UNIVERSITY OF HULL

Improving environmental DNA (eDNA) methods for the detection and monitoring of invasive Dreissenid mussels

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Declaration of authorship

I declare that the work submitted in this thesis is my own (S.P.), except when stated otherwise. Data chapters received contributions from my supervisor (Lori Lawson Handley, L.L.H.), advisors (Ben Aston, B.A.) and collaborators (Nathan Griffiths, N.G.; Rachel Naden, R.N.; Abi Sheriden, A.S.; Rosetta Blackman, R.B.; Jonathan Porter, J.P.; Alan Wan, A.W.; Chris Gerrard, C.G.). The contribution of each person is stated below following the CRediT (Contribution Roles Taxonomy) classification. Any further assistance is acknowledged at the end of each chapter.

Chapter 2

S.P.: conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing – original draft preparation. **N.G.:** investigation. **R.N.:** investigation, writing – review & editing. **B.A.:** funding acquisition, writing – review & editing. **L.L.H:** conceptualization, funding acquisition, project administration, supervision, writing – original draft preparation.

Chapter 3

S.P.: conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing – original draft preparation. **A.S.:** investigation. **B.A.:** funding acquisition. **L.L.H.:** conceptualization, funding acquisition, project administration, supervision, writing – original draft preparation.

Chapter 4

S.P.: conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing – original draft preparation. **R.B.:** methodology, writing – review & editing. **J.P.:** methodology, resources, validation, writing – review & editing. **A.W.:** methodology, resources, validation, writing – review & editing. **C.G.:** resources, writing – review & editing. **B.A.:** funding acquisition, writing – review & editing. **L.L.H.:** conceptualization, project administration, funding acquisition, supervision, writing – original draft preparation.

COVID-19 statement

The COVID-19 pandemic had a significant impact on my research. This impact was partially mitigated by the 6-month extension granted, however not completely. In March 2020 (6 months into my PhD), upon the start of the pandemic and the first UK lockdown I returned to my home country where I remained for 6 months until given authorisation to return. This caused significant delays on fieldwork and sample collection, which was the main focus of the first year. This consequently delayed all the downstream processes in the following years, i.e. lab work and data analysis. In the end, this was translated into insufficient time to complete the fourth data chapter as initially intended.

Open research statement

To adopt open research practices, we intend to publish the three data chapters generated in this thesis in open access journals, where all data and R codes will be accessible and in the public domain. Chapter 4 is already available as a preprint on bioRxiv: https://www.biorxiv.org/content/10.1101/2023.12.18.572119v1. Additionally, all R codes will be annotated to promote reproducibility.

Ethics approval statement

The work conducted in this thesis received ethics approval from the Faculty of Science and Engineering Ethics Committee (application number: FEC_2019_255).

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Abstract

Dreissenid bivalves, zebra mussel (*Dreissena polymorpha*, Pallas, 1771) and quagga mussel (*Dreissena rostriformis bugensis*, Andrusov, 1897), are small freshwater molluscs native to the Ponto-Caspian region in Eastern Europe. Increasing globalization has facilitated their spread and establishment outside of their native range, and they are now widespread in Europe and North America. They are responsible for significant environmental impacts and economic losses, which are more pronounced in water-related companies. The most recent estimates suggest a cost of 5 million pounds for UK water industries. The use of sensitive tools for early detection and monitoring of dreissenid mussels is thus essential. Environmental DNA (eDNA) is described as genetic material that can be obtained from environmental samples. Targeted eDNA assays must meet specific criteria outlined in a 5-level validation scale to ensure reliability of results and be considered operational for use in routine monitoring. In this thesis, I aimed to increase the validation level of two targeted eDNA assays for dreissenid mussels. For zebra mussels, this included assessing the seasonal and spatial variation of eDNA detectability and estimating detection probabilities from statistical modelling. eDNA concentration and detection rates peaked in the summer, consistent with the increase in temperature which triggers mussel spawning. We also observed differences in eDNA concentration between waterbody types, being higher in reservoirs. A new targeted assay for quagga mussels was optimised and used to demonstrate they are more widespread than previously thought, with positive detections in several rivers, recreational lakes and the canal system, highlighting the need to implement urgent biosecurity measures to control their spread. We were able to increase the validation level of both assays tested in this thesis, thus increasing the reliability of their results. The work developed here constitutes therefore an important step towards the operational use of eDNA for routine monitoring of dreissenid mussels.

Table of contents

List of figures

List of tables

Chapter 1

General introduction

1.1 Invasive non-native species

Biological invasions pose a significant threat to wildlife and biodiversity (Mallez & McCartney, 2018), and are a consequence of human activities. In recent decades, the rise in trade and commercial networks facilitated the spread of species to new regions, either intentionally or accidentally (Hulme, 2009; Bellard, Cassey, & Blackburn, 2016). More than 37 thousand species have been introduced worldwide due to humans, from which more than 3500 have become invasive and caused significant negative impacts (IPBES, 2023). Due to ongoing globalisation, the rate of species introductions is increasing at an unprecedented pace, and islands and coastal mainland are acting as hotspots for established non-native species (Dawson et al., 2017).

The process of biological invasion can be divided into four stages – transport, introduction, establishment, and spread – and species have to overcome specific barriers to reach the following stage (Blackburn et al., 2011). Depending on where they are in the invasion process, species terminology varies. Introduced species, also known as alien or non-native species, are those that have been intentionally or unintentionally brought into a new geographic area by humans, where they would not naturally occur. Some introduced species may become established in their new environment without causing significant harm, while others become invasive (Blackburn et al., 2011). Invasive non-native species (INNS) are therefore a subset of introduced species that have the ability to rapidly spread and dominate ecosystems in their new environment, resulting in substantial negative impacts.

Even though introduced/non-native species are more likely to disrupt ecosystems due to lack of coevolutionary history with the invaded ecosystem (Simberloff et al., 2012), native species (i.e. species that naturally occur in a particular environment) can also exhibit invasive behaviours (e.g. colonization of new areas and rapid population growth) under specific circumstances. This often occurs following anthropogenic disturbances to

their environment, such as fire suppression, livestock grazing and eutrophication (Simberloff et al., 2012; Valéry et al., 2009).

The ecological impacts of INNS are complex and vary depending on their trophic level, abundance, and the region and ecosystem they are inserted in (Gallardo et al., 2016; Pyšek et al., 2020). According to Bellard et al. (2016), invasive species are the main driver of vertebrate extinctions, contributing to 54% of all documented extinctions since 1500. Additionally, they can modify trophic networks and community compositions, alter ecosystem productivity and nutrient cycling (Pyšek et al., 2020), and act as vectors for infectious exotic diseases (Herder et al., 2014). Overall, impacts from INNS are amplified in isolated environments such as islands, due to their reduced native populations and high endemism rates (Bacher et al, 2023).

The damage made by INNS not only to natural ecosystems but also to infrastructures, fisheries, livestock and agriculture is reflected in major economic losses every year (Holden, Nyrop, & Ellner, 2016), which have been showed to be in a similar order of magnitude as natural hazards such as storms and earthquakes (Turbelin et al., 2023). The costs of removal, control measures and damage repair associated with INNS have been estimated to be $59.3 billion in Europe and $1.2 trillion worldwide between 1980-2019 (Turbelin et al., 2023), with insects and mammals being the costliest taxonomic groups (Bacher et al., 2023). In an attempt to increase public and political awareness of the impacts of INNS, a public and interactive database (InvaCost) has recently been compiled that describes and summarizes the costs of biological invasions worldwide (Diagne et al., 2020).

In addition to ecological and economic impacts, INNS can also affect human well-being and quality of life. In terrestrial habitats, most impacts to human well-being are caused by plants, mainly to agriculture and livestock lands, while vertebrates and invertebrates have caused the highest impact in freshwater and marine aquatic systems, respectively (Bacher et al., 2023). Impacts can include the transmission of exotic diseases, crop pests that affect agriculture and food production, or changes to water quality (Bacher et al., 2023). Rural communities and indigenous people that are more dependent on nature's contributions are expected to be more affected by these impacts (Bacher et al., 2023).

The implementation of appropriate action plans is therefore crucial for the management of biological invasions, and early detection and rapid response (EDRR) strategies (Reaser, 2020; Reaser et al., 2020) constitute an important component of INNS management. These consist of collaborative networks of different national and international entities and local communities dedicated to monitoring and identifying potential INNS threats at their earliest stages and promptly implementing actions to contain or eradicate outbreaks, before populations become established. The management of biological invasions can include pathway, species or site/ecosystem-focused strategies (Sankaran et al., 2023). Management of introduction pathways is crucial to prevent establishment and can be applied to different areas such as shipping and trade, recreational activities, or tourism, both internationally and domestically. The main goals of species-based management are the containment and suppression of INNS, while site-based approaches focus on INNS removal and site restoration (Sankaran et al., 2023). The most appropriate option will depend on the outcome required and resources available, however integrated approaches that combine different management options are likely to result in a more efficient outcome (Sankaran et al., 2023).

Horizon scanning is an approach for prioritising INNS management that uses information from different sources (e.g. published literature and field experts) to make predictions and highlight potential invaders, therefore helping government and environment agencies to eliminate and/or mitigate the risks of invasion, protecting ecosystems from the threats posed by INNS. This proactive approach has shown great potential to identify introduction pathways and highlight the invasion risk of several INNS (Matthews et al., 2017; Peyton et al., 2019; Tsiamis et al., 2020). For instance, through horizon scanning the freshwater invaders dreissenid mussels have been flagged as a priority species in different regions, such as the Iberian Peninsula (Cano-Barbacil et al., 2023; Oficialdegui et al., 2023) and the British Isles (Gallardo & Aldridge, 2013; Lucy et al., 2020; Roy et al., 2014).

1.2 Dreissenid mussels

Dreissenid mussels belong to the family Dreissenidae, within the class Bivalvia and phylum Mollusca. Studies suggest that their family originated around the Jurassic/Cretaceous periods (Orlova, 2014). Despite inhabiting at first marine and

saltwater environments, they later adapted to freshwater and brackish ecosystems (Karatayev & Burlakova, 2022; Orlova, 2014). This transition is believed to have occurred in the Pliocene epoch due to fluctuations in water salinity levels caused by different geological events, that resulted in alternating periods of lake isolation and connection with the ocean, thus prompting individuals to adapt to novel ecological niches (Orlova, 2014). During this period, the family thrived in the Paratethys basin, which today corresponds to the Black, Caspian and Azov seas, also known as the Ponto-Caspian region (Orlova, 2014).

The family Dreissenidae includes three extant genera – *Dreissena*, *Mytilopsis* and *Congeria* (Karatayev & Burlakova, 2022; Orlova, 2014). Within the genus *Dreissena*, two species have gained global attention due to their negative ecological and economic impacts: the zebra mussel (*Dreissena polymorpha*, Pallas, 1771) and quagga mussel (*Dreissena rostriformis bugensis*, Andrusov, 1897). Despite having similar life histories, their ecology is not identical and they have different environmental tolerances and distributions within waterbodies, for example. While they are both species of interest due to their potential for spread and negative impacts, far more information is available for zebra mussels.

Morphology and identification

Zebra mussels are characterized by their dark and light zebra-like stripes and zigzag patterns, while quagga mussels are usually paler with circular/concentric rings (Figure 1.1). Both species have a triangular shaped-shell, although quagga mussels tend to have a rounder shape (Figure 1.1b). One of the main differences used to distinguish between both dreissenid species is their capacity to stand upright when placed on a flat surface (Figure 1.1a). Zebra mussels are able to stand upright thanks to their flat ventral surface, whereas quagga mussels lack this ability because of their convex ventral surface (Mills et al., 1996). Both species are able to reach a maximum length of approximately 5 cm (Karatayev & Burlakova, 2022).

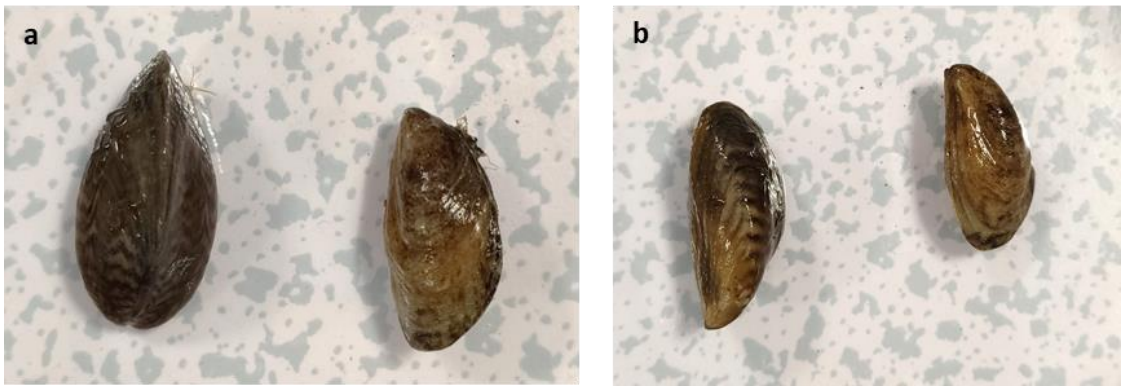

Figure 1.1 Zebra mussel (left) and quagga mussel (right) individuals placed on their ventral surface, demonstrating that quagga mussels cannot be stood upright **a)** and placed on their side **b)**. Photo credit: Sara Peixoto.

Species ecology

Dreissenid mussels originated in marine and saltwater environments, but now inhabit freshwater and brackish ecosystems (Karatayev & Burlakova, 2022; Orlova, 2014). As a result, their life history, which includes a free-floating microscopic larvae (veligers) and an attached adult stage, common in marine mussels, represents a unique ecological feature for freshwater mussels (Karatayev, Burlakova, & Padilla, 2015; Karatayev & Burlakova, 2022). Although similar, their life cycle is not identical and many characteristics differ between the two species. Nevertheless, zebra mussels have received more attention from the scientific community and more studies are available regarding their species ecology when compared to quagga mussels.

Both species reproduce through external fertilization, releasing eggs and sperm into the water column. Water temperature is the key trigger that induces spawning, and zebra and quagga mussels are able to reproduce once water reaches 12°C and 5°C, respectively (Karatayev & Burlakova, 2022). Their initial life stage consists of free-swimming microscopic veligers that settle and attach to the substrate within 10 (Hillbricht-Ilkowska & Stanczykowska, 1969) to 21 (Wright et al., 1996) days, in the case of zebra mussels. Although little information is available for quagga mussels, a study where dreissenid larvae were reared in laboratory conditions showed that quagga mussels took 32 days to settle (Wright et al., 1996). Zebra mussels are known to reach sexual maturity within one year (Thorp & Covich, 2009) and release up to 1 million eggs each season (Sprung, 1991), while no information was found for quagga mussels. Their short maturation, together with their high fecundity rate, allows them to quickly

proliferate and reach high densities in new environments. Both species have an average life span of 3-5 years (Karatayev & Burlakova, 2022).

Several environmental variables influence their establishment and growth rates, such as calcium, pH, temperature, turbidity, conductivity, salinity and dissolved oxygen. Calcium and pH are considered key limiting factors for the establishment of their populations. Dreissenid species have higher calcium requirements when compared to other freshwater bivalves, which could be due to their high permeable skin that reduces the capacity to retain calcium in low calcium waters (Garton, McMahon, & Stoeckmann, 2013). The lower tolerance limits for calcium and pH for zebra mussels are expected to be 23-28 mg/L and 7.3-7.5, respectively (Karatayev & Burlakova, 2022). For quagga mussels, survival and growth has been observed at calcium levels of 12 ppm (equivalent to 12 mg/L; Davis et al., 2015), 17 mg/L (Seitz et al., 2023) and 22 mg/L (Kirkendall et al., 2021). Less information exists regarding their pH thresholds, however Claudi et al. (2012) demonstrated that a pH of 7.1 prevents settlement of quagga mussels in calcium rich waters. As the majority of habitats invaded by quagga mussels were already inhabited by zebra mussels, this suggests the pH tolerance for both species might be similar (Karatayev & Burlakova, 2022). While no lower temperature limits have been found for quagga mussels, zebra mussels cannot tolerate freezing temperatures and their lower limit is thus 0°C (Karatayev & Burlakova, 2022). The upper temperature limit for both species has been demonstrated to be between 28 and 33°C in different studies (Aldridge, Payne, & Miller, 1995; Allen, Thompson, & Ramcharan, 1999; Wong et al., 2012), with zebra mussels being more tolerant of higher temperatures (Karatayev & Burlakova, 2022). Their growth is hampered with temperatures < 10°C and > 30°C. Within this range, their growth rates increase with increasing temperature (Karatayev & Burlakova, 2022). High turbidity levels can inhibit growth and affect oxygen consumption and filtration rates of dreissenid mussels (Garton et al., 2013). MacIsaac and Rocha (1995) demonstrated that when exposed to high turbidity, zebra mussels increased the frequency of valve closures, suggesting irritation. Between both species, quagga mussels are thought to be more tolerant to higher turbidity than zebra mussels due to their ability to maintain higher filtration rates (Garton et al., 2013). Regarding conductivity, colonization potential and growth of zebra mussels is expected to be higher above 83 µS/cm (Chakraborti et al., 2013; O'Neill, 1996), and values are likely similar for quagga

mussels. This preference could be linked to their calcium needs, as high conductivity waters are often an indication of high mineral content such as calcium ions, which are essential for their shell integrity. Zebra and quagga mussels can tolerate salinity levels up to 6 and 4‰, respectively (Karatayev & Burlakova, 2022). The higher tolerance reported for zebra mussels suggest they might be able to more easily invade and survive in estuarine environments when compared to quagga mussels. Both dreissenid species exhibit sensitivity to oxygen depletion, which often prevents them from colonising stratified lakes where periods of hypoxia are frequent (Karatayev & Burlakova, 2022). The low tolerance observed for both species ranks among the lowest levels documented for freshwater bivalves (Garton et al., 2013). Between the two species, quagga mussels are more tolerant to lower oxygen levels, which could explain their ability to inhabit the profundal zone of lakes (Karatayev & Burlakova, 2022).

In addition to environmental variables, their growth and densities are influenced by physical attributes of the habitat such as water velocity, substrate availability and depth. They thrive in moderate water currents due to a constant supply of nutrients and oxygen (Karatayev & Burlakova, 2022), whereas high water velocities can prevent settlement of larvae, inhibit growth and decrease their abundances (Hasler et al., 2019). Between both species, zebra mussels are less susceptible to wave action and velocity (Hasler et al., 2019), likely due to their stronger byssal thread attachment when compared to quagga mussels (Peyer, McCarthy, & Lee, 2009) that minimises dislodgment. Zebra mussels prefer hard substrates, such as rocks, artificial structures or shells of other bivalves, although their attachment to soft substrates such as sand and sediments (e.g. Berkman et al., 1998, 2000) and even aquatic vegetation (e.g. Burlakova, Karatayev, & Padilla, 2006) has previously been recorded, while quagga mussels are able to colonise both soft and hard substrates in high densities (Karatayev et al., 2015). The growth rate of both dreissenid species decreases with increasing depth (Karatayev & Burlakova, 2022), and quagga mussels are expected to be more abundant in deeper waters when compared to zebra mussels (Mills et al., 1996).

Dreissenid mussels are expected to attain higher densities in canals when compared to lakes and reservoirs, due to a constant supply of food and oxygen and availability of stable substrates (Karatayev & Burlakova, 2022). The lowest densities are expected to

occur in rivers due to a high concentration of suspended inorganic matter, mobile substrates and water flow that prevents settlement of larvae (Karatayev & Burlakova, 2022). In lentic habitats, zebra mussel densities are typically higher in polymictic well-mixed lakes, with populations being more abundant in the littoral zone and reaching maximum densities between 1-6 meters, while in deep stratified lakes they are present in lower abundances (Karatayev et al., 2015; Karatayev & Burlakova, 2022). By contrast, quagga mussel densities are expected to be higher in stratified lakes (Karatayev & Burlakova, 2022), and they can be found in both the littoral and profundal zone, being more abundant in the latter (Karatayev et al., 2015).

When both species co-exist in the same site, the more common outcome is the decline of zebra mussel populations (e.g. Haltiner et al., 2022), however the co-existence of both species and even a predominance of zebra over quagga mussels (e.g. Rudstam & Gandino, 2020) have been recorded. The observed pattern will depend on different features such as food availability (Balogh et al., 2023), species-specific predation (Rudstam & Gandino, 2020) and lake morphometry (Karatayev et al., 2021a).

Predators of dreissenid adults include mainly fish (e.g. roach, bream, eel, sturgeon, etc.) and waterfowl (e.g. ducks, pochard, coots, etc.), although other animals such as crayfish, turtles, otters, and muskrats have been described to consume them (Karatayev & Burlakova, 2022). Similarly, dreissenid veligers are an important food source for several fish species, both in their adult (e.g. Chrisafi, Kaspiris, & Katselis, 2007) and larvae (e.g. Marano et al., 2023) stage, as well as for aquatic invertebrates such as shrimp (e.g. Winkler et al., 2007).

Impacts

The range of ecological impacts caused by dreissenid mussels has been widely documented, although more focus has been given to their negative impacts. While fewer studies have been conducted on quagga mussels, both species are considered to have similar impacts (Karatayev & Burlakova, 2022). Upon invasion, their filter feeding activity leads to a reduction of the phytoplankton biomass and a consequent increase in the concentration of phosphorus (Caraco et al., 1997; Effler et al., 2004) and increase in water clarity (Caraco et al., 1997; Effler et al., 2004; Zhu et al., 2006). This in turn

increases light penetration which is followed by an increase in aquatic vegetation (Caraco et al., 2000) and a shift of their abundance to deeper waters as light is able to reach greater depths (Zhu et al., 2006). Their strong filtration capacity also allows them to remove toxic substances such as heavy metals (e.g. Kocabaş, Başaran, & Kocabaş, 2024) and pathogenic organisms (e.g. Mezzanotte et al., 2016) from the water, and dreissenids can therefore be used as bioindicators of water quality in freshwater ecosystems. In contrast, their selective feeding and the excretion of nutrients from metabolic activities can contribute to the formation of toxic algal blooms in invaded sites (Raikow et al., 2004; Vanderploeg et al., 2001).

Invasion by dreissenids also leads to shifts in community composition, causing both positive and negative changes in population densities for different taxonomic groups. Their ability to cause physical changes to the habitat, mainly due to the formation of clumps (druses) at the bottom of the waterbodies, results in modifications of the benthic community of invaded sites (Ward & Ricciardi, 2007, 2013; Zaiko, Daunys, & Olenin, 2009), as these three-dimensional structures can provide refuge and protection from predators and abiotic stressors (Karatayev & Burlakova, 2022). Dreissenid mussels are also part of the diet of several species (see “Species ecology” section), whose populations may experience growth in invaded sites (e.g. Werner et al., 2005) due to an increase in food availability. In contrast, their filter feeding activity can negatively affect other suspension-feeders such as zooplankton (e.g. MacIsaac, Lonnee, & Leach, 1995; Pace, Findlay, & Fischer, 1998), benthic animals (e.g. Strayer & Smith, 2001) and some fish species (e.g. Cunningham & Dunlop, 2023), due to competition for food. Native mussel populations are also significantly impacted following invasion by zebra and quagga mussels. Not only do they compete for food and space, but dreissenids can attach to the shells of native mussels disrupting their valve functions and metabolic processes, which often leads to population declines and local extinctions (Ricciardi, Neves, & Rasmussen, 1998; Strayer & Malcom, 2007). Although most impacts have been documented for unionids (Karatayev et al., 2015; Karatayev & Burlakova, 2022) due to similar habitat preferences, other mussels such as sphaeriids can also be affected (e.g. Lauer & McComish, 2001; Strayer & Malcom, 2007).

Dreissenid mussels are also widely recognized for their fouling of water-related infrastructures such as drinking water treatment facilities and hydroelectric power plants (Prescott, Claudi, & Prescott, 2013), and the economic costs that are associated with it. Biofouling of such facilities and their components can lead to corrosion of pipes and cause water contamination following control operations (Mackie & Claudi, 2009). The accumulation of mussel fouling can reach a thickness of up to 15 centimetres in irrigation pipes, thus significantly reducing their diameter and water flow (Karatayev & Burlakova, 2022). Moreover, the downstream carryover of both live and dead mussels over time can cause clogging of filters and sieves (Karatayev & Burlakova, 2022), affecting the normal operation of drinking-water and power companies. Connelly et al. (2007) estimated that in North America, between 1989 and 2004, the total costs caused by zebra mussels in power plants and water treatment facilities was $267 million. The most recent estimates suggest that the worldwide and European costs of dreissenid mussels between 1980 and 2020 are around $51 billion and $55 million, respectively (Haubrock et al., 2022). Although the economic impacts of dreissenid biofouling are higher in water-related companies, other industries such as recreational facilities, navigation locks and fish hatcheries are also affected to a lesser extent (O'Neill, 1997).

Dispersion pathways

Both dreissenid species use similar pathways for dispersal, which can include either human-mediated (e.g. ballast water, fishing gear, recreational boating, etc) or natural (downstream transport of veligers or attachment to other species/debris) methods. However, their species-specific ecology can offer different advantages. For example, the longer planktonic stage of quagga mussels (Wright et al., 1996) suggest they can remain in the water column for longer periods of time and thus travel longer distances when compared to zebra mussels. On the other hand, zebra mussels are known to have a stronger byssal thread attachment (Collas et al., 2018; Peyer et al., 2009), which offers an advantage for overland dispersion (e.g. attached to boat hulls), as they are less prone to dislodgment.

Human-mediated transport is considered the main vector for dreissenid spread (Mallez & McCartney, 2018). Aquatic recreational activities play a significant role in their dispersion, with individuals being transported attached not only to boats (e.g. De

Ventura et al., 2016) but also to leisure equipment such as fishing gear (Banha et al., 2016). In Switzerland, De Ventura et al. (2016) demonstrated that the transport of recreational boats posed a significant pathway for zebra mussel dispersal within waterbodies, as well as for the invasion of quagga mussels. Similarly, a recent study in Great Britain demonstrated that distance to boat ramps was significant in predicting establishment of zebra mussels, followed by distances to large cities and distance to ports (Rodríguez-Rey et al., 2021). Further to recreational activities, the transport of commercial vessels represents a major pathway, and ballast water has been suggested as the introduction pathway of quagga mussels in Western Europe (Bij de Vaate, 2010).

Although to a lesser extent, natural downstream transport of veligers also represents an important dispersion pathway. This has been demonstrated to be the cause of invasion in different studies (e.g. Horvath et al., 1996; Johnson, Bossenbroek, & Kraft, 2006) and its effect is amplified in areas where there are multiple interconnected waterbodies. Other natural vectors such as the transport by aquatic animals or birds have previously been suggested (Johnson & Carlton, 1996) and recently a zebra mussel was observed attached to the scales of a fish in Canada (Ricciardi & Hill, 2023). Nevertheless, its role in dreissenid spread is expected to be minimal when compared to human-mediated vectors (Banha et al., 2016).

Management and control

Preventive actions are often the most efficient and cost-effective solutions against dreissenid mussels (Karatayev & Burlakova, 2022). This can include the construction of physical barriers between waterbodies, or educational programs for boat owners and the general public (Connelly et al., 2007) to increase awareness of the negative impacts posed by dreissenids. Additionally, the implementation of proper biosecurity measures such as “check, clean, dry” campaigns is essential to minimise their spread. Studies have shown that treatments with aquatic disinfectants (Virasure and Virkon) and thermal shock (immersion in hot water, steam spray, hot air and dry ice) are effective at killing dreissenid adults (Coughlan et al., 2020; Shannon et al., 2018) and should thus be applied to vessels and equipment that is transported between waterbodies. These measures should be a priority in high risk sites, i.e., with the ideal environmental conditions for their establishment and with frequent aquatic leisure activities.

When preventive measures fail and dreissenids are able to invade and establish in a new location, eradication and control measures should focus on areas where economic costs and negative impacts to humans are higher (Karatayev & Burlakova, 2022). Different techniques can be employed to manage their numbers, which can include mechanical, physical, chemical, biological and genetic control measures. Mechanical methods consist mainly of the manual removal of individuals through scrubbing, scraping or water jets, although these can be labour intensive and are only moderately effective (Karatayev & Burlakova, 2022). Physical methods can include manipulating water levels (e.g. Tucker et al., 1997), water velocity, temperature or hypoxia (Karatayev & Burlakova, 2022). The use of chemical treatments has also been commonly used to control dreissenid populations (e.g. Cope, Bartsch, & Marking, 1997; Costa et al., 2008; Waller et al., 1993), however the release of toxic compounds into the water impacts other co-occurring species and has ecological impacts. An environmentally friendly alternative, the "BioBullets" (Aldridge, Elliott, & Moggridge, 2006), encapsulates the active ingredient (potassium chloride) in microscopic edible particles that are filtered by mussels, thus decreasing the risk of water pollution. A biological control approach using a strain of the bacteria *Pseudomonas fluorescens* has also been developed and showed to be effective and specific to dreissenids (Molloy et al., 2013a, 2013b). More recently, the use of genetic biocontrol strategies has been suggested as an alternative and species-specific approach (Elizárraga et al., 2023), however there are still some limitations that need to be addressed before it can be applied in the field. The most appropriate method will depend on the resources available, population densities and site characteristics.

Dreissenids in the UK

The first records of zebra mussel in the UK date back to 1824 in Surrey (Greater London) and Wisbech (Cambridgeshire), where they were supposedly used as bait for fishing perch (Aldridge, Elliott, & Moggridge, 2004; Coughlan, 1998). Between 1831-1834 they were further discovered in Yorkshire, Glasgow and near Edinburgh, and the number of records continued to increase in the following years (Aldridge et al., 2004). Their populations remained stable from 1850-1950, however their distribution started to expand again in the early 2000s, likely due to the increases in water quality or increases

in connectivity between waterways (Aldridge et al., 2004). Currently, they are prevalent and widespread throughout the country (Figure 1.2).

Quagga mussels were first recorded in September 2014, following routine monitoring conducted by the Environment Agency in the Wraysbury River, a tributary of the River Thames, in Surrey (Aldridge, Ho, & Froufe, 2014). As the Wraysbury River at the time was not accessible to the public and did not accommodate recreational activities (e.g. fishing), their invasion pathway was unclear (Aldridge et al., 2014). Following their initial discovery, and as predicted by Aldridge et al. (2014) due to the connectivity of the river Thames with the wider canal and river network, they were later found at several reservoirs and waterways close to London (National Biodiversity Network Trust, 2023) and further north in the East Midlands (Environment Agency, 2020; Aldridge, 2023) (Figure 1.2).

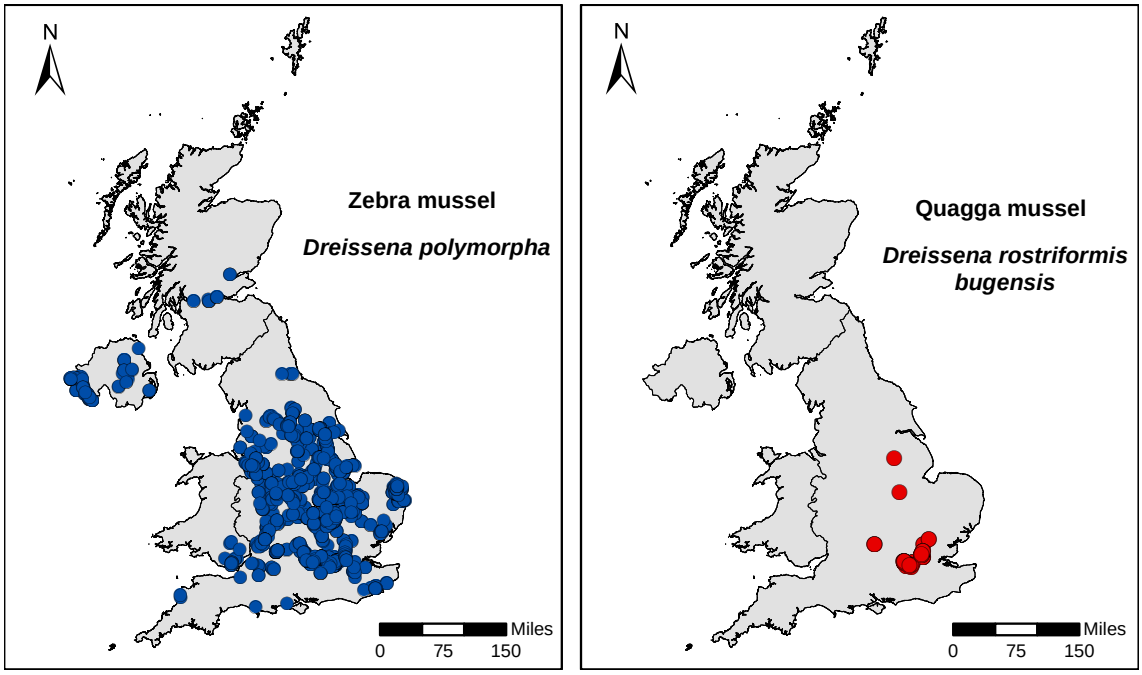

Figure 1.2 Distribution of zebra mussels (left) and quagga mussels (right) in the UK. Except for the two most northern points for quagga mussels (Environment Agency, 2020; Aldridge, 2023), all data for both species was retrieved from the National Biodiversity Network (NBN) Atlas (as of December 6th, 2023).

