope and intercept of each linear model are represented. The blue line represents the linear model fit to the data. As the linear model indicated no significant effect of depth on the fiber concentration (A), this fit was represented as a dotted line.

Table 2. The outputs of the statistical tests for comparisons of the microplastic concentrations and compositions in relation to possible sources and sinks. Where appropriate, a Permanova test (P), Kruskal-Wallis rank sum test (K), or Linear regression model (Lm) was applied.

Difference between waterbodies	Statistical test	Test statistic, degrees of freedom, P-value
Microplastic composition	P	F= 7.7, df= 1, 82, P= 0.001*
Microplastic count total	K	χ^2= 4.8, df= 1, P= 0.029*
Microplastic count fibers	K	χ^2= 11.9, df= 2, P= 0.003*
Microplastic count fragments	K	χ^2= 26.7, df= 2, P< 0.001*

Black Sea

Microplastic link with city, river, shore, grain size and depth

Distance to city	P	F= 1.7, df= 7, P= 0.112
Distance to river	P	F= 1.7, df= 6, P= 0.081
Distance to shore	P	F= 1.6, df=5, P= 0.143
Grain size	P	F= 0.5, df= 4, P= 0.828
Depth	P	F= 2.8, df= 2, P= 0.029*

Depth effect on microplastic shape and color

Fiber ~ Depth continuous	Lm	F= 2.9, df= 1, P= 0.096
Fragment ~ Depth continuous	Lm	F= 6.6, df= 1, P= 0.014*
Fragment ~ Depth and shape	P	F= 1.7, df= 2, P= 0.108
Fragment ~ Depth and color	P	F= 1.7, df= 2, P= 0.094
Fiber ~Depth and color	P	F= 1.1, df= 2, P= 0.375

Within one depth category

Fiber (Lm test)

Parameter	0 – 30 m	31 – 60 m	61 – 100 m
Distance to city	F= 0.6, df=1, 13, P= 0.469, R^2_{adj}= -0.03	F= 0.2, df=1, 8, P= 0.702, R^2_{adj}= -0.10	F= 26.6, df=1, 15, P< 0.001*, R^2_{adj}= 0.62
Distance to river	F= 3.8, df=1, 13, P= 0.072, R^2_{adj}= 0.17	F= 2.3, df=1, 8, P= 0.168, R^2_{adj}= 0.13	F= 0.5, df=1, 15, P= 0.472, R^2_{adj}= 0.03
Distance to shore	F= 2.5, df=1, 13, P= 0.139, R^2_{adj}= 0.10	F= 0.9, df=1, 8, P= 0.370, R^2_{adj}= -0.01	F= 5.5, df=1, 15, P= 0.034*, R^2_{adj}= 0.22
Grain size	F< 0.1, df=1, 10, P= 0.900, R^2_{adj}= -0.10	F= 0.3, df=1, 4, P= 0.604, R^2_{adj}= -0.16	F= 0.1, df=1, 13, P= 0.827, R^2_{adj}= -0.07

Fragment (Lm test)

Distance to city	F= 0.7, df=1, 13, P= 0.419, R^2_{adj}= -0.02	F= 0.2, df=1, 8, P= 0.708, R^2_{adj}= -0.10	F< 0.1, df=1, 15, P= 0.989, R^2_{adj}= -0.07
Distance to river	F= 5.3, df= 1, 13, P= 0.039*, R^2_{adj}= 0.23	F= 14.3, df=1, 8, P= 0.005*, R^2_{adj}= 0.60	F < 0.1, df=1, 15, P= 0.930, R^2_{adj}= -0.07
Distance to shore	F= 3.6, df=1, 13, P= 0.079, R^2_{adj}= 0.16	F= 6.5, df=1, 8, P= 0.034*, R^2_{adj}= 0.38	F= 0.3, df=1, 15, P= 0.567, R^2_{adj}= -0.04
Grain size	F= 2.3, df=1, 10, P= 0.160, R^2_{adj}= 0.11	F< 0.1, df=1, 4, P= 0.958, R^2_{adj}= -0.25	F= 1.4, df=1, 13, P= 0.263, R^2_{adj}= 0.03

Caspian Sea

Microplastic link with city, river, shore, grain size and depth

Fiber	Along depth transect	Along shoreline
Distance to city	F< 0.1, df= 1, 4, P= 0.923	F< 0.1, df=1, 15, P= 0.859
Distance to river	F< 0.1, df=1, 4, P= 0.971	F= 0.1, df=1, 17, P= 0.815
Distance to shore	F< 0.1, df=1, 4, P= 0.915	F< 0.1, df=1, 15, P=0.937
Grain size	F< 0.1, df= 1, 3, P= 0.968	F= 9.8, df=1, 10, P= 0.011*
Depth	F< 0.1, df= 1, 4, P=0.944	NA

Fragment	Along depth transect	Along shoreline
Distance to city	F< 0.1, df= 1, 4, P= 0.828	F= 0.3, df= 1, 15, P= 0.575
Distance to river	F= 0.7, df=1, 4, P= 0.453	F= 0.5, df=1, 17, P= 0.473
Distance to shore	F< 0.1, df= 1, 4, P= 0.832	F= 3.5, df=1, 15, P= 0.081
Grain size	F= 1.0, df= 1, 3, P= 0.737	F= 1.1, df= 1, 10, P= 0.314
Depth	F= 0.1, df= 1, 4, P= 0.771	NA

* Indicates the significant P-values

Table 3. Mean microplastic densities (particles per kilogram of sediment) with standard deviations (SD), as well as minimum (Min) and maximum densities (Max) are shown for different types of plastic and total plastics.

	Fiber/Fragment	Microplastic shape	Mean ± SD	Min	Max	%
Black Sea	Fiber	Fiber	1 515 ± 1 444	80	7 335	94.8
N= 58	Fragment	Angular	5 ± 25	0	180	0.3
		Elongated	47 ± 68	0	389	3.0
		Irregular	22 ± 46	0	280	1.4
		Film	9 ± 29	0	179	0.6
		Sphere	0 ± 0	0	0	0
		Total	**1 599 ± 1 469**	**140**	**7 360**	
Caspian Sea	Fiber	Fiber	834 ± 536	70	2 080	96.7
N= 26	Fragment	Angular	4 ± 7	0	20	0.5
		Elongated	5 ± 8	0	31	0.6
		Irregular	19 ± 25	0	110	2.1
		Film	0 ± 0	0	0	0
		Sphere	0 ± 0	0	0	0
		Total	**862 ± 545**	**80**	**2 095**	

Caspian Sea basin

Depth transect

No significant relationship was found between the fiber and fragment concentration and the water depth, grain size, distance to river, city and shoreline along this transect in the Caspian Sea (P ≥ 0.453, Table 2).

Shoreline transect

The concentration of microplastic fibers along the Caspian Sea shore significantly decreased with increasing sediment grain size (P = 0.011, Table 2). Apart from that, no significant relationships were found between the fiber and fragment concentration and the distance to river, city and shoreline, and the fragment concentration and the sediment grain size (P ≥ 0.081, Table 2).

Discussion

Microplastic types

Fibers were the most abundant shape of microplastic (Figure 4; Table 1). These fibers were found in every sediment sample. In accordance with our results, most other microplastic studies found fibers to be the most abundant shape of microplastic (Lassen et al. 2015, Aytan et al. 2016, Leslie et al. 2017, Filgueiras et al. 2019, Mehdinia et al. 2020). One of the main sources of microplastic fibers in the environment are clothing fibers released during washing (Browne et al. 2011, Lassen et al. 2015, Aytan et al. 2016, Napper and Thompson 2016, Mehdinia et al. 2020). Next to wastewater input from washing machines, fishery activities are most likely a source of fibers in the aquatic environment, as fishing nets and wires break down into fibers (Aytan et al. 2016, Mehdinia et al. 2020).

Spheres or round beads were not found during this study (Table 1). In accordance with our findings, Aytan et al. (2016) did not find microplastic spheres in the Black Sea basins either. This type of plastic beads is most likely derived from cosmetic products like toothpaste, scrubs, hand cleaning and shaving foam (Lassen et al. 2015). The fact that these microplastic beads were not in the Black Sea and Caspian Sea samples might be related to consumer behavior. Eastern European countries (e.g. Bulgaria, Romania) are known to consume less cosmetic products associated with microplastic beads in comparison to Western European countries such as Belgium or The Netherlands (Gouin et al. 2015).

The transparent and white microplastic particles were most abundant in the present study (Figure 5; Table 1). This corresponds to the findings of several other publications (Ballent et al. 2016, Graca et al. 2017, Peng et al. 2017, Wang et al. 2017, Filgueiras et al. 2019, Mataji et al. 2020, Wang et al. 2020a). The high abundance of this color could be explained by the wide use of transparent plastic materials at sea e.g. in fishing nets and lines (Wang et al. 2017). Green was found to be the least abundant color, however, green and yellow may look similar in these small particles, depending on lighting and therefore may be underestimated.

Waterbody comparison

Our study is the first one to assess microplastic in a remote area of the northern part of the Caspian Sea. On average, we found 862 microplastic particles per kilogram of dried sediment (Table 3). Mataji et al. (2020) and Mehdinia et al. (2020) were the first studies to assess microplastic in the sediment of the heavily populated south Caspian coastal zone. Interestingly, the average concentrations in the south Caspian regions were four (Mataji et al. 2020) and eight times (Mehdinia et al. 2020) lower in comparison to our findings in the remote northern area (Table 4). Generally, the south Caspian basin is assumed to be more polluted due to the densely populated shoreline (Mataji et al. 2020, Mehdinia et al. 2020). However, as our microplastic concentrations were four to eight times higher, pollution in the northern part of the Caspian Sea is expected to be higher than in the south. The north Caspian is highly impacted by the Volga river which is the longest European river (De Mora et al. 2004). The Volga has a large drainage basin and transports various pollutants from their sources to the Caspian Sea. Most likely the number of inhabitants in the whole drainage basin has a stronger impact on the microplastic concentration than the inhabitants on the shoreline itself. On average 1599 microplastic particles per kilogram of dried sediment were found in the Black Sea basin (Table 3). Săvucă et al. (2017) found about 30 times less microplastic fibers in sediment in the Romanian part of the Black Sea than the average number in the same area during the present study (Table 4).

The relatively high microplastic concentrations found during the present study might also be due to the higher efficiency of our extraction method. The studies mentioned in Table 4 use a density separation technique without elutriation. We combined an adapted version of the elutriation column developed by Claessens et al. (2013) with density separation by using sodium chloride (NaCl) in the column instead of water. Sodium chloride has a higher density than water, causing the lighter particles to float within this mixture. Moreover, we added an air pump to the construction (Figure 2) creating an additional upward airflow, increasing the chance of microplastic particles to be freed from the sediment particles. Our adaptations increased the efficiency of the elutriation column, as we obtained a 99.9% and 96.6% retrieval rate for LDPE and PVC, respectively.

In case the differences between our study and other publications are not methodology or consumer-related, it might also be caused by the unusually high river discharge into relatively small (semi-)enclosed basins, causing the Black Sea and Caspian Sea to have an increased risk of plastic pollution (Aytan et al. 2016). As the Black Sea and Caspian Sea are (semi-)enclosed waterbodies, it might not be completely valid to compare them to other open-water environments. However, the highest observed average concentrations found in (semi-)enclosed environments like estuaries (1445 kg^{-1}), marine bays (500 kg^{-1}) and harbors (1165 kg^{-1}) are comparable to the average concentrations found during this study (Table 4). Assessments in harbors in Western Europe in the Rhine-Meuse-Scheldt Delta (1885 kg^{-1}) and the Haringvliet (1643 kg^{-1}) resulted in microplastic concentrations comparable to the Black Sea concentrations, while the concentrations in Dutch Wadden Sea harbors (3483 kg^{-1}) were higher (Table 4, D'Hont et al. Unpublished data). Most likely, the microplastic concentrations are influenced by the proximity to possible sources and the sink capacity of the environment. These should thus be taken into account when comparing waterbodies.