In the UK, the costs of zebra and quagga mussels have been estimated to be 12 million and 100 thousand pounds, respectively, in 2021 (Eschen et al., 2023). For water companies, costs of control and removal of zebra mussels increased from 1 million pounds in 2005 to more than 5 million pounds in 2014 (Dobson & Riddick, 2015). Due to

their potential for spread and negative ecological and economic impacts, both species are currently considered high priority for monitoring in the UK (Blackman et al., 2020a), particularly for water companies (Aldous et al., 2016), and the use of sensitive monitoring tools that allow early detection are needed.

1.3 Environmental DNA

Definition and benefits

Environmental DNA (eDNA) is defined as DNA that can be collected and extracted from environmental samples such as water, soil, or air (Barnes & Turner, 2016; Taberlet et al., 2012), as animals interact with their surroundings and leave traces of their DNA behind. The first eDNA studies date back to the 1980s, where researchers were able to extract DNA from sediment and water samples (e.g. Ogram, Sayler, & Barkay, 1987; Somerville et al., 1989; Steffan et al., 1988). However, only at the beginning of the 21st century did eDNA research gained more attention from the scientific community. Initially, Willerslev et al. (2003, 2007) showed it was possible to recover DNA from ice cores and permafrost samples, while Ficetola et al. (2008) was able to detect the invasive American bullfrog (*Rana catesbeiana*) from freshwater samples. Following that, the number of eDNA studies increased exponentially over the years and this technique is now widely adopted for monitoring a large number of species, offering a more sensitive and cost-effective approach than traditional surveys (Fediajevaite et al., 2021).

The analysis of eDNA samples has been previously used for the detection of both dreissenid species (e.g. Amberg et al., 2019; Ardura et al., 2017; Blackman et al., 2020a; De Ventura et al., 2017; Gingera et al., 2017; Sepulveda, Amberg, & Hanson, 2019), offering several advantages over conventional monitoring approaches. The increased sensitivity is one of the main advantages of eDNA methods for dreissenid monitoring, as detection can occur even at low densities (e.g. Blackman et al., 2020a; Gingera et al., 2017), thus providing an early warning system and ensuring a quicker intervention before populations are established. Moreover, detection is possible even during their early microscopical larval stage, which would otherwise remain undetected. Other important benefits of eDNA include the reduced risk of unintentionally facilitating their spread to new locations, as the field equipment used in eDNA-based surveys is sterilised

to minimise contamination (Herder et al., 2014). Additionally, taxonomic expertise is not necessary for species identification as this is based on molecular tools rather than morphological traits (Herder et al., 2014). This is key when monitoring dreissenid mussels, as differentiating between the two species in their adult stage can be challenging due to their similar morphology, while veligers cannot be distinguished visually and require molecular methods for accurate identification.

Ecology of eDNA

The ecology of eDNA includes four main areas – its origin (e.g. reproduction, excretion), state (e.g. intracellular, extracellular), fate (i.e. degradation/persistence) and transport (e.g. downstream, settling) in the environment (Barnes & Turner, 2016). Understanding and accounting for the factors influencing each area of eDNA ecology will enhance our ability to make spatiotemporal inferences about species presence and result in optimized monitoring strategies. Ultimately, this will contribute to a more effective use of eDNA for management of INNS.

eDNA in the environment can originate from various metabolic sources, such as excretion (faeces, urine), reproduction (gametes), secretion (skin, body fluids) or decomposition (carcasses) (Barnes & Turner, 2016). Understanding how eDNA production correlates with other factors, such as biological activity or population sizes, can inform future sampling strategies. For example, the increase in gamete production during the breeding season results in higher eDNA concentrations in the water, and thus higher detection rates. The seasonal variation of eDNA has been shown in several studies (De Souza et al., 2016; Lawson Handley et al., 2019; Spear et al., 2015) and accounting for this can maximize the success of eDNA-based surveys by focusing sampling on specific periods when genetic material accumulates. Similarly, other factors such as biomass and feeding behaviour have also shown to positively influence eDNA production rates (Klymus et al., 2015; Takahara et al., 2012).

Once released into the environment eDNA can be found in different sizes and physical states, and is believed to transition from intracellular to extracellular state over time (Barnes & Turner, 2016). Previous studies in aquatic systems looking at particle size distribution have demonstrated that eDNA is present across several size fractions (e.g.

Barnes et al., 2021; Cooper et al., 2022; Jo et al., 2019; Turner et al., 2014), suggesting a mixture of multiple physical states in the environment (e.g. intact cells, organelles, freely dissolved DNA, bound to particles). An understanding of the state and size distribution of eDNA and other non-target particles such as PCR inhibitors in the environment can help select appropriate filter pore sizes and sampling methods that allow the capture of target eDNA while avoiding unwanted particles, thus maximising efficiency (Barnes & Turner, 2016).

The fate of eDNA in aquatic systems, i.e. its degradation and persistence rates, can be influenced by several environmental variables, such as temperature, pH, microbial and enzymatic activity, UV radiation and sediments. The effect of temperature on eDNA has been extensively investigated and studies consistently demonstrate that higher temperatures promote eDNA degradation (Eichmiller, Best, & Sorensen, 2016; Goldberg, Strickler, & Fremier, 2018; Kasai et al., 2020; Strickler, Fremier, & Goldberg, 2015; Tsuji et al., 2017). Studies have shown that temperatures above 20°C (Strickler et al., 2015) and 25°C (Goldberg et al., 2018) increase eDNA degradation, while decay rates are expected to be lower at 5°C (Eichmiller et al., 2016; Strickler et al., 2015). The effects of pH on eDNA have also been commonly investigated and results have been consistent across studies. Lower eDNA degradation rates are expected under alkaline settings (Strickler et al., 2015), while acidic conditions promote eDNA decay rates (Goldberg et al., 2018; Seymour et al., 2018). The effects of temperature and pH on eDNA persistence in the environment have been attributed to interactions with other environmental factors such as microbial and enzymatic activity (Joseph et al., 2022). Recent studies have shown that the presence of microbial communities and high microbial activity promote eDNA degradation (Beattie et al., 2023; Zulkefli, Kim, & Hwang, 2019). Similarly, Saito & Doi (2021) demonstrated that the absence of microbes and extracellular enzymes in purified water resulted in lower eDNA degradation rates when compared to sea and pond water samples. The effects of microbial and enzymatic activity on eDNA persistence are partially linked. Higher microbial activity is likely followed by an increase in DNase production that degrades DNA, and components such as carbon, nitrogen and phosphorus from DNA molecules are used by microbes for their metabolisms (Beattie et al., 2023; Zulkefli et al., 2019). Additionally, excretion products resulting from microbial activity can disrupt covalent bonds in DNA molecules promoting its

degradation (Joseph et al., 2022). UV radiation can also influence eDNA persistence in the environment by inducing different types of damage to DNA molecules (Cadet, Sage, & Douki, 2005) thus accelerating degradation. Previous findings show that exposure to UV radiation significantly reduces eDNA availability when compared to samples protected from the light (Pilliod et al., 2014). Similarly, Strickler et al. (2015) demonstrated that lower UV levels result in lower eDNA degradation rates. The fate of eDNA in the environment can also be affected by the presence of sediments. As they bind to eDNA molecules, which protects them from nuclease activity and other external stressors, and simultaneously bind to nucleases themselves, inactivating them (Barnes et al., 2014; Joseph et al., 2022), the persistence of eDNA in the environment is extended. Additionally, since different sediment types have different DNA binding capacities (Levy-Booth et al., 2007), with finer sediments expected to retain DNA more efficiently that coarse substrates (Jerde et al., 2016), the presence of specific sediment types can equally impact the persistence of eDNA.

The transport of eDNA in aquatic systems includes both horizontal (i.e. with the water current, downstream) and vertical (i.e. in the water column, settling) dispersion. Horizontal transport is usually restricted in lentic systems such as lakes, ponds and reservoirs (Harrison, Sunday, & Rogers, 2019), where eDNA tends to accumulate near the target organism (e.g. Dunker et al., 2016). In contrast, flowing waters such as rivers, canals and streams are characterized by higher dispersion and dilution of eDNA (Herder et al., 2014). Previous studies in rivers and streams have reported the downstream transport of eDNA over distances of 12.3 km (Deiner & Altermatt, 2014) and 22.8 km (Villacorta-Rath et al., 2021) from the source population, as well as a dilution in detection rates with increasing distance to the eDNA source (Blackman et al., 2020b; Deiner & Altermatt, 2014). The horizontal transport of eDNA is thus expected to have a bigger impact in lotic systems. Conversely, the vertical transport of eDNA is likely more important in lentic systems, with a previous study demonstrating that in experimental ponds eDNA accumulated in aquatic sediments when compared to surface water (Turner, Uy, & Everhart, 2015). As eDNA exists in different physical forms (Barnes & Turner, 2016), the settling rate will differ according to the density and size of particles (Harrison et al., 2019). Water stratification can also impact the vertical movement of eDNA and sampling at different depths can result in the detection of different species in

both freshwater (e.g. Lawson Handley et al., 2019) and marine (e.g. Jeunen et al., 2020) environments.

In addition to the ecology of eDNA, other factors can equally impact eDNA detectability. For example, high levels of sediments and suspended matter might accelerate clogging of filters, thus reducing the volume of water filtered (Peixoto et al., 2021) and consequently the concentration of eDNA captured and detection probabilities. Downstream laboratory procedures can also be negatively affected by turbid waters, as sediments can clog extraction columns and the high concentration of PCR inhibitors can decrease PCR efficiency (Harper et al., 2019a), thus decreasing detection rates. Likewise, calcium-rich waters might result in lower detection rates as calcium ions are able to inhibit the activity of polymerase enzymes due to competition with the cofactor magnesium, thus decreasing PCR amplification success (Opel, Chung, & McCord, 2010).

Previous dreissenid research

Several eDNA assays already exist for the detection of zebra and quagga mussels (Feist & Lance, 2021), and these can be categorized into targeted or passive approaches. Targeted methods focus on the detection of a single species, with quantitative PCR (qPCR) being the most common method used for dreissenid monitoring (e.g. Amberg et al., 2019; Blackman et al. 2020b; Gingera et al., 2017; Sepulveda et al., 2019) due to its high sensitivity. Other targeted methods such as standard PCR (Blackman et al. 2020a), droplet digital PCR (Watts, 2020) and loop-mediated isothermal amplification (Williams et al., 2017) have also been successfully used. Passive approaches such as high-throughput sequencing (HTS), by contrast, are able to amplify DNA from a broad range of species, and this method is also commonly used for dreissenid detection (e.g. Blackman et al. 2020b; Klymus, Marshall, & Stepien, 2017; Marshall & Stepien, 2019).

Research gaps

As research on dreissenid mussels using eDNA methods progresses, there are still several gaps and areas where further investigation is needed. For example, understanding how different environmental variables affect eDNA degradation and detection needs to be further explored, as well as investigating the temporal and spatial dynamics of dreissenid eDNA, i.e. how it degrades over time and space, which can help

inform future monitoring campaigns. Exploring the advantages offered by the combined use of eDNA and environmental RNA (eRNA) for dreissenid monitoring is also lacking. Additionally, due to their potential for negative impacts, focusing on techniques with higher sensitivity (e.g. digital PCR) and faster turnaround times (e.g. on-site testing) is also needed, thus allowing early species detection and prompting faster responses, respectively. Further unexplored areas for investigation include the use of eDNA to identify key pathways for dreissenid spread, evaluating the impact of dreissenid mussels on the structure and function of invaded ecosystems at the whole ecosystem level, and improving techniques to quantify eDNA concentration and correlate it with mussel population sizes and densities, to better estimate actual abundances.

In addition to the research questions previously mentioned, the need for further standardization and validation of targeted eDNA assays has also been recently highlighted as an outstanding challenge (Thalinger et al., 2021), although this is not dreissenid-specific. In their work, Thalinger et al. (2021) emphasised the lack of specific guidelines to help end-users assess the validation and suitability of eDNA assays, therefore preventing their incorporation in routine monitoring. To tackle this gap, the authors developed a 5-level validation scale where the readiness of targeted eDNA assays for use in routine monitoring can be defined as ranging from “incomplete” (level 1) to “operational” (level 5), depending on the criteria they meet (Figure 1.3). The greater the validation level attributed to an assay, the higher the confidence in the results. For example, failure to detect the target species at level 3 (“essential”) does not indicate its absence and a positive detection requires additional steps to validate the result (e.g. sequencing amplicons), while at levels 4 and 5 a positive and negative detection are a strong indication that the target species is present or absent, respectively.

Together with the development of the validation scale, Thalinger et al. (2021) conducted a meta-analysis to assess the validation level of all targeted eDNA assays published up until April 2019. For zebra mussels, the majority of assays were placed at level 3, i.e. “essential”. From these, the species-specific qPCR assay developed by Gingera et al. (2017) targeting the cytochrome b (*Cyt* b) gene meets all minimum criteria for level 4 (“substantial”) with the exception of the calculation of limits of detection. For quagga

mussels, surprisingly, half of the assays analysed by the authors failed to meet the minimum criteria for level 1. Other species-specific assays targeting quagga mussels have since been developed and showed great promise. Specifically, the DRB1 assay targeting the cytochrome oxidase I gene (COI) currently meets the minimum requirements of levels 1-3 (Blackman et al., 2020a, 2020b) and its validation level is thus considered as "essential".

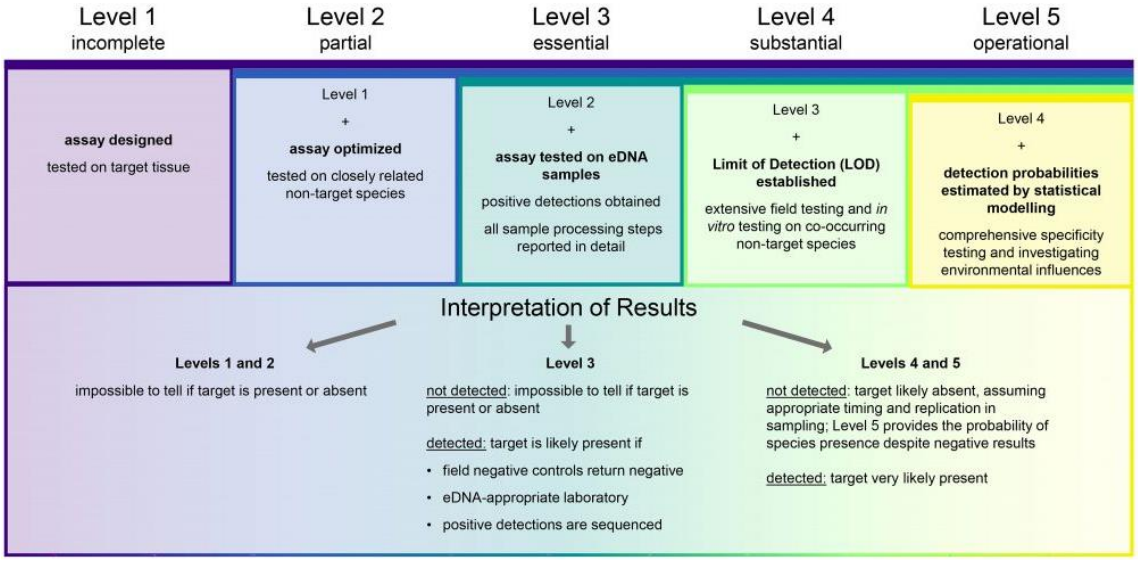

Figure 1.3 Overview of the 5-level validation scale, including the requirements needed to achieve each validation level and how to interpret the results at each level. Figure retrieved from Thalinger et al. (2021) under a Creative Commons Attribution-NonCommercial license (CC BY-NC).

The development of the validation scale will aid stakeholders and managers to make informed decisions when selecting eDNA assays to use in monitoring programs, ensuring the results are reliable. For dreissenid mussels, continuous surveillance programs using operational assays can work as early warning systems and flag up the presence of individuals in newly invaded sites, allowing to quickly implement biosecurity and control measures on site. Moreover, information obtained from eDNA-based surveys can aid water companies and regulatory agencies to more effectively evaluate the environmental impacts of activities that may promote the spread of dreissenid mussels (e.g. water transfers between waterbodies, fishing licences, etc). Ultimately, this will contribute to a more effective use of eDNA for management of invasive species, thus protecting ecosystems and infrastructures from their negative impact and reducing costs.

1.4 Thesis aim and overview

In this thesis, I aimed to increase the readiness of targeted eDNA assays for routine monitoring of both dreissenid species. For zebra mussels, this included establishing limits of detection, assessing the influence of species seasonal activity, waterbody type and several environmental variables on eDNA detectability, calculating detection probabilities and conducting *in silico* tests with the Gingera et al. (2017) species-specific *Cyt* b assay. For quagga mussels, the DRB1 assay (Blackman et al., 2020a, 2020b) was further developed to a probe-based qPCR assay in order to increase specificity. Following this, *in vitro* tests, extensive field testing and assessment of limits of detection were conducted. We were able to increase the validation level of both assays to level 5 and 4, respectively, which increases the reliability of results and allows their use in a management context with increased confidence. The work developed here is thus an important step towards the operational use of eDNA for monitoring dreissenid mussels and will help inform future eDNA monitoring campaigns.

Chapter 2

In the first data chapter, we aimed to establish limits of detection for the Gingera et al. (2017) species-specific *Cyt* b assay, assess the temporal dynamics of zebra mussel eDNA in relation to mussel activity, and investigate the influence on eDNA concentration of environmental variables that are relevant to both zebra mussel and eDNA ecology, as part of the requirements for levels 4 and 5 of the validation scale (Thalinger et al., 2021). Water samples were collected every month, for one year, from a river and a reservoir in Yorkshire, England. We hypothesize that eDNA detection and concentration would peak in the summer due to spawning and veliger production, and be greater in the reservoir than the river due to differences in eDNA transport and dilution.

Chapter 3

Following the findings from our previous chapter that highlighted differences in eDNA detectability between the river and reservoir, in this chapter we aimed to increase the number of sampling sites and waterbody types to provide more spatial variation and higher statistical power, in order to investigate the influence of environmental variables on eDNA detection and concentration. As required for level 5 of the validation scale (Thalinger et al., 2021), we also used statistical modelling to estimate detection

probabilities and conducted *in silico* tests to further confirm assay specificity. Water samples were collected from 20 sites spread throughout England, including lakes, reservoirs, rivers and canals. We hypothesize that detection rates will be mostly influenced by waterbody and substrate type, being higher in lentic than lotic systems sites due to differences in eDNA transport and dilution, and close to hard substrates due to zebra mussel preference.

Chapter 4

In the third data chapter, we focused on quagga mussels and further developed the DRB1 assay (Blackman et al., 2020a, 2020b) from a dye-based to a probe-based qPCR assay. Following this, we performed *in vitro* tests on non-target species, estimated limits of detection and conducted extensive field testing, as required for level 4 of the validation scale (Thalinger et al., 2021). Samples were collected from 24 sites spread throughout England, including sites with established populations near London and sites spread across the East Midlands and East Anglia regions, and we were able to obtain an updated distribution of quagga mussels in England.

Chapter 5

In the final chapter, I summarize the progress achieved for both assays tested in this thesis and outline further research needed. I discuss promising technologies for future monitoring of zebra and quagga mussels, as well as the incorporation of eDNA-based outcomes into INNS management. Finally, I provide recommendations for future dreissenid eDNA monitoring campaigns based on our observations.

Chapter 2

Seasonal variation in zebra mussel (*Dreissena polymorpha*) detection with environmental DNA: detection rates are mostly influenced by temperature in a reservoir and river

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2.1 Abstract

Invasive species are a worldwide threat to wildlife, ecosystems and infrastructure. Zebra mussels (*Dreissena polymorpha*) are considered a priority species for monitoring due to their rapid spread and multiple ecological and economic impacts on invaded ecosystems. The use of sensitive monitoring techniques such as environmental DNA (eDNA) is essential to ensure early detection and is highly promising for zebra mussel monitoring. eDNA production and persistence in the environment is influenced by several biotic and abiotic factors. Understanding how these factors affect species detection is crucial to minimise false negative results. Here we investigated the temporal dynamics of zebra mussel eDNA and the influence of several environmental variables on species detection. Ten water samples were collected monthly across one year from a river and a reservoir with known zebra mussel populations, along with several environmental variables, and tested with a species-specific qPCR assay. Zebra mussels were detected throughout the year in both sites, with a strong peak in average eDNA concentration in the summer, corresponding to peak temperatures for spawning and larval production. Average eDNA concentration was positively correlated with temperature and volume filtered in both waterbodies. Turbidity and conductivity were positively correlated with average eDNA concentration in the reservoir but negatively correlated in the river. Temperature was the only significant predictor in linear mixed-effects models, and it was only significant

for the reservoir. Our results indicate that zebra mussels can be detected all year round but monitoring in the summer maximises detection. We also highlight a difference in detection levels between sites, more consistent in the reservoir, likely due to differences in eDNA transport and dilution, or substrate type impacting mussel attachment.

2.2 Introduction

Invasive non-native species (INNS) are one of the five major drivers of global biodiversity loss (IPBES, 2023). Impacts of INNS are complex and vary depending on the species, trophic levels and ecosystems they invade, and can include changes to nutrient cycling, ecosystem productivity, community composition and trophic networks (Pyšek et al., 2020). Moreover, they predate on native species and outcompete them for food and resources, which ultimately can lead to their extinction (Bellard et al., 2016). The global economic cost of INNS has been estimated to be $1.2 trillion between 1980-2019, a similar order of magnitude as natural hazards such as floods and earthquakes (Turbelin et al., 2023).

The zebra mussel (*Dreissena polymorpha,* Pallas, 1771) is a freshwater mussel species native to the Ponto-Caspian region in Eastern Europe, but is currently widespread and invasive in most of Europe and North America. Their success as invaders is partially associated with their life cycle. Once water temperature reaches 12°C, zebra mussels start spawning (Marsden, 1992). During this period, females can release between 300 thousand and 1 million eggs into the water column (Karatayev, Burlakova, & Padilla, 2013; Karatayev & Burlakova, 2022) and zebra mussel larvae (veligers) can reach densities of up to 9000 individuals per litre in areas with high adult mussel density (Karatayev & Burlakova, 2022). In addition, individuals reach sexual maturity and are able to reproduce again within 3-11 months (Lvova & Makarova, 1994). These characteristics allow zebra mussels to rapidly proliferate and establish in recently invaded ecosystems.

Zebra mussels are also ecosystem engineers and are able to significantly modify the availability of resources for other species and alter the physical structure of ecosystems. They are highly efficient suspension feeders, reducing suspended particulate matter and plankton availability in the water column, which can impact other species that rely on

the plankton community as their food source (e.g. Cunningham & Dunlop, 2023). Moreover, adult mussels attach to hard substrate and form three-dimensional structures at the bottom of waterbodies, which can affect benthic communities (Ward & Ricciardi, 2007). Zebra mussels are arguably most notorious for their fouling of water-related infrastructures including water treatment facilities, irrigation systems and power stations, in which they can greatly reduce the diameter of pipes, accelerate corrosion and cause water contamination (Karatayev & Burlakova, 2022). Recent estimates suggest a global cost of zebra mussels of $19.3 billion between 1980 and 2020 (Haubrock et al., 2022). Due to their negative impacts and rapid spread, zebra mussels are considered a priority species for management in several countries (Blackman et al., 2020a) and continuous monitoring is essential.

Common monitoring methods for zebra mussels include searching for veligers in the water column during their breeding season and searching for adults attached to the substrate or hard structures (Karatayev & Burlakova, 2022). More recently, the use of videography has been suggested (Karatayev et al., 2021b) as an additional tool to assess the distribution and density of zebra mussels. While these methods are useful, searching for veligers is restricted to the summer, when they are present in the water in detectable densities, and the use of videography can be restricted in sites with limited visibility (Karatayev et al., 2021b). Moreover, expertise is needed to correctly identify veligers and adults. The use of sensitive techniques that allow early detection is thus essential for monitoring of zebra mussels in order to prevent establishment, minimise their impacts and reduce eradication and control costs.

Environmental DNA (eDNA) is described as DNA that is released into the environment, as organisms interact with their surroundings. It can be collected from a range of different habitats, such as marine (e.g. Aglieri et al., 2021), freshwater (e.g., Goldberg et al., 2018), aerial (e.g. Lynggaard et al., 2022) and soil (e.g. Evrard et al., 2019). The use of eDNA methods for INNS monitoring is a cost-effective and sensitive approach, often outperforming traditional monitoring techniques (e.g. Blackman et al., 2020b). Due to its benefits, the use of eDNA for monitoring INNS has rapidly increased over the past years and it has been successfully applied in different ecosystems and targeting species

from several taxonomic groups (Blackman, Hänfling, & Lawson-Handley, 2018), demonstrating the adaptability and potential of this tool.

Several eDNA assays have already been developed and successfully used for the detection of zebra mussels from aquatic systems (Feist & Lance, 2021). The most common detection methods include quantitative PCR (qPCR) and high-throughput sequencing, however standard PCR (Blackman et al. 2020a) and loop-mediated isothermal amplification have also been successful (Williams et al., 2017). Probe-based qPCR assays targeting zebra mussels were initially developed by Tucker (2014) and Bollens, Rollwagen-Bollens, & Goldberg (2015), however they were not zebra mussel-specific and have never been tested in zebra mussel-infested waters, respectively. More recently, assays developed by Gingera et al. (2017) and Amberg et al. (2019) have proven to be species specific and highly sensitive, and gained the most popularity.

Wider testing and understanding of eDNA detectability are required in order for these assays to be used in operational settings. Thalinger et al. (2021) developed a 5-level validation scale where the readiness of eDNA assays for use in routine monitoring can be evaluated using a set of specific criteria. Assays can be defined ranging from “incomplete” (level 1) to “operational” (level 5), and the greater the level of validation, the higher the confidence in the results. Understanding the ecological and physical factors influencing detection of eDNA in the environment is a key requirement for level 5 validation, which together with statistical modelling of detection probabilities will render the assays operational.

Several environmental variables such as (but not limited to) waterbody type, temperature, pH, calcium and turbidity can jointly impact both the growth and longevity of zebra mussels (Karayatev & Burlakova, 2022) and the persistence/degradation of eDNA in the environment (Barnes & Turner, 2016). Understanding the influence of environmental variables on the detection and concentration of zebra mussel eDNA is therefore essential for designing and interpreting eDNA surveys and minimising false negatives. Densities of zebra mussels are expected to be higher in reservoirs than in rivers due to the lower availability of hard substrates in the latter. Rivers are more commonly (but not exclusively) constituted by soft substrates such as silt, clay, and sand,

while reservoirs are generally man-made and thus contain harder substrates such as concrete. The downstream transport of veligers in rivers also prevents their settlement and the establishment of high population densities (Karayatev & Burlakova, 2022). Moreover, the dispersion and dilution of eDNA in flowing waters such as rivers can result in lower detectability (Herder et al., 2014), when compared to reservoirs. Increases in water temperature are key for triggering zebra mussel reproduction, but have also been shown to have a positive effect on DNA degradation, and could therefore negatively impact detection (e.g. Tsuji et al., 2017; Kasai et al., 2020). pH and calcium are considered the most important parameters for zebra mussel establishment, with mussels having low tolerance for pH < 7.3-7.5 and calcium < 23-28 mg/L (Karayatev & Burlakova, 2022). As eDNA degradation is also more rapid in acidic conditions (Seymour et al., 2018) it is fair to assume that zebra mussel eDNA detection will be reduced at low pH. The relationship between detection of zebra mussel eDNA and turbidity is potentially more complex. Low turbidity is characteristic of high mussel densities due to their filter feeding capacity (Karatayev & Burlakova, 2022). However, suspended sediments in high-turbid sites can bind to and protect DNA from degradation (Barnes et al., 2014), potentially leading to higher detection rates, while simultaneously can cause filters to clog more quickly, thus reducing the volume filtered and lowering detection rates.

In addition to environmental variables, the seasonal activity of zebra mussels can equally influence eDNA availability in the water. Previous studies have observed higher zebra mussel eDNA detection levels in the autumn compared to the spring (Gingera et al., 2017; Peñarrubia et al., 2016), likely due to a high density of veligers in the water following spawning in the summer. A similar study found that dreissenid detection probabilities were higher in July, when water temperature favoured spawning, compared to June and October (Sepulveda et al., 2019). Despite providing valuable results that can be used to inform future monitoring campaigns, the temporal replication in these studies was reduced and detection rates beyond these temporal windows are not well known.

To our knowledge, no studies have carried out continuous sampling throughout the year to better understand the seasonal variation of zebra mussel eDNA. Moreover, environmental variables relevant to zebra mussel growth and biology as well as eDNA

persistence, such as water temperature, pH, calcium or turbidity have not been thoroughly explored yet. In this study, we aimed to increase the readiness of the Gingera et al. (2017) cytochrome b (*Cyt* b) assay for routine monitoring, by investigating the influence of environmental variables on eDNA concentration, as required for level 5 validation on the scale of Thalinger et al. (2021), and assess how zebra mussel eDNA fluctuates throughout the year. Water samples were collected every month, over the course of one year, from one river and one reservoir in Yorkshire, England. We hypothesize that eDNA detection and concentration would 1) be greater in the reservoir than the river, 2) peak in the summer and be positively associated with temperature in relation to spawning and veliger production, 3) be positively associated with pH, calcium, and species abundance, and 4) be influenced to some extent by turbidity and volume filtered. The work conducted here will help inform future eDNA monitoring campaigns by providing details on the seasonal variation of zebra mussel eDNA and provide an understanding of how environmental variables influence zebra mussel detection.

2.3 Materials and methods

Sample collection and processing

Two sites in Yorkshire, England, were selected for this study: Eccup Reservoir (Leeds; 53.87095, -1.54606) and the River Hull (Tickton; 53.85529, -0.39353) (Figure 2.1). Eccup Reservoir is a storage reservoir with a surface area of 79 hectares, a maximum depth of 19 metres and able to retain approximately 7 million m^3 of water. The River Hull is a navigable river that runs through East Riding of Yorkshire for approximately 40 kilometres and flows into the Humber Estuary. Both sites have confirmed zebra mussel presence, with the first records dating back to 2015 and 2017, respectively (National Biodiversity Network Trust, 2023). At each site ten sampling locations were selected (Figure 2.1) and samples were collected every month from each location, from December 2020 until November 2021. Sampling locations at Eccup Reservoir were chosen based on accessibility to the water and in order to maximise the perimeter of the reservoir sampled. At the River Hull, samples were collected every 100 meters over a 1 kilometre stretch of a publicly accessible area.

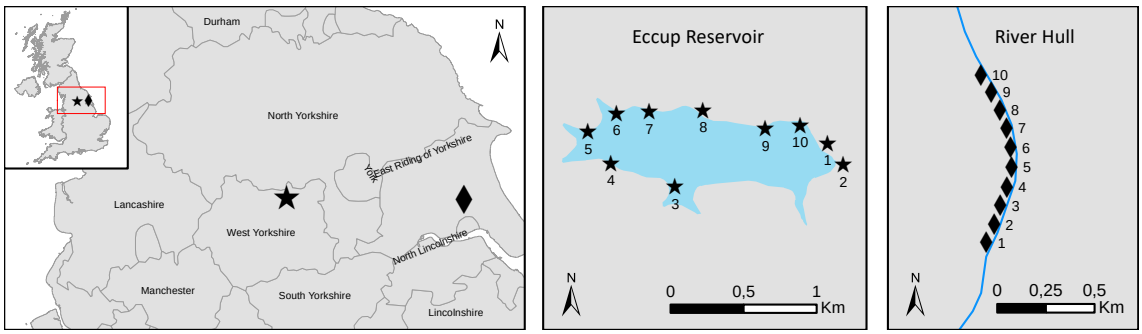

Figure. 2.1 (Left) Location within the UK of the two sampling sites selected for this study – Eccup Reservoir (star) and River Hull (diamond); (middle and right) ten sampling locations selected for Eccup Reservoir and River Hull, respectively.

At each sampling location, 4 subsamples of approximately 500 mL of surface water were collected into a sterile 2 L plastic bottle (Gosselin Square HDPE Graduated 2 L Bottles; Fisher Scientific, UK). Samples were stored in a bleach-sterilised cool box with ice packs and transported to the laboratory on the same day of collection. To monitor for contamination, 2 L of purified water were brought to the field each month and for each site (n = 24), and treated the same as eDNA samples. Gloves were worn at all times and changed between samples. Additionally, sample collection was done from downstream to upstream at the River Hull to avoid downstream contamination of the samples.

All samples and field negative controls were vacuum-filtered within 24h of collection in a dedicated laboratory. Workbenches and surfaces were cleaned with 10% bleach prior to filtration. All filtration equipment (i.e. filtration cups and tweezers) was sterilised by emersion in 10% bleach for 10 minutes, followed by 5-10 minutes in 5% lipsol detergent to remove bleach residues, and lastly rinsed through purified water. Two 0.45 µm cellulose filters (47 mm, Cytiva Whatman Mixed Cellulose Ester Membranes; Fisher Scientific, UK) were used for each sample in order to maximise the volume filtered, and information on volume filtered was recorded for each sample. Following filtration, sterile tweezers were used to store the filters in UV-sterilised 5 mL tubes (Axygen Screw Cap Transport Tubes; Fisher Scientific, UK) containing garnet grit (0.8-1.2 g of both 0.15 mm and 1-1.4 mm diameter sterile beads, following Sellers et al., 2018), and filters were stored at -20°C until DNA extraction.

Field metadata

Several environmental variables were measured at each of the ten sampling points, each month, in both sites. Water temperature, pH, conductivity and turbidity were obtained using a HI-98130 meter (Hanna Instruments) and calcium levels were measured with the LAQUATwin Calcium Ion Ca-11 meter (Camlab, UK). Due to technical problems with the probes, we were unable to collect calcium data in December, and turbidity and conductivity data in February.

Water levels were recorded each month at Eccup Reservoir by consulting a reverse water depth gauge board installed on site. This meter included an inverted scale (with the number zero on top), which is commonly used to measure how far the water level has dropped in reservoirs. Similarly, water levels at the River Hull on the day of sampling were obtained from a monitoring station close to the sampling location (Beverley Shipyard; https://check-for-flooding.service.gov.uk/station/8281?direction=u). Values for river level were obtained by averaging the water level at the start and at the end of the fieldwork session each month.

Abundance of zebra mussel adults was collected on each sampling day and at all sampling points for both sites, following collection of eDNA samples. Adult mussels were searched for using kick or dip nets, depending on water accessibility, for approximately 1 minute. This was complemented with visual observation of shorelines, exposed structures and/or rocks, when possible. Both live and dead individuals (i.e. empty shells) were recorded. The number of juveniles was also recorded at Eccup Reservoir through the installation of colonisation platforms. This was not possible on the River Hull due to the lack of suitable areas for installation. Colonisation platforms were installed in May, ahead of the breeding season, and consisted of three grey PVC plates deployed in series (Marsden, 1992; Figure S1.1, appendix 1). After installation, the platforms were inspected every month using a magnifying glass (x10) and the number of individuals on each plate was recorded. Although aimed at recording juveniles, some adults were also observed on the platforms and were recorded as well. Juveniles and adults were distinguished based on their size, and individuals bigger than ~0.5 cm were counted as adults, following Ackerman et al. (1994). Each month after inspection all plates were

cleaned with scrapers. In cases of uncertainty regarding species identity, particularly concerning very small specimens, individuals were not included for species count.

Biosecurity

Two sets of kick nets were brought to the field each month and a different set was used at each site. At the end of the fieldwork day, both nets were submerged for at least 15 minutes in hot tap water (Shannon et al., 2018) and thoroughly dried before usage the following month. Similarly, the same pair of boots/wellies were never used on both sites on the same day and were cleaned and dried between months following the same procedure.

DNA extraction

All water samples were processed in a dedicated laboratory for eDNA samples only. DNA extraction was performed following the water protocol described in Sellers et al. (2018). Samples from each site and each month were extracted in separate batches (n = 24). A negative extraction control with only reagents was included in each batch to monitor for contamination during the extraction process.

Inhibition tests

All eDNA samples were tested for inhibition by real-time qPCR, using an exogenous positive control (TaqMan Exogenous Internal Positive Control Reagents; Fisher Scientific, UK). Final qPCR conditions consisted of 7.5 µL of TaqMan Environmental Master Mix 2.0 (Fisher Scientific, UK), 1.5 µL of 10X Exo IPC Mix, 0.3 µL of 50X Exo IPC DNA, 3.7 µL of molecular grade water and 2 µL of eDNA sample, in a total reaction volume of 15 µL. The thermal cycler conditions were as follows: 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. All eDNA samples were tested in duplicate, with replicates from the same site being run in the same plate to be able to directly compare. Samples were considered to be inhibited if the difference between the average cycle threshold (Cq) value of the sample and the average Cq value of the no template reaction was more than 2 cycles (e.g. Tillotson et al., 2018). All reactions were performed on a StepOnePlus Real-Time PCR machine.

eDNA samples

Species-specific qPCR reactions were conducted using the zebra mussel-specific *Cyt* b assay from Gingera et al. (2017) with minor modifications, namely reducing the total volume of the reaction from 20 to 15 µL (but maintaining original primer and probe concentrations), reducing the volume of sample added to 2 µL, and increasing the number of cycles from 40 to 45. Preliminary tests showed that the efficiency of the assay was sub-optimal (i.e., less than 90%) and steps were taken to improve it (see appendix 1 for further details). Despite the efforts, efficiency remained under 90%. Following this, a subset of samples was used to test the repeatability of the assay and ensure the assay yielded consistent results across runs (see appendix 1 for further details), despite the low efficiency.

Final qPCR conditions for screening of eDNA samples included 1x TaqMan Environmental Master Mix 2.0 (Fisher Scientific, UK), 0.2 µM of each primer (forward and reverse), 0.1 µM of probe, 4.75 µL of molecular grade water and 2 µL of sample. The qPCR program consisted of an initial step at 50°C for 10 min and 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 1 min and annealing and extension at 60°C for 1 min. Six replicates were performed for all eDNA samples, field and extraction controls. In addition, to monitor for contamination during qPCR preparation, three negative controls with only reagents were included in each plate. All reactions were performed on a StepOnePlus Real-Time PCR machine.

To accurately quantify DNA levels, a standard curve was generated using a 126 bp gBlock fragment (Integrated DNA Technologies, Belgium) that included the annealing sites for both forward and reverse primers and the probe. Extra base pairs were added (see appendix 1 for full sequence) to allow a distinction between the synthetic fragment and the real *D. polymorpha* DNA sequence, in case any contamination was detected. The gBlock fragment was quantified with a Qubit fluorometer (Thermo Fisher Scientific) using the double stranded high sensitivity assay and following manufacturer's instructions. Copy numbers were calculated following Sint, Raso, & Traugott (2012), using the formula copies/µL = (ng/µL x $6.02214179 \times 10^{23}$) / (weight x 10^9), where *ng/µL* corresponds to the DNA concentration previously obtained from Qubit, and *weight* corresponds to the molecular weight of the target double-stranded fragment. A

standard curve was then generated by doing seven 10-fold dilutions ranging from $3x10^7$ to $3x10^1$ copies/µL. Each standard dilution was included in triplicate, and a different standard curve was generated for each plate.

Data analysis

All analyses and plots were made using R version 4.2.1 (R Core Team, 2022) and R studio (Posit team, 2023). Except when mentioned otherwise, figures were produced using the ggplot2 3.4.2 package (Wickham, 2016). The variables analysed were average DNA copies/µL (of the 6 qPCR replicates for each sample) as response variable and water temperature, pH, conductivity, turbidity, calcium**,** water level**,** mussel abundance (data for juveniles and adults was combined) and volume filtered as explanatory variables. We investigated the relationships between average DNA copies/µL and the remaining variables through a principal component analysis (PCA), correlations and linear mixed-effects models as detailed below.

Correlation between variables was investigated to assess the relationship between average DNA copies/µL and explanatory variables, as well as collinearity between explanatory variables. The “cor()” function from the stats 4.2.1 package (R Core Team, 2022) was used to calculate Pearson correlation coefficients (r), followed by the “cor.mtest()” function from the corrplot 0.92 package (Wei & Simko, 2021) to calculate respective p-values. Due to the existence of missing values (calcium data for December and turbidity/conductivity data for February), the argument “use” was set as "pairwise.complete.obs", where pairs of variables with missing values are excluded from the calculation and only pairs with no non-missing values are considered. The correlation plots were made using the “corrplot()” function.

A PCA was performed using the “prcomp()” function from the stats 4.2.1 package (R Core Team, 2022). All variables were scaled and centered and rows with missing values were excluded. A biplot was then made using “fviz_pca_biplot()” from the package factoextra 1.0.7 (Kassambara & Mundt, 2020) to visualize the relationships between average DNA copies/µL and explanatory variables.

Occupancy models were initially considered to analyse the effects of the explanatory variables on eDNA detection rates. For qPCR data, such models rely on presence/absence information (e.g. unmarked package (Fiske & Chandler, 2011), eDNAoccupancy package (Dorazio & Erickson, 2018)). However, our data was not well suited to this approach since we obtained almost 100% positive detection in Eccup Reservoir (Table 2.1) and the effects of explanatory variables would not be significant. As a result, linear mixed-effects models were used instead. These were conducted using the "lmer()" function from the package lme4 1.1.33 (Bates et al., 2015). The logarithm of the average DNA copies/µL was used as the response variable. To overcome the presence of zeros in the River Hull data set, as the logarithm of zero does not exist, a constant value of one - log10 (DNA + 1) - was added to each data point. For consistency, the same procedure was applied to the Eccup Reservoir data set, although this did not affect the results of the model (data not shown). Due to high collinearity between explanatory variables, conductivity was removed from the analysis for Eccup Reservoir, while conductivity, turbidity and volume filtered were removed for River Hull. All remaining explanatory variables were scaled prior to the models and two random factors were included – month and sampling location within each site. The residuals of each model were assessed to ensure normality (verified with density and Q-Q plots), homogeneity of variances (verified with fitted vs residuals plot) and no autocorrelation (verified with an autocorrelation plot). The variance inflation factor (VIF) was also calculated using the "vif()" function from the car 3.1.2 package (Fox & Weisberg, 2019). This measures how the variance of the estimated coefficient of a specific independent variable is affected by multicollinearity with other independent variables, i.e., by the presence of multiple correlated predictors in a regression model.

2.4 Results

Assay performance

All qPCR standards amplified in 100% of the replicates with the exception of the lowest one (30 copies/µL) which amplified in 86% of the replicates. The lowest standard with at least 95% amplification, i.e. the limit of detection (LOD), was therefore 300 copies/µL. qPCR assays exhibited an average efficiency of 81.7% (76.4 – 87.2) and R^2 of 0.995 (0.983 – 0.998) for Eccup Reservoir, and average efficiency of 82.0% (76.6 – 87.3) and R^2 of 0.994 (0.981 – 0.999) for River Hull samples. The repeatability test exhibited similar Cq

values between the same samples across both runs (Table S1.1, appendix 1). Further information about the assay and MIQE checklist (Bustin et al., 2009) can be found in the supplementary material (Table S1.2, appendix 1).

None of the eDNA samples showed any PCR inhibition when tested using an exogenous internal positive control, with inhibition values (i.e. the difference between the average Cq of each sample and the no template reaction) ranging from 0 – 0.66 ($\bar{x}$ = 0.24, SD = 0.14) for Eccup Reservoir samples and 0 – 0.57 ($\tilde{x}$ = 0.18, SD = 0.14) for River Hull samples (Table S1.3, appendix 1). No contamination was observed in any of the field, extraction or PCR negative controls for neither of the sites. All replicates and samples from Eccup Reservoir amplified successfully, with the exception of a single replicate from one sample collected in April, that failed to amplify (Table S1.4, appendix 1). For the River Hull, the number of replicates and samples amplified varied greatly throughout the year, ranging from 3.3% positive replicates and 20% positive samples in January, to 100% amplification in July and August (Table 2.1; Table S1.4, appendix 1).

Table 2.1 Percentage of positive replicates and samples, as well as average DNA copies/µL obtained per month and site.

Season	Month	% positive replicates	% positive samples	Average DNA copies/µL
Eccup Reservoir				
Winter	Dec	100	100	1.89 E+02
	Jan	100	100	1.41 E+03
	Feb	100	100	1.28 E+02
Spring	March	100	100	1.84 E+02
	April	98.3	100	9.88 E+01
	May	100	100	6.15 E+02
Summer	June	100	100	1.71 E+04
	July	100	100	1.03 E+05
	Aug	100	100	1.26 E+05

Autumn	Sept	100	100	3.31 E+04
	Oct	100	100	3.35 E+02
	Nov	100	100	4.65 E+02
River Hull				
Winter	Dec	61.7	100	2.95 E+01
	Jan	3.3	20	6.22 E-01
	Feb	10	40	3.97 E+00
Spring	March	30	80	5.08 E+00
	April	50	100	7.37 E+01
	May	45	100	1.41 E+01
Summer	June	80	100	9.87 E+02
	July	100	100	4.11 E+04
	Aug	100	100	1.18 E+04
Autumn	Sept	86.7	100	1.23 E+03
	Oct	96.7	100	1.13 E+02
	Nov	93.3	100	1.40 E+02

Temporal variation in eDNA concentration

A strong temporal pattern was observed at both sites, with higher DNA concentrations for the summer months compared to the rest of the year (Figure 2.2a and 2.2b). At Eccup Reservoir, average DNA concentration per month ranged from 9.88 E+01 copies/µL in April to 1.26 E+05 copies/µL in August (Table 2.1). At the River Hull, values ranged from 6.22 E-01 copies/µL in January to 4.11 E+04 copies/µL in July. There was also a slight variation in average DNA copies/µL between the ten sampling locations each month at both sites (Figure 2.2c and 2.2d).

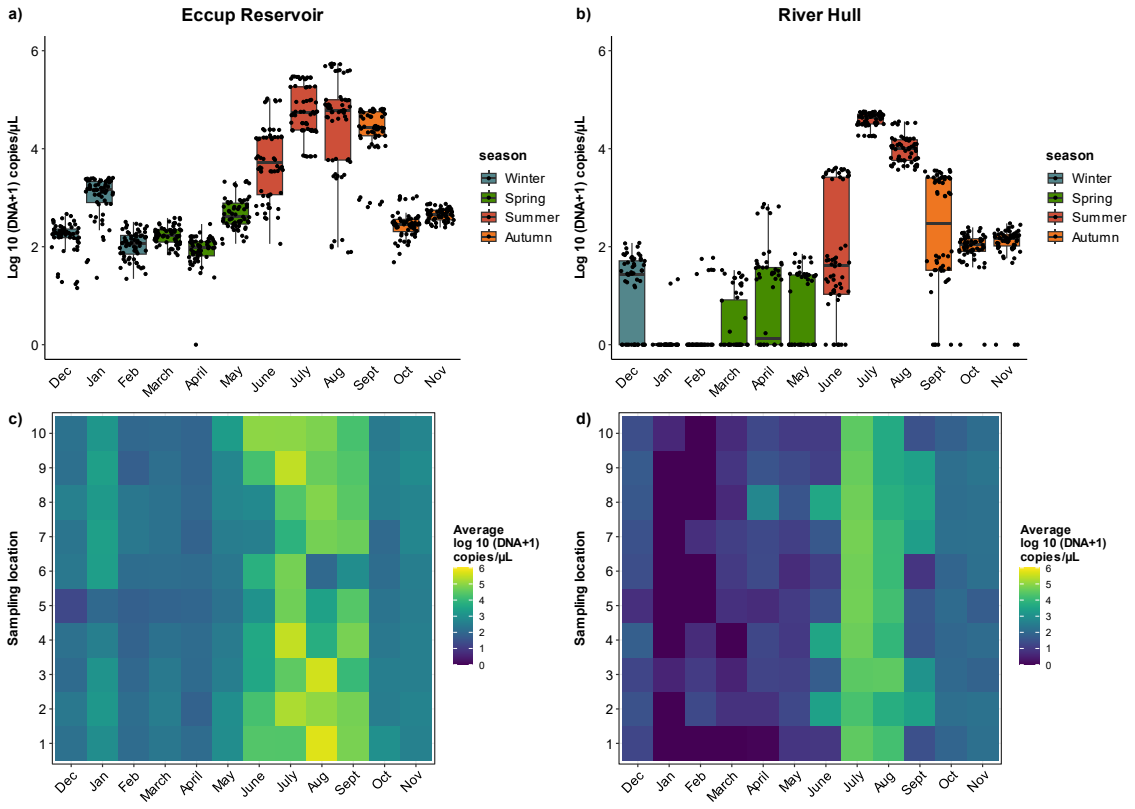

Figure 2.2 Log 10 (DNA+1) copies/µL obtained for each month at **a)** Eccup Reservoir and **b)** and River Hull; heatmap of the average log 10 (DNA+1) copies/µL obtained for each month and sampling location (1-10) at **c)** Eccup Reservoir and **d)** River Hull.

Temporal variation in field metadata

Environmental variables varied across the year at both locations (Figure 2.3; Table S1.3, appendix 1). Calcium ranged from 39 – 170 ppm (mg/L) ($\bar{x}$ = 105.6, SD = 33.5) at Eccup Reservoir and 100 – 410 ppm (mg/L) ($\bar{x}$ = 310.2, SD = 78.2) at the River Hull (Figure 2.3a). Conductivity ranged from 0.16 – 0.6 mS ($\bar{x}$ = 0.36, SD = 0.09) at Eccup Reservoir and 0.56 – 0.73 mS ($\bar{x}$ = 0.64, SD = 0.04) at the River Hull, registering the highest value in December in both sites (Figure 2.3b). pH ranged from 7.46 – 9.87 ($\bar{x}$ = 8.26, SD = 0.43) at Eccup Reservoir and 7.68 – 8.85 ($\bar{x}$ = 8.13, SD = 0.25) at the River Hull (Figure 2.3c), while temperature ranged from 4.4 – 22.4°C ($\bar{x}$ = 12.3, SD = 5.5) at Eccup Reservoir and 6.3 – 20.6°C ($\bar{x}$ = 12.6, SD = 4.4) at the River Hull, peaking in June and July, respectively (Figure 2.3d). Turbidity ranged from 0.11 – 0.37 ppt ($\bar{x}$ = 0.19, SD = 0.05) at Eccup Reservoir and 0.28 – 0.36 ppt ($\bar{x}$ = 0.32, SD = 0.02) at the River Hull, following a similar trajectory to conductivity and also exhibiting the highest value in December in both sites (Figure 2.3e). Volume filtered per sample ranged from 500 – 2000 mL ($\bar{x}$ = 1652.5, SD = 393.4) at Eccup Reservoir, and 300 – 2000 mL ($\bar{x}$ = 1444.6, SD = 567.8) at the River Hull, with the lowest

values registered in February and January, respectively (Figure 2.3f). Lastly, water level ranged from -0.42 to -1.98 m ($\bar{x}$ = -1.10, SD = 0.46) at Eccup Reservoir with a peak in June, and from 1.7 – 3.5 m ($\bar{x}$ = 2.31, SD = 0.59) at the River Hull with the highest values recorded in January and February (Figure 2.3g).

Zebra mussel juveniles were found on the platforms at Eccup Reservoir from July to November. Similarly, adults were recovered in March and then from July to November at Eccup Reservoir through the combination of different techniques, with the highest number recorded in September (Table 2.2). No individuals were found at the River Hull.

Table 2.2 Number of juvenile and adult individuals observed at Eccup Reservoir for each month.

Month	Juveniles (platforms)	Adults			Total
		Visual obs.	Kick/dip nets	Platforms	
Dec	NA	0	0	NA	0
Jan	NA	0	0	NA	0
Feb	NA	0	0	NA	0
March	NA	0	3	NA	3
April	NA	0	0	NA	0
May	NA	0	0	NA	0
June	0	0	0	0	0
July	18	2	8	2	30
August	39	0	0	25	64
Sept	1	882	22	14	919
Oct	53	0	0	2	55
Nov	4	12	0	1	17

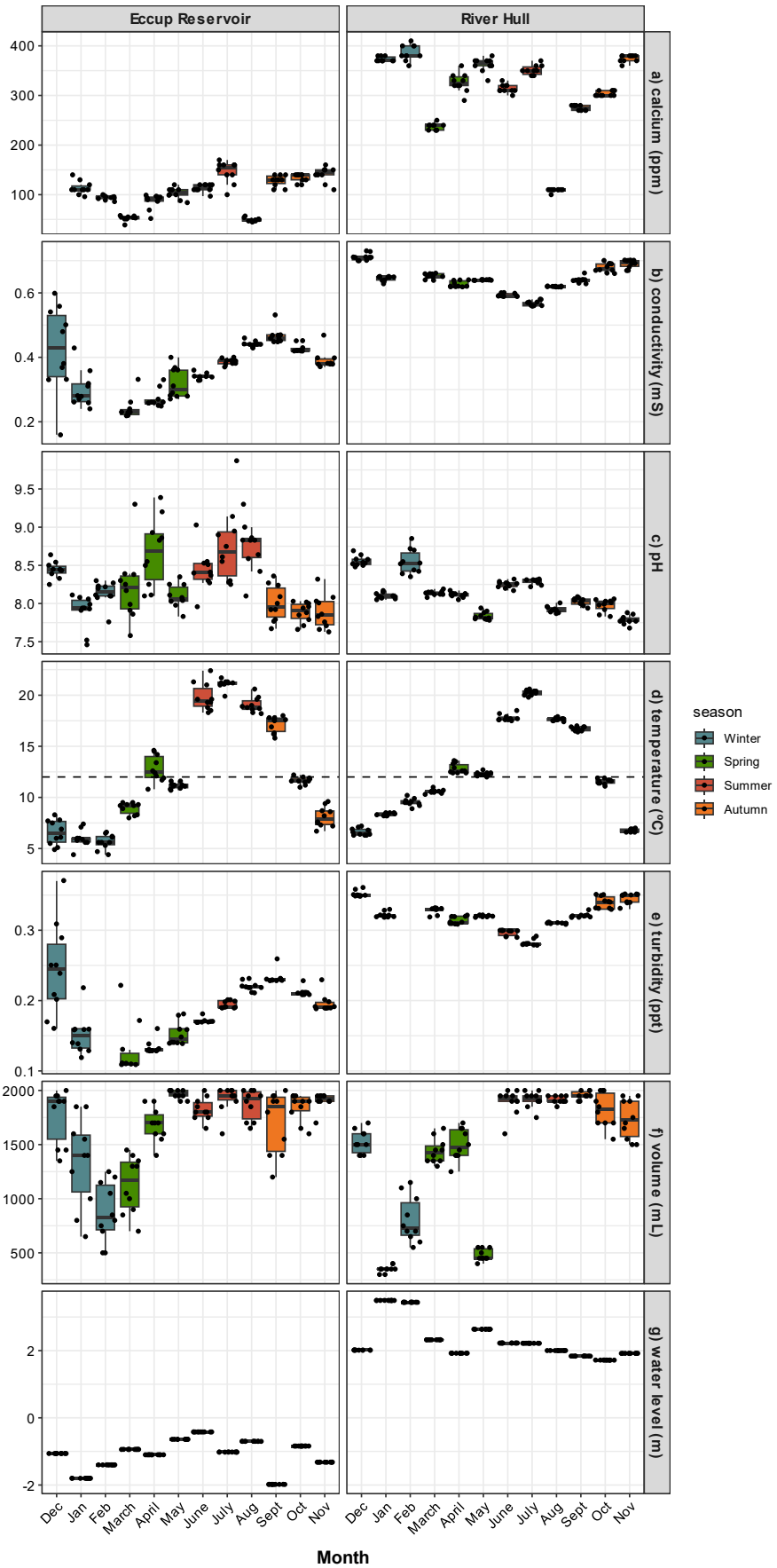

Figure 2.3 Variation in field metadata collected at Eccup Reservoir and River Hull throughout the months. The dashed line in the temperature grid (d) indicates the 12°C mark.

Influence of environmental variables on eDNA concentration

For both sites, the PCA biplots exhibited four separate clusters, despite some overlap, each corresponding to a season (Figure 2.4a and 2.4b). For Eccup Reservoir, the first principal component explained 35.4% of the variance and was strongly associated with turbidity and conductivity, while for the River Hull almost half of the variance (49.7%) was explained by the first principal component, which was positively associated with water temperature, pH and average DNA copies/µL, and negatively associated with turbidity and conductivity. According to the biplots, higher DNA levels and water temperature are associated with the summer season at both Eccup Reservoir and River Hull. A similar pattern was observed for pH and volume filtered, which are expected to be higher in the summer for both sites.

Average DNA copies/µL was significantly correlated with the same variables at both sites, namely water temperature, turbidity, conductivity and volume filtered (Figure 2.4c and 2.4d). At Eccup Reservoir, all of these correlations were positive – $r(118) = 0.425$ and $p = 1.27 E-06$, $r(108) = 0.190$ and $p = 0.046$, $r(108) = 0.226$ and $p = 0.018$, $r(118) = 0.200$ and $p = 0.029$, respectively (Table 2.3). At the River Hull, average DNA copies/µL was negatively correlated to turbidity ($r(108) = -0.631$ and $p = 1.41 E-13$) and conductivity ($r(108) = -0.603$ and $p = 3.23 E-12$), but positively correlated with water temperature ($r(118) = 0.598$ and $p = 5.29 E-13$) and volume ($r(118) = 0.314$ and $p = 4.87 E-04$) (Table 2.3). No significant correlation was observed between average DNA copies/µL and the number of individuals at Eccup reservoir (Figure 2.4c). Several significant correlations were observed among the explanatory variables, indicating collinearity. The strongest correlation was between turbidity and conductivity, which were highly positively correlated at both Eccup Reservoir ($r(108) = 0.798$ and $p = 1.72 E-25$) and the River Hull ($r(108) = 0.985$ and $p = 1.59 E-84$). These two variables were also negatively correlated with temperature at the River Hull ($r(108) = -0.847$ and $p = 2.27 E-31$, $r(108) = -0.854$ and $p = 1.93 E-32$, respectively).

Table 2.3 Pearson correlation coefficients (top) and p-values (bottom) for each combination of variables for both sites. Significant correlations are shown in bold.

Eccup Reservoir									
-	**Average DNA copies/µL**	**Temperature**	**pH**	**Turbidity**	**Conductivity**	**Calcium**	**Volume**	**Water level**	**N individuals**
Average DNA copies/µL	-	**0.425**	0.177	**0.190**	**0.226**	-0.044	**0.200**	0.108	0.075
Temperature	**1.27E-06**	-	**0.443**	0.149	**0.239**	0.106	**0.454**	**0.349**	**0.304**
pH	5.27E-02	**4.10E-07**	-	0.040	-0.032	**-0.308**	0.134	**0.343**	-0.172
Turbidity	**4.65E-02**	1.21E-01	6.81E-01	-	**0.798**	**0.291**	**0.357**	-0.137	**0.368**
Conductivity	**1.77E-02**	**1.18E-02**	7.42E-01	**1.72E-25**	-	**0.313**	**0.424**	-0.134	**0.421**
Calcium	6.49E-01	2.72E-01	**1.06E-03**	**3.26E-03**	**1.52E-03**	-	**0.322**	**-0.270**	**0.212**
Volume	**2.88E-02**	**1.86E-07**	1.46E-01	**1.28E-04**	**4.03E-06**	**5.94E-04**	-	**0.335**	0.071
Water level	2.41E-01	**9.18E-05**	**1.23E-04**	1.54E-01	1.62E-01	**4.32E-03**	**1.87E-04**	-	**-0.578**
N individuals	4.18E-01	**7.35E-04**	5.99E-02	**7.53E-05**	**4.77E-06**	**2.62E-02**	4.42E-01	**4.64E-12**	-

River Hull									
-	**Average DNA copies/µL**	**Temperature**	**pH**	**Turbidity**	**Conductivity**	**Calcium**	**Volume**	**Water level**	**Individuals**
Average DNA copies/µL	-	**0.598**	0.109	**-0.631**	**-0.603**	-0.070	**0.314**	-0.100	-
Temperature	**5.29E-13**	-	-0.084	**-0.847**	**-0.854**	**-0.438**	**0.494**	**-0.291**	-
pH	2.37E-01	3.60E-01	-	-0.105	-0.069	**0.230**	-0.067	**0.319**	-
Turbidity	**1.41E-13**	**2.27E-31**	2.73E-01	-	**0.985**	0.080	-0.168	-0.160	-
Conductivity	**3.23E-12**	**1.93E-32**	4.75E-01	**1.59E-84**	-	0.101	-0.170	-0.156	-
Calcium	4.67E-01	**1.69E-06**	**1.57E-02**	4.31E-01	3.17E-01	-	**-0.479**	**0.445**	-
Volume	**4.87E-04**	**9.57E-09**	4.67E-01	7.93E-02	7.64E-02	**1.21E-07**	-	**-0.827**	-
Water level	2.76E-01	**1.26E-03**	**3.85E-04**	9.53E-02	1.04E-01	**1.13E-06**	**2.77E-31**	-	-

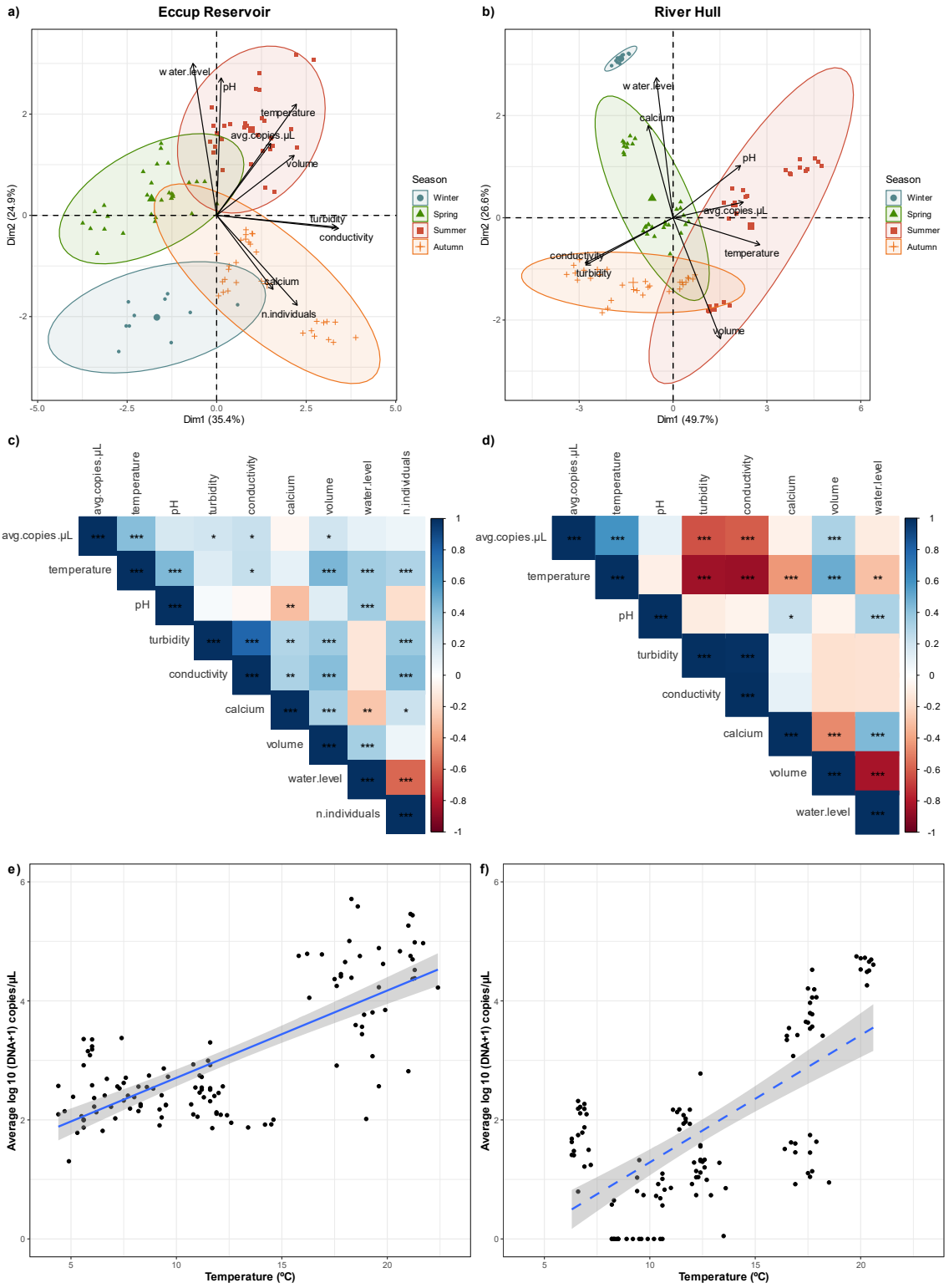

Figure 2.4 PCA biplots of environmental variables and average DNA copies/µL for **a)** Eccup Reservoir and **b)** River Hull; the ellipses for each season were drawn with a confidence level of 0.95 (default value). Correlation plots for **c)** Eccup Reservoir and **d)** River Hull; colours indicate the strength of each pairwise correlation, with stronger colours representing a stronger correlation, whether positive (blue) or negative (red); stars indicate the significance level and lack of significance is depicted by empty squares. Scatterplots showing the effect of water temperature on average log 10 (DNA+1) copies/µL at **e)** Eccup

Reservoir and **f)** River Hull; the linear regression line is shown in blue, either solid (indicating significance) or dashed (no significance), and grey shading represents the confidence interval.

Model assumptions, i.e., normality, homogeneity of variances and autocorrelation, were met for both Eccup Reservoir and the River Hull (Figures S1.2 and S1.3, appendix 1). Additionally, VIF values were under 5 for all variables on both models (Table 2.4), indicating low multicollinearity with other predictor variables (James et al., 2013). This suggests that while there might be correlations between variables, the level of multicollinearity among predictors in the model is not severe enough to cause problems such as inflated standard errors or unstable coefficients. The random effect of month had an estimated variance approximately 5 and 25 times higher than the random effect of sampling location for Eccup Reservoir and River Hull, respectively (Table 2.4). Only water temperature significantly affected the amount of DNA obtained at Eccup Reservoir (estimate = 0.784, SE = 0.258, df = 13.337, t = 3.044, p = 0.009), with warmer temperatures favouring higher DNA copies/µL (Figure 2.4e). None of the variables tested significantly influenced DNA levels at the River Hull (Table 2.4), even though there is a (non-significant) trend for increasing DNA copies/µL with temperature (Figure 2.4f). Scatterplots of the average DNA copies/µL plotted against the remaining explanatory variables for both sites can be found in appendix 1 (Figure S1.4 and Figure S1.5).

Table 2.4 VIF values and linear mixed-effects model parameters for both Eccup Reservoir and River Hull.