The present study uncovered two times higher microplastic concentrations in the Black Sea in comparison to the Caspian Sea (Figure 6). This might be explained by the proximity of microplastic sources. River input is considered to be one of the main sources of microplastic in the marine environment (Lechner et al. 2014, Ballent et al. 2016, van Calcar and van Emmerik 2019). The Black Sea samples were collected at on average 37 km from the river mouth, while the Caspian Sea samples were collected at on average 185 km from the closest river mouth. The distance from the river mouth might also be an explanation for the difference in concentration found between the Black Sea and Caspian Sea samples (Secrieru and Secrieru 2002, Baltas et al. 2017).

Table 4. An overview of the average microplastic concentrations (items per kilogram dried sediment) found in studies conducted over the last decade throughout the globe in estuaries, lakes, marine bays, marine beaches, harbors, offshore and deltas.

Waterbody	**Study area**	**Environment**	**Average items kg^{-1} (dw)**	**Reference**
Changjiang Estuary	China	Estuary	121	Peng et al. 2017
Hangzhou Bay	China	Estuary	84.3	Wang et al. 2020b
Jagir Estuary	Indonesia	Estuary	345.2	Muhammad et al. 2019
Lagoon of Venice	Italy	Estuary	1445.2	Vianello et al. 2013
Lake Ontario	Canada	Lake	760	Ballent et al. 2016
Yellow Sea	China	Marine Bay	500	Zhang et al. 2019a
Yellow Sea	China	Marine Bay	427.8	Sun et al. 2021
Yellow Sea	China	Marine Bay	15	Zheng et al. 2019
Deep bay	Hong Kong	Marine Bay	263	Tsang et al. 2017
Banten Bay	Indonesia	Marine Bay	267	Falahudin et al. 2020
Bay of Brest	France	Marine Bay	0.97	Frère et al. 2017
Mediterranean	Spain	Marine Bay	270	Alomar et al. 2016
North Sea	Germany	Marine Beach	2	Dekiff et al. 2014
Atlantic Ocean	Canada	Marine Beach	5000	Mathalon and Hill 2014
North Sea	Belgium	Marine Harbor	166.7	Claessens et al. 2011
Indian Ocean	South Africa	Marine Harbor	1165	Naidoo et al. 2015
Black Sea	Romania	Marine Littoral	49	Săvucă et al. 2017
Yellow Sea	China	Marine Offshore	134	Zhang et al. 2019b
Yellow Sea	China	Marine Offshore	371.9	Sun et al. 2021
Bohai Sea	China	Marine Offshore	171.8	Zhao et al. 2018
Yellow Sea	China	Marine Offshore	72	Zhao et al. 2018
Yellow Sea	China	Marine Offshore	37.1	Zhu et al. 2018
North Sea	Belgium	Marine Offshore	97.2	Claessens et al. 2011
Mediterranean	Tunisia	Marine Offshore	316	Abidli et al. 2018
Mediterranean	Spain	Marine Offshore	113.2	Filgueiras et al. 2019
Mediterranean	North Africa	Marine Offshore	649.3	Tata et al. 2020
Atlantic Ocean	Portugal	Marine Offshore	55	Frias et al. 2016
Caspian Sea	Iran	Marine Offshore	210	Mataji et al. 2020
Caspian Sea	Iran	Marine Offshore	107.6	Mehdinia et al. 2020
Black Sea	Romania, Bulgaria	Marine Offshore	1599	This study
Caspian Sea	Kazakhstan	Marine Offshore	862	This study
Rhine-Meuse-Scheldt Delta	Netherlands	Delta Harbors	1885	D'Hont et al. Unpublished data
Haringvliet	Netherlands	Delta Estuary	1643	D'Hont et al. Unpublished data
Wadden Sea	Netherlands	Marine Harbor	3483	D'Hont et al. Unpublished data

Adapted from Sun et al. (2021) and Ballent et al. (2016)

Water depth

The concentration of fragments decreased with water depth, while the concentration of fibers was independent of depth. A similar pattern was observed by Pohl et al. (2020). On the other hand, both Wang et al. (2019) and Frias et al. (2016) found an increasing microplastic concentration with an increasing depth in the Yellow Sea and the Atlantic Ocean, respectively. However, the latter studies did not assess microplastic fibers and fragments separately. As fibers are by far the most abundant type found in these studies, they probably recorded a positive trend for microplastic fibers and depth, canceling out a possible negative trend for fragments (Figure 7).

The decreasing concentration in fragments with depth and the independence of fiber concentration with depth might be explained by settling processes. The shape of a microplastic particle is known to have an influence on their settlement velocity and behavior (Khatmullina and Isachenko 2017, Zhang 2017, Filgueiras et al. 2019). Cylindrical particles will start rotating, oscillating, and tumbling during the fall, which decreases the sinking velocity (Kowalski et al. 2016, Khatmullina and Isachenko 2017). Long cylindrical particles, like fibers, tend to settle with their long axis perpendicular to the direction of fall, slowing down their settling velocity due to higher friction drag (Khatmullina and Isachenko 2017). Several studies have indicated that microplastic fibers have a significantly slower settling velocity in comparison to microplastic fragments (Kowalski et al. 2016, Khatmullina and Isachenko 2017, Waldschläger and Schüttrumpf 2019). Therefore, the turbulence of water affects the settlement of (microplastic) particles (Pohl et al. 2020). A decreasing level of turbulence along the depth gradient, causes deeper areas to become a sink for fine and slowly settling microplastic particles like fibers (Enders et al. 2019). On the other hand, fragments most likely settle closer to their sources in shallow environments with higher hydrodynamic energy (Enders et al. 2019, Pohl et al. 2020). The interaction between their shape and the ocean dynamics (waves, wind, tides, currents, etc.) explains why microplastic fragment concentrations decrease with depth and why the fiber concentration would either increase with depth or would be divided evenly along the depth gradient (Frias et al. 2016, Wang et al. 2019, Pohl et al. 2020). Next to the particle shape and size, the particle density and fluid density have an impact on the sinking velocity of a microplastic particle as well (Kowalski et al. 2016, Zhang 2017). However, during the present study, the particle size and density were not taken into account.

The angular, film and elongated fragment shapes did not significantly relate to the depth of the sampling site. The category “irregular” was not included in the analyses as it was hard to classify this type of shape. Elongated shapes were the only type of fragments found in a substantial number of samples (N = 36 samples). Angular and film fragments were only found in six and four samples, respectively. This low retrieval rate makes it hard to analyze the pattern for fragment shapes and might be the reason why we cannot find a significant pattern in relation to the water depth. Additionally, there was no significant relationship between the color of the fibers or fragments and the depth of the sampling site.

How to explain deviations from the depth pattern

Generally, the concentration of microplastic fragments significantly decreased with water depth, while the microplastic fiber concentration did not change along the depth gradient (Figure 7). However, some samples exhibited slight deviations from this pattern (Figure 1, Supplementary 2). Deviations of the fragment pattern can be seen in transect B, C, F and G (Figure 1, Supplementary 2). There are a few possible explanations for this phenomenon: alterations of particle sinking behavior, microplastic particle size and strong currents created by rivers or wind (Ballent et al. 2013, Kowalski et al. 2016, Khatmullina and Isachenko 2017, Pohl et al. 2020). The sinking behavior of a microplastic particle may be altered considerably by weathering and biofouling and therefore by alteration of the shape of the particle (Kowalski et al. 2016). As we described before, the shape of the particle has an influence on the sinking pattern and velocity of these particles (Kowalski et al. 2016, Khatmullina and Isachenko 2017).

Deviations from the depth pattern might be explained by the microplastic particle size as well. The difference in settling velocity between both fibers and fragments is impacted by the size of the microplastic particle. The difference in settling velocity between both fibers and fragments becomes larger with increasing particle size, as a larger particle can be affected by a stronger drag force (Kowalski et al. 2016, Khatmullina and Isachenko 2017). Microplastic fibers and fragments that do not follow the observed trend (Figure 7) might have been relatively small in size. During the present study the particle size and density were not taken into account, therefore, we are not able to falsify this hypothesis.

Other possible explanations for deviations from the depth related pattern can be related to currents (Kowalski et al. 2016). Higher current velocities cause increased sediment and microplastic transport (Ballent et al. 2013). Large currents, as can be found in proximity to river mouths, flush microplastic particles further out at sea and counteract the precipitation of plastic particles. This causes low microplastic concentrations in the sediment for samples in proximity to river mouths (e.g. transect B, samples number 14-19) for both microplastic types (Figure 1, Supplementary 2).

Distance to river

The relationship between the microplastic concentration in sediment and the distances to the river mouth has been assessed taking all Black Sea open water samples (number 11-52) into account and for each depth category (0-30 m, 31-60 m, 61-100 m) separately. No significant effect of the distance to the river was found when assessing all samples, perpendicular to the bathymetric lines. However, the currents in this area of the Black Sea basin run southwards more or less parallel to the shore instead of perpendicular to it (Aytan et al.

2016). When assessing the microplastic concentrations parallel to the shore, we found that the concentration of microplastic fragments increased when sampling further away from the river mouth in the 0-30 m and 31-60 m depth categories. This result opposes the findings of Luo et al. (2019) and Falahudin et al. (2020) as they found a decrease in microplastic with an increasing distance from the river mouth. Close to the river high currents might cause resuspension of plastic particles and inhibit them from settling down (Filgueiras et al. 2019). This might explain the inverted pattern for microplastic fragment concentrations in sediment. However, the fact that the microplastic concentration does relate to the distance from the river in these depth categories suggests that the river is a source of microplastic in our sediment samples. Rivers are believed to be the main source and transport pathway of plastic in the aquatic environment (Lechner et al. 2014, Ballent et al. 2016, van Calcar and van Emmerik 2019).

Distance to shore

The microplastic concentration did not relate to the distance to the shore when assessing the samples perpendicular to the shore throughout all depth categories. We did find some relationships when assessing shoreline effects in the separate depth categories. The concentration of microplastic fragments increased in the 31-60 m depth category when sampling further away from the shore, while the fiber concentration decreased further away from the shore in the 61-100 m depth category. The relationship between the distance to the shore and the microplastic concentration is thus not very distinct. This corresponds to findings in literature as some studies found no effect of distance to the coast (Claessens et al. 2011, Filgueiras et al. 2019, Wang et al. 2020a). Other studies did find a decrease in microplastic concentration further away from the shore as we did for fibers in the deepest sample category (Vianello et al. 2013, Graca et al. 2017, Zhang et al. 2019a, Zhang et al. 2019b, Wang et al. 2020a). As fibers are the most abundant microplastic type, they might neutralize the concentration gradient for fragments. The fact that we found no effect close to the shore might be related to high wave activity and currents close to the shore inhibiting microplastic settlement (Pohl et al. 2020). The effect of the shore onto the microplastic concentration is also related to the sources present along the shoreline (Ballent et al. 2016, Waldschläger et al. 2020). The studies that did not find an effect of the shore onto the microplastic concentration might be conducted at sites with an uneven source distribution along the shoreline, cancelling out the possible shore effects.

Distance to city

No effect from distance to the city was found when considering all samples at once. When splitting these samples up into three depth categories, we found the concentration of fibers to decrease when sampling further away from the nearest upstream city in the 61-100 m depth category. A similar result was found by Ballent et al. (2016), Wang et al. (2017) and Waldschläger et al. (2020). However, other studies found no such effect (Claessens et al. 2011, Filgueiras et al. 2019). The outcome of this assessment might depend on the type of analysis and whether or not other parameters, like depth, were taken into account during the analyses. Moreover, hydrodynamic activity in the study area can cancel out relationships between microplastic concentrations and the distance to a city. The fact that we could not find such relationships closer to shore in the other depth categories might be related to high wave activity inhibiting microplastic settlement (Pohl et al. 2020). Nevertheless, our results suggest that distance to cities can to some extent explain microplastic concentration in sediments. Most likely the number of inhabitants in the whole drainage basin and their behavior as plastic consumers have a stronger impact on the microplastic concentration than the distance to cities themselves.