Eccup Reservoir						
Random effects						
	Variance	**Std. dev.**				
Sampling location	0.067	0.259				
Month	0.405	0.636				
Residual	0.225	0.474				
Fixed effects						
	Estimate	**Std. error**	**df**	**t value**	**Pr(>|t|)**	**VIF**
(Intercept)	3.058	0.229	5.757	13.343	0.000	-
Temperature	0.784	0.258	13.337	3.044	0.009	1.771
pH	-0.133	0.077	85.927	-1.731	0.087	1.204

Turbidity	0.076	0.137	82.680	0.553	0.582	1.171
Calcium	0.100	0.141	38.172	0.713	0.480	1.224
Volume	0.134	0.101	86.840	1.328	0.188	1.088
Water level	-0.279	0.315	6.200	-0.887	0.408	2.649
N individuals	-0.052	0.304	6.087	-0.172	0.869	2.513
River Hull						
Random effects						
	Variance	**Std. dev.**				
Sampling location	0.041	0.202				
Month	0.988	0.994				
Residual	0.285	0.534				
Fixed effects						
	Estimate	**Std. error**	**df**	**t value**	**Pr(>|t|)**	**VIF**
(Intercept)	1.843	0.317	7.025	5.806	0.001	-
Temperature	0.605	0.348	9.103	1.737	0.116	1.307
pH	0.075	0.188	87.367	0.398	0.692	1.125
Calcium	0.105	0.283	16.350	0.370	0.716	1.253
Water level	-0.629	0.349	7.787	-1.801	0.110	1.393

2.5 Discussion

In this study, two different freshwater systems - a river and a reservoir - were sampled to investigate the temporal dynamics of zebra mussel eDNA and the effects of environmental variables on species detection. As predicted, there was a difference between sites regarding eDNA concentration and detection levels throughout the year, with higher DNA concentrations and more consistent species detection in Eccup Reservoir than the River Hull. Both sites exhibited a peak in eDNA concentration in the summer, consistent with the increase in temperature, which stimulates spawning thus increasing eDNA concentration in the water. Temperature and volume filtered were significantly positively correlated with eDNA concentration at both sites. Turbidity and conductivity were both positively correlated with eDNA concentration in the reservoir. By contrast, in the river, both variables were negatively correlated with eDNA

concentration. Temperature was the only significant variable in the linear mixed-effects models, and for Eccup only. Our results support a growing body of work demonstrating that concentration of zebra mussel eDNA is strongly influenced by the species' seasonal activity, namely veliger production, but also show that waterbody characteristics influence detection.

Temporal and spatial variation in eDNA concentration and detection rates

Both sites exhibited a similar temporal pattern, with a peak in eDNA concentration in the summer. This is consistent with our hypothesis based on the species' biology, as spawning is stimulated when water temperatures reach 12^{o}C (Marsden, 1992), increasing DNA levels in the water. In our study, this threshold was reached in April, and water temperatures remained above 12^{o}C until September in both sites, apart from in May at Eccup Reservoir. Higher detectability of zebra mussel eDNA during the summer and late spring has also been demonstrated in others studies in North America, with more restricted temporal sampling (Gingera et al., 2017; Sepulveda et al., 2019). Interestingly, Gingera et al. (2017) did not detect zebra mussels in May in some of their sampling locations, and suggested their assay might not be sensitive enough to detect mussels before the spawning season. By contrast, mussels were detected all year round in our study, even when the water temperature was as low as 4°C in Eccup Reservoir and 6°C in the River Hull. This suggests that while summer months are optimal to maximise zebra mussel detection, detection is possible year-round if enough replication is performed and when populations are established.

Despite exhibiting similar temporal patterns, the detection rates were different between sites, with higher detectability observed at Eccup Reservoir compared to the River Hull. At Eccup, 100% amplification was observed for 11 months and only one replicate from one sample failed to amplify in April. By contrast, detection in River Hull samples varied greatly throughout the year, with amplification success ranging from 3% and 10% of positive replicates in January and February, respectively, to 100% detection in July and August. The difference in detection between sites is consistent with our expectations based on the habitat preferences of zebra mussels and the different dynamics of eDNA in lentic and lotic systems. Zebra mussels are thought to rarely reach high densities in rivers due to water flow, which carries larvae downstream, and due to

the lack of suitable hard substrates, which prevent attachment. By contrast, shallow reservoirs provide ample hard substrates for attachment, and zebra mussels can attain high densities if conditions such as pH and calcium are favourable (Karatayev & Burlakova, 2022). Lower detection is also expected in rivers due to greater DNA dispersion and dilution (Herder et al., 2014). Our data indicate the optimal sampling window might be wider in lentic (July-September) than lotic (July-August) systems, but this might be confounded by differences in mussel density between our study sites. Greater sampling and/or replication depth might also be needed in lotic compared to lentic systems to achieve comparable detection rates and this warrants further investigation.

We found little spatial variation in eDNA concentration between samples taken in the same month at each site. Previous work on quagga mussels, *Dreissena rostriformis bugensis,* demonstrated a clear dilution of eDNA concentration downstream from the main source population over a distance of only 2.75 km (Blackman et al., 2020b). No similar dilution patterns were seen here on the River Hull, which could be due to a combination of the close proximity of sampling locations and/or more homogeneous distribution of mussels. Challenges still remain in interpreting concentrations of eDNA in rivers due to the combination of eDNA production, transport, and removal, and hydrological models are needed to facilitate this (e.g. Carraro et al., 2018). The lack of spatial variation is perhaps more surprising at Eccup Reservoir, where several substrate types can be found. Zebra mussels are known to prefer hard substrates and to vary in density over small scales due to differences in substrate type (Depew et al., 2021; Karatayev, Burlakova, & Padilla, 1998). Nevertheless, shallow reservoirs are subject to considerable water-level fluctuations, wind-induced circulation and subsequent mixing, which could homogenise the spatial distribution of eDNA, as has been found in eDNA studies of fish communities (e.g. Evans et al., 2017).

Effects of environmental variables on eDNA concentration

Zebra mussel eDNA concentration was mostly influenced by temperature, as evidenced by the close proximity/overlap in the PCA biplots and significant positive correlations for both sites. Additionally, linear mixed-effects models showed the effect of temperature to be statistically significant on eDNA concentration, although for Eccup Reservoir only.

For the River Hull, the lack of significant predictors in linear mixed-effects models could be due to a combination of only sampling 1 kilometre stretch of the river and/or the close proximity of sampling locations, that possibly resulted in less environmental variability and thus less statistical power. While the increase in temperature in the summer seems to play an important role in the amount of eDNA available in the water, which is related to the species' biology as discussed above, other variables were also noteworthy.

As predicted, eDNA concentration was positively correlated with the volume of water filtered for both sites, but the association was stronger for the River Hull than for Eccup Reservoir. Previous studies have also demonstrated that volume filtered is positively associated with eDNA detection rates (Mächler et al., 2016; Peixoto et al., 2023), since the probabilities of capturing eDNA of the target species increase as more water is sampled. Higher water volumes might also increase the concentration of inhibitors in samples (Herder et al., 2014), however this does not seem to be the case in our study, as we found no evidence of inhibition.

The relationship between eDNA concentration, turbidity and conductivity is more complex. Turbidity and conductivity were significantly associated with eDNA concentration, but opposite patterns were found for the different sites. At Eccup Reservoir, turbidity and conductivity were weakly positively correlated with eDNA concentration, while there was a negative and strong correlation for the River Hull. High levels of turbidity can increase eDNA concentration, as DNA molecules bind to sediments which protects them from degradation (Barnes et al., 2014), but can also accelerate clogging of filters, reducing the volume filtered (Peixoto et al., 2021) and decreasing DNA concentration. Here, volume filtered was positively correlated to turbidity at Eccup Reservoir and there was no relationship between these variables at the River Hull. It is possible that the amount of sediment influenced the result, as turbidity was notably higher in the river than the reservoir. Conductivity is a product of both Total Dissolved Solids (TDS) and sediment type, and it is therefore not surprising that it was positively correlated with turbidity. Conductivity was much higher in the River Hull than in Eccup Reservoir, suggesting river substrate could be more clay-dominated compared to the reservoir. Although few studies have addressed the effects of

conductivity, it has previously been shown to negatively affect eDNA detection (Harper et al., 2019b; Sengupta et al., 2019), as found here for the River Hull, while other studies have failed to observe a significant effect (Goldberg et al., 2018; Keskin, 2014). The opposite patterns observed here for both variables seem to be site-dependent and could be related to other abiotic factors, as both turbidity and conductivity were significantly correlated with other variables at both sites. More importantly, turbidity and conductivity were strongly correlated with each other, making it challenging to separate their individual effects. The relationship between waterbody type, sediment load, and conductivity should be the subject of further research to disentangle these effects.

Although not significant in our analyses, our data qualitatively suggests that water level could have influenced species detection. At the River Hull, lower PCR amplification success in January and February compared to the rest of the year could have been influenced by high river levels. Intense rain caused the river to flood in both months, likely increasing eDNA dilution. A similar observation has been made for eDNA concentrations and detectability of the invasive clam, *Corbicula fluminea,* in streams in the USA (Curtis et al., 2021). By contrast, low water levels in January and September at Eccup Reservoir could have resulted in higher eDNA concentrations relative to other months of the respective seasons.

The relationship between eDNA concentration and species abundance is not straightforward. While dreissenid eDNA concentration has been shown to correlate with species biomass in controlled mesocosm experiments (Blackman et al., 2020a), field tests have failed to detect such relationship (Amberg et al., 2019). In our study, mussel abundance was not correlated with eDNA concentration at Eccup Reservoir. The sampling was restricted to the edges of the waterbody and our abundance data might not reflect true population densities throughout the reservoir, perhaps explaining the lack of correlation observed.

While pH and calcium are expected to have a strong influence on zebra mussel establishment (Karayatev & Burlakova, 2022), with lower pH levels increasing eDNA degradation rates (Seymour et al., 2018), neither pH nor calcium were correlated to

eDNA concentrations in our study. The values recorded here at both sites were above the lower limit needed for zebra mussel establishment (pH > 7.3 and calcium > 23 mg/L or ppm) and eDNA persistence, which could explain the lack of significant correlation observed. Future studies with an increasing number of sites might yield more statistical power to detect the influence of these variables.

Assay performance and potential limitations

In this study, we used a previously-designed qPCR assay (Gingera et al., 2017) that has demonstrated to provide repeatable and reproducible results (Sepulveda et al., 2020a). Although the assay performed well here in terms of species detection, efficiency values were lower (76.4 - 87.3%) than those reported in the original publication (90.22%) and the preferred range of 90-110% (Raymaekers et al., 2009; Taylor et al., 2010). Several factors can affect standard curves and influence qPCR efficiency, such as the reagents used and their concentrations, the number of replicates, or even the machine itself (Svec et al., 2015). Better designed assays are less susceptible to any changes, while some can be very sensitive to it. Nonetheless, several steps were taken here to increase efficiency values, although unsuccessfully.

The low efficiency observed here could have resulted in reduced sensitivity of the assay. In fact, the LOD obtained for the assay was 300 copies/µL, which is higher than expected when compared to other eDNA-based qPCR studies (e.g. Alzaylaee et al., 2020; Nolan et al., 2023). While this can result in higher rates of false negatives, it seems unlikely to have occurred in our study, given the very high detection rates we obtained overall. In addition, the assay was found to be highly repeatable across runs, which provides additional confidence in our data. The low efficiency of the assay and consequent high LOD is perhaps more problematic in circumstances where species detection is likely to be more challenging, i.e. at lower population densities. In such cases, higher replication levels are recommended to offset this caveat. Regardless, the low efficiency observed here should be further explored and optimised to achieve values within the range of 90-110%, and potentially increase the LOD and sensitivity of the assay.

Conclusions, recommendations and future work

To our knowledge, this is the first study to assess the influence of variables that are important for zebra mussel biology on eDNA concentration, as well as assessing the seasonal variation of zebra mussel eDNA continuously throughout the year. Overall, our results suggest that eDNA concentration is mostly influenced by temperature, and future eDNA monitoring campaigns should conduct surveys during the summer to maximise detection. We also found a difference in detection levels between sites, potentially due to a combination of differences in eDNA transport and dilution, and substrate type impacting mussel attachment.

The work conducted in this study aimed to increase the readiness of the zebra mussel eDNA assay to an operational level (Thalinger et al., 2021). While our study provides information about the role of environmental variables that have an important temporal component, greater understanding of the spatial variation in eDNA detection would be beneficial, since there are clear differences in detection between waterbody types. The estimation of detection probabilities from statistical modelling is also needed for level 5 validation (Thalinger et al., 2021). This was not possible in the current study because currently available modelling approaches rely on presence/absence data (e.g. Fiske & Chandler, 2011; Dorazio & Erickson, 2018; Griffin et al., 2020) or do not accommodate temporal datasets (e.g. Espe et al., 2022). Further work, including more sites and statistical modelling of detection probabilities is needed for the assay to be considered fully operational.

2.6 Acknowledgments

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Chapter 3

Spatial dynamics of zebra mussel (*Dreissena polymorpha*) environmental DNA: do environmental variables influence eDNA concentration?

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3.1 Abstract

Invasive non-native species (INNS) are one of the main threats to biodiversity. Zebra mussels (*Dreissena polymorpha*) are freshwater molluscs native to the Ponto-Caspian region but are widespread and invasive in several regions of the world. Their negative impacts to the environment and infrastructure makes them a priority species for monitoring. Several environmental DNA (eDNA) assays targeting zebra mussels have been developed, and previous research has demonstrated that detection rates are highest in summer, relating to mussel spawning. However, research is lacking on the performance of assays across different waterbody types and environmental conditions. To address this, water samples were collected in the summer from twenty sites (canals, lakes, reservoirs, and rivers) in the UK with established zebra mussel populations. Environmental variables were collected alongside and species detection was conducted using a species-specific qPCR assay. Detection rates were consistently high, with 85-100% positive replicates and 100% positive samples for all sites. Average eDNA concentration was higher in reservoirs than in lakes, rivers or canals, and weakly influenced by substrate type. Correlations between average eDNA concentration per sample and environmental variables showed a negative relationship with turbidity and conductivity. However, mixed-effects models indicated volume filtered to be the only significant variable, with positive effects on uncensored ln[eDNA]. Exploration of detection probabilities suggest that the filtration of just two 250 mL samples could provide a 95% probability of detection. Our results demonstrate that detection probabilities are high

across different waterbodies and environmental conditions when samples are collected during the mussel reproductive season and when populations are established, but eDNA concentration is influenced by waterbody type and volume filtered, and weakly influenced by substrate type. The assay can now be considered operational for routine monitoring, however testing of newly invaded sites would be beneficial to understand detection rates at lower population densities.

3.2 Introduction

Invasive non-native species (INNS) are considered one of the main threats to biodiversity, being responsible for local and worldwide extinctions (Bacher et al., 2023; Bellard et al., 2016). Their introduction to new locations, either intentionally or accidentally, allows them to overcome biogeographical barriers otherwise inaccessible to them (Roy et al., 2023). Due to the lack of coevolutionary history with the invaded ecosystem, their introduction can cause significant disruptions (Simberloff et al., 2012). The most common impacts include changes to ecosystem properties (e.g. soil and water chemistry, nutrient cycling), followed by deterioration of the performance of native species (e.g. their reproduction, growth and survival rates; Bacher et al., 2023). Additionally, the costs of biological invasions have been estimated to be $1.2 trillion worldwide between 1980-2019 (Turbelin et al., 2023).

Zebra mussels (*Dreissena polymorpha,* Pallas, 1771) are small freshwater molluscs native to the Ponto-Caspian region in Eastern Europe. Although initially originating in the marine and saltwater environment, they later adapted to freshwater and brackish ecosystems (Orlova, 2014) and can now be found in a range of waterbodies, such as rivers, reservoirs, canals and lakes. Zebra mussels are known to prefer hard substrates for attachment, such as rocks (e.g. Hetherington et al., 2019), artificial hard substrates, or even shells of other bivalves (e.g. Larson et al., 2022), although they are also able to attach to soft substrates such as sand/mud sediments (e.g. Berkman et al., 1998, 2000) and aquatic vegetation (e.g. Burlakova et al., 2006). In lentic systems, zebra mussels are more abundant in polymictic lakes, where they are limited to the littoral zone and reach maximum densities between 1-6 meters (Karatayev et al., 2015; Karatayev & Burlakova, 2022). By contrast, they are expected to be less abundant in stratified lakes, where the strong wave action in the shallow areas prevents the settlement of larvae (Karatayev &

Burlakova, 2022). Within lotic habitats, densities are generally much higher in canals than rivers due to more availability of stable substrates for attachment and reduced levels of suspended inorganic particles, while the typically higher water flow in rivers reduces recruitment of larvae as they are transported downstream (Karatayev & Burlakova, 2022). Densities are thus usually low in rivers, however in the outflow area of adjacent invaded reservoirs or lakes, and in the lower course of rivers and deltas abundances can be high (Karatayev & Burlakova, 2022).

Several chemical variables can impact the establishment, growth and survival of zebra mussels. Calcium and pH are considered the most important variables that limit establishment, and populations are thought to be limited by calcium concentrations lower than 23-28 mg/L and pH lower than 7.3-7.5 (Karatayev & Burlakova, 2022). The effect of both variables on mussel populations is linked, as pH tolerance can vary with calcium concentrations (Garton et al., 2013). Temperature thresholds for zebra mussel survival range between 0°C and around 30°C (Karatayev & Burlakova, 2022), with 12-24°C being the optimal range for spawning and larval development (Garton et al., 2013). Their colonization potential and growth are expected to be higher in sites with conductivity levels above 83 µS/cm (Chakraborti et al., 2013; O'Neill, 1996), while high turbidity values can negatively affect their metabolic functions such as oxygen consumption and filtration rates, although no limits have been established yet (Garton et al., 2013). Once populations are established, turbidity levels tend to decrease due to their filtering activity, which reduces phytoplankton biomass, thus increasing water clarity (e.g. Karatayev et al., 2014). Similarly, high mussel densities have also been shown to result in lower conductivity, alkalinity and pH, possibly due to the removal of calcium and carbonate ions from the water column for their shells (Beshkova et al., 2017; Jones & Montz, 2020).

The magnitude of zebra mussel impacts on invaded ecosystems depends on their abundance, distribution in the waterbody, and time since invasion, with the strongest impacts occurring in the first 5-10 years (Karatayev et al., 2021c). Their role as suspension feeders, where they effectively reduce plankton biomass, negatively affects native filter feeders such as molluscs, amphipods (e.g. Watkins et al., 2007) and fish (e.g. Cunningham & Dunlop, 2023). Their ability to attach to hard substrates such as shells of

other bivalves (e.g. Larson et al., 2022) via byssal threads can lead to population declines, as they disrupt their valve functions and prevent metabolic processes, while also competing for food and space. Toxic algal blooms can also occur following zebra mussel invasion (e.g. Raikow et al., 2004). Additionally, fouling of water supply infrastructures by zebra mussels interferes with the normal functioning of the systems by clogging, corroding and reducing the thickness of pipes (Karatayev & Burlakova, 2022). Estimates suggest that the worldwide costs of zebra mussels in the last four decades was around $19 billion (Haubrock et al., 2022). Sensitive tools such as environmental DNA (eDNA) are thus needed for early detection and monitoring of spread of zebra mussels to help mitigate their negative impacts to the environment and infrastructures.

Environmental DNA refers to DNA that can be collected from environmental samples, such as water, soil or air (Barnes & Turner, 2016; Taberlet et al., 2012). The analysis of eDNA samples has shown great promise for monitoring of INNS (Blackman et al., 2018), offering a cheaper and more sensitive approach compared to traditional monitoring methods (Fediajevaite et al., 2021). Understanding the ecology of eDNA, i.e. its generation and persistence in the environment, is essential for interpreting results from eDNA-based surveys (Barnes & Turner, 2016). Several environmental variables have been shown to influence eDNA degradation and persistence. For example, higher water temperature (e.g. Eichmiller et al., 2016) and acidic pH levels (e.g. Goldberg et al., 2018) are commonly associated with higher eDNA degradation rates, likely due to an increase in microbial and enzymatic activity under these conditions (Joseph et al., 2022). In turn, high suspended matter and turbidity can increase eDNA persistence in the environment, as sediments bind to both DNA molecules, protecting them from degradation, and to exonucleases, inactivating them (Barnes et al., 2014; Joseph et al., 2022). The type of sediment is also expected to influence eDNA persistence in the environment, as the DNA binding capacities differ among sediment types (Levy-Booth et al., 2007). Additionally, the persistence of eDNA is expected to be lower in lotic systems due to higher dispersion and downstream transport when compared to lentic systems (Harrison et al., 2019).

Several eDNA assays have already been developed and successfully applied for eDNA-based surveys of dreissenid mussels (Feist & Lance, 2021), and highly-specific quantitative PCR (qPCR) assays developed by Gingera et al. (2017) have showed to

provide repeatable and reproducible results (Sepulveda et al., 2020a). In order for these assays to be used in routine monitoring, wider testing and understanding of eDNA detectability are required. The 5-level validation scale introduced by Thalinger et al. (2021) helps researchers and end-users understand the validation level of targeted eDNA assays and how much testing is needed for assays to be considered operational and ready for use in routine monitoring. The authors suggest minimum criteria that need to be achieved to reach each validation level, that range from incomplete to operational. Understanding the effects of environmental variables on eDNA concentration and estimating detection probabilities from statistical modelling are two of the requirements for assays to reach the highest level of validation and thus be considered fit for operational use.

Previous studies have already assessed the influence of environmental variables on zebra mussel eDNA detection, to some extent. For example, research in Denmark found that zebra mussel eDNA concentration was positively influenced by water velocity and nutrient concentration (Shogren et al., 2019), while Amberg et al. (2019) found that eDNA concentration was higher at greater depths and near soft substrates, such as silt and sand. This contradicts what is known regarding zebra mussel preferences for hard substrates and shallower depths, and the authors suggested this could have been due to wind-driven currents that transported zebra mussel eDNA into deeper areas, abundant in soft substrates, where colder temperatures and less UV light makes DNA less susceptible to degradation (Strickler et al., 2015). In our previous work (chapter 2) we investigated the seasonal variation of zebra mussel eDNA in a reservoir and a river, and assessed the influence of several environmental variables on eDNA concentration and detection rates over the course of a year. We found that eDNA concentration at both sites peaked in the summer and was mostly influenced by temperature, which stimulates spawning and thus the increase in eDNA in the water. Turbidity and conductivity were also correlated with eDNA concentration, although we observed opposite patterns for the two sites, suggesting a complex relationship between the three variables. We also observed differences in detection rates between the river and reservoir, with higher eDNA concentration and more consistent detection rates observed in the reservoir, likely due to the increased DNA dilution in the river that reduces detectability (Herder et al., 2014).

Findings from our previous study helped shed some light on the influence of seasonal variation and environmental variables on zebra mussel eDNA detectability, and highlighted differences between waterbody types. Here, we increased the number and type of sites to provide greater spatial variation and statistical power in order to further investigate the impact of environmental variables, while sampling in the summer when detection rates are at their highest. We also use statistical modelling to estimate detection probabilities and conduct *in silico* tests**,** as required for level 5 ("operational") of the validation scale proposed by Thalinger et al. (2021). Water samples were collected from 20 sites with known zebra mussel presence. Sites were spread throughout England and included canals, lakes, reservoirs and rivers. We expect detection rates and eDNA concentration to be 1) influenced by waterbody and substrate type, being higher in lentic (lakes/reservoirs) than lotic (canals/rivers) sites due to lower eDNA transport and dilution, and close to hard substrates where mussels are predicted to be more abundant, 2) positively associated with pH, calcium, species abundance and volume filtered, 3) influenced by turbidity and conductivity to some extent, and 4) less influenced by temperature when compared to the previous study, due to the shorter sampling period.

3.3 Materials and methods

Sample collection and processing

Twenty sites spread throughout England and with known zebra mussel presence were sampled during July and August 2021 (Figure 3.1; Table 3.1). The sites included different waterbody types, such as canals, lakes, reservoirs and rivers (5 sites of each type), in order to cover different environmental conditions. All lakes were small and shallow, under 40 hectares (surface area) and with an average depth between 1.7-10 meters (Table 3.1). Reservoirs exceeded 40 hectares in size, with the exception of Ulley Reservoir. Zebra mussel presence was assessed by checking the National Biodiversity Network Atlas (National Biodiversity Network Trust, 2023) and Environment Agency records, and further confirmed by contacting site managers. At each site, ten sampling locations were selected and one water sample was collected from the shoreline at each location (coordinates for each sample are provided in Table S2.1, appendix 2). For canals and rivers, samples were taken approximately every 100 metres over a 1 kilometre stretch, in an upstream direction to minimise downstream contamination. For lakes and

reservoirs, sampling locations were chosen in order to maximize the perimeter of the waterbody covered.

Water was collected directly from the waterbody with a disposable syringe when easily accessible, while a sterile Whirl-Pak® bag and a litter picker were used to collect water from inaccessible sites. Samples were filtered using a 100 mL luer lock syringe (Nature Metrics, UK) and an enclosed filter with a polyethersulfone membrane and 0.8 µm pore size (Nature Metrics, UK). Water was pushed through the filter with the syringe and repeated as many times possible until the filter clogged, and the volume filtered was registered. Air was then pushed through the filter to remove excess water, 1 mL of Longmire's buffer (Longmire, Maltbie, & Baker, 1997) was added to preserve the DNA, and samples were stored at room temperature. To control for contamination in the field, 1 L of purified water brought from the laboratory was filtered at each site, after all ten samples had been collected. Disposable gloves were worn at all times and changed between samples. At the end of each fieldwork campaign (duration between 1-5 days) samples were stored at -20°C until DNA extraction.

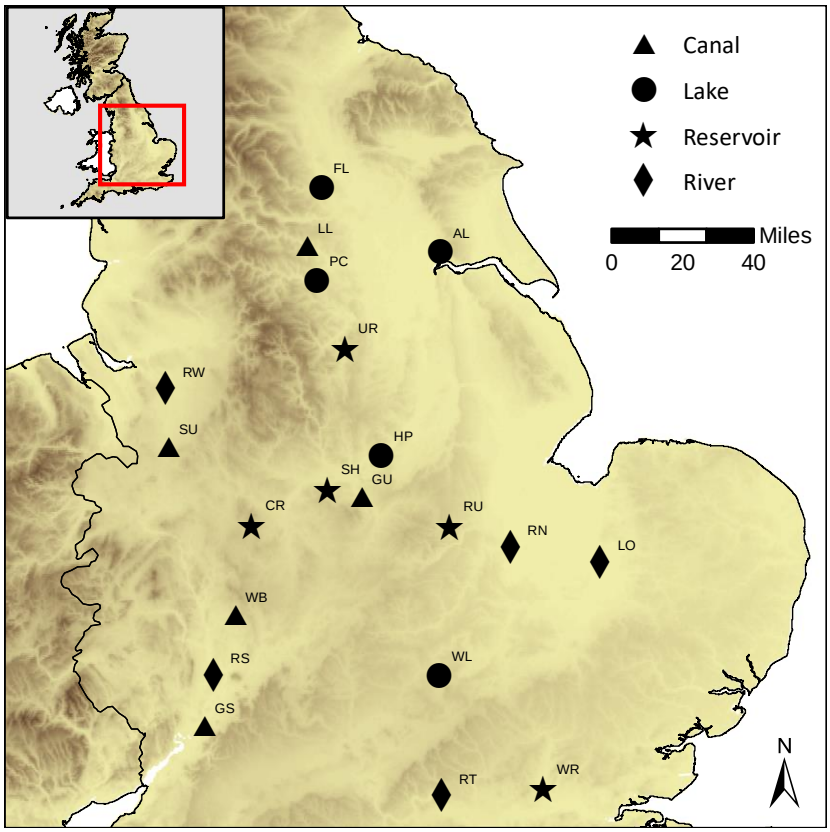

Figure 3.1 Location of the 20 sites selected for this study, identified by waterbody type. Site codes are the same as in Table 3.1.

Table 3.1 Details of the 20 sites sampled in this study. Site coordinates correspond to the middle of the transect (i.e. sample 5) for canals and rivers, while for lakes and reservoirs coordinates correspond approximately to the middle of the waterbody. For canals and rivers size information correspond to their total length, while surface area is indicated for lakes and reservoirs. Zebra mussel (ZM) records were obtained from both the National Biodiversity Network Atlas (National Biodiversity Network Trust, 2023) and Environment Agency records.

Waterbody	Site name	Site code	Latitude	Longitude	Location	Sampling date	Size	Mean depth	First ZM records
canal	Leeds & Liverpool Canal	LL	53.800	-1.571	Leeds, West Yorkshire	21/07/2021	127 miles	-	1966
	Gloucester & Sharpness Canal	GS	51.856	-2.259	Gloucester, Gloucestershire	03/08/2021	16 miles	-	1989
	Worcester & Birmingham Canal	WB	52.304	-2.061	Bromsgrove, Worcestershire	04/08/2021	30 miles	-	2004
	Grand Union Canal	GU	52.780	-1.216	Loughborough, Leicestershire	05/08/2021	137 miles	-	1969
	Shropshire Union Canal	SU	52.983	-2.509	Crewe, Cheshire	11/08/2021	66.5 miles	-	1971
lake	Farnham Lake	FL	54.035	-1.469	Farnham, North Yorkshire	15/07/2021	21 ha	3.9 m	2012
	Eight Acre Lake	AL	53.769	-0.658	North Cave, East Riding of Yorkshire	15/07/2021	3 ha	10 m	pers. comm. (Domino Joyce)
	Pugneys Country park	PC	53.657	-1.505	Wakefield, West Yorkshire	21/07/2021	28 ha	3.4 m	2019
	Holme Pierrepont	HP	52.948	-1.082	Nottingham, Nottinghamshire	05/08/2021	31 ha	2.4-3 m	pers. comm. (EA)
	Willen Lake (south)	WL	52.051	-0.718	Milton Keynes, Buckinghamshire	18/08/2021	38 ha	1.7 m	2017

reservoir	Ulley Reservoir	UR	53.383	-1.317	Aughton, South Yorkshire	23/07/2021	8 ha	3.4 m	2014
	Staunton Harold Reservoir	SH	52.810	-1.443	Melbourne, Derbyshire	02/08/2021	79 ha	7.8 m	2011
	Chasewater Reservoir	CR	52.667	-1.950	Burntwood, Staffordshire	04/08/2021	82 ha	3.7 m	2005
	Rutland Water	RU	52.652	-0.626	Oakham, Rutland	16/08/2021	1212 ha	10.9 m	2005
	Walthamstow Reservoirs	WR	51.582	-0.052	London	19/08/2021	43 ha	1-4.9 m	2016
river	River Severn	RS	52.062	-2.205	Worcester, Worcestershire	03/08/2021	220 miles	-	1870
	River Weaver	RW	53.222	-2.531	Northwich, Cheshire	10/08/2021	71 miles	-	1882
	River Nene	RN	52.566	-0.227	Peterborough, Cambridgeshire	17/08/2021	100 miles	-	1882
	Little Ouse River	LO	52.495	0.368	Southery, Norfolk	17/08/2021	37 miles	-	2017
	River Thames	RT	51.567	-0.713	Bourne End, Buckinghamshire	18/08/2021	215 miles	-	1980

Field metadata

Information on water chemistry and environmental data was recorded at each site and sampling location following collection of eDNA samples. Water temperature, pH, conductivity and turbidity were measured with a HI-98130 meter (Hanna Instruments) and the LAQUATwin Calcium Ion Ca-11 meter (Camlab, UK) was used to register calcium levels. Information on substrate type was divided into four categories: boulders (B), gravel (G), silt (S), and sand (SA). The depth at each sampling point was obtained by dipping a long pole into the water and measuring with a tape until the water mark. Number of adult individuals at each sampling location was recorded using a 1-minute kick/dip netting, depending on water accessibility. This was complemented with a 1-minute visual observation of rocks, shorelines and exposed structures nearby. When mussel abundance was too high to feasibly count in the field, a 5x5 quadrat was placed on the ground, photographed, and individuals counted later.

Biosecurity

To ensure proper biosecurity procedures, different sets of equipment (i.e. kick/dip nets and pole to measure depth) were used at each site, and all equipment that came in contact with water was cleaned at the end of each fieldwork day. For kick/dip nets this included submerging them in hot tap water for at least 15 minutes, while the pole to measure depth was rinsed repeatedly with hot tap water for a few minutes (Shannon et al., 2018). Following this, equipment was left to dry thoroughly overnight.

DNA extraction

Water samples were extracted using a modified version of the DNeasy Blood & Tissue Kit (Qiagen, UK), in a dedicated laboratory for eDNA samples. Samples from each site were extracted separately and a negative control was included in each extraction batch to monitor for contamination (n = 20). Following extractions, DNA purity and concentration was checked using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). The extraction protocol can be found in appendix 2.

Inhibition tests

All samples were tested for inhibition following the same protocol described in chapter 2. Briefly, an exogenous positive control (Fisher Scientific, UK) was used following

manufacturer's instructions, however reducing the total reaction volume to 15 µL and the sample volume to 2 µL. All samples were tested in duplicate and if the average cycle threshold (Cq) of a sample was higher than the no template reaction by more than 2 cycles, the sample was considered inhibited. All reactions were performed on a StepOnePlus Real-Time PCR machine.

Species-specific qPCR

In silico testing of the Gingera et al. (2017) cytochrome b (*Cyt* b) assay was conducted using the Primer-BLAST tool (Ye et al., 2012). Specificity was assessed by testing both primers against the entire Mollusca nucleotide database, with default settings.