Grain size

The microplastic concentration was not related to the sediment grain size. This contradicts the results of Zhang et al. (2019b), as sediment grain size is known to affect the content and distribution of substances such as organic carbon, total nitrogen, biogenic opal, carbohydrates and lignin in sediments (Alomar et al. 2016). Moreover, small particles of sediment and plastic are both likely to be transported by the flow of water and be deposited in areas where water movement is low (Browne et al. 2010). Nevertheless, our results concurred with the results of several studies finding no effect of sediment grain size on the microplastic concentration (Browne et al. 2010, Browne et al. 2011, Mathalon and Hill 2014, Alomar et al. 2016, Peng et al. 2017, Filgueiras et al. 2019). Enders et al. (2019) found a significant correlation between the microplastic size fractions and sediment grain size. As we did not assess the microplastic particle size, we were not able to assess such a link with the sediment grain size. Another explanation for the lack of grain size effect might be related to the fact that most studies (including ours) assessed the number of microplastic particles in a kilogram dried sediment. Clayey soil contains much more water than sandy soil, so we assessed a larger volume of small-grained sediments in comparison to larger grained sediments. We could thus assume there were fewer microplastic particles present in clay soil of the same surface area as sandy soil with a larger grain size.

Microplastic sources and sinks in the Caspian Sea

The microplastic concentrations in sediment samples from the Caspian Sea did not show a relationship with water depth. There were no microplastic sources in the proximity of these sampling sites. The nearest river is about 150 km away from the sampling sites. This might explain why no relationship was found between microplastic particles and the depth at which they were collected, as we believe that the microplastic concentration is determined by an interaction between present sources (rivers, cities) and sinks (sediment).

Unlike the Black Sea pattern, the sediment grain size in the Caspian Sea was negatively related to the concentration of microplastic fibers. This can be explained by the difference in hydrodynamic (turbulence) regimes, as fine-grained sediments are found in low-energy environments while coarser sediments are found in high-energy environments (Enders et al. 2019). Turbulent water will most likely cause resuspension of plastic particles and prevent them from settling down.

Conclusions

Fibers and transparent or white microplastic particles were the most abundant shape and color in the Caspian and Black Sea basins. For both basins, we found a higher microplastic concentration in comparison to earlier studies. Moreover, we found two times higher microplastic concentrations in the Black Sea in comparison to the Caspian Sea. These differences in microplastic concentrations might be explained by the higher efficiency of our extraction method, consumer behavior or by the proximity of sources, like rivers.

The concentration of microplastic fragments decreased with water depth, while the concentration of fibers was independent of depth. This is most likely related to their shape as this has an influence on their settlement velocity and behavior. Deviations from this depth pattern might be related to alterations of particle sinking behavior, microplastic particle size and strong currents created by rivers or wind. Nevertheless, the water depth influences the microplastic sink capacity of sediment.

Within some depth classes, microplastic concentrations of microplastic fragments increased when sampling further away from the river mouth, which suggests that rivers are a microplastic source in our sediment samples. Moreover, within some depth classes, concentrations were related to the distance from shores and cities. Microplastic concentration in sediment was not related to sediment grain size. The concentration of microplastic in sediment of both basins results from interactions between their sinks (water depth) and sources (rivers, shore, cities).

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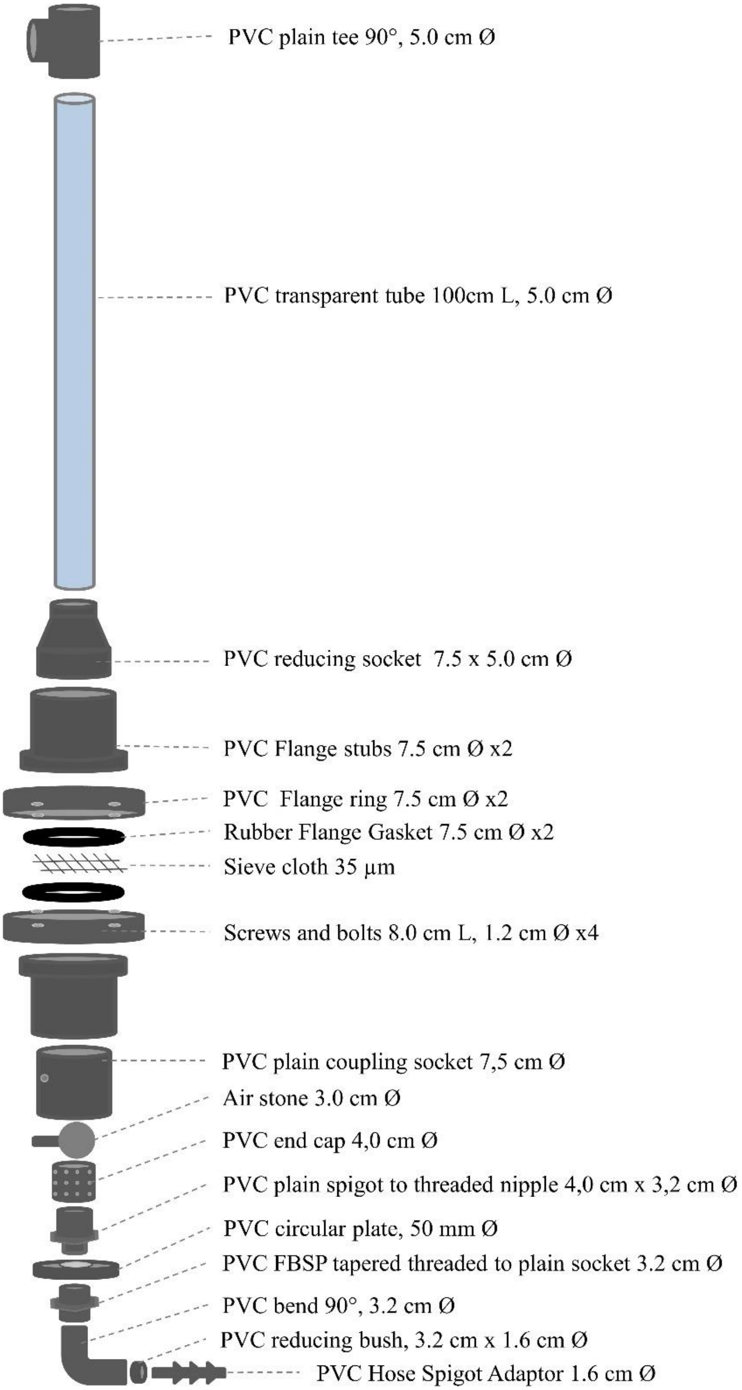
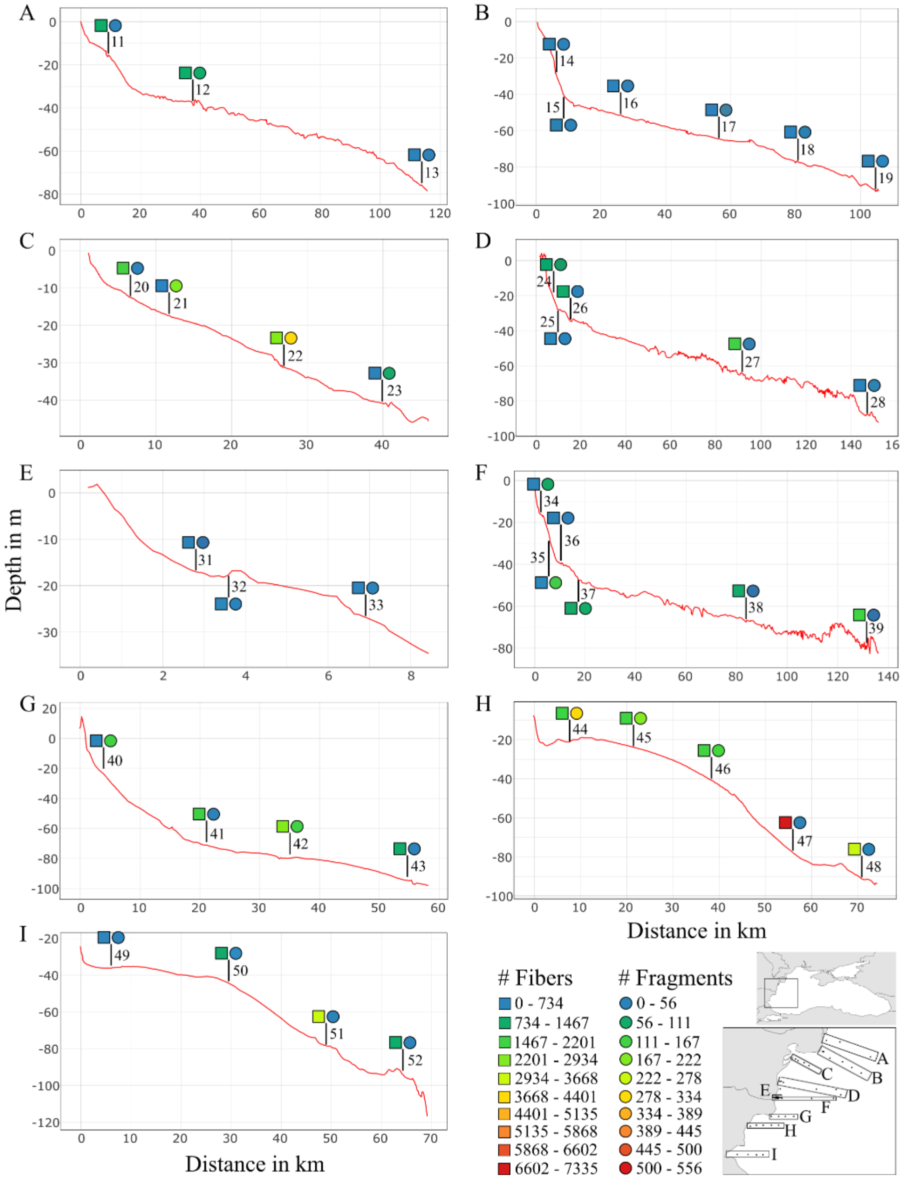


Supplementary 1. An overview of all construction parts of the elutriation column.



Supplementary 2. The depth profiles of the Black Seafloor where the open water samples were collected (A-I). The sample numbers are indicated in each of the figures (Figure 1). The concentration of fibers (squares) and fragments (circles) are indicated at their sample locations on the depth profile. The samples were color coded according to their concentration.

Chapter 6

Synthesis

Use it or lose it - Factors affecting the fitness and dominance shifts of dreissenid mussels

Introduction

The Ponto-Caspian mussels *Dreissena polymorpha* and *D. bugensis* spread out of their native area to invade hard freshwater surfaces in large parts of the northern hemisphere. In both their native and introduced areas dominance shifts are observed going from the earliest invader *D. polymorpha* to *D. bugensis* as the dominant species in these areas. However, the factors driving these dominance shifts remain uncertain. This thesis aims at identifying species traits or external factors affecting these species traits leading to higher fitness and competitive benefits for either one of the dreissenid species. This way these species traits or external factors could contribute to the dreissenid dominance shifts. We aimed to achieve this by comparing life-history traits, attachment-detachment traits and mobility traits for both species. Additionally, we assessed whether external factors like temperature, salinity, light and pollution differentially affected the above-mentioned species traits. This way we aimed at identifying the potential drivers of the dreissenid dominance shifts.

Dreissenids as nuisance species within and outside of the Ponto-Caspian area

Both Ponto-Caspian molluscs *D. polymorpha* and *D. bugensis* cause extensive ecological (Grigorovich and Shevtsova 1995) and economic (Schonenberg and Gittenberger 2008) damage in their native regions (Popa and Murariu 2009) and introduced areas (Pimentel et al. 2005, Binimelis et al. 2008, Fahnenstiel et al. 2010). One of the causes of these ecological changes is a dramatic increase in water filtration (Karatayev et al. 1997, Collas et al. 2020). High amounts of water filtration cause a reduction in food and a shift in water chemistry (Ojaveer et al. 2002, Binimelis et al. 2008, Fahnenstiel et al. 2010, Collas et al. 2020). Moreover, high water filtration causes a shift in species composition, in the relative abundances and biomass of functional groups throughout all trophic levels within the aquatic ecosystem (Karatayev et al. 1997, Ojaveer et al. 2002). Dreissenids also cause problems as fouling organisms. Rapid declines of native (unionid) mussel communities have been reported due to fouling of the dreissenids onto native mussels but also due to competition for food (Ojaveer et al. 2002). The dreissenid shells create shelter and habitats for numerous invertebrates. The shell deposits of dreissenids transform soft bottoms into shell gravel, creating a local hard substrate for sessile species to settle onto (Ojaveer et al. 2002). Additionally, dreissenids can cause sludge formation due to the production of large quantities of (pseudo)faeces. Moreover, dreissenids can withstand, and thus accumulate high pollutant levels (Hendriks et al. 1998, Carrasco et al. 2008). As a primary food source for some waterfowl (e.g. common coot, ducks, gulls) and fish (e.g. whitefish, eel, carp, bream, sturgeon, flounder), dreissenids transfer heavy metals and organic contaminants through the food chain causing bioaccumulation of those contaminants in their predators and eventually an increased exposure for humans (Hendriks et al. 1998). Economic damages caused by dreissenids include an increase in maintenance costs of overgrown man-made structures like boats, hydraulic infrastructures and pipes in cooling water circuits of industry and power plants (Binimelis et al. 2008, Schonenberg and Gittenberger 2008). Moreover, their sharp shells can cause harm to humans (Schonenberg and Gittenberger 2008). However, the presence of dreissenids can be advantageous in some situations as well, e.g. counteracting eutrophication, filtration of effluents from wastewater treatment plants, food source for birds in wetlands and biodiversity increases on hard substrates (Collas et al. 2020).

Differential fitness of both dreissenid species possibly driving the dominance shift

Species traits influencing the dreissenids' fitness

During the last decade, a dominance shift has been observed from the early invader *D. polymorpha* to its successor *D. bugensis* as the dominant species on hard substrates in freshwater ecosystems (Stoeckmann 2003, Therriault et al. 2005, Matthews et al. 2014). This phenomenon was observed in both their native and introduced regions. Chapters 2-4 aimed at identifying species traits influencing the fitness of these species and possibly acting as drivers of the dominance shift between both species. The assessed species traits have been summarised in table 1.

Dreissena polymorpha can settle earlier in the season, is more salinity tolerant, and is predated less upon than *D. bugensis* (Chapter 2). Additionally, a higher number of *D. polymorpha* individuals attach to hard substrates than *D. bugensis* individuals do, which can help when trying to withstand currents and predators (Chapter 3). About 15 years after the introduction of *D. bugensis*, both species still co-occur and *D. bugensis* was not able to fully outcompete *D. polymorpha* (Zhulidov et al. 2005, Grutters et al. 2012). The above mentioned *D. polymorpha* species traits likely provide this species with competitive benefits over *D. bugensis*. This could thus counteract a complete species shift and cause a co-occurrence of both species.

On the other hand, *D. bugensis* can settle in a wider range of habitats and adjust its growth and settlement better to seasonally and annually varying salinities and temperatures (Chapter 2). Moreover, this species has lower winter mortality and settles in higher numbers when *D. polymorpha* individuals are already present on the substrate (Chapter 2). A higher attachment strength is found for *D. bugensis* compared to *D. polymorpha* in natural dreissenid assemblages (Chapter 3). *Dreissena bugensis* had some advantages related to mobility as well. After a detachment, *D. bugensis* has a higher top speed than *D. polymorpha* (Chapter 4). Moreover, *D. bugensis* has a significantly higher number of individuals moving at any point in time than *D. polymorpha* did (Chapter 4). Finally, *D. bugensis* is known to have a higher assimilation efficiency, a higher activity of certain enzymes (e.g., thiaminase), higher filtration rates, lower respiration rates and can persist at greater depths and in lower nutrition environments than *D. polymorpha* (Ram et al. 2012).

Table 1. Traits affecting the fitness of dreissenid species as found during our study. "+" traits posing an advantage for this species in comparison to the other dreissenid, "-" traits posing no advantage for this species in comparison to the other dreissenid, "0" No observed differences between both species.

	 ***D. polymorpha***	 ***D. bugensis***	**Reference**
Settlement	**+** Earlier settlement	**+** Settle in higher numbers Wider range of habitats Adjust better to varying salinities, temperatures	Chapter 2
Growth	**-** Grows slower	**+** Grows faster Adjust better to varying salinities, temperatures	Chapter 2
Maximum shell size	**-** Remains smaller	**+** Larger max. shell size	Chapter 2
Harm due to predation	**+** Lesser predation pressure	**-** Higher predation pressure	Chapter 2
Winter mortality	**-** Higher winter mortality	**+** Lower winter mortality	Chapter 2
Attachment strength	**-** Significantly lower for smaller individuals in natural dreissenid field assemblage	**+** Significantly higher for larger individuals in natural dreissenid field assemblage	Chapter 3
Attachment rate	**+** Significantly higher	**-** Significantly lower	Chapter 3
Byssus width	**-** Two times thinner	**+** Two times thicker	Chapter 3
Byssus count	**-** Half the amount of byssus threads	**+** Double the amount of byssus threads	Chapter 3
Movement speed	**-** On average equal	**+** Higher top speed	Chapter 4
Distance moved	**0** On average equal	**0**	Chapter 4
Movement duration	**0** On average equal	**0**	Chapter 4
Movement frequency	**-** Significantly less individuals moving	**+** Significantly more individuals moving	Chapter 4
Harm due to microplastic pollution	**-** Lower tolerance	**+** Higher accumulation rate	Magni et al. 2018 Magni et al. 2019 Baldwin et al. 2020 Pedersen et al. 2020 Pastorino et al. 2021
Harm due to heavy metal pollution	**+** Defence mechanism	**-** Cellular damage	Potet et al. 2018 Louis et al. 2019

Contribution of species traits to the observed dominance shift

Dreissena bugensis seems to thrive in a wider range of habitats than *D. polymorpha*, as it occurs in a wider salinity, temperature and light range (i.e. settlement and growth) (Chapter 2). Additionally, the presence of *D. polymorpha* on a hard substrate enhances the settlement rate of *D. bugensis* individuals. *Dreissena polymorpha* individuals thus seemed to facilitate *D. bugensis* settlement. Having more suitable habitats and being able to settle in a higher number, especially when *D. polymorpha* is already present on the substrate, provides *D. bugensis* with a strong competitive benefit over *D. polymorpha*.

The attachment strength of dreissenids turned out to be related to their shell size (Chapter 3). *Dreissena bugensis* grows faster and larger than *D. polymorpha* does (Figure 1, Chapter 2). Therefore, in a natural assemblage with larger *D. bugensis*, the *D. bugensis* individuals will have a higher attachment strength than the smaller *D. polymorpha* individuals (Figure 1, Chapter 3). This corresponds with the two times thicker and twice as many byssal threads for *D. bugensis* (Chapter 3). However, significantly more *D. polymorpha* individuals attached to the substrate than *D. bugensis*. Additionally, *D. bugensis* specimens had a higher mobility rate than *D. polymorpha* specimens (Figure 1, Chapter 4). Detachment and mobility of sessile mussel species can be useful to avoid unfavourable environmental conditions. We conclude that *D. bugensis* individuals tend to look longer for an optimal environment, and once there, attach stronger to the substrate than the generally smaller *D. polymorpha*. A higher attachment strength may give *D. bugensis* an advantage over *D. polymorpha* when it comes to withstanding predators and disturbances such as currents and navigation-induced changes in flow velocity (Chapter 3). These species traits pose competitive benefits for *D. bugensis* over *D. polymorpha* and could potentially contribute to the dominance shift between both species.

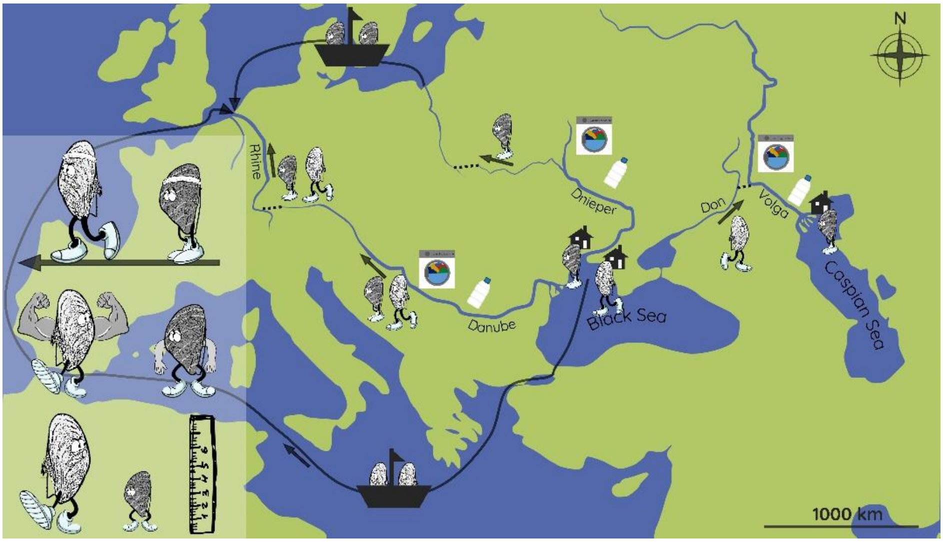


Figure 1. *Dreissena polymorpha* and *D. bugensis* originate from the north Black Sea and Caspian river delta's. These dreissenids increased their geographical range along rivers, attached to boat hulls and in ballast water. In both their native range and introduced areas ongoing dominance shifts are observed. These dominance shifts are most likely driven by parameters like higher mobility and speed, stronger attachment and faster and larger growth for *D. bugensis*. Dreissenids often dominate polluted (e.g. microplastic originating from washing machines and plastic waste) and eutrophic environments, as rivers are one of the main transport pathways for pollutants. Microplastic pollution seems to have less impact on *D. bugensis*, yet accumulates more in this species, in comparison to *D. polymorpha*.

Microplastic pollution in the dreissenids' native and invaded regions

Microplastic pollution in aquatic sediments

Our study assessed the microplastic pollution in the dreissenids' native region, the Ponto-Caspian area. We focussed on the microplastic particles in sediment, as sediment is a sink for microplastic (Law et al. 2010, Cózar et al. 2014, Van Cauwenberghe et al. 2015). On average about 1600 microplastic items per kilogram of dried sediment were found in the open Black Sea basin. The microplastic concentration in this basin was related to both their sources (rivers, shorelines, cities) and sinks (water depth) (Chapter 5). The concentrations found during our study were considerably higher than concentrations observed in comparable studies. During our study we found on average about 1600 items per kilogram dried sediment in the Black Sea where Săvucă et al. (2017) found about 30 times less microplastic particles. Similarly, we found on average about 900 microplastic particles per kilogram dried sediment in the northern Caspian Sea while comparable studies in the southern Caspian Sea found four to eight times less microplastic particles (Mataji et al. 2020, Mehdinia et al. 2020). At the dreissenid study sites in The Netherlands in Numansdorp and Tiengemeten (Chapters 2-4) we found an average microplastic concentration in sediment of about 1600 microplastic items per kilogram dried sediment (unpublished data). In the Black Sea basin, fibers and transparent or white microplastic particles were the most abundant microplastic shape and colour (Chapter 5). Fibers discharged by washing machines are an important primary source for microplastic ending up in rivers (Browne et al. 2011, Napper and Thompson 2016). Rivers are considered to be the main source and transport pathway of plastic in the aquatic environment (Ballent et al. 2016, Van Calcar and Van Emmerik 2019). However, high water turbulence causes a decrease in microplastic concentration in sediment, as they tend to settle in low energy environments (Enders et al. 2019).