Species-specific qPCR reactions were performed following the protocol described in chapter 2. In short, a repeatability test was initially conducted with a subset of 12 samples (see appendix 2 for further details) to ensure the assay generated consistent results across runs. Following this, screening of eDNA samples was conducted using the *Cyt* b assay with minor modifications. This included reducing the total volume of the reaction and the volume of sample added to 15 µL and 2 µL, respectively, as well as increasing the number of cycles to 45. Six replicates were performed for all eDNA samples, field and extraction negatives, while three replicates of PCR negative controls were included in each qPCR plate to monitor for contamination at the qPCR stage. To estimate DNA concentration, a standard curve with seven 10-fold dilutions ranging from $3x10^7$ to $3x10^1$ copies/µL was run simultaneously, and each standard dilution was performed in triplicate. All reactions were performed on a StepOnePlus Real-Time PCR machine.

Data analysis

All analyses and figures were made using R version 4.2.1 (R Core Team, 2022) and R Studio (Posit team, 2023). Unless specified otherwise, figures were generated using the ggplot2 3.4.2 package (Wickham, 2016). Explanatory variables included water temperature, pH, conductivity, turbidity, calcium, depth, substrate, waterbody type, mussel abundance (i.e. number of individuals counted) and volume filtered. Response variables were different for the exploratory analyses and mixed-effects models, and are further explained below. For sites where contamination of negative controls was

observed (n = 6), the number of DNA copies/µL detected in the negative control was subtracted from all samples of the respective site. If more than one replicate amplified in the negative control, the highest value was chosen and the same procedure was followed. While the data used in the mixed-effects models was not corrected for contamination (as the models require Cq values instead of DNA levels as the response variable; see below for more information), models were repeated with and without sites with more than one positive replicate in the field negative controls (n = 4) and results were identical.

The response variable used for the exploratory data analysis (i.e. Kruskall-Wallis tests, correlation, and PCA) was the average DNA copies/µL per sample (i.e., average of the 6 qPCR replicates, hereafter just referred to as "average DNA copies/µL" for brevity). A Kruskal Wallis test was conducted using the "kruskal.test()" function from the stats 4.2.1 package (R Core Team, 2022) to test for differences in average DNA copies/µL between substrate (B, G, S, SA) and waterbody (canal, lake, river, reservoir) types. If significant, a post-hoc Dunn's test was performed using the function "dunn.test()" from the package dunn.test 1.3.5 (Dinno, 2017), to evaluate differences between groups. Correlations and PCA were used to explore the relationship between average DNA copies/µL and explanatory variables, and between the explanatory variables. Correlations were performed with the stats 4.2.1 (R Core Team, 2022) and corrplot 0.92 (Wei & Simko, 2021) packages. The functions "cor()" and "cor.mtest()" were used to calculate Pearson correlation coefficients (r) and p-values, respectively, and the "corrplot()" function was used to plot the correlations. The PCA was conducted using the "prcomp()" function from the stats 4.2.1 package (R Core Team, 2022). All variables were scaled and centred prior to the analysis and a PCA biplot was made with the "fviz_pca_biplot()" function from the package factoextra 1.0.7 (Kassambara & Mundt, 2020). For both correlation and PCA analysis, only continuous variables were included.

Next, the effect of continuous explanatory variables on eDNA concentration was investigated using the artemis 2.0.3 package (Espe et al., 2022). The models implemented in this package use a Bayesian framework that account for censored data, i.e., when a sample or replicate does not amplify within the specified threshold (i.e. number of PCR cycles). In such cases, negative replicates will be reported as having no

DNA instead of their true (albeit likely low) DNA concentration value, thus generating censored data. If not accounted for, this can lead to biases in the model estimates, which are proportional to the amount of censorship. Additionally, these models allow the calculation of detection probabilities and to assess the influence of environmental variables using DNA concentration as the response variable, instead of simply presence/absence information. This is advantageous when detection rates are high, such as in this study, as the impact of variables would not be detected if only using detection/non-detection data. Mixed-effects models were performed using the "eDNA_lmer()" function in artemis, which uses a similar formula as the "lmer" function from the lme4 package (Bates et al., 2015). For this analysis, the Cq values of each qPCR replicate (rather than the average per sample as in the other analysis) and the slope and intercept of each standard curve were provided as required by the model to internally calculate eDNA concentration levels (specifically the natural logarithmic concentration of eDNA, hereafter referred to as "ln[eDNA]"), which was then used as response variable. Explanatory variables were scaled, and site and sample (i.e. sampling location) were included as random factors, with sample nested within site. Due to high collinearity between turbidity and conductivity, the latter was removed from the analysis. To find the model that was the best fit to our data, a full model with all explanatory variables (except conductivity) was initially performed and non-significant variables were removed sequentially until only significant variables remained. The fit and performance of all models was assessed using the loo 2.6.0 package (Vehtari, Gelman & Gabry, 2017; Yao et al., 2018), as in Espe et al. (2022). This package allows the calculation of the leave-one-out information criterion (looic), a measure used in Bayesian models for model checking and model comparison. This was achieved using the "loo()"and "loo_compare()" functions, respectively. In the final model, we considered explanatory variables to have a significant effect on DNA concentration when 95% credible intervals did not overlap zero. Detection probabilities were then calculated separately for different volumes (250, 500, 750, 1000 mL) using the "est_p_detect()" function in artemis, keeping the remaining variables constant (i.e. assigning each variable its mean value across the entire dataset).

3.4 Results

Assay performance

In silico tests showed species-specific amplification of zebra mussels, without cross-amplification of other species.

All qPCR standards exhibited 100% detection, except the lowest one (30 copies/µL) that amplified in 85% of the replicates. The limit of detection (LOD), i.e., the lowest standard with at least 95% detection, was therefore 300 copies/µL. The qPCR assay exhibited an average efficiency of 81.3% (76.9 - 90.6) and average R^2 of 0.996 (0.988 – 0.999). The 12 samples used in the repeatability test exhibited identical Cq values across both runs (Table S2.2, appendix 2). Further details about the assay and MIQE checklist (Bustin et al., 2009) are available in the supplementary material (Table S2.3, appendix 2).

No eDNA samples exhibited signs of PCR inhibition when tested with the exogenous internal positive control, with inhibition values ranging from 0 – 2.08 ($\bar{x}$ = 0.11, SD = 0.17) (Table S2.1, appendix 2). Contamination of field negative controls was observed for six sites, with amplification ranging between one and four (out of six) replicates (Table S2.4, appendix 2). No contamination was detected for any of the extraction or PCR negative controls. High detection levels were observed overall, with all sites exhibiting 100% positive samples and positive replicates ranging from 85% to 100% (Table 3.2; Table S2.4, appendix 2).

Table 3.2 Percentage of positive replicates, positive samples and average DNA copies/µL obtained for each site.

Waterbody	Site	% positive replicates	% positive samples	Average DNA copies/µL
canal	Leeds & Liverpool Canal	100	100	6.62 E+03
	Gloucester & Sharpness Canal	100	100	7.27 E+04
	Worcester & Birmingham Canal	97	100	3.46 E+03
	Grand Union Canal	100	100	3.35 E+04
	Shropshire Union Canal	100	100	2.52 E+03

lake	Farnham Lake	100	100	1.96 E+05
	Eight Acre Lake	100	100	3.97 E+04
	Pugneys Country Park	85	100	5.83 E+02
	Holme Pierrepont	100	100	2.11 E+03
	Willen Lake (south)	100	100	2.16 E+02
reservoir	Ulley Reservoir	100	100	9.12 E+04
	Staunton Harold Reservoir	100	100	2.14 E+03
	Chasewater Reservoir	100	100	5.08 E+05
	Rutland Water	98	100	6.12 E+04
	Walthamstow Reservoirs	97	100	4.69 E+05
river	River Severn	88	100	5.83 E+01
	River Weaver	100	100	8.70 E+03
	River Nene	100	100	3.66 E+03
	Little Ouse River	100	100	1.85 E+03
	River Thames	100	100	1.40 E+05

Spatial variation in eDNA concentration

The variation in eDNA concentration between the 20 sites is shown in Figure 3.2a. Average eDNA concentration per site ranged from 5.83 E+01 copies/µL at River Severn to 5.08 E+05 copies/µL at Chasewater Reservoir (Table 3.2). There was also a slight variation in eDNA concentration between the ten samples collected in each site, however this was less noteworthy than the variation observed between sites (Figure 3.2b).

Average DNA copies/µL was significantly influenced by waterbody (Kruskal-Wallis chi-squared = 27.527, df = 3, p-value = 4.565E-06). The post-hoc Dunn's test showed significant differences between reservoir and all remaining waterbody types, and no significant difference between lakes, canals or rivers (Table 3.3; Figure 3.3a). Substrate type weakly influenced average DNA copies/µL (Kruskal-Wallis chi-squared = 9.445, df = 3, p-value = 0.024), with highest median concentration close to gravel (Figure 3.3b), and

the only significant difference in the post-hoc Dunn's test between gravel and silt (Table 3.3).

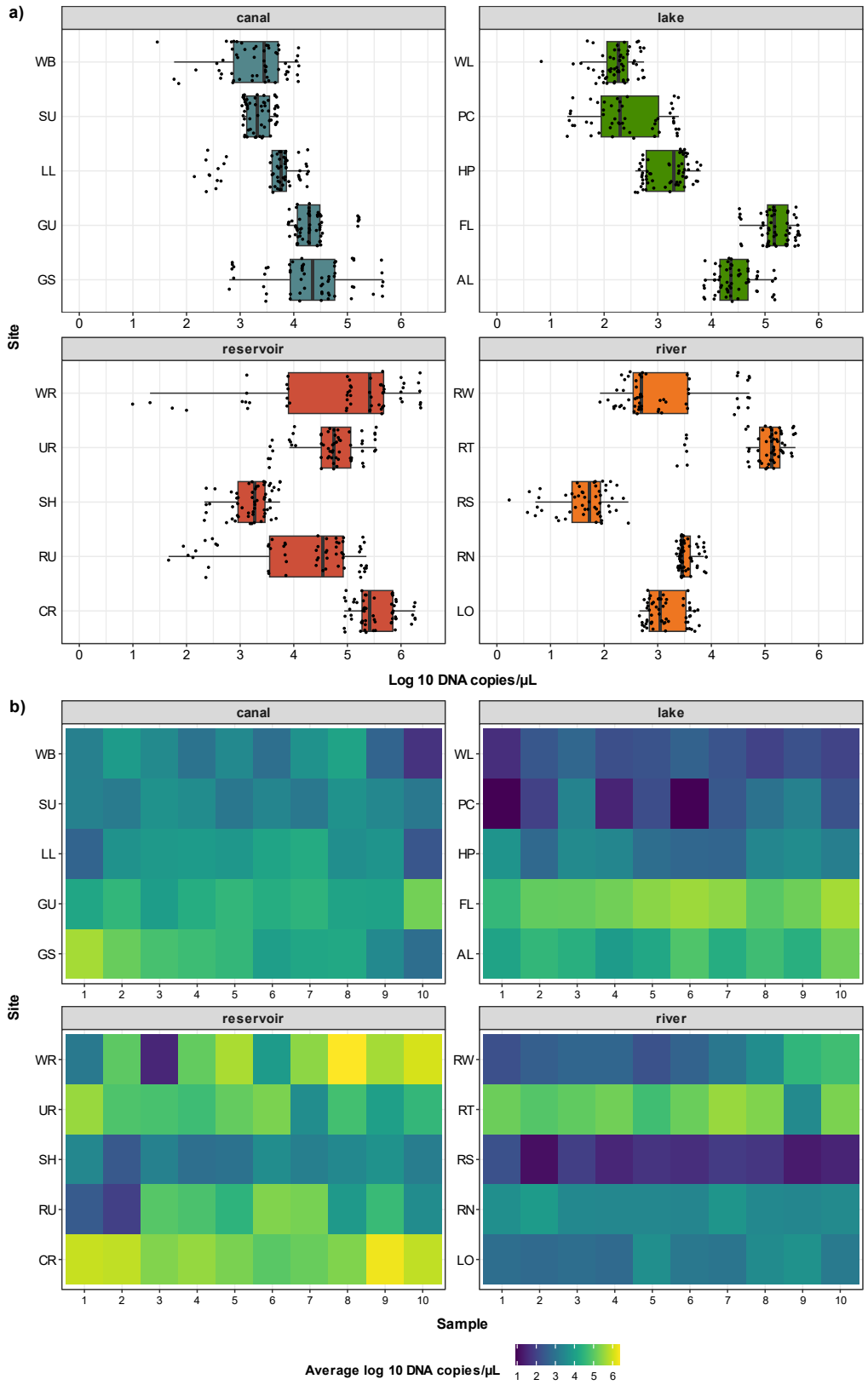

Figure 3.2 Log 10 DNA copies/µL obtained for each site **a)** and heatmap of the average log 10 DNA copies/µL obtained for each site **b)**. Site codes are the same as in Table 3.1. For visual purposes log 10 DNA copies/µL is being used instead of non-transformed data.

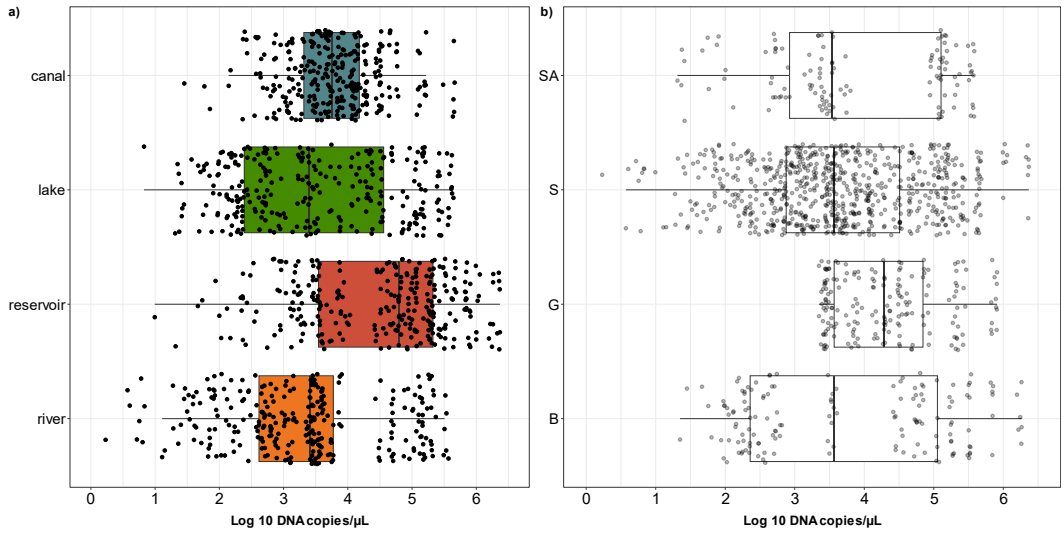

Figure 3.3 Log 10 DNA copies/µL recovered per waterbody type **a)** and substrate type (boulders - B, gravel - G, silt - S, sand - SA) **b)**. Log 10 DNA copies/µL was used instead of non-transformed data for visual purposes.

Table 3.3 Adjusted p-values of the post-hoc Dunn's test used to assess differences between groups of waterbody and substrate (boulders - B, gravel - G, silt - S, sand - SA). Stars indicate statistical significance (i.e. $p < 0.05$). For each group, average and standard deviation (SD), median, and median inter quartile range (IQR) are provided.

waterbody				
	canal	lake	river	reservoir
canal	-			
lake	0.533	-		
river	0.114	1.000	-	
reservoir	0.016*	0.000*	0.000*	-
Average + SD	2.38E+04 + 6.46E+04	4.78E+04 + 9.55E+04	3.09E+04 + 6.86E+04	2.26E+05 + 4.36E+05
Median	5.89E+03	2.41E+03	2.51E+03	6.22E+04
Median IQR	1.26E+04	3.41E+04	5.05E+03	2.08E+05
substrate				
	B	G	S	SA
B	-			
G	0.110	-		
S	1.000	0.007*	-	
SA	1.000	0.263	1.000	-
Average + SD	1.47E+05 + 3.61E+05	9.24E+04 +1.89E+05	6.73E+04 + 2.38E+05	7.38E+04 + 1.13E+05
Median	3.43E+03	1.92E+04	3.60E+03	3.22E+03
Median IQR	1.01E+05	5.59E+04	2.91E+04	1.27E+05

Variation in field metadata

The variation in environmental variables is shown in Figure 3.4. The number of mussels recorded ranged from 0 – 285 ($\bar{x}$ = 14.54, SD = 38.54), with the highest abundance observed in a canal (Figure 3.4a). Calcium ranged from 35 – 560 ppm (mg/L) ($\bar{x}$ = 245.62, SD = 157.07) and values were more consistent in reservoirs and more variable in lakes (Figure 3.4b). Conductivity ranged from 0.32 – 0.97 mS ($\bar{x}$ = 0.71, SD = 0.16), with the highest value observed in a lake (Figure 3.4c). Depth ranged from 4 – 339 cm ($\bar{x}$ = 76.25, SD = 63.25) and pH ranged from 7.64 – 10.64 ($\bar{x}$ = 8.39, SD = 0.62), both showing highest variability in lakes (Figure 3.4d and 3.4e, respectively). Water temperature ranged from 17.1 – 29.6°C ($\bar{x}$ = 20.13, SD = 2.46), with the highest values registered in lakes (Figure 3.4f). Turbidity values ranged from 0.16 – 0.48 ppt ($\bar{x}$ = 0.35, SD = 0.08) and followed the same pattern as conductivity (Figure 3.4g). Volume filtered ranged from 200 – 1000 mL ($\bar{x}$ = 787, SD = 259.75), with more water filtered in rivers (Figure 3.4h). Information on field metadata for each sample can be found in appendix 2 (Table S2.1).

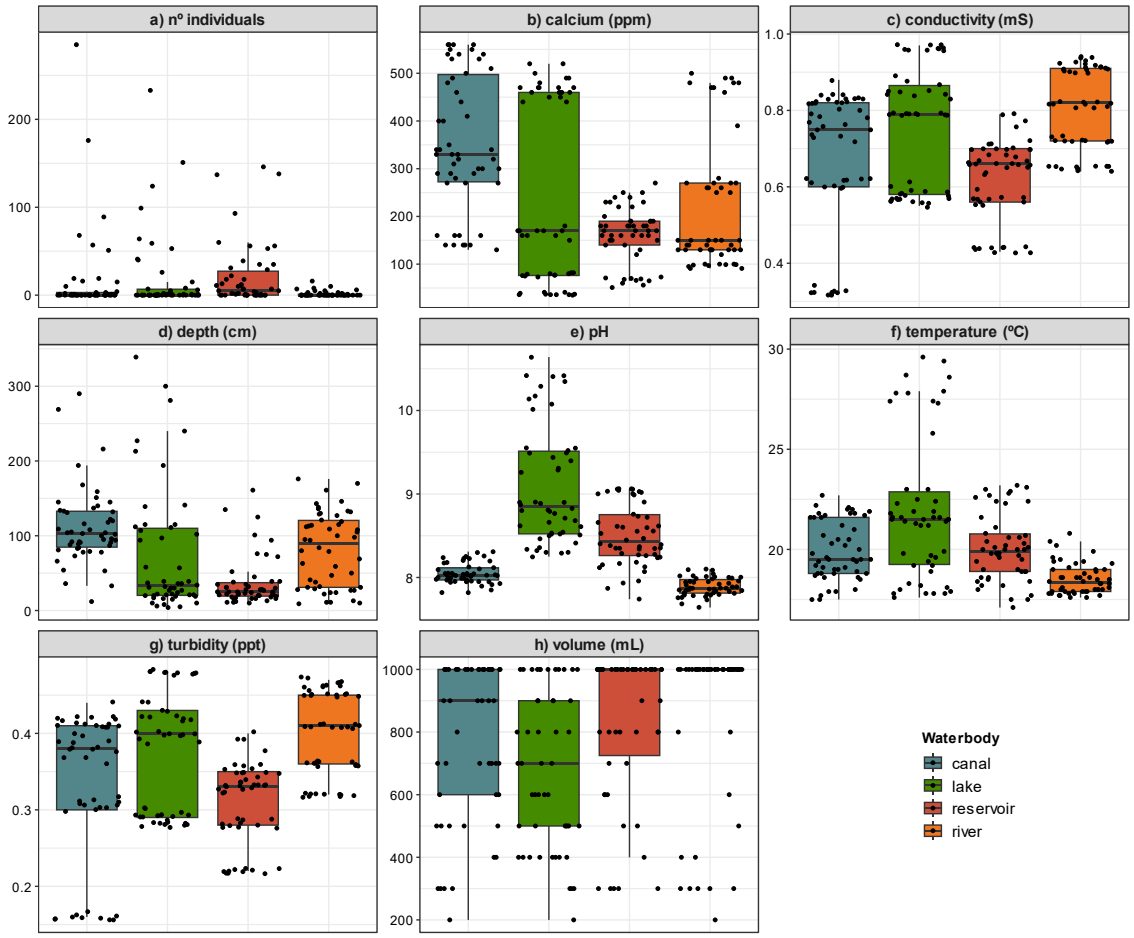

Figure 3.4 Variation in number of individuals and environmental variables per waterbody type. Each point corresponds to a unique sample.

Influence of environmental variables on DNA concentration

In the PCA biplot, the first principal component explained 32.9% of the variance and was mostly influenced by turbidity and conductivity, while 15.7% of the variance was explained by the second principal component, with temperature and pH having the biggest contribution (Figure 3.5a). The four clusters representing each waterbody type overlapped and were not distinguishable, with data from rivers being more tightly grouped, and lakes exhibiting higher variability. No variables were in close proximity with average DNA copies/µL suggesting no positive relationship with any explanatory variable, while turbidity, conductivity, calcium and depth are suggested to have a negative relationship with average DNA copies/µL as they are pointing in opposite directions.

Average DNA copies/µL was negatively correlated with both turbidity ($r(198) = -0.184$ and $p = 9.10\text{ E-}03$) and conductivity ($r(198) = -0.186$ and $p = 8.28\text{E-}03$) (Figure 3.5b; Table 3.4). These variables were also significantly correlated with each other ($r(198) = 0.998$ and $p = 7.25\text{E-}250$) and with all remaining variables except the number of individuals. The correlation coefficient and significance level with other variables were similar for both turbidity and conductivity. They were, respectively, negatively correlated with temperature ($r(198) = -0.345$ and $p = 5.59\text{E-}07$; $r(198) = -0.345$ and $p = 5.82\text{E-}07$) and pH ($r(198) = -0.176$ and $p = 1.28\text{E-}02$; $r(198) = -0.178$ and $p = 1.16\text{E-}02$), and positively correlated with volume filtered ($r(198) = 0.403$ and $p = 3.26\text{E-}09$; $r(198) = 0.401$ and $p = 4.00\text{E-}09$), calcium ($r(198) = 0.423$ and $p = 4.49\text{E-}10$; $r(198) = 0.429$ and $p = 2.39\text{E-}10$) and depth ($r(198) = 0.169$ and $p = 1.70\text{E-}02$; $r(198) = 0.175$ and $p = 1.31\text{E-}02$).

Scatterplots of the average DNA copies/µL plotted against the different explanatory variables can be found in appendix 2 (Figure S2.1).

Table 3.4 Pearson correlation coefficients (top) and p-values (bottom) for each combination of variables. Significant correlations are highlighted in bold.

	Average DNA copies/µL	**Volume**	**Temperature**	**pH**	**Turbidity**	**Conductivity**	**Calcium**	**Depth**	**Individuals**
Average DNA copies/µL	-	0.037	0.010	0.011	**-0.184**	**-0.186**	-0.092	-0.133	0.066
Volume	6.04E-01	-	**-0.256**	**-0.156**	**0.403**	**0.401**	**0.201**	0.046	-0.137
Temperature	8.86E-01	**2.54E-04**	-	**0.692**	**-0.345**	**-0.345**	**-0.154**	-0.012	0.091
pH	8.73E-01	**2.76E-02**	**7.37E-30**	-	**-0.176**	**-0.178**	**-0.195**	**-0.181**	0.039
Turbidity	**9.10E-03**	**3.26E-09**	**5.59E-07**	**1.28E-02**	-	**0.998**	**0.423**	**0.169**	0.011
Conductivity	**8.28E-03**	**4.00E-09**	**5.82E-07**	**1.16E-02**	**7.25E-250**	-	**0.429**	**0.175**	0.012
Calcium	1.97E-01	**4.39E-03**	**2.98E-02**	**5.77E-03**	**4.49E-10**	**2.39E-10**	-	**0.165**	-0.074
Depth	5.98E-02	5.15E-01	8.61E-01	**1.04E-02**	**1.70E-02**	**1.31E-02**	**1.99E-02**	-	**0.233**
Individuals	3.53E-01	5.23E-02	2.02E-01	5.81E-01	8.74E-01	8.71E-01	2.97E-01	**9.20E-04**	-

The best mixed-effects model, explored using artemis, included the explanatory variables volume filtered, calcium, number of individuals, pH and turbidity (looic value -359.7), and excluded depth, temperature and conductivity. Model fit values for all other models are available in the supplementary material (Table S2.5, appendix 2). The final model showed a significant positive effect of volume filtered on ln[eDNA] (Figure 3.5c; Table 3.5). Credible intervals for the remaining variables tested – pH, turbidity, number of individuals and calcium - overlap zero, indicating no significant effects (Figure 3.5c; Table 3.5).

Exploration of detection probabilities with different filtered volumes indicated that the filtration of at least 250 mL of water provides a 95% detection probability with as low as two samples (Figure 3.5d; Table S2.6, appendix 2). If filtering 500 mL, 99% detection probabilities are reached with three samples, while only two samples are need to reach 99% detection probability when filtering 750 mL and 1000 mL (Table S2.6, appendix 2).

Table 3.5 Model estimates (mean and 95% credible interval) for the effects of continuous variables on ln[eDNA] based on mixed-effects models performed in artemis, as plotted in Figure 3.5c. Variables were considered significant when 95% credible intervals did not overlap zero.

Variable	Model estimates
Volume	0.219 (0.084, 0.363)
pH	-0.148 (-0.485, 0.215)
Turbidity	-0.008 (-0.512, 0.539)
Individuals	0.065 (-0.058, 0.184)
Calcium	0.237 (-0.315, 0.819)

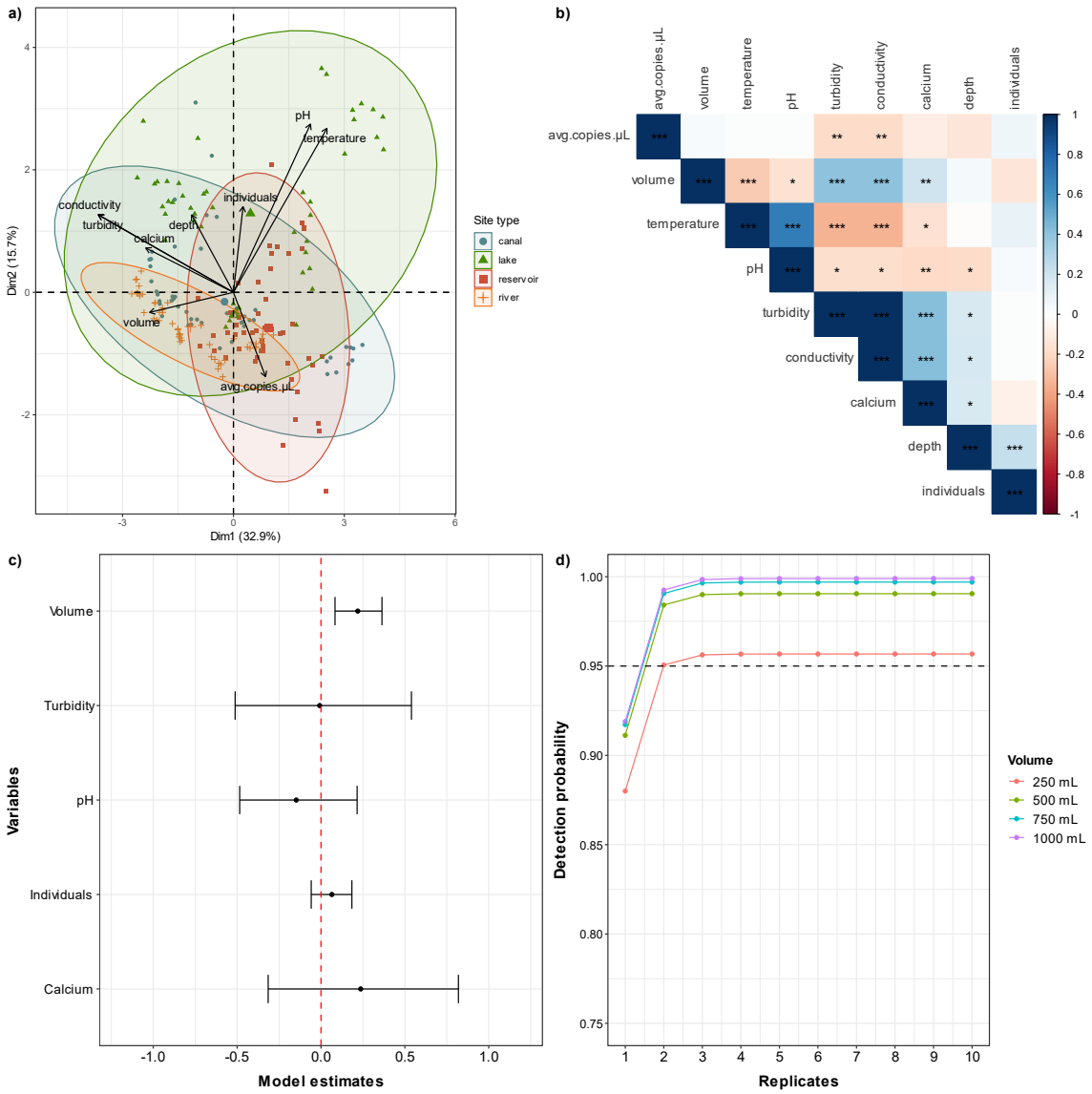

Figure 3.5 a) PCA biplot and **b)** correlation plot, showing the relationship between variables, where "avg.copies.µL" corresponds to average DNA copies/µL; the PCA ellipses were drawn with a confidence level of 0.95 (default value); correlation colours indicate the strength of each pairwise correlation, with stronger colours representing a stronger correlation, whether positive (blue) or negative (red); stars indicate the significance level, with lack of significance depicted by empty squares. **c)** Model estimates for the effects of continuous variables on ln[eDNA] based on mixed-effects models performed in artemis; dots represent the mean and error bars represent the 95% credible intervals. **d)** Zebra mussel detection probabilities depending on the volume filtered and number of replicates, estimated in artemis; the black dashed line represents the 95% detection probability; the 95% credible intervals for each value can be found in the supplementary material (Table S2.6, appendix 2).

3.5 Discussion

In this study, 20 sites covering four waterbody types and varying environmental conditions were sampled in order to assess the influence of environmental variables on

concentration of zebra mussel eDNA and determine detection probabilities. Detection rates were high for all sites, with 85-100% positive replicates and 100% positive samples for all sites. Average eDNA concentration per sample was influenced by waterbody type, being higher in reservoirs than in lakes, rivers or canals, and weakly influenced by substrate type. Correlations between average eDNA concentration and environmental variables indicated a negative relationship with turbidity and conductivity. However, this was not supported by the mixed-effects models conducted with artemis, that used uncensored ln[eDNA] as the response variable (Espe et al., 2022). The best model included volume filtered, calcium, number of individuals, pH and turbidity, but only volume significantly influenced ln[eDNA]. Our results demonstrate that zebra mussel detection rates are consistently high across waterbodies and environmental conditions, at least when sampling is carried out in the summer and with established populations, but that eDNA concentration is influenced by waterbody type and volume filtered, and weakly influenced by substrate type.

Spatial variation in eDNA concentration and detection rates

Zebra mussel densities are expected to be higher in canals than in other waterbodies due to the combination of stable sediments and continuous water current that delivers food and oxygen (Karatayev & Burlakova, 2022), but eDNA concentration is predicted to be lower in lotic than in lentic systems due to horizontal transport and dilution (Harrison et al., 2019; Herder et al., 2014). Here, average eDNA concentration was significantly affected by waterbody type, being higher in reservoirs than lakes, canals and rivers. This is similar to findings from our previous study (chapter 2), where eDNA concentrations were higher in a reservoir compared to a river. However, contrary to our expectations, eDNA concentrations were similar between lakes and lotic systems. Within lentic systems, one could hypothesize that the difference between reservoirs and lakes could relate to availability of substrate. However, as the percentage of hard substrate (i.e., boulders and gravel combined) in reservoirs and lakes was similar (42 and 40%, respectively), this is unlikely to explain the difference observed. Other factors such as site morphometry could be influencing species abundance and consequently eDNA availability. While all reservoirs and lakes were shallow and similar in average depth, reservoirs were typically larger in terms of surface area (apart from Ulley Reservoir), which could provide more extensive surfaces for attachment and food sources, and thus

harbour higher zebra mussel densities. Despite the differences in eDNA concentrations, detection rates were high for all waterbody types.

We predicted that eDNA concentration would be highest close to hard substrates, where mussels are expected to be more abundant (Karatayev & Burlakova, 2022). However, average eDNA concentration was only weakly influenced by substrate type, with the highest concentrations close to gravel, and the only significant difference between substrates found between gravel and silt. This is in contrast to a previous study that reported higher concentration of zebra mussel eDNA associated with soft substrates (flock, silt and sand) in a lake (Amberg et al., 2019). This suggests that different waterbodies and substrates can interact in unique ways to influence the persistence and distribution of eDNA. In our study, the uneven distribution of substrates across all sites (see Table S2.7, appendix 2) may have influenced the results obtained. The relationship between waterbody and substrate is thus crucial in understanding the dynamics of eDNA concentration in the environment and should be considered for effective eDNA sampling design. This finding, together with the limited spatial variation in eDNA concentration found here within site, further suggests that zebra mussel eDNA is distributed relatively uniformly across a waterbody and that sampling over softer sediments, where mussels are less likely to settle, is as effective as sampling over hard substrates (Amberg et al., 2019).