Contaminants in sediment versus organisms

The concentrations of such pollutants (e.g. microplastic, heavy metals and persistent organic chemicals) in aquatic sediments are comparable to the concentrations found in filter-feeding organisms, as they accumulate pollutants from the water column. This way they become pollutant sinks, just like aquatic sediments. Therefore, pollutant concentrations measured within sediment pose as good indicators for concentrations in organisms (Baldwin et al. 2020, Pedersen et al. 2020).

Dreissenids fulfil the requirements of biomonitoring organisms: they have a long lifespan (up to 5 years), are sedentary, easy to collect and handle, have a wide distribution and the ability to withstand high pollution levels, without suffering mortality (Kraak et al. 1991). Moreover, dreissenids filter large volumes of water, up to 280 litres per gram dry weight per day (Pedersen et al. 2020).

Effects of pollutants on dreissenids' fitness

Microplastic accumulation

Both dreissenid species filter selectively when exposed to contamination or harmful particles (Pedersen et al. 2020). They can selectively feed by ingesting preferred particles (e.g. nutritious phytoplankton) and expelling rejected particles (e.g. toxic cyanobacteria) through their siphons or as pseudofeces (Vanderploeg et al. 2001). However, microplastic particles have been found to accumulate in dreissenids (Pastorino et al. 2021). This means they do not recognise microplastic particles as harmful or useless and ingest them, or their selection mechanism is not (yet or enough) adjusted to distinguish microplastic from sediment particles (Pedersen et al. 2020).

Dreissenids were found to retain particles larger than 150 μm and were even able to filter particles up to 2300 μm (Pastorino et al. 2021). During our microplastic study, we assessed particles with a size ranging from 35 μm to 5000 μm. Most observed particles had a size range between 50 μm and 1000 μm. These particles are thus within the absorption range for dreissenids. Field studies assessing microplastic concentrations and composition in dreissenids are still very scarce. During these kinds of field studies, microplastic particles have been found in dreissenids (Baldwin et al. 2020, Pastorino et al. 2021). In the field, microplastic concentrations in *D. bugensis* ranged from 2.70 to 13.00 items per specimen from Lake Mead National Recreation Area, United States of America, while levels in *D. polymorpha* from Lake Iseo, Italy, were between 0.03–0.27 items per specimen (Baldwin et al. 2020, Pastorino et al. 2021). The microplastic concentrations in organisms were related to the concentrations in water (Pastorino et al. 2021). *Dreissena bugensis* individuals are known to have a higher filtration rate than *D. polymorpha* (Ram et al. 2012). We could thus hypothesise that *D. bugensis* can filter and accumulate more microplastic particles from the water column than *D. polymorpha*.

Effects of ingested microplastic on filter-feeding marine bivalves

The effects of microplastic ingestion have been widely studied during the last few years (e.g. Magni et al. 2018, Magni et al. 2019, Li et al. 2019a, Gautam et al. 2020, Pedersen et al. 2020, Weber et al. 2020, Seuront et al. 2021). Scientific evidence indicated that exposure to microplastic can cause feeding disruption, reduced filtering activity, inflammatory responses, histological changes, lysosomal membrane destabilisation, neurotoxic effects, alterations of antioxidant systems, increase in haemocyte mortality, dysplasia, physical adsorption, energy metabolism disruption, genotoxicity and transcriptional responses on filter-feeding organisms such as *Mytilus edulis*, *M. galloprovincialis*, *Mytilus* spp. and *Perna perna* (Li et al. 2019a, Gautam et al. 2020).

Additionally, some studies reported the effects of microplastic on the attachment and mobility of mussel species. In the presence of high microplastic concentrations in seawater, some species produced more byssal threads (*Perna perna* and *Mytilus galloprovincialis*), while other species reduced motility and aggregation behaviour (*M. edulis* and *Choromytilus meridionalis*) (Seuront et al. 2021).

Effects of ingested microplastic on dreissenids

Dreissena bugensis decreased filtration if high concentrations of microplastic were ingested (Pedersen et al. 2020). However, despite large-scale microplastic ingestion, no effects on survival, reproduction rates or oxygen consumption were recorded (Pedersen et al. 2020). Their resilience to microplastic exposure might be additional evidence of their tolerance for a wide range of habitats.

In *D. polymorpha*, the ingestion of microplastic caused variations in the activity of the enzymes catalase and glutathione peroxidase, an increase in the concentration of the neurotransmitter dopamine and modulation of 78 different proteins mainly related to the response against oxidative stress (Magni et al. 2018, Magni et al. 2019). Additionally, microplastic particles can fuse with the byssal threads of dreissenids, as has already been observed for *Mytilus* spp. (Li et al. 2019b). A preliminary study on *D. polymorpha* indicated that the fusion of microplastic with the byssal threads had a significant impact on the dreissenids' attachment strength to the substrate (Paul et al. 2019). In the absence of sufficient studies, interruption of attachment ability by the fusion of microplastic with byssal threads might perhaps have a higher impact on *D. bugensis* than on *D. polymorpha*. The higher attachment strength of *D. bugensis* in their natural environment provides this species with a competitive benefit over *D. polymorpha* (Chapter 3). Interrupting their attachment strength might thus eliminate this competitive benefit for *D. bugensis*.

No effect was found on the cellular stress level, mussel activity, food clearance activity, glycogen content, lipid content, total energy content, lipid peroxidation and immunological state of the dreissenids (Magni et al. 2018, Magni et al. 2019, Weber et al. 2020). So far, only minor effects of microplastic ingestion were found on dreissenids leading to a slight reduction of overall fitness over time. Comparative analyses assessing the impact of microplastic on both *D. bugensis* and *D. polymorpha* are still lacking, making it difficult to assess which species suffers most from microplastic pollution. We would thus recommend future studies to assess the effect of microplastic on both species.

Laboratory versus field conditions

The above described microplastic effects should be treated with caution as the effects were studied during laboratory experiments with unrealistically high doses of microplastic (Redondo-Hasselerharm et al. 2018, Li et al. 2019a, Binelli et al. 2020). The microplastic concentration used during experiments were about 200 000 times higher than concentrations measured in the field (Magni et al. 2018, Zhang et al. 2018). Moreover, many experiments used plastic polymers that are relatively rare in a natural environment (Li et al. 2019a).

Therefore, these laboratory experiments are not a very good representation of realistic natural conditions (Binelli et al. 2020). Generally, so far no biological effects of microplastic were found on filter feeders at environmental concentrations (Revel et al. 2020). Moreover, both synthetic microplastic particles and natural suspended sediment particles caused an increase in byssal thread production in the Mediterranean mussel *Mytilus galloprovincialis* (Yap et al. 2020). Synthetic particles might thus mimic effects of natural particles on filter feeders. Consequently, the present-day body of literature expects a low risk for the benthic community of freshwater systems. However, microplastic concentrations are expected to increase in the environment, which implies that these effects cannot be excluded in the future (Redondo-Hasselerharm et al. 2018). Additionally, in our own study, we usually found much higher microplastic concentrations in sediment than indicated by other studies. The resuspension of these microplastic particles (e.g. by ship induced currents, high run-off and discharge during heavy rainfall) might cause temporarily high microplastic concentrations in the aquatic environment which can become available for filter feeders like dreissenids. In view of these uncertainties, microplastic pollution still needs to be considered as one of the possible factors affecting dreissenids' fitness.

Macroplastic as a vector for dispersal of dreissenids

Plastic can affect dreissenids in other ways as well. Larger plastic items can serve as a substrate for dreissenids and can transport them through an aquatic system. *Dreissena bugensis* individuals settle in higher numbers on PVC plates than *D. polymorpha* individuals do (Chapter 2), possibly resulting in higher colonisation of plastic debris by this species. This would provide *D. bugensis* with a fast and easy dispersal vector. As *D. bugensis* has a higher tolerance to changing environmental conditions (e.g. salinity and temperature fluctuations) than *D. polymorpha* (Chapter 2), *D. bugensis* individuals would prevail from this mode of dispersal during long-distance transport through several aquatic habitats with exposure to fluctuating environmental conditions.

Microplastic as a vector for other pollutants

Furthermore, plastic debris can contain or adsorb several environmental contaminants (e.g. heavy metals, pesticides, hydrocarbons, flame retardants, dioxins and plasticisers) and can be colonised by microorganisms and proteins which can alter the uptake, infiltration and accumulation of microplastic in tissues and their toxicity (Akhbarizadeh et al. 2017, Li et al. 2018, Li et al. 2019a, Binelli et al. 2020). Contaminant concentrations in or on plastic particles are several times higher than the concentrations found in the water column and can thus become toxic (Brennecke et al. 2016). When ingested, the contaminants can desorb from the (micro)plastic and can be absorbed by the organisms' digestive tract resulting in decreased nutrient absorption and potential bioaccumulation (Schessl et al. 2019). In most ecosystems, ingested contaminants, like e.g. heavy metals, will likely not lead to acute toxicity phenomena, but long-term exposure is very likely to lead to chronic toxicity effects, derived from biomagnification (Brennecke et al. 2016). However, there seemed to be no difference in contaminant absorption between pollutants transported by microplastic particles, food particles or natural particles (Bakir et al. 2016). Therefore, the contribution of microplastic particles in heavy metal transport, absorption and accumulation might be relatively small for benthic invertebrates.

Differential effect of heavy metals

For dreissenids, exposure to heavy metals can affect growth, cause filtration inhibition, prevent attachment by byssal threads and increase their mobility (Kobak et al. 2002, Kwan et al. 2003, Mendoza 2014, Wang et al. 2021). Dreissenids accumulate various heavy metals differently. *Dreissena bugensis* accumulates significantly more aluminium, iron and lead than *D. polymorpha* (Matthews et al. 2015). On the other hand, *D. polymorpha* contains significantly more zinc66, cadmium111, copper, nickel, cobalt and molybdenum than *D. bugensis* (Matthews et al. 2015). Generally, *D. bugensis* accumulates less heavy metals than *D. polymorpha* does.

However, even though *D. bugensis* accumulates less heavy metals than *D. polymorpha,* it seems to experience stronger heavy metal impacts. While *D. polymorpha* individuals induce their defence mechanisms, *D. bugensis* suffers more cellular damage (Potet et al. 2018, Louis et al. 2019). Overall, Potet et al. (2018) expect *D. polymorpha* populations to be more tolerant to high heavy metal concentrations than *D. bugensis*.

Environmental pollutants as potential drivers of the dominance shift

Comparative studies on the effect of pollutants on both dreissenid species are scarce, making it hard to assess the overall impact on both species. Microplastic seemed to affect *D. polymorpha* more than it affected *D. bugensis*. However, more studies have been conducted on *D. polymorpha* than *D. bugensis*, possibly causing bias in our knowledge on the effect of microplastic on both species. Nevertheless, we could hypothesise that *D. bugensis* is less impacted by microplastic pollution, yet seems to accumulate more microplastic particles in the field. A higher microplastic accumulation causes a higher exposure to microplastic adsorbed contaminants, like heavy metals.

In general, *D. bugensis* accumulates fewer heavy metals than *D. polymorpha* does. However, *D. polymorpha* individuals seem to be more tolerant to high heavy metal concentrations than *D. bugensis*. *Dreissena polymorpha* induces defence mechanisms, while *D. bugensis* suffers more cellular damage.

We would expect that high microplastic pollution causes less deterioration in *D. bugensis*, while heavy metal pollution causes less deterioration in *D. polymorpha.* However, higher mobility for *D. bugensis* allows them to move away from unfavourable environmental conditions (e.g. a polluted site after accidental emission). This ability provides *D. bugensis* with a competitive benefit over *D. polymorpha* in a polluted environment. Nevertheless, to prove this hypothesis, the effect of pollutants on the mobility of both dreissenid species should be assessed.