Effects of environmental variables on eDNA concentration

Results from our PCA and correlations combined indicated that turbidity and conductivity were significantly negatively correlated with average eDNA concentration, and positively correlated with one another. Average eDNA concentration was not correlated with any other variables. The mixed-effects model, using uncensored ln[eDNA] as the response variable, provided quite different insights. The best model included volume filtered, turbidity, number of individuals, pH and calcium, and only volume filtered had a clear positive effect on ln[eDNA], as the 95% credible intervals of the remaining variables overlap with zero. We consider the mixed-effects model results to be the most informative since they are not subject to the problems of data censorship (Espe et al., 2022). The following discussion therefore focusses on interpreting the model results.

The probability of capturing target eDNA increases as more water is filtered, and the positive effect of volume on eDNA concentration and detection rates has been consistently demonstrated (e.g. Goldberg et al., 2018; Mächler et al., 2016; Peixoto et al., 2023). In our previous study, investigating seasonal variation in zebra mussel eDNA, volume filtered was positively correlated with eDNA concentration in both a river and reservoir (chapter 2). Here, volume filtered and average eDNA concentration were not correlated, but the mixed-effects model estimates showed that ln[eDNA] was positively influenced by volume filtered, as expected. While previous studies have shown that low volumes such as 200 mL could limit eDNA detection rates (e.g. Goldberg et al., 2018), here the filtration of at least 250 mL with as few as two samples would be enough to provide a 95% detection probability, suggesting that zebra mussel eDNA was sufficiently abundant in our sites to be detected even with such small volumes. This could be the result of high densities across all our sites and/or high eDNA concentration during the sampling period due to spawning.

Turbidity was significantly negatively correlated with average eDNA concentration, and the PCA plot also suggested a negative relationship. High turbidity is generally thought to present a challenge for detecting eDNA because suspended particles can quickly clog filters, reducing the volume of water filtered, and concentrating PCR inhibitors such as humic acids (Takasaki et al., 2021; Williams, Huyvaert, & Piaggio, 2017). However, higher turbidity did not increase filter clogging in our study, as turbidity and volume filtered were positively correlated. We also found no evidence of inhibition in our samples, suggesting turbidity was not associated with humic acids or particulate inhibitors. The negative correlation between turbidity and average eDNA concentration was not reflected in the mixed-effects model. The 95% credible intervals for turbidity overlap zero, indicating no significant effect on ln[eDNA]. As the mixed-effects model account for data censorship, this result is likely more informative. Corresponding scatterplots also indicate the relationship between turbidity and average eDNA concentration to be weak. In our previous work, we found that turbidity was positively correlated with eDNA concentration in a reservoir but negatively correlated in a river (chapter 2). This, combined with the findings of the current study, suggest that the relationship between eDNA concentration and turbidity is complex. Additional analyses based on waterbody type (i.e. rivers, canals, lakes, reservoirs) could offer further insights on the relationship

between these variables, but more sites per waterbody are required than included in the present study, to increase statistical power.

Zebra mussel abundance had no significant effect in the mixed-effects model, and the exploratory analysis (PCA and correlation) also suggest no relationship with average eDNA concentration. This result is consistent with our previous work in a single reservoir over the course of one year (chapter 2) and with a study conducted in two North American lakes (Amberg et al., 2019), where no correlation was found between zebra mussel density and eDNA concentration. Other studies have reported a positive but weak relationship between eDNA concentration and zebra mussel numbers in a Danish river system (Shogren et al., 2019). As in our previous work, sampling of adults was also restricted to the edges of waterbodies. Consequently, our abundance data might not accurately represent true population densities, which could explain the pattern observed. In fact, we were able to detect zebra mussel eDNA in sites where no adult mussels were recorded through dip/kick netting and visual observation. Alternative methods not restricted to the periphery of the waterbody, such as scuba diving or the use of videography (Karatayev et al., 2021b), could provide a more precise assessment of densities for comparison with DNA concentration, although this was not feasible in our study due to logistic reasons.

Calcium and pH are considered the most important variables that influence the establishment of viable zebra mussel populations (Karayatev & Burlakova, 2022), however neither of the variables significantly influenced ln[eDNA] in the mixed-effects model. Additionally, correlation plots also did not suggest any significant relationship of either variable with average eDNA concentration. Calcium is needed for maintaining shell integrity and we therefore expected a positive influence of calcium on mussel abundance, and therefore eDNA concentration. Our previous study (chapter 2) found no correlation between eDNA concentration and calcium, however the range of values was limited due to only sampling two waterbodies (131 and 310 ppm, for Eccup Reservoir and the River Hull, respectively). The 20 waterbodies sampled here increased the range of calcium values to 525 ppm, however its effect on ln[eDNA] was still not significant, and there was no correlation between calcium and the number of individuals. The effect of calcium on eDNA concentration has been rarely explored, but a previous

study has also failed to observe a significant correlation between the two variables (Calata et al., 2019). Its effect may be complicated by the interaction with other ions such as sodium, chloride, potassium and magnesium (Garton et al., 2013), and this warrants further investigation. As acidic conditions promote eDNA degradation (e.g. Goldberg et al., 2018), we expected a positive influence of pH on eDNA concentration. pH of the waterbodies sampled here ranged from 7.64-10.64 and, consistent with our previous work (chapter 2), did not influence ln[eDNA]. Zebra mussels are limited to alkaline waters, with their pH tolerance estimated to be between 7.3-7.5 (Karatayev & Burlakova, 2022) and 9.3-9.6 (Bowman & Bailey, 1998). The narrow pH range within which zebra mussels thrive, characterized by low eDNA decay rates (e.g. Strickler et al., 2015), suggests that any degradation that does occur is unlikely to be primarily influenced by pH levels, thus possibly explaining the lack of effect observed in this and the previous study.

While conductivity was not included in the mixed-effects model due to high collinearity with turbidity, the PCA and correlation plots both suggest a negative relationship with average eDNA concentration, which is consistent with previous studies (Harper et al., 2019b; Peixoto et al., 2023). For zebra mussel-infested sites, this could potentially be due to a decrease in calcium and carbonate ions in the water column that is used for their shells (Jones & Montz, 2020), however our results do not indicate a significant negative relationship between number of zebra mussels and calcium levels. Alternatively, it is possible that waterbody type influenced the result to some extent, as conductivity was lower in reservoirs, where more eDNA was recovered from.

Finally, water depth and temperature were also excluded from the final mixed-effects model, and the correlation plots do not indicate any significant relationship of these variables with average eDNA concentration. A previous study found that zebra mussel eDNA concentration was positively correlated with depth in a heavily infested lake, but not in a newly-established site (Amberg et al., 2019). The sampling depths recorded in this study were within a slightly narrower range (4-339 cm), than those of Amberg et al. (2019; 1-6 m) which could have affected our ability to detect changes. Our result might also be attributed to the proximity of our sampling locations to the shoreline, where eDNA is likely to be mixed by wave movement. In contrast, eDNA distribution is likely to

be more heterogeneous in deeper, stratified lakes due to reduced water mixing (e.g. Lawson Handley et al., 2019). The results obtained for water temperature contradict the findings from our previous study (chapter 2), which found a strong positive relationship between temperature and eDNA concentration when sampling was conducted over the course of a year, with a peak in eDNA concentration during the summer relating to mussel spawning. However, a smaller effect (if any) was expected in the present study due to the shorter sampling period (two versus twelve months) which specifically targeted the peak reproductive period for zebra mussels, thus resulting in a narrower range of temperatures (temperature range of 12.5°C here, compared to a range of 14.3 and 18°C in chapter 2, for the River Hull and Eccup Reservoir, respectively) and thus less statistical power. Previous studies demonstrating the effect of temperature have tested larger temperature ranges such as 20°C (Tsuji et al., 2017; Kasai et al., 2020) and 30°C (Strickler et al., 2015).

Conclusions, recommendations and future work

Relative to our previous work, the increased spatial variation in this study provides new insights about the influence of environmental variables on zebra mussel eDNA detectability. Overall, our results suggest that eDNA concentration is mostly influenced by waterbody type and volume filtered, and to a lesser extent by substrate type. Nevertheless, detection rates were consistently high for all sites, demonstrating that zebra mussel eDNA is consistently detected across a wide range of waterbody types and environmental conditions when populations are established and sampling is carried out in the summer, corresponding to the peak mussel reproductive season.

This study aimed to increase the readiness of the Gingera et al. (2017) *Cyt* b assay to an operational level (Thalinger et al., 2021). By investigating the effects of environmental variables on eDNA availability, estimating detection probabilities and conducting further *in silico* tests, we argue the assay can now be considered operational for use in routine monitoring of established zebra mussel populations, as long as good sampling and experimental design is performed, including appropriate replication at the sampling and technical levels. However, we recommend that further research is conducted to understand how the assay performs at lower population densities, in newly established sites.

3.6 Acknowledgments

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Chapter 4

Mussels on the move: new records of the invasive non-native quagga mussel (*Dreissena rostriformis bugensis*) in England using eDNA and a new probe-based qPCR assay

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4.1 Abstract

Invasive non-native species (INNS) pose a worldwide environmental threat, negatively impacting invaded ecosystems on an ecological and economical scale. In recent decades, quagga mussels (*Dreissena rostriformis bugensis*) have successfully invaded several countries in Western Europe from the Ponto-Caspian region, being recorded for the first time in England in 2014, in Wraysbury, near London. In recent years, environmental DNA (eDNA) analysis has proven to be a sensitive and effective method for early detection and monitoring of a number of INNS. Previously, a dye-based quantitative PCR (qPCR) assay was developed for the detection of quagga mussels from eDNA samples. Here, a target-specific probe was designed to further increase the specificity of this assay and used to obtain an updated distribution of this species in England. Twenty-four sites were sampled, including sites with established populations near London and sites spread across the East Midlands and East Anglia regions. Positive detections were obtained for 11 of the 24 sites, and these were widely spread, as far as Nottingham (East Midlands) and Norfolk (East Anglia). Detection rates were 100% at the three sites with known

established populations, while rates were lower (3-50% of positive replicates) in the eight newly-identified sites, consistent with an early stage of invasion. Of particular concern was the detection of quagga mussels in major waterways and in popular recreational sites, highlighting urgent measures are needed to control pathways and spread. Our study demonstrates that quagga mussels are considerably more widespread in England than previously thought and provides a much-needed step towards operational use of eDNA for monitoring quagga mussels.

4.2 Introduction

Invasive non-native species (INNS) pose a worldwide environmental threat, causing ecological and economic impacts on invaded ecosystems. Ponto-Caspian invaders such as the quagga mussel (*Dreissena rostriformis bugensis,* Andrusov, 1897) are of special concern due their successful large-scale invasion into Western Europe in recent decades. The first observation of quagga mussels in Western Europe dates back to 2006 in The Netherlands (Molloy et al., 2007). This introduction was suggested to be either via the Main-Danube Canal (Molloy et al., 2007) or due to the discharge of contaminated ballast water in the port of Rotterdam (Velde & Platvoet, 2007). River connectivity within Europe allowed for further spread, and in the following years more records of quagga mussels were documented, with first detections in Germany in 2007 (Velde & Platvoet, 2007), Belgium in 2010 (Marescaux & Van Doninck, 2012), France in 2011 (Bij de Vaate & Beisel, 2011), Switzerland in 2015 (Haltiner et al., 2022), and Italy in 2022 (Salmaso et al., 2022).

In England, the first quagga mussel record was documented during routine monitoring in 2014 in Wraysbury River, a tributary of the River Thames, near London (Aldridge et al., 2014). This observation occurred shortly after a horizon scanning study identified quagga mussels as the non-native species with the highest risk of invasion, establishment, and impacts in Great Britain (GB), posing major threats to Britain's freshwater biodiversity (Roy et al., 2014). After almost a decade, quagga mussel populations are now well established in the Thames and its tributaries. With the River Thames serving as a major corridor to the wider canal and river network (Aldridge et al., 2014), populations of quagga mussels have since been discovered in reservoirs and other waterways north of London (National Biodiversity Network Trust, 2023; Figure

4.1). More recently, they have been found further north in Rutland Water reservoir (Environment Agency, 2020) and in a water treatment facility in Lincoln (Aldridge, 2023; Figure 4.1), making the latter the northernmost point they have been recorded so far.

Species distribution models based on bioclimatic data predict that the distribution of quagga mussels could extend to much of England, central Scotland and southern Wales (Gallardo and Aldridge, 2013). It is often assumed that their distribution will be similar to that of zebra mussels, which have been established in GB for over 100 years and are widespread (Aldridge et al., 2014). Nevertheless, environmental models suggest that there are important differences in the two species' niches, with quagga mussels preferring higher temperatures and lower precipitation (Quinn, Gallardo & Aldridge, 2014), and being more tolerant of low oxygen (Karatayev et al., 1998). Moreover, while zebra mussels require hard substrates and are more abundant in the littoral zone, quagga mussels are also able to attach to and colonise soft substrates such as silty sediments, and favour the profundal zones of lakes (Karatayev et al., 2015; Karatayev & Burlakova, 2022).

Quagga mussels are generally more competitive than zebra mussels, and in most sites where the two species co-occur they can quickly outcompete and displace zebra mussels (Haltiner et al., 2022; Karatayev et al., 2021a; Strayer et al., 2019). A study in which larvae of both species were reared in controlled conditions showed that the planktonic stage of quagga mussels (veligers) took more time to settle (Wright et al., 1996), suggesting an extended presence in the water column which could allow them to disperse over longer distances. Moreover, quagga mussels are able to reproduce at lower temperatures than zebra mussels (Karatayev & Burlakova, 2022) and larvae are usually found year-round in invaded sites (e.g. Haltiner et al., 2022). Their ability to survive and grow at lower temperatures and with less food is reflected in greater ecological impacts on invaded ecosystems and for longer periods of time (Karatayev & Burlakova, 2022).

Both dreissenid species are ecosystem engineers due to their high water filtering capacity, which has important direct and indirect effects on invaded systems (MacIsaac, 1996; Roy et al., 2014). Quagga mussels quickly proliferate to become dominant, causing

ecological impacts that range from changes to the density and richness of macroinvertebrate communities (Mills, Chadwick & Francis, 2019; Ward and Ricciardi, 2007), fouling and suffocation of unionid mussels (Larson et al., 2022; Schloesser et al., 2006), and modifications of the river's geomorphic processes such as sediment mobility (Sanders et al., 2022). In addition to ecological impacts, the biofouling of infrastructures on invaded sites poses a problem, particularly for water companies (Chakraborti et al., 2013). The presence of quagga mussels on infested water treatment plants requires increased maintenance to keep the components (e.g., pipes, tanks, intake structure) clean and this is associated with elevated costs (Chakraborti, Madon & Kaur, 2016; Connelly et al., 2007). The potential for spread and high impact, both ecologically and economically, means that the quagga mussel is recognised as a top priority species for monitoring and mitigation (Roy et al., 2014).

Sensitive tools that allow early detection and rapid response are crucial for monitoring invasive species such as quagga mussels. The analysis of environmental DNA (eDNA) samples (e.g. soil, water, air) has proven to be an efficient method for the detection of INNS from different taxonomic groups and different environments, often outperforming traditional methods (Blackman et al., 2018; Fonseca et al., 2023; Lawson Handley, 2015). Likewise, quantitative PCR (qPCR) has proven to be an efficient and sensitive method and has been the technique of choice over the years for the detection of several INNS, across different taxonomic groups (e.g. Gingera et al., 2017; Prabhakaran et al., 2023; Roux et al., 2020).

Several targeted eDNA assays already exist for the detection of dreissenid mussels from eDNA samples (Feist & Lance, 2021), however some of these co-amplify both quagga and zebra mussels (e.g. Gingera et al., 2017; Peñarrubia et al., 2016). Recently, sensitive and species-specific conventional PCR and dye-based qPCR assays ("DRB1") were developed for quagga mussels and tested *in silico*, *in vitro*, in mesocosm experiments and field trials (Blackman et al., 2020a, 2020b). Both the conventional and dye-based qPCR assays, from here referred to as cDRB1 and dDRB1 respectively, outperformed kick-sampling and eDNA metabarcoding for the detection of quagga mussels in field trials conducted in the Wraysbury River, and the qPCR assay had the advantage of

providing information on the decreasing signal of DNA concentration with increasing distance from the main source population (Blackman et al., 2020b).

Understanding the uncertainties and limitations associated with targeted eDNA assays, such as the rate of false positives and negatives, helps policymakers and end-users to choose the best assay for routine monitoring. With this in mind, a 5-level validation scale for targeted eDNA assays was developed by Thalinger et al. (2021), with minimum criteria defined for each level, ranging from level 1 ("incomplete") to level 5 ("operational"). The dDRB1 assay currently meets the minimum requirements of levels 1-3, i.e., *in silico* analysis, *in vitro* testing on target tissue and closely related species, and detection from environmental samples (Blackman et al., 2020a, 2020b). However, it is well recognised that inclusion of a target-specific probe during qPCR increases assay specificity and reduces the chance of false positives, providing more confidence in the results (Thalinger et al., 2021). Further development and testing of the DRB1 assay is therefore required to improve the readiness of the assay for routine monitoring. The goals of this study were thus to 1) further improve the dDRB1 assay, by designing a probe and estimating limits of detection (LOD), and 2) use the probe-based qPCR assay (from here referred to as pDRB1) to screen for quagga mussels in several locations in England, in order to update their current distribution.

4.3 Materials and methods

Development of pDRB1 assay

The quagga mussel-specific DRB1 assay (Blackman et al., 2020a, 2020b), targeting a 188 bp fragment of the cytochrome oxidase I (COI) gene, was further developed by designing a target-specific probe (Table 4.1). A TaqMan probe was designed using the PrimerQuest tool (IDT, www.idtdna.com) in conjunction with alignments (Clustal Omega) of quagga and related mussel COI sequences from the EBI database. Candidate probe sequences were confirmed *in silico* using the EBI database with the consensus target sequences used for eventual quantification standards (Table 4.1).

Table 4.1 Details of the species-specific pDRB1 assay, including primers, probe, and ultramers (used for the standard curve).

Oligo name	Sequence (5'- 3')	Size (bp)
Primer forward	GGA AAC TGG TTG GTC CCG AT	20
Primer reverse	GGC CCT GAA TGC CCC ATA AT	20
Probe	6FAM - TCG GCG TTT AGT GAG GGC GGA TTT - QSY	24
Ultramer forward	GGAAACTGGTTGGTCCCGATAATACTAAGTCTTCCTGATATAGGTTTC CCTCGTTTAAATAATGTAAGATTTTGGGTTCTACCTATCTCTATRGCCT TATTATTCTGTTCGGCGTTTAGTGAGGGCGGATTTGGTGGGGGTTGA ACATTATATCCACYGTTATCCAGGATTATGGGGCATTCAGGGCC	188
Ultramer reverse	GGCCCTGAATGCCCCATAATCCTGGATAACRGTGGATATAATGTTCAA CCCCCACCAAATCCGCCCTCACTAAACGCCGAACAGAATAATAAGGCY ATAGAGATAGGTAGAACCCAAAATCTTACATTATTTAAACGAGGGAA ACCTATATCAGGAAGACTTAGTATTATCGGGACCAACCAGTTTCC	188

The new primer and probe combination was then optimised *in vitro* by varying annealing temperatures against tissue of quagga mussels, closely related taxa - zebra mussel (*Dreissena polymorpha*), killer shrimp (*Dikerogammarus villosus*) and demon shrimp (*Dikerogammarus haemobaphes*) - and common species found in England - pea mussel (*Sphaerium corneum*), blue mussel (*Mytilus edulis*), European oyster (*Ostrea edulis*), common periwinkle (*Littorina littorea*) and the common limpet (*Patella vulgata*). Standards made of the target amplicon, which included the primer and probe binding sites (ultramers; Table 4.1), and with known copy numbers were used to assess the assay's efficiency. For this, four 10-fold dilutions ranging from 10^1 to 10^4 copies/µL numbers were run together with the tissue samples. Each standard was run twice and tissue samples four times. qPCR reactions were performed with 12.5 µL of TaqMan Universal PCR Master Mix (Fisher Scientific, UK), 1.6 µM of primers forward and reverse combined, 0.05 µM of probe, 0.64 mg/mL of BSA and 2 µL of sample. The qPCR thermal profile consisted of an initial step at 50°C for 10 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 seconds and annealing for 1 min at 60, 62 and 63°C.

eDNA water samples from Wraysbury River, previously collected and tested with the dDRB1 assay by Blackman et al. (2020b), were tested with the new probe-based assay to compare the performance of both assays. Three samples were selected from three different locations - Wraysbury Bridge, Wraysbury Gardens, Wraysbury Weir. A total of nine samples were chosen based on DNA copy numbers, in order to include a range of low, medium and high DNA concentrations. Final qPCR conditions used for eDNA samples were as described above but with 45 cycles and 62°C as the annealing temperature. Six replicates were performed for each sample.

eDNA sample collection

Sample collection was conducted in England, with a focus on the area between the River Thames (at Bourne End, Buckinghamshire) in the south, to the River Ancholme (Lincolnshire) in the north, and from Staunton Harold Reservoir (Derbyshire) in the west to Norfolk and Suffolk in the east (Figure 4.1). Sampling locations were chosen to include known established populations of quagga mussels (i.e. Rutland Water, Walthamstow Reservoirs, River Thames), priority sites with suspected but unconfirmed reports (i.e. Holme Pierrepont water park and Grand Union Canal), sites upstream of water company intakes and sites adjacent to already invaded waterbodies, while providing a broad coverage across the East Midlands and East Anglian regions. Water samples were collected from a total of 24 sites including 1 canal, 2 lakes, 5 reservoirs and 16 rivers (Figure 4.1; Table 4.2). Five water samples were collected at each site, with the exception of Ardleigh (ARD) where only three samples were collected due to lack of access. Samples were collected from five locations spaced around 100 m, although this was not always possible due to safety reasons, and in such cases samples were collected within smaller distances or in the same location (see Table S3.1 in appendix 3 for coordinates for each individual sample). Water was collected using a sterile Whirl-Pak bag, without disturbing the sediment. Gloves were worn at all times during handling of samples to minimise contamination and new sterile equipment was used for each sample. A field negative control of purified water brought from the lab or shop-bought water was filtered at each site.

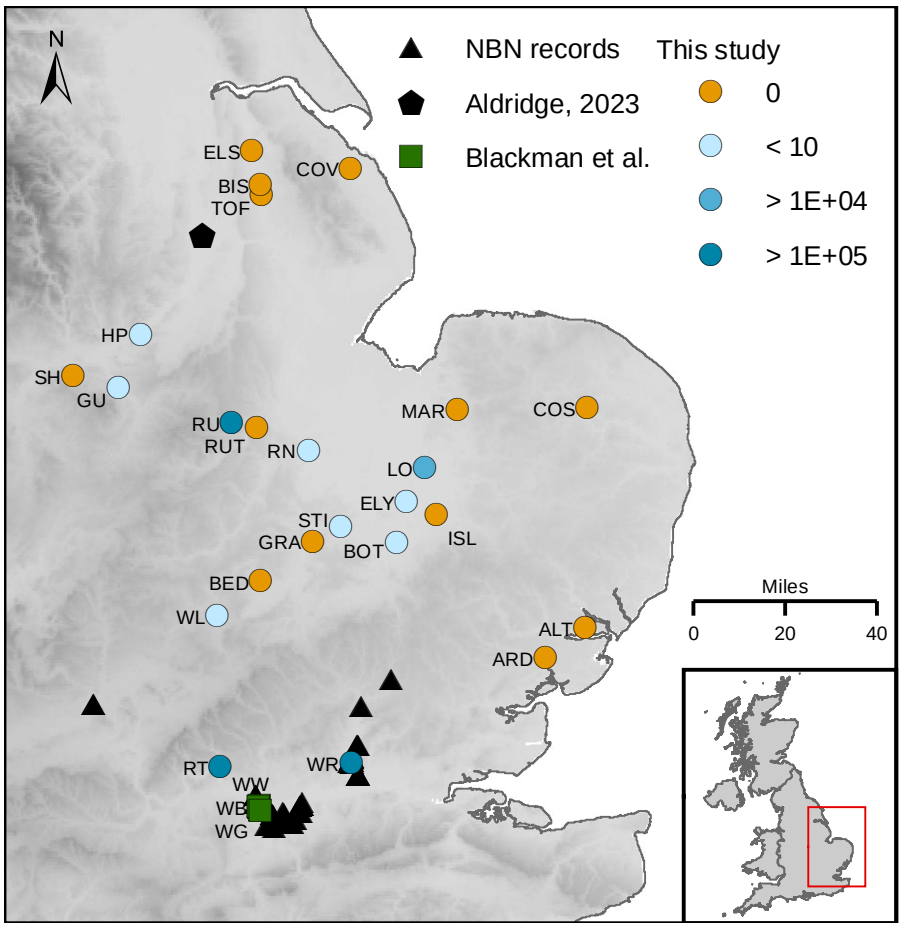

Figure 4.1 Location of the 24 sites sampled in England for this study (circles) and respective average DNA copies/µL recovered. Green squares represent the locations of the eDNA samples collected by Blackman et al. (2020b) and used here for assay optimization. Site codes are the same as in Table 4.2. Black data points correspond to the current known distribution of quagga mussels, obtained from the National Biodiversity Network (NBN) Atlas, as of April 5th 2023 (triangles) and from Aldridge (2023) (pentagon). They have also been recorded at Rutland Water (RU), however this is not depicted in the map due to overlap with data points from this study.

Table 4.2 Details of the 24 locations sampled in this study as well as the three sites at Wraysbury River (sampled by Blackman et al., 2020b) that were used for testing the new pDRB1 assay. Sites are ordered alphabetically.

Site name	Location	Site code	Type	Latitude and longitude	Sampling date	Average DNA copies/µL
Alton	Holbrook, Suffolk	ALT	river	51.978844 1.158257	22/08/2022	0
Ardleigh	Lower Salary Brook, Essex	ARD	river	51.890300 0.951479	22/08/2022	0

Bedford WTW direct intake	River Great Ouse, Bedfordshire	BED	river	52.158551 -0.490244	20/09/2022	0
Bishopbridge	River Ancholme, Lincolnshire	BIS	river	53.406411 -0.449692	08/08/2022	0
Bottisham Lock	River Cam, Cambridgeshire	BOT	river	52.268628 0.208697	04/08/2022	0.73
Costessey Pits	River Wensum, Norfolk	COS	river	52.668337 1.217589	13/08/2022	0
Covenham	Louth, Lincolnshire	COV	reservoir	53.448377 0.028919	04/08/2022	0
Elsham	River Ancholme, Lincolnshire	ELS	river	53.513170 -0.491932	08/08/2022	0
Ely Waterfront/ Marina	River Great Ouse, Cambridgeshire	ELY	river	52.394688 0.269283	04/08/2022	1.44
Grafham	River Great Ouse, Cambridgeshire	GRA	river	52.280178 -0.221880	18/08/2022	0
Grand Union Canal	Loughborough, Nottinghamshire	GU	canal	52.774600 -1.210940	05/08/2021	7.75
Holme Pierrepont park	Nottingham, Nottinghamshire	HP	lake	52.941480 -1.091560	05/08/2021	1.35
Isleham Marina	River Lack, Cambridgeshire	ISL	river	52.354417 0.419963	04/08/2022	0
Little Ouse River	Southery, Norfolk	LO	river	52.500028 0.367306	17/08/2021	5.65E+04
Marham	River Nar, Norfolk	MAR	river	52.678289 0.547812	12/08/2022	0
River Nene	Peterborough, Cambridgeshire	RN	river	52.566774 -0.231123	17/08/2021	0.87
River Thames	Bourne End, Buckinghamshire	RT	river	51.576788 -0.717745	18/08/2021	1.99E+05
Rutland - Tinwell	River Welland, Rutland	RUT	river	52.642440 -0.496663	07/09/2022	0
Rutland Water	Oakham, Rutland	RU	reservoir	52.658162 -0.625246	16/08/2021	9.06E+05

Staunton Harold Reservoir	Melbourne, Derbyshire	SH	reservoir	52.812690 -1.442850	02/08/2021	0
St Ives	River Great Ouse, Cambridgeshire	STI	river	52.322793 -0.074799	04/08/2022	4.11
Toft Newton Reservoir	Market Rasen, Lincolnshire	TOF	reservoir	53.373865 -0.445297	08/08/2022	0
Walthamstow Reservoirs	Greater London	WR	reservoir	51.579039 -0.051376	19/08/2021	1.53E+05
Willen Lake	Milton Keynes, Buckinghamshire	WL	lake	52.051308 -0.717679	18/08/2021	0.08
Wraysbury Bridge	Wraysbury River, Surrey	WB	river	51.448497 -0.523809	April 2015	9.76E+05
Wraysbury Gardens	Wraysbury River, Surrey	WG	river	51.436737 -0.514678	April 2015	9.66E+03
Wraysbury Weir	Wraysbury River, Surrey	WW	river	51.452367 -0.520532	April 2015	1.39E+06

For nine of the 24 sites, sample collection was conducted in August 2021 (Table 4.2). Water samples were filtered using a 100 mL luer lock syringe (Nature Metrics, UK) and an enclosed filter with a polyethersulfone membrane and 0.8 µm pore size (Nature Metrics, UK). Water was pushed through the filter as many times as possible until the filter clogged, and the volume filtered was recorded (Table S3.1, appendix 3). Air was passed through the filter to dry it and 1 mL of Longmire's buffer (Longmire et al., 1997) was added to each filter to preserve the sample. Following sample collection, filters were stored at room temperature until return to the laboratory, where they were stored at -20°C until DNA extraction. For the remaining 15 sites, sample collection was conducted during August and September 2022 (Table 4.2). Water was collected and preserved as previously described, but in this case, due to unavailability of the filters formerly used, filtration was conducted using Sterivex filters with 0.45 µm pore size and a PVDF (polyvinylidene difluoride) membrane (Merck Millipore, UK) and a 60 mL luer lock syringe (Fisher Scientific, UK).

DNA extraction

Samples collected with Nature Metrics filters were extracted using a modified version of the DNeasy Blood & Tissue Kit (Qiagen, UK), whereas samples obtained with Sterivex filters were extracted using a modified version of the Mu-DNA protocol (Di Muri et al., 2020; Sellers et al., 2018). Both protocols are available in the supplementary material, in appendix 2 and 3, respectively. All DNA extractions were conducted in a dedicated laboratory for processing eDNA samples and a negative control was included in each extraction batch to monitor for contamination. Following extractions, the purity and concentration of all samples were assessed using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, UK).

Inhibition tests

All samples used in this study (i.e. samples collected in the present study and samples from Blackman et al., 2020b) were tested for inhibition using the Applied Biosystems TaqMan Exogenous Internal Positive Control Reagents (Fisher Scientific, UK). A similar protocol was applied for all samples with the only difference being the type of master mix used (Table S3.1, appendix 3). For samples collected with Nature Metrics filters and extracted with the Qiagen kit, the TaqMan Environmental Master Mix 2.0 (Fisher Scientific, UK) was used, while for the remaining samples the TaqMan Universal Master Mix 2.0 (Fisher Scientific, UK) was used instead. This was due to the inefficiency of dilution in overcoming inhibition for the first set of samples when using the TaqMan Universal Master Mix 2.0, which was only overcome when using the TaqMan Environmental Master Mix 2.0.

Reaction volumes consisted of 7.5 µL of TaqMan Environmental or Universal Master Mix 2.0 (Fisher Scientific, UK), 1.5 µL of 10X Exo IPC Mix, 0.3 µL of 50X Exo IPC DNA, 3.7 µL of molecular grade water and 2 µL of sample. Reactions were run on a StepOnePlus Real-Time PCR machine with the following thermal cycler conditions: 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. All eDNA samples were tested in duplicate and samples were considered to be inhibited if the average cycle threshold (Cq) of a sample was higher than the no template reaction by 2 or more cycles (e.g. Tillotson et al., 2018). All samples that showed inhibition were diluted 10x and re-run with the respective master mix to confirm whether inhibition was overcome

(Table S3.1, appendix 3). If amplification with the species-specific qPCR assay was detected for diluted samples, a 10x correction factor was applied to their final DNA concentration.

eDNA sample screening

Following inhibition tests, eDNA samples were analysed with the newly developed pDRB1 assay. Final qPCR conditions for eDNA samples consisted of 12.5 µL of TaqMan Environmental or Universal Master Mix 2.0 (the same used for the inhibition step), 1.6 µM of primers (forward and reverse combined), 0.05 µM of probe (Table 4.1), 0.64 mg/mL of BSA, 5.45 µL of water and 2 µL of sample. The qPCR program consisted of an initial step at 50°C for 10 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 seconds and 62°C for 1 min on a StepOnePlus Real-Time PCR machine. Six replicates were performed for each eDNA sample and respective field and extraction negative controls. Eight qPCR negative controls were included in each plate to monitor for contamination during PCR preparation.

Standards of known concentration were included in each plate, in triplicate, to accurately quantify the DNA concentration of eDNA samples. For this, two single stranded DNA sequences of 188 bp each (Table 4.1; Integrated DNA Technologies, Belgium) were combined and serially diluted to obtain four 10-fold dilutions ranging from 10^4 to 10^1 copies/µL.