Implications for the management of dreissenids species

It is very hard to manage a dreissenid population in natural open water systems. Once they have been established, it is impossible to eradicate them (Sepulveda et al. 2019). Therefore, management of open water systems have to focus on preventative measures. To prevent the spread of dreissenids, boats have to be cleaned before transportation to another waterbody. This can be done by rinsing the boats with hot (60 °C) high-pressure water (Britton 2015). During our study, we found that *D. bugensis* had a higher tolerance for temperature and a stronger attachment than *D. polymorpha* (Chapter 2, 3). We could thus hypothesise that *D. bugensis* has a higher resistance against the cleaning of boat hulls than *D. polymorpha*. However, they will not be able to withstand this kind of hot high-pressure water. Nevertheless, if these preventative measurements do not happen thoroughly, *D. bugensis* is most likely the one to survive. Another preventative management type applicable to natural open water systems is ballast water management. The spread of invasive species through ballast water can be prevented by exchanging ballast water at sea or by applying physical (e.g. filtration, hydro cyclones), mechanical (e.g. ultraviolet radiation (UV), thermal, ultrasound (US), magnetic, electrical treatment) and chemical (e.g. use of biocides, chlorine, ozone, hydrogen peroxide, chlorine dioxide) treatments to ballast water (Tsolaki and Diamadopoulos 2010).

Most management techniques are focused on (closed) anthropogenic infrastructures. The management strategies for dreissenids in these systems are typically subdivided into three categories: preventative, proactive, and reactive management (Chakraborti et al. 2013). Preventative management is designed to prevent or delay the establishment of dreissenids by public education, regulation, inspections and relocating pipelines of cooling water systems. Preventative management is by far the most effective treatment against a dreissenid invasion.

Our study can provide some more insight in the effectiveness of proactive and reactive management treatments. Some proactive control techniques include (1) chemical treatments using oxidising or non-oxidising chemicals; (2) antifouling coatings on infrastructure surfaces; (3) various filter types; (4) UV radiation; (5) high water currents (> 2 m/s) to minimise veliger settlement (Chakraborti et al. 2013). When dreissenids are already abundant and widely established within a system, reactive management is implemented. Reactive management techniques may involve (1) chemical treatments using oxidising or non-oxidising chemicals; (2) physical removal of adult individuals using mechanical forces like power washing, scraping, and pigging; (3) desiccation, i.e. water level drawdown, draining and/or drying of a reservoir; (4) shock treatment using a thermal shock or freezing; (5) oxygen deprivation by inducing hypoxic or anoxic conditions (Chakraborti et al. 2013).

Proactive management treatments will be most efficient if the start and duration of dreissenid settlement are taken into account. Our study indicated that the *D. polymorpha* settlement already starts in spring and lasts throughout summer, while the *D. bugensis* settlement starts later, during summer alone (Chapter 2). Therefore, proactive management treatments are only necessary during spring and summer, not year-round.

When exposed to pollutants, *D. polymorpha* will activate defence mechanisms, while *D. bugensis* suffers more cellular damage (Potet et al. 2018, Louis et al. 2019). We can thus hypothesise that *D. bugensis* will experience a stronger impact when exposed to chemical treatments. On the other hand, *D. bugensis* has a higher capacity to move away from unfavourable environmental conditions (Chapter 4). Exposing them to chemical treatments might cause them to temporarily retreat to a safe area and return once the chemical treatment is over. In that case, *D. polymorpha* individuals have a higher chance of being killed by chemical management treatments. The same goes for desiccation and oxygen depletion treatments as these are unfavourable environmental conditions for dreissenids.

Antifouling and physical removal treatments relate to the attachment of dreissenids. The attachment strength of dreissenids is linearly related to their shell size (Chapter 3). As *D. bugensis* grows faster and becomes larger than *D. polymorpha*, *D. bugensis* individuals generally have a higher attachment strength in a natural assemblage (Chapter 3). Removing *D. bugensis* individuals from a substrate thus will take more effort in comparison to *D. polymorpha*. Individuals of the latter species attach more often to a substrate than *D. bugensis* individuals do. Additionally, the attachment strength of dreissenids is highly related to the type of substrate. Their attachment strength will be much higher on a coarse substrate in comparison to a smooth surface (Chapter 3). To decrease mechanical or chemical cleaning efforts, it is therefore recommended to opt for a smooth infrastructure surface.

Thermal shock treatment (i.e. heat shocks or freezing) is a final type of reaction management. Our study shows that *D. bugensis* has a higher tolerance for low winter temperatures than *D. polymorpha* and is better at handling fluctuating temperatures. We could thus hypothesise that shock treatments will be more efficient when dealing with *D. polymorpha* in comparison to *D. bugensis* individuals.

In general, we expect a higher cost efficiency when trying to manage a *D. bugensis* infestation in comparison to a *D. polymorpha* infestation. This indicates the importance of comparative species trait studies for understanding the ongoing dominance shifts from *D. bugensis* to *D. polymorpha* as the dominant fouling species on hard substrates in freshwater ecosystems.

Conclusions – species traits causing a competitive benefit for either species

Since its recent introduction, *D. bugensis* seems to be outcompeting the already present *D. polymorpha*. However, mechanisms driving this dominance shift are still not completely understood. This thesis aimed at unravelling some of the drivers of this observed dominance shift between *D. polymorpha* and *D. bugensis.*

Species traits differentially leading to higher fitness and competitive benefits

- *Dreissena polymorpha* can settle earlier in the season and is predated less upon than *D. bugensis*.

- *Dreissena polymorpha* individuals attach more often to hard substrates than *D. bugensis* individuals do, which can help when trying to withstand currents and predators.

- *Dreissena bugensis* settles in higher densities when *D. polymorpha* individuals are already present on the substrate.

- *Dreissena bugensis* has a higher attachment strength compared to *D. polymorpha* in natural dreissenid assemblages. This allows *D. bugensis* to better withstand currents and predators than *D. polymorpha*.

- After detachment, *D. bugensis* mussels move at a higher top speed than *D. polymorpha*. Additionally, this species has a significantly higher number of individuals moving at any point in time than *D. polymorpha*. *Dreissena bugensis* individuals tend to avoid unfavourable environmental conditions and spend more time searching for the optimal environment after detachment.

External factors differentially affecting dreissenid fitness

- *Dreissena polymorpha* has a higher salinity tolerance.

- *Dreissena bugensis* can settle in a wider range of habitats and adjusts its growth and settlement better to seasonally and annually varying salinities and temperatures.

- *Dreissena bugensis* shows lower winter mortality than *D. polymorpha*.

- Microplastic pollution appears to have less impact on *D. bugensis*, while heavy metal pollution appears to have less impact on *D. polymorpha.* However, ambient concentrations in microplastic and microplastic adsorbed pollutants (e.g. heavy metal) have limited effects. Comparative ecotoxicological studies are needed to confirm differential sensitivities of both species.

Knowledge gaps and recommendations for future research

- Most knowledge on dreissenids is derived from single-species studies, mostly on *D. polymorpha*. A general recommendation is to perform more comparative analyses on both species.

- Comparative studies assessing the impact of pollution on both species are scarce. We would thus recommend assessing the differential impact of pollutants like microplastic and heavy metals on the settlement, growth, survival, movement and attachment of both species.

- Knowledge on the relationship between the microplastic concentrations in sediment and the microplastic concentrations in organisms (e.g. dreissenids) is scarce. We would recommend future studies to focus on this issue in both the native range (Ponto-Caspian rivers and estuaries) and invaded areas of dreissenids.

- The heterogeneity of the environment leads to the coexistence of both dreissenid species on a regional scale. We do not have enough knowledge on the drivers of the dominance shift to make up a total balance at the population level yet. We would recommend future studies to focus on drivers of dominance shifts at the population level in various environmental contexts.

Recommendations for management

- Preventative measures for reducing dispersal via boat hulls in natural waterbodies (e.g. cleaning boat hulls with hot high-pressure water) should be applied thoroughly. If not, *D. bugensis* is most likely the one to survive. This is not favourable, as *D. bugensis* is the strongest invader with the highest expected environmental and economic impact.

- Proactive management treatments in anthropogenic infrastructures will be most efficient when the start and duration of dreissenid settlement are taken into account. *Dreissena polymorpha* settlement already starts in spring and lasts until summer, while *D. bugensis* settlement only happens during summer. Proactive treatments could thus be applied from the beginning of spring until the end of summer and will not be required year-round.

- When applying management techniques in anthropogenic infrastructures (i.e. chemical treatments, desiccation and oxygen depletion), it is important to make sure there are no hideout spots where dreissenids can hide from the treatment. *Dreissena bugensis* has a higher capacity to move away from unfavourable environmental conditions (like the above-mentioned treatments) than *D. polymorpha*.

- The dreissenid attachment strength is much higher on coarse substrates in comparison to smooth surfaces. To decrease detachment efforts, it is therefore recommended to opt for smooth surfaces in anthropogenic infrastructures.

- Thermal shock treatments (i.e. heat shocks or freezing) will be most effective on *D. polymorpha* populations, as *D. bugensis* is more resistant to various temperature conditions.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Summary

The freshwater mussels *Dreissena polymorpha* (zebra mussel) and *D. bugensis* (quagga mussel) originate from rivers and their estuaries in the Northern part of the Ponto-Caspian basins (Black Sea and Caspian Sea). Attached to boat hulls and in ballast water, they expanded their geographical range to large parts of the Northern hemisphere. *Dreissena polymorpha* started spreading out of its native area in the beginning of the 19th century and became a dominant species on hard surfaces in many freshwater ecosystems. At the end of the 20th century, *D. bugensis* followed in its footsteps. Both dreissenid species cause extensive ecological and economic damage in their native regions and introduced areas. During the last decade, there have been widespread observations of the replacement of *D. polymorpha* by *D. bugensis* as the dominant species on hard substrates. These ongoing dominance shifts have been observed in both their native and introduced regions. However, *D. bugensis* is not always able to fully outcompete *D. polymorpha* leading to the co-existence of both species. To date, the drivers of these dominance shifts are still largely unknown.

To identify drivers of the dominance shifts between both dreissenids, comparative analyses were conducted to identify species traits and external factors leading to higher fitness and competitive benefits for either one of these species. We assessed which life-history traits (Chapter 2), attachment and detachment traits (Chapter 3) and mobility traits (Chapter 4) provided a competitive benefit for one dreissenid species over another. Additionally, we studied the effects of external factors like temperature, salinity and light on the above-mentioned species traits (Chapters 2, 3) and the concentration of pollutants and their effect on the dreissenids' fitness (Chapters 5, 6).

Firstly, we assessed which species traits led to a higher fitness and competitive benefits for either one of the species. *Dreissena polymorpha* can settle earlier in the season and is predated less upon than *D. bugensis* (Chapter 2). Additionally, *D. polymorpha* individuals attach more often to hard substrates than *D. bugensis* individuals do, which can help when trying to withstand strong currents and predators (Chapter 3). These species traits cause competitive benefits for *D. polymorpha* possibly leading to the co-existence of both species. *Dreissena bugensis*, on the other hand, settles in higher densities when *D. polymorpha* individuals are already present on the substrate (Chapter 2). Additionally, *D. bugensis* has a higher attachment strength compared to *D. polymorpha* in natural dreissenid assemblages (Chapter 3). This allows *D. bugensis* to better withstand strong currents and predators than *D. polymorpha*. Mobility traits cause additional benefits for *D. bugensis*. After detachment, *D. bugensis* mussels have a higher top speed than *D. polymorpha*. *Dreissena bugensis* also has a significantly higher number of individuals moving at any point in time than *D. polymorpha* (Chapter 4). This way, *D. bugensis* individuals can avoid unfavourable environmental conditions and spend more time searching for the optimal environment where they can thrive. These species traits cause competitive benefits for *D. bugensis* and could be possible drivers of the dominance shift between both species.

Secondly, we assessed whether external factors differentially affected dreissenid fitness. *Dreissena polymorpha* has a higher salinity tolerance than *D. bugensis* (Chapter 2). A higher average salinity of the dreissenids' environment might therefore lead to a competitive benefit for *D. polymorpha* and could cause the co-existence of both species. On the other hand, *D. bugensis* can settle in a wider range of habitats and adjusts its growth and settlement better to seasonally and annually varying salinities and temperatures than *D. polymorpha* does. Additionally, *D. bugensis* has lower winter mortality rates than *D. polymorpha* (Chapter 2).

Dreissenids often live in polluted environments, as rivers are one of the main pathways for transporting pollutants from sources to their sinks. Heavy metals are well-known pollutants in river systems. Additionally, (micro-)plastic represents one of those, still largely unknown, pollutants. Not much is known about microplastic concentrations in the Ponto-Caspian area. Therefore, we first determined whether microplastic could potentially reach harmful concentrations in the

Black Sea and Caspian Sea. In Chapter 5, we showed that the microplastic concentration in the Black Sea was twice as high as in the Caspian Sea. Moreover, the microplastic concentrations were related to both their sources (rivers, shorelines, cities) and sinks (water depth). We usually found much higher microplastic concentrations in sediment than indicated by other studies in the same or comparable habitats (Chapter 5). The resuspension of these microplastic particles (e.g. by ship induced currents, high run-off and discharge during heavy rainfall) might temporarily cause high microplastic concentrations in the aquatic environment which can become available for filter feeders like dreissenids. We, therefore, recommend considering microplastic pollution as one of the possible factors affecting dreissenids' fitness. Based on recent literature, we expect microplastic pollution to have less impact on *D. bugensis*, while heavy metal pollution has less impact on *D. polymorpha* (Chapter 6). Nevertheless, comparative ecotoxicological studies are needed to confirm differential sensitivities of both species. The assessed external factors cause competitive benefits for *D. bugensis* and could therefore be possible drivers of the dominance shift between both species.

Our studies resulted in some recommendations for dreissenid management. Preventative measures (e.g. cleaning boat hulls with hot high-pressure water) should be applied thoroughly; if not *D. bugensis* is most likely the one to survive. Proactive management treatments of (cooling) water facilities will be most efficient when the start and duration of dreissenid settlement are taken into account. Dreissenids settle during spring (*D. polymorpha*) and summer (*D. polymorpha* and *D. bugensis*). Therefore, proactive settlement treatments will not be required year-round. *Dreissena bugensis* has a higher capacity to move away from unfavourable environmental conditions than *D. polymorpha*, giving it the opportunity to hide or move away from chemical treatments, desiccation or oxygen depletion. Additionally, dreissenids have a higher attachment strength on rough substrates in comparison to smooth surfaces. Therefore, it is recommended to opt for smooth surfaces in cooling water infrastructures and other water facilities. Finally, thermal shock treatments (i.e. heat shocks or freezing) will be most effective on *D. polymorpha* populations, as *D. bugensis* is more resistant to various temperature conditions.

Generally, most knowledge on dreissenids is derived from single-species studies, which are mostly performed with *D. polymorpha*. Therefore, we recommend performing more comparative analyses, for instance on the differential impact of pollutants like microplastic and heavy metals on the settlement, growth, survival, movement and attachment of both species.

Samenvatting

De driehoeksmossel (*Dreissena polymorpha*, ook wel zebramossel genoemd) en quaggamossel (*D. bugensis*) zijn invasieve zoetwatermosselen die veel ecologische en economische schade veroorzaken. Oorspronkelijk kwamen beide soorten enkel voor in rivieren en riviermondingen ten noorden van de Zwarte en Kaspische Zee. Beide soorten hebben zich echter doorheen de tijd verspreid over bijna het gehele noordelijke halfrond, vastgehecht aan scheepsrompen en in het ballastwater van schepen. De zebramossel begon al met deze verspreiding aan het begin van de 19^e eeuw en werd een dominante soort op harde oppervlakken in veel zoetwaterecosystemen. Aan het einde van de 20^e eeuw trad de quaggamossel in zijn voetsporen. Gedurende het laatste decennium waren er verscheidene waarnemingen van een overname door de quaggamossel als de dominante soort op harde zoetwatersubstraten. Deze verschuiving in dominantie is waargenomen in zowel hun herkomstgebied als in nieuwe verspreidingsgebieden. De quaggamossel is echter niet in staat gebleken om de zebramossel volledig weg te concurreren, wat ertoe heeft geleid dat op veel plekken beide soorten samenleven. Tot voor kort was onbekend waarom de quaggamossel zo dominant kon worden, met andere woorden, waarom hij beter was in de competitie.

Om te verklaren waarom de quaggamossel zo dominant kon worden ten opzichte van de zebramossel zijn enkele experimenten uitgevoerd. Zo is uitgezocht welke soortkenmerken (bijvoorbeeld grootte en kracht) en welke omgevingsfactoren hierbij een rol spelen. We onderzochten welke kenmerken uit de levensgeschiedenis (Hoofdstuk 2), aanhechtingskenmerken (Hoofdstuk 3) en mobiliteitskenmerken (Hoofdstuk 4) een competitief voordeel opleverden van de ene ten opzichte van de andere mosselsoort. Daarnaast bestudeerden we het effect van omgevingsfactoren zoals temperatuur, zoutgehalte en licht op de bovengenoemde soortkenmerken (Hoofdstuk 2, 3), de concentratie van microplastics (Hoofdstuk 5) en het effect van deze vervuiling op de fitness van beide mosselsoorten (Hoofdstuk 6). Met de fitness van een soort wordt niet bedoeld "de fitste organismen", maar de organismen die het best zijn aangepast (“to fit”) aan hun leefomgeving.

Quaggamossels vestigen zich in hogere aantallen wanneer reeds driehoeksmossels op het oppervlak aanwezig zijn (Hoofdstuk 2). Bovendien hebben quaggamossels onder natuurlijke omstandigheden een hogere aanhechtingskracht dan driehoeksmossels (Hoofdstuk 3). Daarnaast hebben quaggamossels nog een aantal voordelige mobiliteitskenmerken. Ze kunnen bij verplaatsingen een hogere snelheid behalen dan driehoeksmossels. Tijdens onze experimenten merkten we ook op dat meer quaggamossels in beweging waren in vergelijking met driehoeksmossels (Hoofdstuk 4). Op deze manier kunnen quaggamossels ongunstige omgevingsomstandigheden beter vermijden en meer tijd besteden aan de zoektocht naar de optimale leefomgeving waarin ze kunnen gedijen. Al deze soortkenmerken veroorzaken mogelijk een competitief voordeel voor de quaggamossel, waardoor deze soort op veel plaatsen dominanter aanwezig is dan de driehoeksmossel.

De quaggamossel is er echter niet in geslaagd om de driehoeksmossel volledig te verdringen. De resultaten van onze experimenten boden ook een mogelijke verklaring hiervoor. Zo bleek dat driehoeksmossels zich vroeger in het seizoen kunnen vestigen en dat de predatie door roofdieren waarschijnlijk lager is dan bij de quaggamossel (Hoofdstuk 2). Bovendien hechten driehoeksmossels zich vaker aan harde oppervlakken (Hoofdstuk 3). Driehoeksmossels kunnen onder bepaalde omstandigheden meer weerstand bieden tegen stroming en roofdieren, waardoor ze toch samen met quaggamossels kunnen voorkomen in dergelijke leefgebieden.

Daarnaast onderzochten we welke omgevingsfactoren een invloed hadden op de fitness van beide mosselsoorten. Driehoeksmossels zijn toleranter tegen zout water dan quaggamossels (Hoofdstuk 2). Een hoger zoutgehalte van hun leefomgeving kan daarom leiden tot een competitief voordeel voor de driehoeksmossel. De quaggamossel is in een zoutere leefomgevingen niet in staat om de zebramossel volledig weg te concurreren. Aan de andere kant kunnen quaggamossels hun groei en vestiging beter aanpassen aan variabele (maar dus niet te hoge) zoutgehaltes en temperaturen dan driehoeksmossels. Bovendien overleeft een groter percentage quaggamossels de winter, waardoor ze in het voorjaar met meer individuen kunnen starten met de voortplanting (Hoofdstuk 2).

Beide mosselsoorten overleven ook in vervuilde omgevingen. Veel voorkomende verontreiniging in rivieren zijn bijvoorbeeld zware metalen. Een andere, nog grotendeels onbekende, vervuiling is (micro-)plastic. Microscopisch kleine plasticdeeltjes komen in de rivieren terecht door bijvoorbeeld afvalwater van wasmachines, cosmetische producten of de afbraak van groter plastic afval. Er was nog niet veel bekend over hoeveel microplastic voorkwam in de Zwarte en Kaspische Zee. We ontdekten dat microplastic concentraties in het sediment van de Zwarte Zee twee keer zo hoog lagen dan in sediment van de Kaspische Zee (Hoofdstuk 5). Bovendien waren de microplastic concentraties gerelateerd aan zowel de afstand tot hun vermoedelijke bronnen (rivieren, kustlijnen en steden) als de waterdiepte. Tot slot vonden we meestal veel hogere microplastic concentraties dan aangegeven door andere studies in dezelfde of vergelijkbare gebieden (Hoofdstuk 5). Het opwoelen van deze microplastic deeltjes in het sediment (bijvoorbeeld door sterke waterstroming tijdens hevige regenval of stroming veroorzaakt door scheepsvaart) kan tijdelijk hoge concentraties in het (zee)water veroorzaken. Deze deeltjes worden dan opgenomen door filtervoeders zoals de driehoeksmossel en quaggamossel.

De kans bestaat dus dat microplastic een effect heeft op de fitness van deze mosselsoorten. Op basis van onderzoeksresultaten van andere wetenschappers verwachten we dat vervuiling door microplastic een kleinere impact zal hebben op quaggamossels, terwijl vervuiling door zware metalen dan weer een kleinere impact zal hebben op driehoeksmossels (Hoofdstuk 6). Om dit met zekerheid vast te stellen, moeten in de toekomst studies worden uitgevoerd die de effecten van deze vervuiling op beide soorten vergelijken. We kunnen wel besluiten dat de eerdergenoemde milieufactoren (temperatuur, zoutgehalte, microplastic) competitieve voordelen veroorzaken voor quaggamossels en waarschijnlijk bijdragen aan hun dominantie.

Op basis van de resultaten van onze studies zijn ook enkele aanbevelingen geformuleerd voor het beheer van de invasieve driehoeksmossel en quaggamossel. Preventieve maatregelen (zoals het reinigen van scheepsrompen met heet water onder hoge druk) moeten grondig worden toegepast. Indien dit niet gebeurt zullen quaggamossels hoogstwaarschijnlijk overleven. Aangroei van de driehoeksmossel en quaggamossel in (koel)watersystemen van bedrijven en elektriciteitscentrales worden bestreden met behulp van proactieve maatregelen zoals behandelingen met chemische stoffen en door middel van uitdroging en zuurstoflimitatie. Daarbij moet rekening worden gehouden dat quaggamossels beter in staat zijn om ongunstige omgevingen (zoals we die creëren door behandeling van de omgeving) te verlaten. Thermische schokbehandelingen (door verhitting of bevriezing) zijn het meest effectief bij driehoeksmossels, omdat die minder bestand zijn tegen temperatuurfluctuaties en extreme temperaturen dan quaggamossels. Proactieve maatregelen zullen het meest efficiënt zijn wanneer rekening wordt gehouden met het begin en de duur van de vestigingsperiode van beide soorten. We weten dat de mosselen zich vestigen tijdens de lente (driehoeksmossel) en de zomer (driehoeksmossel en quaggamossel), waardoor proactieve maatregelen niet doorheen het hele jaar vereist zijn. Ten slotte hebben beide mosselsoorten een hogere aanhechtingskracht op ruwe oppervlakken dan op gladde oppervlakken. Het is daarom aan te raden te kiezen voor gladde oppervlakken bij de aanleg van bijvoorbeeld koelwatersystemen, steigers of pijpleidingen.

Doorheen de geschiedenis werden de meeste onderzoeken uitgevoerd op de driehoeksmossel. Studies die beide soorten met elkaar vergelijken zijn eerder zeldzaam. Daarom raden we aan om in de toekomst meer vergelijkende studies uit te voeren op bijvoorbeeld de impact van verontreinigende stoffen zoals microplastic en zware metalen op de vestiging, groei, overleving, beweging en aanhechting van beide soorten.