Data analyses

To compare the performance of the dDRB1 and pDRB1 assays, a non-parametric Wilcoxon signed-rank test was used. For this, the average DNA copies/µL for each of the nine samples collected by Blackman et al. (2020b) and each assay was calculated and tested with the “wilcox.test()” function from the stats 4.2.1 package in R (R Core Team, 2022). The map was created with ArcMap 10.8.2 and the remaining figures were made using the ggplot2 3.4.2 R package (Wickham, 2016).

4.4 Results

Development of pDRB1 assay

All annealing temperatures tested showed species-specific amplification of quagga mussel with no cross-amplification of other species. qPCR amplification efficiency values were 102.9, 99.1 and 63.4% for annealing temperatures of 60, 62 and 63°C, respectively. At 63°C, the lowest standard (10^1) did not show any amplification, which explains the lower efficiency value. Cq values for quagga mussel amplification between 60 and 62°C were very similar (21.65 and 21.61, respectively). All annealing temperatures showed little or lack of a plateau stage when run for 40 cycles only. The final retained conditions included 62°C as the annealing temperature and 45 cycles.

There was a statistically significant difference between the average DNA copies/µL obtained for dDRB1 ($\bar{x}$ = 3.87+05, SD = 5.84+05) and pDRB1 ($\bar{x}$ = 7.93+05, SD = 1.07+06) assays (Wilcoxon signed-rank test: V = 0, p = 0.004, n = 9), with the latter consistently yielding higher DNA levels (Figure 4.2). Details on Cq values and copies/µL for each sample and assay is provided in the supplementary material (Table S3.2, appendix 3).

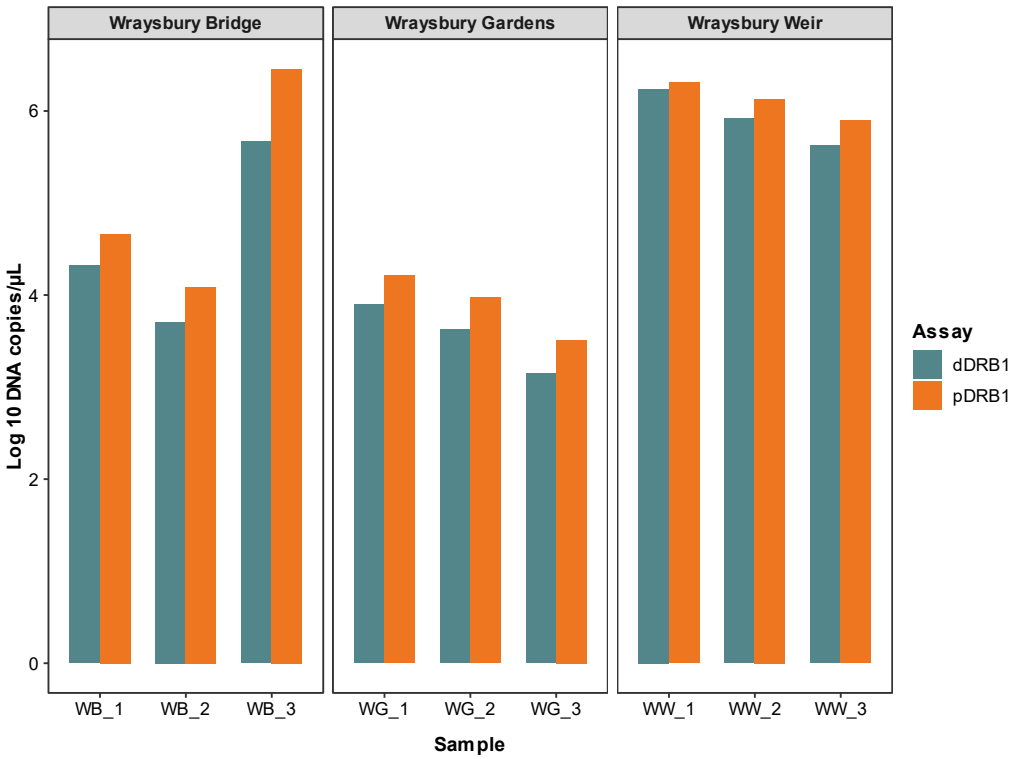

Figure 4.2 Number of log 10 DNA copies/µL obtained for both DRB1 assays for each of the 9 samples tested from Blackman et al. (2020b). For visual purposes log 10 DNA copies/µL is being used instead of non-transformed data.

Further information about the assay and MIQE checklist (Bustin et al., 2009) can be found in the supplementary material (Table S3.3, appendix 3).

Inhibition tests

Out of the 118 samples from this study, inhibition was observed in 21 samples from nine sites (inhibition values ranging from 2.53 – 26.83; $\bar{x}$ = 12.17, SD = 10.61), while the remaining samples showed no sign of inhibition (Table S3.1, appendix 3). In addition, one sample from the nine collected by Blackman et al. (2020b) showed inhibition (inhibition value of 2.77). The 10x dilution was successful in overcoming inhibition for all the samples.

eDNA sample screening

All standards amplified in 100% of the replicates with the exception of the lowest one (10 copies/µL) which amplified in 93% of the replicates. The LOD, i.e. the lowest standard with at least 95% amplification, was therefore 100 copies/µL. qPCR assays exhibited an average efficiency of 97.2% (91.8 – 104.8) and R^2 of 0.993 (0.984-0.998).

From the 24 sites sampled in our study, 11 sites had detectable quagga mussel DNA (Figure 4.1 and 4.3), with volume filtered ranging from 33 – 1000 mL ($\bar{x}$ = 540.64, SD = 310.59) (Table S3.1, appendix 3). All samples and replicates amplified for three sites - Rutland Water, Walthamstow Reservoirs and River Thames - with average DNA copies/µL ranging from 1.5E+05 to 9.1E+05 copies/µL (Figure 4.1; Table 4.2). Two further sites were positive for all 5 samples - Grand Union Canal and Little Ouse River - although with only 15 and 14 positive replicates out of 30, respectively (Figure 4.3). The additional six sites with detectable quagga mussel DNA exhibited very low detection levels, with a maximum of 3/5 (60%) samples and 7/30 (23%) of replicates amplifying (Figure 4.3). No amplification was observed for any sample of the remaining 13 sites (Figure 4.1). Information on Cq values and copies/µL for each sample and replicate is provided in the supplementary material (Table S3.4, appendix 3).

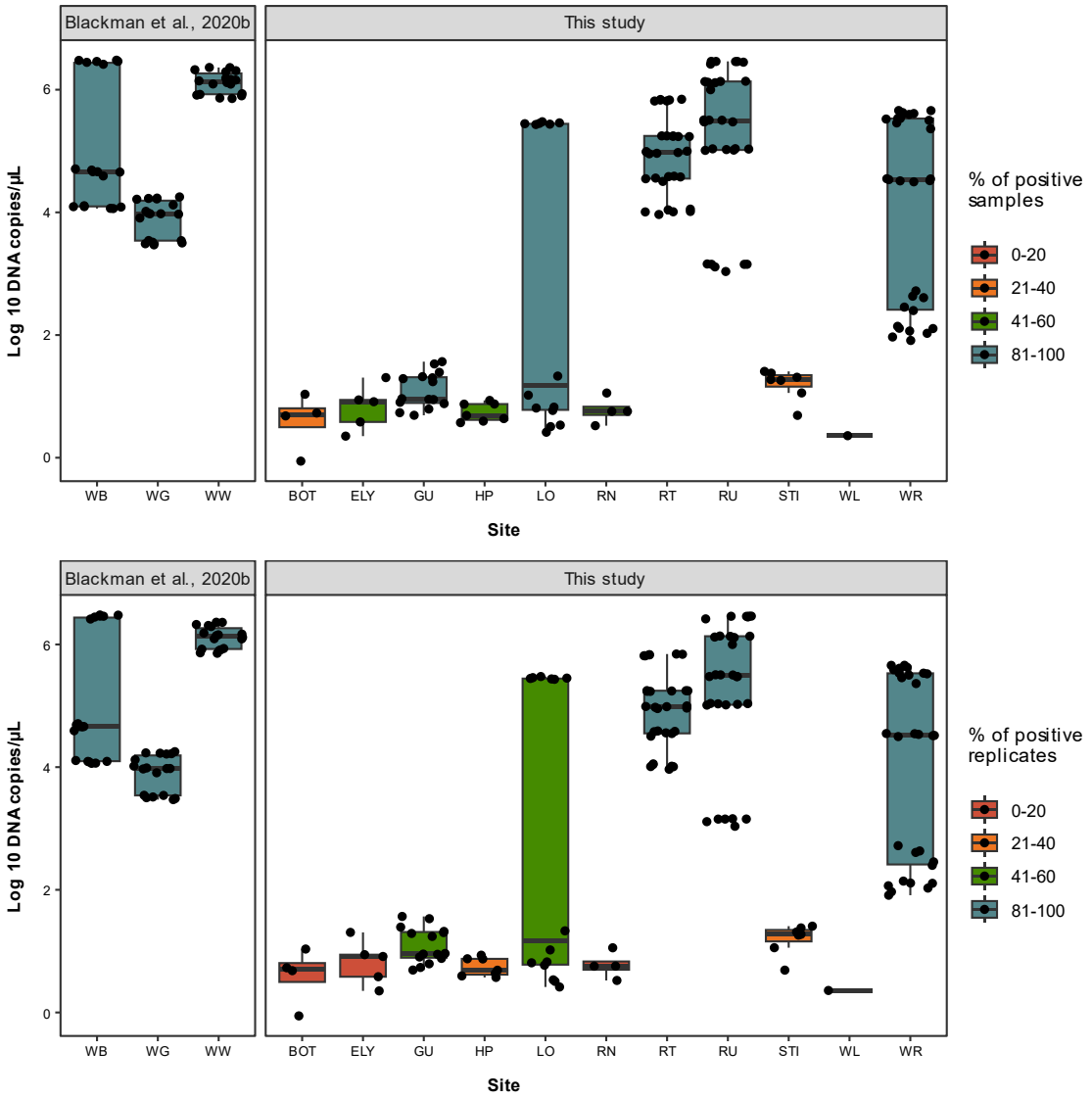

Figure 4.3 Log 10 DNA copies/µL and percentage of positive samples (top) and replicates (bottom) for samples from Blackman et al. (2020b) and from this study (total number of samples = 3 and 5, respectively; total number of replicates = 18 and 30, respectively). Only sites where quagga mussel amplification occurred are represented and site codes are the same as in Table 4.2 and Figure 4.1. Each black dot represents a positive replicate. For visual purposes log 10 DNA copies/µL is being used instead of non-transformed data.

Regarding contamination, one negative field control (Rutland Water) showed amplification. However, contamination was only detected in one replicate (out of six) and at very low levels (Cq of 38.4) compared to the samples (Cq 19.9 – 31.4) and as such no data was discarded. All remaining field, extraction and PCR negative controls did not display any amplification.

4.5 Discussion

In this study, the dDRB1 qPCR assay developed by Blackman et al. (2020b) was further developed to a probe-based assay (pDRB1) and successfully used to screen for quagga mussels in several locations in England, demonstrating its efficiency in detecting this INNS. The LOD for the pDRB1 assay was defined as 100 copies/µL and the DNA concentration obtained for the nine samples from Wraysbury River was significantly higher for the pDRB1 assay. Positive detections were obtained for 11 out of the 24 sites sampled in this study, with a stronger signal observed in locations where established populations were already known - Rutland Water, River Thames, and Walthamstow Reservoirs. Sites with low number of samples and/or replicates amplified as well as low copy numbers suggest a recent invasion and thus should be closely monitored to prevent population expansion. Our results demonstrate that quagga mussels are considerably more widespread in England than previously thought, with one of our positive sites – Holme Pierrepont water park – being the second furthest north they have been recorded so far. The detection of this species in a number of different rivers, recreational lakes and the canal system highlights the urgent need to implement biosecurity measures and limit further spread.

pDRB1 assay

Thalinger et al. (2021) described a 5-level validation scale for targeted eDNA assays, ranging from "incomplete" (level 1) to "operational" (level 5). Initial testing was completed for the dDRB1 assay by Blackman et al. (2020b) through a series of mesocosm experiments and field trials, and the assay currently meets the criteria for level 3 ("essential"). In the present study, specificity and sensitivity of the new pDRB1 assay were further confirmed by testing in non-target and closely related species (zebra mussel, killer shrimp, demon shrimp, pea mussel, blue mussel, European oyster, common periwinkle and the common limpet), together with extensive field testing and determining the LOD. The additional development and testing of the DRB1 assay reported here brings the assay to level 4 ("substantial") of the 5-level validation scale (Thalinger et al., 2021). By their definition, a positive detection at level 4 can be interpreted as the target being "very likely present", whereas a negative detection means the target is likely to be absent, "assuming appropriate timing and replication in sampling". To reach the highest level of validation, the influence of ecological and

physical factors on eDNA availability and an estimation of detection probabilities from statistical modelling is needed, however this was beyond the scope of our study.

The nine samples previously collected and tested with the dDRB1 assay by Blackman et al. (2020b) and tested again here with the pDRB1 assay showed significantly different DNA concentrations. The probe-based assay yielded on average more than twice as many DNA copies, demonstrating an increased efficiency in amplifying the target DNA compared to the dye-based assay. This could be related to differences in PCR conditions. The initial denaturation time of the pDRB1 assay was longer when compared to the dDRB1 assay (10 vs 2 minutes). This step separates the double-stranded DNA into single strands and activates the DNA polymerase enzymes. Hence, a longer denaturation step possibly resulted in more DNA template for PCR amplification and a higher percentage of enzymes activated. The LOD obtained for the pDRB1 assay in this study was 100 copies/µL. While Blackman et al. (2020b) had previously reported a LOD of 1E-04 ng/µL for the dDRB1 assay with SYBR green dye, and Marshall, Vanderploeg & Chaganti (2022) found the LOD to be 9.8E-05 ng/reaction for the same assay with the EvaGreen dye, the different units used to assess limits of detection (ng/µL and copies/µL) renders the results not comparable.

New records with eDNA

Quagga mussels are native to the Ponto-Caspian region in Eastern Europe, but in recent decades human activities allowed their introduction and colonization of the rest of the continent. They were first detected in England in 2014 in the Wraysbury River (Aldridge et al., 2014) and they are now well established in the River Thames and its tributaries. Several locations within the Thames network are used to supply water to the Walthamstow Reservoirs, and quagga mussels are now established at this location too. Moreover, in 2020 they were found further north in the East Midlands in Rutland Water (Environment Agency, 2020). The detection rates for both samples and replicates at these three locations was 100%, and average DNA copy number per site was in the order of 1.5E+05 to 9.1E+05 copies/µL, consistent with a high abundance of quagga mussels in these established locations.

Positive detections were obtained at an additional eight sites where quagga mussels were suspected and/or expected, but not previously confirmed. These sites are spread across four additional counties (Buckinghamshire, Cambridgeshire, Norfolk, Nottinghamshire) and include four rivers (the Cam, Great Ouse, Little Ouse and Nene), two popular water sports lakes (Holme Pierrepont and Willen Lake), and the Grand Union Canal. This new distribution indicates that the species is now widespread and present in several river catchments as well as key recreational sites.

Of these eight new sites, detection rates were higher in the Grand Union Canal at Loughborough, and on the Little Ouse River at Southery, Norfolk. For these sites, positive detections were obtained in all five samples and in 47-50% of all replicates. The average DNA copy number at Southery was one order of magnitude lower than that of the three established populations, but still quite high compared to the remaining sites. The stretch of the Little Ouse River where the samples were collected is used for boat mooring, and a high number of boats were present at the time of sample collection. It is possible that mussels were attached to boats and/or mooring structures, which could have increased the concentration of quagga mussel eDNA at this location.

The remaining seven new sites displayed an average DNA copy number lower than the LOD defined for the pDRB1 assay. All field, extraction and PCR negative controls associated with these locations were negative, suggesting these were not false positives. According to Klymus et al. (2020), detections below the LOD can be expected to occur in eDNA studies due to the rarity of the target species, and should still be considered and not treated as noise. This could be an indication that the species is at an early point of invasion and population densities are not too high yet in these locations. Continuous monitoring at these sites is crucial and surveys at adjacent sites should be conducted frequently to prevent their further spread.

Despite the positive detections in several new locations, we were unable to detect quagga mussels in 13 sites out of the 24 sampled. According to the 5-level validation scale proposed by Thalinger et al. (2021), when the target species is not detected with a level 4 assay, it is likely that the target is absent “assuming appropriate timing and replication in sampling” has been conducted. The timing of sampling is unlikely to have

caused any false negatives. Samples were collected during the summer period, whose temperatures favour breeding by quagga mussels. This has been shown to be associated with peaks in eDNA concentration in the environment (Spear et al., 2015). If quagga mussels were present at these sites, eDNA availability in the water would be expected to be high, thus increasing the likelihood of capturing eDNA during sample collection and their subsequent detection in the lab. Nevertheless, replication levels were not ideal for some of the sites. Due to lack of further access and safety concerns, samples were sometimes collected within a very small space (e.g. all samples were collected within 10 meters of each other at both Bishopbridge and Marham). If present, this could have prevented the collection and detection of quagga mussel eDNA at those sites, as eDNA is not homogeneously distributed in the water and collecting samples at different locations in the same site helps increase detection probabilities. Further surveys in more accessible areas and increased replication are needed at these locations to determine the population status of quagga mussels.

Pathways

The extensive canal network in England is a major potential pathway for the spread of quagga mussels. By attaching to boat hulls through their strong byssal threads, quagga mussels can easily reach and colonise new locations, and this is thought to be one of the main vectors for their spread (Karatayev & Burlakova, 2022). Moreover, canals are often connected to hotspots of human activity such as marinas, ports and industrial areas, facilitating further spread of INNS (Chapman et al., 2020). The detection of quagga mussel eDNA in samples from the Grand Union Canal in this study poses a serious concern. Due to its extension, being the longest canal in England (approximately 220 km long) connecting London and Birmingham, it could facilitate the further spread of this species in the southern part of the country. Recent lessons have been learnt from another Ponto-Caspian species, the demon shrimp (*D. haemobaphes*), which was first recorded in GB in 2012 and quickly became widespread once it entered the canal network (Johns et al., 2018).

Although to a lesser extent, rivers are also subject to human activities and as such are also potential routes that can be used by quagga mussels to spread further. Despite the previous belief that this species prefers canals and would not be found in fast flowing

rivers (Aldridge et al., 2014), we were able to detect them in four new rivers - Cam, Great Ouse, Little Ouse and Nene - in addition to the River Thames. Even though the high flow rates of rivers when compared to canals might prevent quagga mussel veligers from settling in and establishing, they can still be used as pathways to reach new locations and as such the role of rivers on quagga mussel expansion cannot be overlooked.

Recreational activities such as water sports and fishing are also commonly associated with the spread of dreissenid mussels. Adults are able to strongly attach to hard structures such as boats, and veligers can be accidentally transported in nets and equipment, thus being introduced to new locations otherwise inaccessible to them. In addition to previously known reservoir sites, quagga mussels were detected at Holme Pierrepont water park in Nottingham and Willen Lake in Milton Keynes, both popular water sports facilities. The detection of quagga mussels in these locations highlights the urgent need for better biosecurity and "check, clean, dry" awareness campaigns at these sites, as it has been implemented at Grafham Water reservoir for example.

Conclusions and future work

In the present study, the additional development and testing of the DRB1 assay brought the assay to level 4 of the 5-level validation scale defined by Thalinger et al. (2021), allowing to improve the readiness of the assay for routine monitoring and increasing the confidence in the results. At this level, a positive or negative eDNA result is a strong indication that the species is present or absent, respectively. Nevertheless, despite the improvements in the DRB1 assay, further work is needed before it can achieve the highest level (level 5) and be considered fully operational. This includes estimating detection probabilities via statistical modelling and investigating the influence of environmental and physical factors on eDNA detection.

Positive quagga mussel detections were obtained at 11 out of the 24 sites sampled, with a stronger signal being observed in locations where established populations were already known. Locations with lower eDNA concentrations could be at an early point of invasion and should be monitored closely to prevent further spread to adjacent waterbodies. The 13 sites where quagga mussels were not detected should be considered high risk of invasion and therefore continued surveillance is needed, with

the addition of more accessible areas and increased replication level in order to confirm if the species is absent or not in these locations.

Overall, our results demonstrate that quagga mussels are considerably more widespread in England than previously thought. Positive detections were obtained at eight new sites, spread across four counties, and including several rivers, recreational lakes and the canal system. Due to their high ecological and economic impacts, this highlights the urgent need to continuously monitor priority sites and potential pathways using eDNA and assays such as the pDRB1, in order to closely monitor their expansion. Information obtained from surveillance surveys can then be used by regulatory bodies and water companies to better assess the environmental impacts of risk activities (i.e. that can favour quagga mussel dissemination) such as water transfers between reservoirs or the authorization of fishing licences, ultimately improving site-specific biosecurity measures and minimising their further spread. Additionally, modelling environmental factors and hydrological connectivity together with eDNA data will help identify priority areas and thus focus monitoring and biosecurity efforts.

4.6 Acknowledgements

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Chapter 5

General discussion

Dreissenid mussels pose a significant risk to ecosystems and water-related infrastructures, both in the UK and worldwide. Continuous monitoring is therefore essential using sensitive tools such as eDNA, which have proven to be efficient through targeted assays. In this thesis, I increased the readiness and validation level of two targeted qPCR eDNA assays for both dreissenid species. Following this work, the Gingera et al. (2017) *Cyt* b assay, for zebra mussels, and the DRB1 assay (Blackman et al., 2020a, 2020b), for quagga mussels, are ranked among the highest validated assays for dreissenid species, thus increasing the reliability of results and their suitability for routine monitoring. In this chapter, I summarize the progress achieved for each assay and outline further research needed. Emerging detection technologies that can be useful for future dreissenid monitoring and the incorporation of eDNA-based surveys into INNS management are also discussed here. Finally, I provide recommendations for future zebra mussel eDNA monitoring campaigns.

5.1 Targeted eDNA assays for dreissenid monitoring

Several targeted eDNA assays have already been developed and successfully applied for dreissenid detection. However, they vary in their level of validation, which can impact the interpretation and credibility of results (Thalinger et al., 2021). Among the species-specific qPCR assays available for zebra mussels, the probe-based and dye-based assays developed by Gingera et al. (2017) and De Ventura et al. (2017), respectively, were the ones further advanced in the validation scale, being ranked at level 3 ("essential"), prior to the present study (Thalinger et al., 2021). For quagga mussels, based on the minimum criteria defined by Thalinger et al. (2021), the species-specific dye-based qPCR assays developed by De Ventura et al. (2017) and Blackman et al. (2020a, 2020b) were equally placed at level 3 ("essential") of the validation scale, while the probe-based assay developed by Sepulveda et al. (2019) ranked at level 4 ("substantial").

The work conducted in this study targeting zebra mussels with the Gingera et al. (2017) species-specific *Cyt* b assay increased its validation level to level 5, rendering the assay

operational (Table 5.1). This included establishing limits of detection, conducting further *in silico* tests, assessing the influence of several ecological and physical factors on eDNA in the environment, and estimating detection probabilities from statistical modelling. Results from the temporal (chapter 2) and spatial (chapter 3) studies combined suggest that zebra mussel eDNA concentration is mainly influenced by temperature and the seasonal activity of organisms. For both sites sampled in chapter 2, we observed a peak in eDNA concentration in the summer, and the mixed models indicated a significant influence of temperature. This is because increases in water temperature trigger mussel spawning and the release of gametes into the water, thus increasing eDNA concentration. The effect of temperature was less significant in the spatial study, as expected, due to the shorter sampling period. We also observed that eDNA concentration was influenced by waterbody type, being higher in reservoirs in both chapters, and weakly influenced by substrate. Additionally, results from both chapters revealed that filtering higher volumes increased eDNA concentration. Altogether our results suggest that sampling in the summer maximises detection of zebra mussel eDNA, and when doing so detection rates are expected to be high across different waterbodies and environmental conditions, at least if populations are established. While the work conducted here renders the assay operational for use in routine monitoring of zebra mussels, caution is still needed due to the low qPCR efficiency observed. Additionally, the implementation of appropriate sampling designs and replication levels remains crucial for maintaining best practices and ensuring reliability of results. Future research focusing in areas where species detection is likely to be more challenging, such as sites at the invasion front that have only been recently invaded, could help understand how detection rates vary at lower population densities.

For quagga mussels, the work conducted in this study allowed the development of the dye-based DRB1 assay (Blackman et al., 2020a, 2020b) to a probe-based assay. Following this, we performed *in vitro* tests, extensive field testing and assessed limits of detection, increasing the validation level of the assay to level 4 (“substantial”) (Table 5.1). Field testing included sampling of 24 sites spread throughout England, which allowed us to obtain an updated distribution of quagga mussels in the country and demonstrate that they are considerably more widespread than previously thought. We detected quagga mussels at three sites with established populations and at eight additional sites with no

previous records of their presence. These sites were spread across four counties and included several rivers, recreational lakes and the canal system, highlighting the need for urgent measures to control their spread. Further work is needed to consider the assay operational, specifically understanding ecological and physical factors influencing eDNA in the environment and assessing detection probabilities (Table 5.1) as done for zebra mussels in the temporal and seasonal studies (chapters 2 and 3). Further to this, a wider understanding of the species ecology and the effects of environmental variables on their life history, growth and longevity is needed as most literature focus on zebra mussels and less information is available for quagga mussels (Karatayev & Burlakova, 2022). Doing so will help make predictions and better interpret how environmental variables might influence eDNA concentration and detectability in natural settings.

Table 5.1 Variable blocks and minimum criteria for each level of the 5-level validation scale, as defined by Thalinger et al. (2021). For each level, a scoring of 1 or 0 indicate if criteria have been met or not, respectively, for each assay optimized in this thesis: the Gingera et al. (2017) *Cyt* b assay for zebra mussels and the DRB1 assay (Blackman et al., 2020a, 2020b) for quagga mussels.

Validation level	Variable blocks	Minimum criterion	Gingera et al. (2017)	Blackman et al. (2020a, 2020b)
Level 1 Incomplete	*In silico* analysis	Target species	1	1
	Target tissue testing	Target tissue	1	1
	Target tissue PCR	Primer (and probe) sequence	1	1
Level 2 Partial	Comprehensive reporting of PCR conditions	DNA extract volume in PCR	1	1
	In vitro testing on closely related non-target species	Any *in vitro* non-target testing	1	1
Level 3 Essential	Extraction method performed on eDNA samples	Method of extraction	1	1
	Concentration of eDNA from environmental samples	Filter type or precipitation chemicals	1	1

	Detection obtained from environmental samples	Detection from an environmental sample (artificial or natural habitat)	1	1
Level 4 Substantial	Limit of Detection (LOD)	LOD determined	1	1
	Extensive field testing of environmental samples	Multiple locations or multiple samples	1	1
	In vitro testing on co-occurring non-target species	Any advanced *in vitro* testing	1	1
Level 5 Operational	Comprehensive specificity testing	Non-co-occurring/closely related species checked from *in silico*	1	0
	Detection probability estimation from statistical modelling	Any effort made towards detection probability estimation	1	0
	Understanding ecological and physical factors influencing eDNA in the environment	Any factor influencing eDNA in the environment tested	1	0
Current validation level:			5	4

Statistical modelling

Further improvements to the statistical methods available are needed in the future, in order to facilitate the estimation of detection probabilities and investigate the influence of biotic and abiotic factors on eDNA concentration, as needed for level 5 validation. Occupancy models typically rely on presence/absence data (e.g. unmarked package (Fiske & Chandler, 2011), eDNAoccupancy package (Dorazio & Erickson, 2018)), which means that important information about strength of eDNA signal is lost. For datasets such as ours, where detection rates were quite high across sites, this type of model is unsuitable as it restricts the ability to discern the impacts of environmental variables,

given the consistent nature of the response variable. Future models should thus consider eDNA concentration as the response variable, instead of simply detection/non-detection. Although not an occupancy approach, the recent Bayesian model developed by Espe et al. (2022), employed in chapter 3, allows to assess the influence of environmental variables on eDNA concentration and estimate detection probabilities under specific conditions, while using Cq values as response variable. This model further accounts for the biases that might arise from data censorship, i.e., when eDNA detection occurs at or below the assay's detection threshold, which is generally overlooked. The implementation of a similar framework in occupancy models, where imperfect detection is also accounted for, is thus needed in the future. To address this, a team of statisticians from the University of Kent (UK) is currently developing such models using the dataset generated in chapter 3 of this thesis (A. Diana and E. Matechou, *pers. comm)*. Additionally, the extension of these models to temporal datasets is also needed.

5.2 Emerging technologies for improved dreissenid monitoring and management

The use of molecular tools has proved to be a valuable asset for INNS detection, often outperforming traditional monitoring techniques. While this thesis focused mainly on the analysis of eDNA samples with species-specific qPCR assays, other emerging technologies have recently gained attention from the eDNA community and their potential for dreissenid surveillance and management show promise. Some of these tools include digital PCR (dPCR), Oxford Nanopore sequencing platforms (e.g. MinION), portable field instruments and on site-testing, automated passive samplers, the analysis of environmental RNA (eRNA) samples and the combination of species distribution modelling with eDNA data to predict spread and focus biosecurity and control efforts.

Digital PCR (dPCR) techniques partition PCR reactions into multiple individual reactions that are analysed separately for the presence of the target organism (Vogelstein & Kinzler, 1999). Droplet digital PCR (ddPCR) is a specific type of dPCR, that uses water-in-oil droplets to partition DNA samples and PCR reagents into approximately 20 thousand individual reactions (Hindson et al., 2011). When compared to qPCR, ddPCR is less affected by PCR inhibition (Doi et al., 2015), while offering higher sensitivity and

precision (Hindson et al., 2011), which is crucial for dreissenid monitoring to minimise false negatives. Moreover, it does not require the use of a standard curve to obtain DNA concentration, thus reducing the workload and increasing the number of samples in each run. Previous studies have already reported the use of this technique to detect various species from eDNA samples (Capo et al., 2019; Doi et al., 2015; Steiner et al., 2022). For dreissenid mussels, Watts (2020) was able to detect dreissenid eDNA in different times of the year and different lakes in a pilot study conducted in North America. The successful application of this technique for dreissenid detection and the benefits offered by ddPCR suggest it can be a useful tool for future monitoring campaigns. Other less common dPCR approaches include chip-based or microfluidic chamber-based dPCR, however no dreissenid assays have been developed for these yet.

Future dreissenid surveillance campaigns can also benefit from the use of Oxford Nanopore Technologies (ONT) sequencing platforms such as the MinION or Flongle. As with other passive detection approaches, ONT enables the description of the wider community, as well as detection of the target taxa, and is therefore suited to detecting previously undetected species and, for example, studying impacts of INNS on communities. When compared to other sequencing platforms, ONT has reduced turnaround times and allows the sequencing of longer reads. The drawbacks of this technology, as yet, are related to the higher rate of sequencing errors and higher costs per sample when compared, for example, to the more common Miseq (Illumina) platform (Egeter et al., 2022). To date, only one study has used the MinION platform for the detection of dreissenid mussels (Egeter et al., 2022). In their study, authors were able to successfully detect zebra mussel eDNA from several lakes in Italy, while demonstrating that this approach is also faster than the MiSeq. For dreissenid management, this allows a more rapid response, such as the implementation of appropriate biosecurity measures. A preliminary collaborative investigation into the use of ONT platforms for detecting quagga mussels was initiated during the period of this thesis and is still in progress. Twenty-seven samples from Wraysbury River, with positive and negative quagga mussel detections from metabarcoding (Blackman et al., 2020b), were PCR amplified using standard COI barcoding primers (Folmer et al., 1994) and sequenced on an ONT Flongle using v14 chemistry. The run produced 187K reads, with an average read length of 744 bp. Bioinformatics and downstream data analyses are

ongoing on this dataset, but it is hoped this will provide insights on whether detection rates for ONT, MiSeq and qPCR platforms are comparable.

The ultimate goal for dreissenid monitoring is to be able to obtain reliable on-site results, thus reducing turnaround time and prompting a fast response. The use of portable field instruments and field-friendly techniques have the potential to increase the ability to perform on-site surveillance and improve future dreissenid monitoring and management programs. For example, loop-mediated isothermal amplification (LAMP) techniques do not require the use of thermal cycler machines, as amplification occurs at a single temperature, making it suitable for on-site or field-based testing. This technique amplifies target DNA using a strand displacement approach that forms loop DNA structures (Notomi et al., 2000). The high tolerance to PCR inhibitors of the polymerase used in LAMP techniques (Koloren, Sotiriadou, & Karanis, 2011) offers an additional advantage. Recent studies have successfully applied this approach for the detection of dreissenid species from environmental samples, with positive detections even when sampling outside of their reproductive season (where species activity and veliger abundance are lower) and with fast turnaround times (Carvalho et al., 2021; Williams et al., 2017). In fact, when coupled with a field portable device, Williams et al. (2017) was able to get results within 90 minutes of sample collection.

Although not thoroughly explored yet, the combination of PCR techniques and lateral flow assays has also showed potential for species detection from environmental samples (e.g. Doyle & Uthicke, 2021). In this approach, a sample with labelled amplicons (obtained via PCR) is loaded into an absorbent pad and moves through capillary action. If present, the target molecules (amplicons) bind to specific antibodies present on the pad, resulting in a visible line which indicates a positive result. In their study, Doyle & Uthicke (2021) demonstrated that PCR combined with lateral flow assays can be as sensitive as ddPCR, in addition to being more cost-effective and less time-consuming. Moreover, under field conditions, detection was possible in sites with low population densities (Doyle & Uthicke, 2021). When combined with field portable instruments, that allow DNA extraction, purification and amplification on-site (e.g. Bento Lab, Bento Bioworks Ltd, London, UK), these tools can provide fast results.