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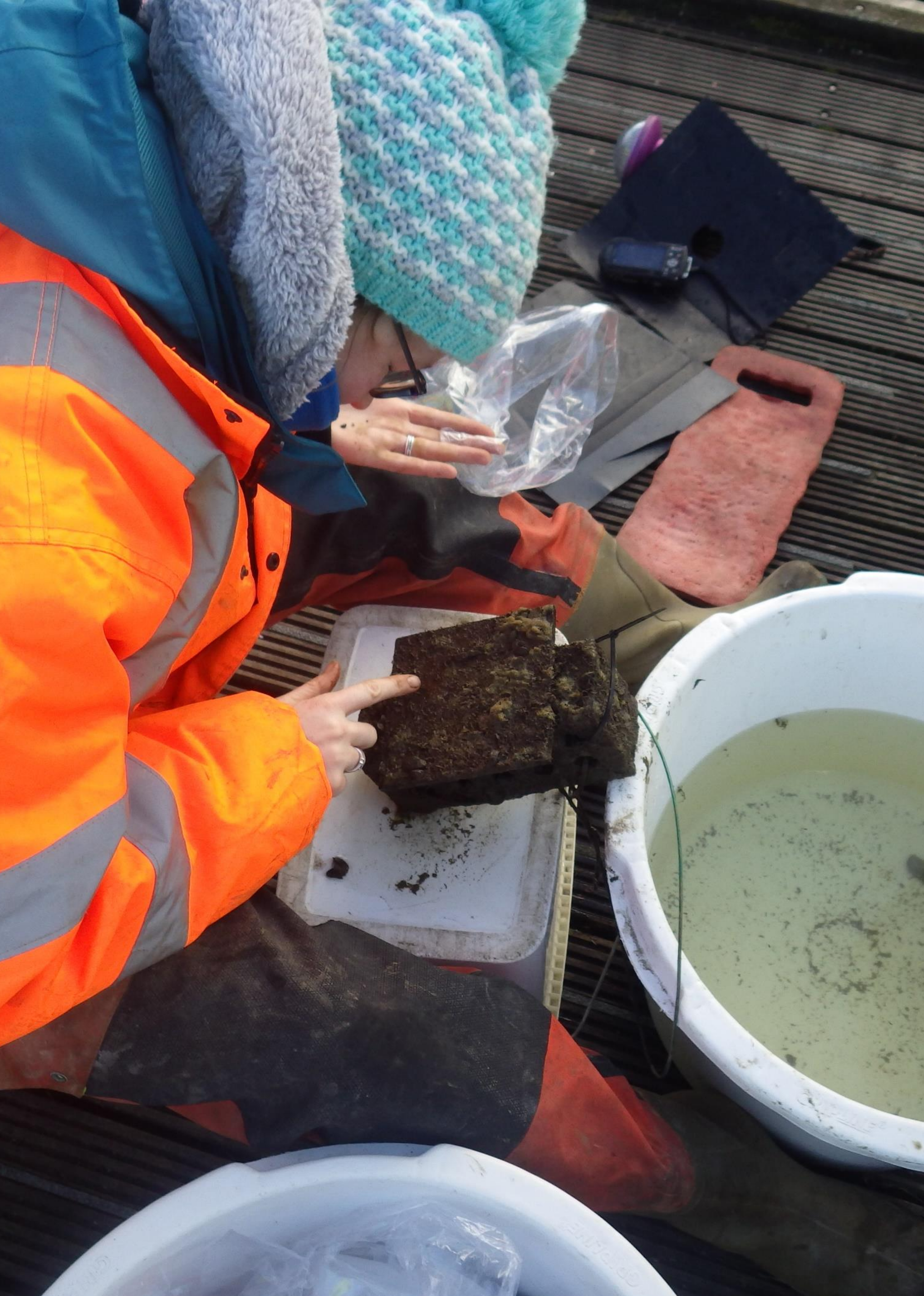
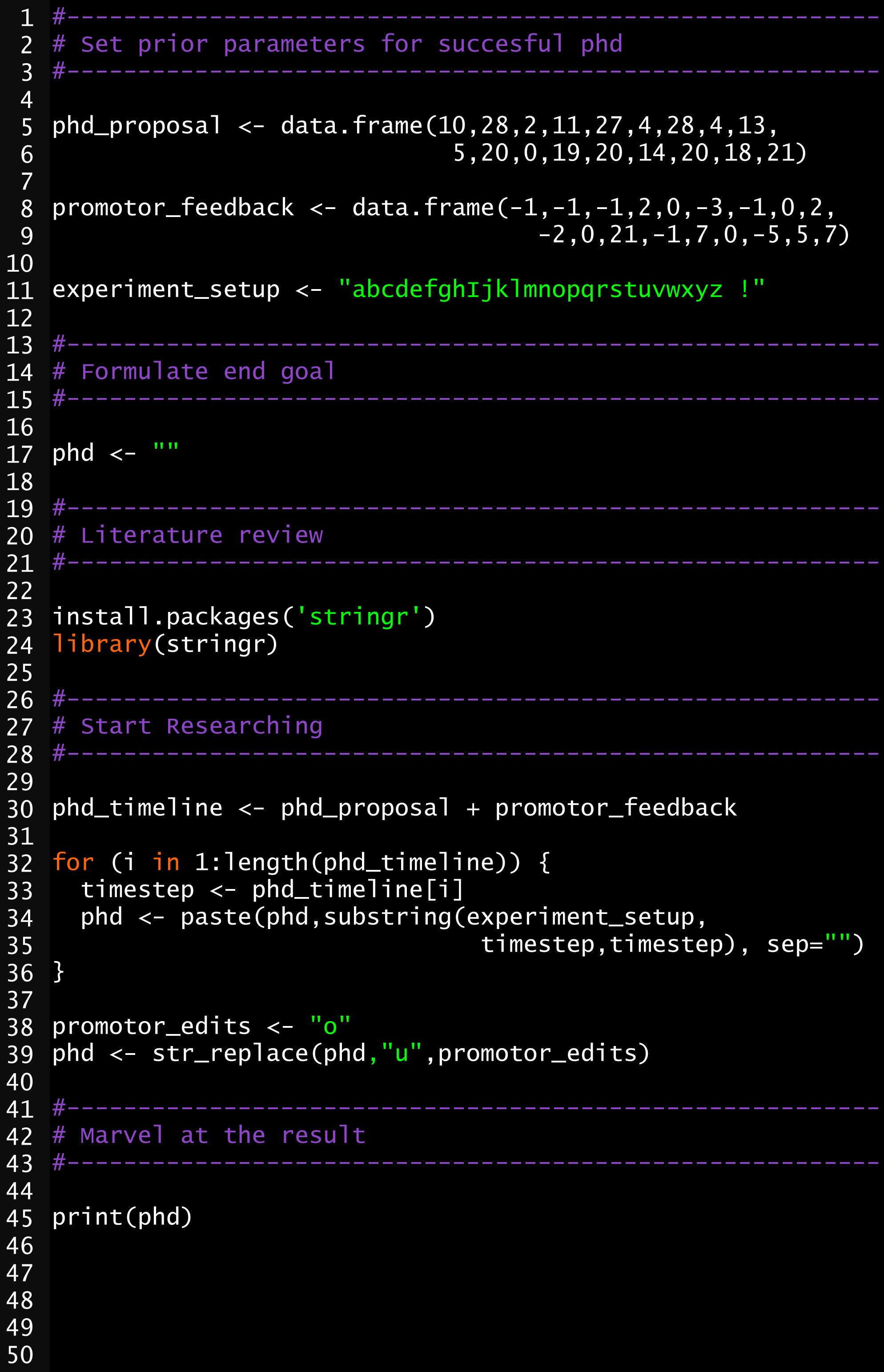


About the author

Anouk D'Hont was born on the 22nd of August, 1991 in Leuven (Belgium). She attended the Mater Dei institute in Leuven from 2003 until her graduation in 2009. She started her scientific career by selecting the science-math options during high school. She continued this path by starting her Bachelor of Science education in Biology at the KULeuven in 2009. In 2012, she graduated her Bachelor cum laude and decided to pursue a more marine oriented career. Anouk started her Erasmus mundus Master of Science in Marine Biodiversity and Conservation in 2012, studying at the Universidade Do Algarve in Portugal. She conducted her research internship with Prof. Dr. Ester Serrao and Dr. Aschwin Engelen, studying the response of *Ulva* spp. to temperature changes. During the summer of 2013, she attended a summer school in Crete (Greece), researching the effect of temperature and salinity on rock pool communities. In the fall of 2013, Anouk started her second master year at the Galway-Mayo Institute of Technology in Ireland. During the last part of her master, she finished her master thesis on the effect of ocean acidification on meiofauna in Papua New Guinea. She completed this research at the Marine Biology Department of the University of Ghent, Belgium, supervised by Prof. Dr. Ann Vanreusel and Dr. Katja Guilini. Anouk obtained her master degree in 2014, graduating magna cum laude and started working in the molecular phylogenetic laboratory of the National Botanic Garden Meise in Belgium. In 2015, she started her PhD research at the Department of Environmental Science and the Department of Animal Ecology and Physiology of the Radboud University in Nijmegen, The Netherlands. This resulted in the present thesis. The first three years of her PhD were funded by European Union's Horizon 2020 research and innovation programme and the Innovative Training Network 2015–2019 Drivers of Pontocaspian Biodiversity Rise & Demise (PRIDE) under the Marie Sklodowska-Curie grant agreement No 642973. As of the start of her PhD in 2015, she was employed and supported by the marine inventory company GiMaRIS in Sassenheim (The Netherlands). At this company, she coordinated the international fouling community study SETL (SETtLement), conducted SASI (Schelpdier Afhankelijke Soorten Inventarisatie) monitoring on the import of mussels and oysters in The

Netherlands and conducted harbour inventories within the framework of the Ballast Water Management Convention. In 2021, her career came full circle, when she was hired by the KULeuven as an information specialist for the Faculty of Science and Technology.



```r
#---------------------------------------------------------
# Set prior parameters for succesful phd
#---------------------------------------------------------

phd_proposal <- data.frame(10,28,2,11,27,4,28,4,13,
                            5,20,0,19,20,14,20,18,21)

promotor_feedback <- data.frame(-1,-1,-1,2,0,-3,-1,0,2,
                                  -2,0,21,-1,7,0,-5,5,7)

experiment_setup <- "abcdefghIjklmnopqrstuvwxyz !"

#---------------------------------------------------------
# Formulate end goal
#---------------------------------------------------------

phd <- ""

#---------------------------------------------------------
# Literature review
#---------------------------------------------------------

install.packages('stringr')
library(stringr)

#---------------------------------------------------------
# Start Researching
#---------------------------------------------------------

phd_timeline <- phd_proposal + promotor_feedback

for (i in 1:length(phd_timeline)) {
  timestep <- phd_timeline[i]
  phd <- paste(phd,substring(experiment_setup,
                              timestep,timestep), sep="")
}

promotor_edits <- "o"
phd <- str_replace(phd,"u",promotor_edits)

#---------------------------------------------------------
# Marvel at the result
#---------------------------------------------------------

print(phd)
```

Research Data Management

This thesis research has been carried out under the RDM policy of the Institute for Water and Wetland Research, version 8-Jan-2021 accessed at www.ru.nl/iwwr/organisation/data-management/

Chapter 2

D'Hont A, Gittenberger A, Hendriks AJ, Leuven RSEW (2018) Data for: Drivers of dominance shifts between invasive Ponto-Caspian dreissenids *Dreissena polymorpha* (Pallas, 1771) and *Dreissena rostriformis bugensis* (Andrusov, 1897). DANS EASY [Dataset]. https://doi.org/10.17026/dans-z5z-cp2f

Chapter 3

D'Hont A, Gittenberger A, Hendriks AJ, Leuven RSEW (2020) Data for: Dreissenids' breaking loose: Attachment strength as a driver of the dominance shift between two invasive mussel species. DANS EASY [Dataset]. https://doi.org/10.17026/dans-xzn-aen9

Chapter 4

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Chapter 5

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MSA

Publication list

Peer reviewed articles

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