The use of automated passive samplers also holds great potential for dreissenid monitoring, as they are capable of collecting and preserving eDNA samples remotely and autonomously, without the need for human intervention (Barnes & Turner, 2016). The applicability of these devices has been demonstrated in both marine (Hendricks et al., 2023; Preston et al., 2011) and freshwater (Sepulveda et al., 2020b) environments. The most advanced devices are capable of performing water filtration, DNA extraction and DNA amplification (i.e. PCR), while transmitting the results in real-time (Hansen et al., 2020; Preston et al., 2011). This shows immense promise for biodiversity monitoring, especially in remote and inaccessible sites. For dreissenid monitoring specifically, the deployment of automated passive samplers at high priority sites and important pathways can serve as early warning systems, thus prompting a faster response and preventing the establishment of populations.

While eDNA has been the main focus of molecular-based studies targeting dreissenids, recent research shows that environmental RNA (eRNA) could be an equally useful tool. The main difference between both types of nucleic acids is their structure, which affects their stability and degradation rates in the environment. As RNA molecules are single-stranded, they are less stable under laboratory conditions and are expected to be more prone to degradation in field settings (Yates, Derry, & Cristescu, 2021), thus reflecting more recent communities. Only one study so far focused on eRNA detection of dreissenid mussels (Marshall, Vanderploeg, & Chaganti, 2021). In their study, the authors observed that initial eRNA concentration following removal of the target species was considerably higher than eDNA, although eRNA degradation increased faster with time, suggesting that the ratio of eRNA:eDNA can be used to provide more accurate estimates of the age of genetic material (Marshall et al., 2021). As such, rather than a replacement, the analysis of eDNA and eRNA simultaneously can be advantageous and provide better spatiotemporal inferences of species presence. In the case of dreissenid monitoring, this can help discriminate whether the genetic material results from contemporary (i.e. local organisms/populations, live individuals) or non-local (e.g. transported from an invaded site upstream) sources (Feist & Lance, 2021). The analysis of eRNA samples can offer further advantages over eDNA, such as the ability to distinguish life stages (e.g. juveniles vs adults), sexes and phenotypes, as well as assess the health of individuals and communities, as the genes expressed and their expression

rate varies depending on the life stage and physiological condition of organisms (Yates et al., 2021).

Finally, the combined use of species distribution modelling (SDM) with eDNA data can provide better predictions of dreissenid spread. These models use data from environmental variables and human-related predictors to assess habitat suitability and predict the distribution of a species. For INNS, this approach is often used to identify sites at risk of invasion, thus concentrating biosecurity and monitoring efforts and mitigating their impacts. Previous studies focusing on dreissenids have shown the utility of modelling approaches to predict invasion risk based on water quality (Sepulveda et al., 2023a) and recreational activities (Timar & Phaneuf, 2009). The incorporation of eDNA data with SDMs has been suggested to improve these models, as eDNA-based surveys provide solutions for some of the limitations posed by conventional monitoring techniques (Muha et al., 2017). Future collaborations between statisticians and molecular ecologists could thus generate more accurate predictions of dreissenid spread and prevent their establishment.

These emerging technologies have the potential to improve our capacity to monitor and manage invasive species such as dreissenid mussels, that have unquestionable negative impacts. However, before they can be applied in routine monitoring, further lab and field trials are needed to better understand the limitations of each technique and reduce their error and uncertainty. This will ensure they can reliably be applied in real-word settings and allow for precise and informed monitoring and management strategies, thus minimising the detrimental effects of dreissenid species on ecosystems.

5.3 Incorporating eDNA-based outcomes into INNS management

As the main focus of eDNA research shifts to biodiversity monitoring rather than investigating methodological and ecological aspects of eDNA (Schenekar, 2023), the incorporation of eDNA-based surveys into INNS monitoring and management strategies becomes essential. This will allow early detection of INNS, thus prompting a faster response and preventing the establishment of populations. While Sepulveda et al. (2020c) suggested that eDNA methods can reliably be used for the management of INNS, the authors argue that the gap between eDNA results and management actions still

needs to be addressed, as INNS managers struggle with the uncertainty and errors of eDNA. Most of the uncertainty is associated with false positives (Feist & Lance, 2021; Sepulveda et al., 2020c), that can result in misuse of time and resources. A greater collaboration between eDNA practitioners and INNS managers is thus needed for a more effective use of eDNA for management of INNS. To address this, decision-making frameworks have been developed to help managers decide whether early detections with eDNA are actionable, based on the species potential impacts and the containment measures in place (Sepulveda et al., 2023b). The development of the 5-level validation scale for targeted eDNA assays (Thalinger et al., 2021) serves a similar purpose, as it will help end-users and INNS managers to make an informed choice when selecting the assay for routine monitoring, thus increasing the reliability of results. Lastly, the creation of specific recommendations by eDNA researchers can provide valuable guidance to INNS managers when implementing eDNA methods in operational settings.

The creation of a structured decision-making framework has proved useful for the management of dreissenid mussels in a reservoir with socio-economic importance in North America (Sepulveda et al., 2022). Working with stakeholders and INNS managers, the authors were able to evaluate the suitability of different management responses following eDNA detection of dreissenids. They found that the best approach, i.e., the one with bigger benefits and lower risks, was delayed containment measures. This consisted of the immediate (within one month) confirmation of positive eDNA detections using non-molecular methods, followed by a visual inspection (i.e. looking for mussel-fouled boats) of all watercrafts leaving the reservoir to prevent their spread to other waterbodies. A similar approach could thus be applied at a national or local scale in order to find the best management actions following positive eDNA detections, that will depend on site-specific features and the final objectives of stakeholders and INNS managers.

The increase in the validation level of both the Gingera et al. (2017) and the Blackman et al. (2020a, 2020b) species-specific assays achieved in this thesis further facilitates the incorporation of eDNA-based surveys into dreissenid management strategies. These assays are currently ranked among the highest validated assays for dreissenid species and can now be confidently used for routine monitoring of both species.

Recommendations for future eDNA monitoring campaigns

Based on our observations, the main factors likely to influence zebra mussel eDNA detectability, assuming populations are established, are the timing of sampling and waterbody type. Sampling strategies and replication levels should thus be adjusted according to these factors (Table 5.2). In our study, we observed that if populations were established and sampling was conducted in the summer (chapter 3), detection rates were consistently high across different waterbodies and environmental conditions, and thus fewer samples and replicates would be needed to ensure detection (Table 5.2). If sampling in the winter with established populations (chapter 2), detection rates still remained high in the reservoir. However, the River Hull displayed only 20% and 40% of positive samples in January and February, respectively, highlighting that even with established populations, sampling in the winter in rivers drastically reduces detection rates. Future monitoring campaigns under the same circumstances should employ a similar approach to ours to ensure detection, i.e. collecting at least 10 water samples evenly spaced and performing at least 6 qPCR replicates (Table 5.2). Even though we only sampled 1 kilometre of the river due to logistic reasons, sampling larger distances is advised, if possible to do so, as it can increase detection probabilities, especially in larger rivers. While our work offers valuable insights about detection rates when populations are established, monitoring campaigns often target sites with low or unknown population status. In such cases, detection probabilities are expected to be lower and replication levels need to be increased accordingly to minimise false negatives (Table 5.2). Additional factors such as the volume filtered can also influence detection rates, as demonstrated here (chapter 2 and 3) and previous studies (e.g. Goldberg et al., 2018; Mächler et al., 2016). Based on our results, in sites with high and moderate detection probabilities, the filtration of at least 200-250 and 500 mL, respectively, would be advised, while sites with low detection probabilities would benefit from the filtration of 1-2 L. Additionally, while we observed that environmental variables (e.g. pH, calcium, etc) do not affect detection rates when sampling in the summer and with established populations, the effects of turbidity and conductivity on eDNA concentration remain unclear, as we observed opposite patterns both per site (chapter 2) and analytical approach (chapter 3). Despite this, the assay can still be considered operational for use in routine monitoring, as the influence of other factors has been tested and understood. The effect of the environmental variables tested here (including turbidity and

conductivity) can likely be disregarded in sites with high detection probability as those in our study, as we saw that it did not affect detection rates. Nevertheless, their influence in situations where detection probabilities are expected to be lower needs to be further investigated. Until such studies are conducted, it is advisable to use high replication levels to minimize false negatives.

For quagga mussels, even though we did not investigate what factors influence eDNA detection, population status (i.e. established/non-established) and waterbody type (lentic vs lotic) are likely to equally influence their detection rates. However, as quagga mussels are able to reproduce at lower temperatures than zebra mussels (5°C vs 12°C, respectively; Karatayev & Burlakova, 2022), with veligers usually found year-round in invaded sites (e.g. Haltiner et al., 2022), the timing of sampling is potentially less important, however future research is needed to test this hypothesis.

Table 5.2 Prediciton of detection probabilities and suggestion of the number of samples/PCR replicates (in brackets) needed to ensure detection of zebra mussel eDNA under different conditions.

	Established		Non-established/Unknown	
Summer	High (5/3)	High (5/3)	Moderate (10/3)	Moderate (10/3)
Winter	Moderate (10/3)	Low (10/6)	Low (10/6)	Very low (10-15/12)
	Lentic	Lotic	Lentic	Lotic

5.4 Final remarks

The work developed in this thesis constitutes an important step towards the operational use of eDNA for monitoring and management of dreissenid mussels. The further testing and optimization of zebra mussel (chapters 2 and 3) and quagga mussel (chapter 4) species-specific qPCR assays increased their validation level to levels 5 and 4, respectively, thus ensuring high confidence in eDNA results. In turn, this will increase the confidence of INNS managers and stakeholders in eDNA methods and ensure their adoption into dreissenid routine monitoring campaigns. The zebra mussel assay can now be considered operational for use in routine monitoring, although sampling strategies should be adjusted based on factors such as waterbody type, timing of sampling and

population status. For quagga mussels, further optimization in the future will render the assay operational. Additionally, gathering more information about quagga mussel ecology will help predict and interpret how environmental variables might influence their eDNA concentration and detectability. While this thesis primarily focused on qPCR, other emerging technologies such as dPCR and automated passive samplers have the potential to improve how we monitor and manage dreissenid mussels in the future, as they can provide faster turnaround times and on-site results, prompting a faster response. Until these methods can reliably be applied in routine monitoring, the work developed in this thesis stands as a valuable asset for future surveillance and management of dreissenid species.

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Appendix 1: Additional information for chapter 2

Gingera et al. (2017) assay: efficiency optimization

Initial attempts to increase the efficiency of the Gingera assay included using three different products for the standard curve – extracted DNA, PCR product and synthetic DNA (gBlock). Standards were run in triplicate for all tests. First, DNA was extracted from tissue of one zebra mussel individual using the MuDNA protocol (Sellers et al., 2018), quantified on a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific) and diluted accordingly to create a standard curve (seven 10-fold dilutions, from 10 to $1x10^{-5}$ ng/µL). PCR and thermal cycling conditions included 1x TaqMan Environmental Master Mix 2.0 (Fisher Scientific, UK), 0.2 µM of each primer (forward and reverse), 0.1 µM of probe, 4.75 µL of molecular grade water and 2 µL of sample, in a total of 15 µL. The PCR program consisted of an initial step at 50°C for 10 min and 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 1 min and annealing and extension at 60°C for 1 min. Following this, the same PCR and thermal cycling conditions were tested using both PCR product and synthetic DNA (gBlock) instead of extracted DNA. The PCR product was generated using zebra mussel tissue and the Gingera et al. (2017) *Cyt* b assay, while synthetic DNA consisted of a 126 bp fragment of gBlock (Integrated DNA Technologies, Belgium) that included the annealing sites for both primers and the probe. Both were quantified with Qubit (Thermo Fisher Scientific) using the double stranded high sensitivity assay, the number of DNA copies for each was calculated following Sint et al. (2012) and both were diluted to create a standard curve with eight 10-fold dilutions, from $3x10^{7}$ to $3x10^{0}$ copies/µL.

Following these attempts, further tests were carried out, all of them using eight 10-fold dilutions ($3x10^{7}$ to $3x10^{0}$ copies/µL) of gBlock in triplicate for the standard curve and thermal cycling conditions as described above. First, the original PCR conditions described in Gingera et al. (2017) were tested, i.e., similar to the above-mentioned conditions but using 20 µL as the total PCR volume instead of 15 µL, as well as 5 µL of sample instead of just 2 µL, while maintaining the primer and probe concentrations. Next, three different combinations of primer/probe concentrations were tested – 0.9/0.25 µM, 0.6/0.17 µM and 0.3/0.9 µM – in a total volume of 10 µL and 2 µL of sample.

The first concentrations (0.9/0.25 µM) are recommended by ThermoFisher and Applied Biosystems for qPCRs, and from that lower concentrations using similar primer to probe ratios were calculated. Finally, reagents (i.e. aliquots) and PCR conditions from a separate lab, reporting efficiencies above 90%, were tested. PCR volumes included 1x of TaqMan Universal Master Mix 2.0, 0.2 µM of primers (forward and reverse combined), 0.1 µM of probe, 0.6 mg/mL of BSA, 7.9 µL of water and 1 µL of sample.

Manually adjusting the baseline in the qPCR software (instead of using the default values) was also tested in some plates but it didn't improve efficiency. Experimenting with changes in thermal cycling conditions were also considered but not tested as it could have had implications in the specificity of the assay.

Gingera et al. (2017) assay: repeatability test

To ensure repeatability of the assay, despite the low efficiency, a subset of ten samples were run twice, separately. Five samples from each site (Eccup Reservoir and River Hull) were selected, including samples from different months and a range of different DNA copy number, i.e., amplifying at high and low cycles. PCR conditions included 1x TaqMan Environmental Master Mix 2.0 (Fisher Scientific, UK), 0.2 µM of each primer (forward and reverse), 0.1 µM of probe, 4.75 µL of molecular grade water and 2 µL of sample, in a total of 15 µL. The PCR program consisted of an initial step at 50°C for 10 min and 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 1 min and annealing and extension at 60°C for 1 min.

Gingera et al. (2017) assay: DNA sequences (5'-3')

Primer forward: CAT TTT CTT ATA CCT TTT ATT TTA TTA GTG CTT TT

Primer reverse: CGG GAC AGT TTG AGT AGA AGT ATC A

Probe: FAM-TAG GTT TTC TTC ATA CTA CTG GC-MGBNFQ

gBlock fragment (bold letters indicate the extra bases added to the original *D. polymorpha* sequence):

CATTTTCTTATACCTTTTATTTTATTAGTGCTTTTAATAGTAC**TTTTTTGGGGGG**ATGTAGGTTTT
CTTCATACTACTGGCTCTAGAAACCCTTTAGGGATTGATACTTCTACTCAAACTGTCCCG

Figure S1.1 Colonisation platforms installed at Eccup Reservoir to record numbers of zebra mussel juveniles.

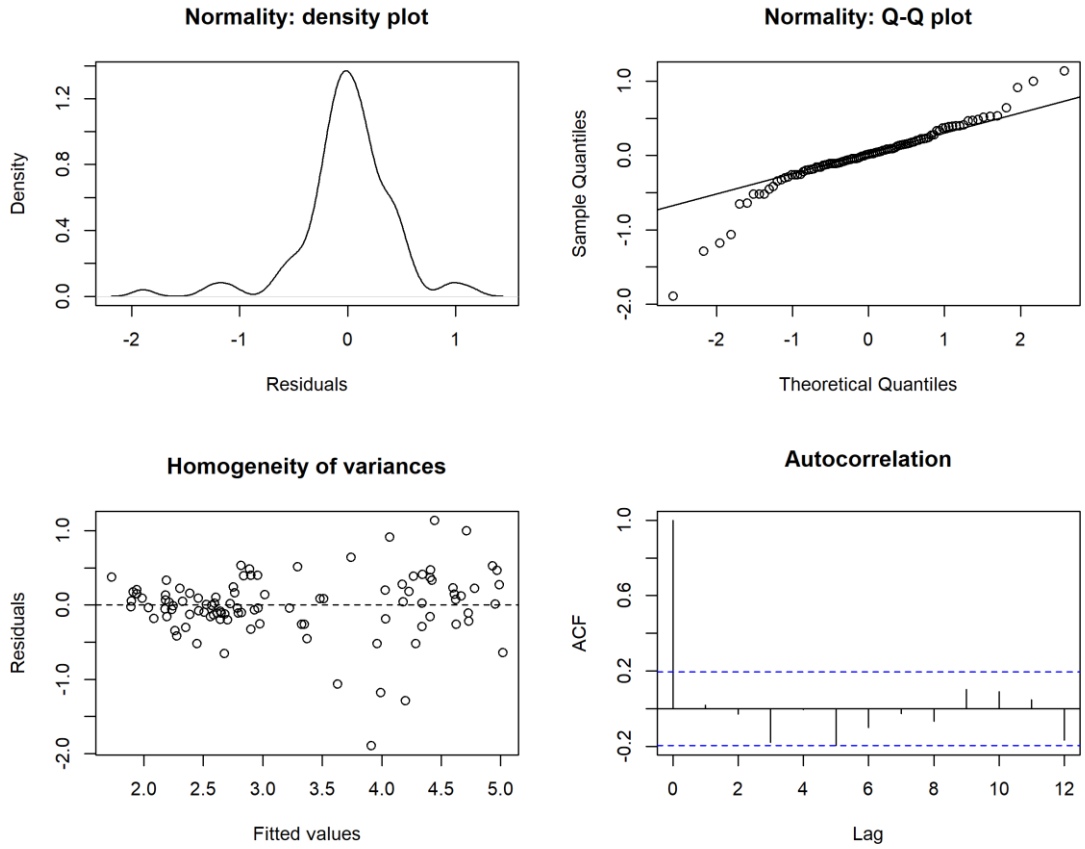

Figure S1.2 Normality, homogeneity of variances and autocorrelation plots used to assess model assumptions for Eccup Reservoir data.

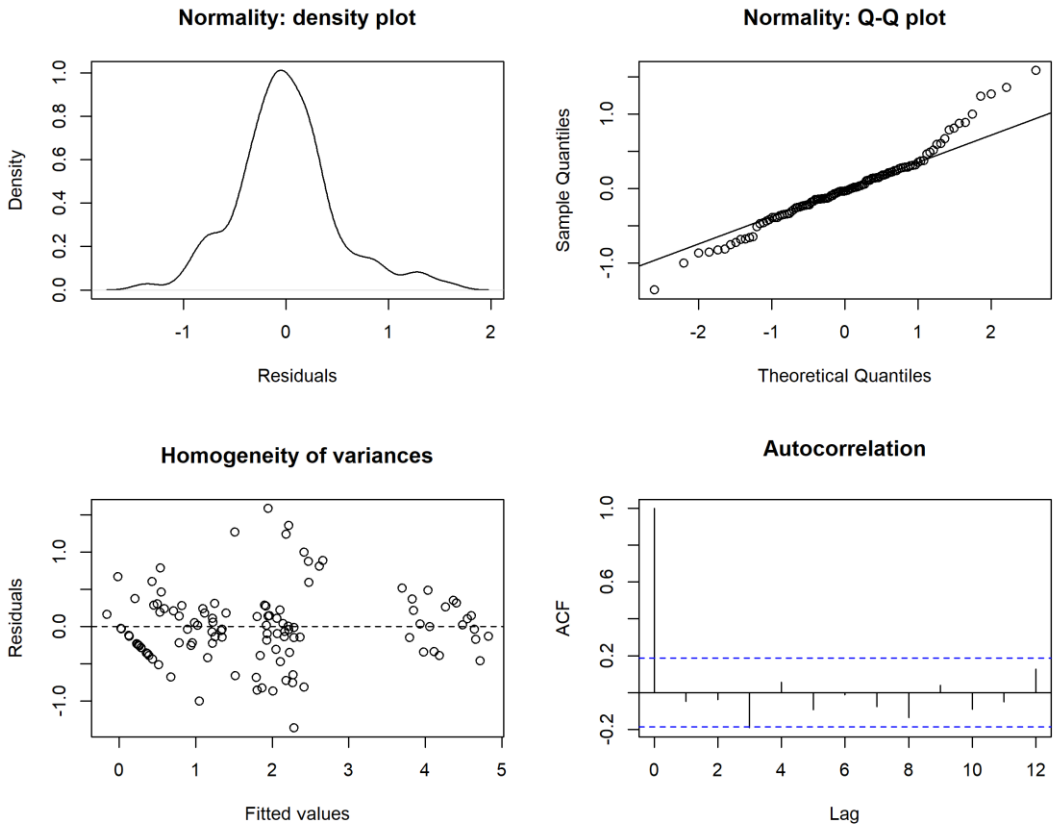

Figure S1.3 Normality, homogeneity of variances and autocorrelation plots used to assess model assumptions for River Hull data.

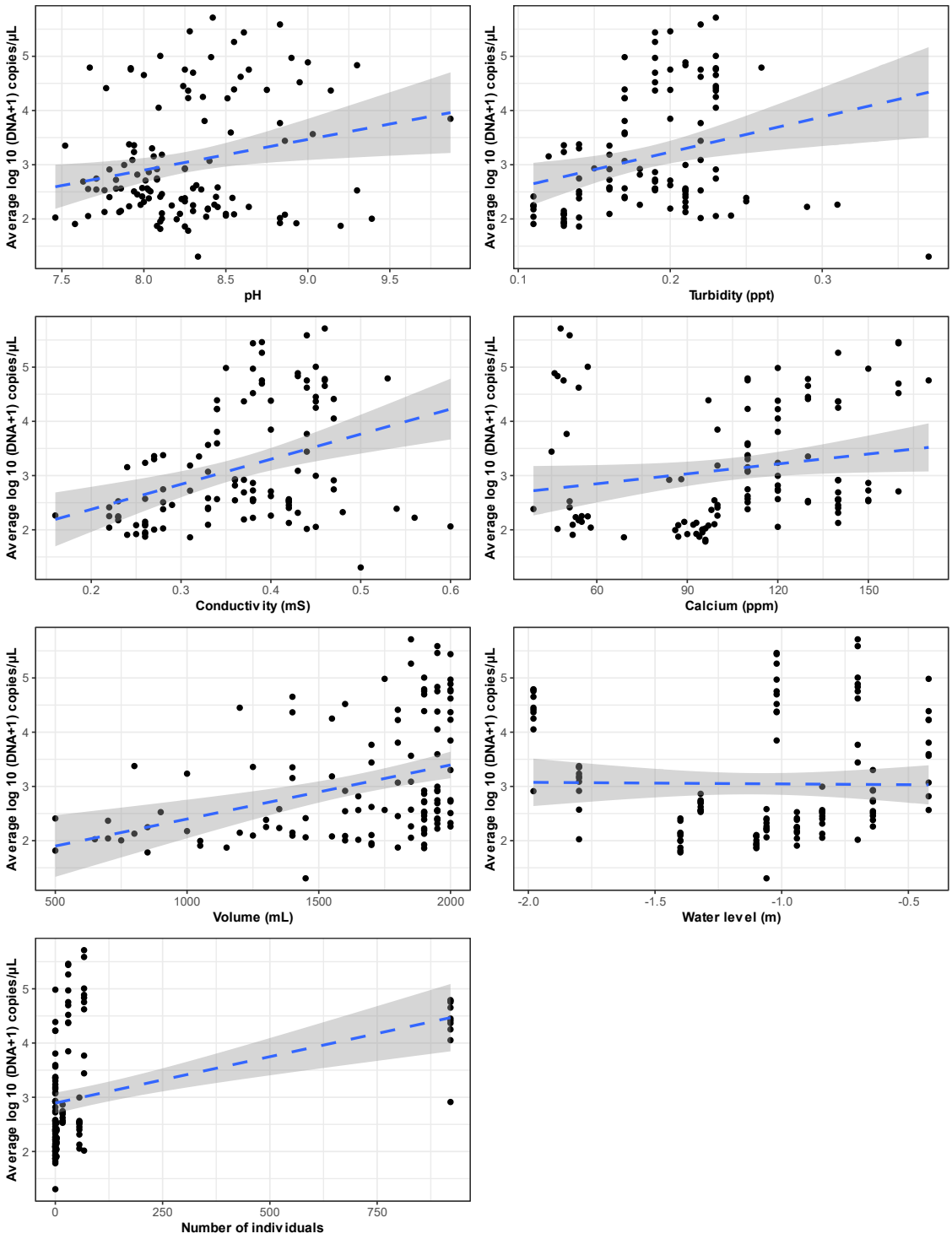

Figure S1.4 Scatterplots showing the effect of environmental variables on average log 10 (DNA+1) copies/µL at Eccup Reservoir. The dashed blue line corresponds to the non-significant linear regression line and grey shading represents the confidence intervals.

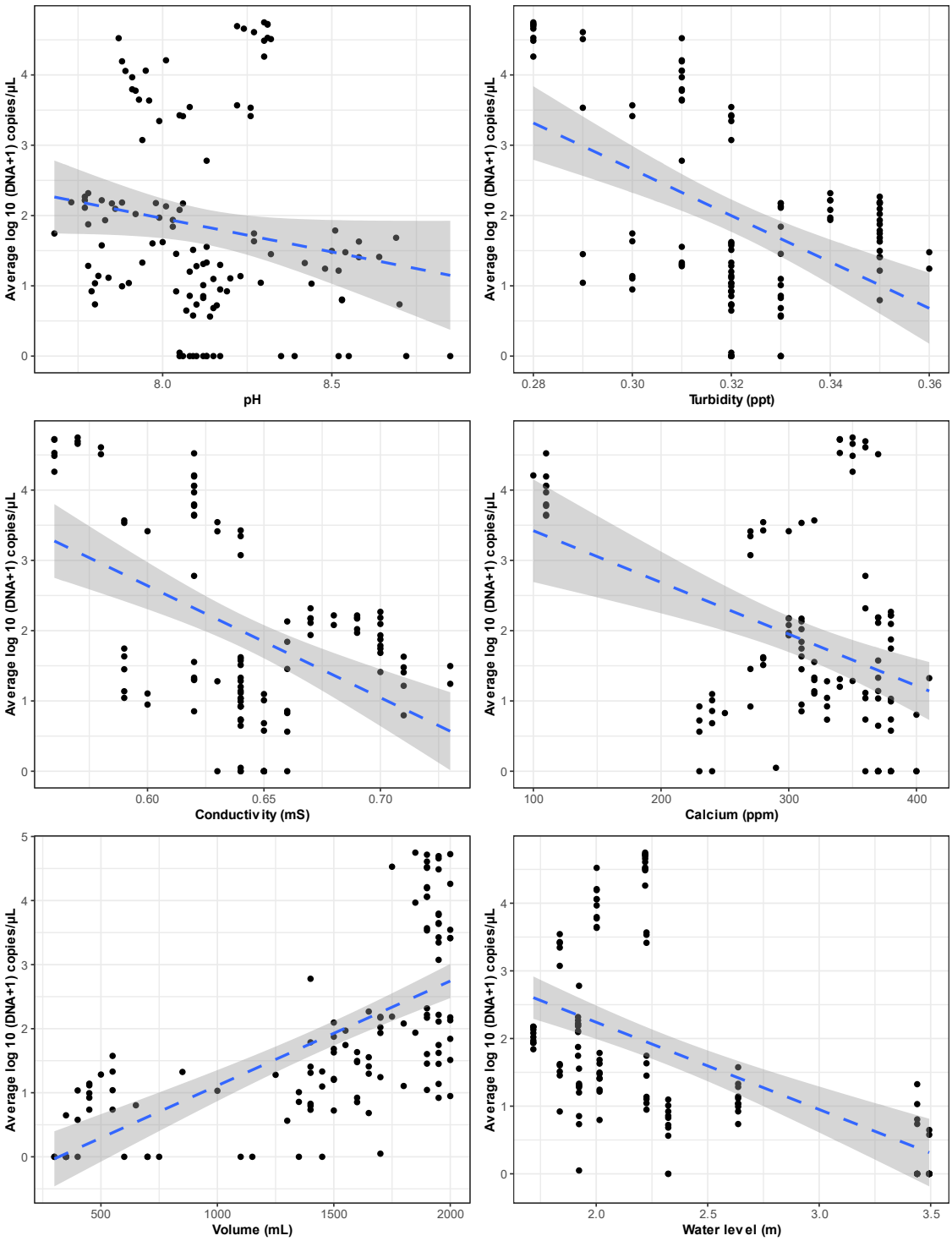

Figure S1.5 Scatterplots showing the effect of environmental variables on average log 10 (DNA+1) copies/µL at River Hull. The dashed blue line corresponds to the non-significant linear regression line and grey shading represents the confidence intervals.

Table S1.1 Individual and average Cq values obtained for each replicate and sample, respectively, for each run of the repeatability test. The standard deviation (SD) for each sample is also provided.

Site	Season	Month	Sample code	Run	Cq	Average Cq + SD
Eccup Reservoir	Winter	Dec	ER17	1	33.845	33.736 + 0.126
Eccup Reservoir	Winter	Dec	ER17	1	33.649	
Eccup Reservoir	Winter	Dec	ER17	1	33.809	
Eccup Reservoir	Winter	Dec	ER17	1	33.745	
Eccup Reservoir	Winter	Dec	ER17	1	33.526	
Eccup Reservoir	Winter	Dec	ER17	1	33.840	
Eccup Reservoir	Winter	Dec	ER17	2	33.567	33.841 + 0.237
Eccup Reservoir	Winter	Dec	ER17	2	33.972	
Eccup Reservoir	Winter	Dec	ER17	2	33.983	
Eccup Reservoir	Winter	Jan	ER29	1	30.781	30.765 + 0.134
Eccup Reservoir	Winter	Jan	ER29	1	30.617	
Eccup Reservoir	Winter	Jan	ER29	1	30.872	
Eccup Reservoir	Winter	Jan	ER29	1	30.580	
Eccup Reservoir	Winter	Jan	ER29	1	30.872	
Eccup Reservoir	Winter	Jan	ER29	1	30.869	
Eccup Reservoir	Winter	Jan	ER29	2	30.286	30.452 + 0.154
Eccup Reservoir	Winter	Jan	ER29	2	30.480	
Eccup Reservoir	Winter	Jan	ER29	2	30.590	
Eccup Reservoir	Spring	March	ER42	1	34.400	34.201 + 0.248
Eccup Reservoir	Spring	March	ER42	1	33.944	
Eccup Reservoir	Spring	March	ER42	1	33.939	
Eccup Reservoir	Spring	March	ER42	1	34.388	
Eccup Reservoir	Spring	March	ER42	1	34.054	
Eccup Reservoir	Spring	March	ER42	1	34.477	
Eccup Reservoir	Spring	March	ER42	2	34.460	34.245 + 0.643
Eccup Reservoir	Spring	March	ER42	2	34.752	
Eccup Reservoir	Spring	March	ER42	2	33.522	
Eccup Reservoir	Summer	July	ER84	1	22.516	22.490 + 0.042

Eccup Reservoir	Summer	July	ER84	1	22.540	
Eccup Reservoir	Summer	July	ER84	1	22.497	
Eccup Reservoir	Summer	July	ER84	1	22.440	
Eccup Reservoir	Summer	July	ER84	1	22.439	
Eccup Reservoir	Summer	July	ER84	1	22.510	
Eccup Reservoir	Summer	July	ER84	2	22.019	22.151 + 0.116
Eccup Reservoir	Summer	July	ER84	2	22.196	
Eccup Reservoir	Summer	July	ER84	2	22.237	
Eccup Reservoir	Autumn	Sept	ER107	1	26.668	26.776 + 0.080
Eccup Reservoir	Autumn	Sept	ER107	1	26.760	
Eccup Reservoir	Autumn	Sept	ER107	1	26.751	
Eccup Reservoir	Autumn	Sept	ER107	1	26.781	
Eccup Reservoir	Autumn	Sept	ER107	1	26.778	
Eccup Reservoir	Autumn	Sept	ER107	1	26.917	
Eccup Reservoir	Autumn	Sept	ER107	2	26.295	26.370 + 0.092
Eccup Reservoir	Autumn	Sept	ER107	2	26.342	
Eccup Reservoir	Autumn	Sept	ER107	2	26.473	
River Hull	Winter	Dec	RH16	1	NA	37.984 + 0.453
River Hull	Winter	Dec	RH16	1	NA	
River Hull	Winter	Dec	RH16	1	37.901	
River Hull	Winter	Dec	RH16	1	38.444	
River Hull	Winter	Dec	RH16	1	38.198	
River Hull	Winter	Dec	RH16	1	37.392	
River Hull	Winter	Dec	RH16	2	36.991	38.417 + 1.235
River Hull	Winter	Dec	RH16	2	39.115	
River Hull	Winter	Dec	RH16	2	39.145	
River Hull	Spring	April	RH58	1	30.822	30.943 + 0.296
River Hull	Spring	April	RH58	1	31.310	
River Hull	Spring	April	RH58	1	30.760	
River Hull	Spring	April	RH58	1	30.567	
River Hull	Spring	April	RH58	1	30.919	

River Hull	Spring	April	RH58	1	31.281	
River Hull	Spring	April	RH58	2	32.514	32.674 + 0.181
River Hull	Spring	April	RH58	2	32.638	
River Hull	Spring	April	RH58	2	32.871	
River Hull	Summer	July	RH86	1	26.098	26.244 + 0.104
River Hull	Summer	July	RH86	1	26.128	
River Hull	Summer	July	RH86	1	26.303	
River Hull	Summer	July	RH86	1	26.316	
River Hull	Summer	July	RH86	1	26.342	
River Hull	Summer	July	RH86	1	26.280	
River Hull	Summer	July	RH86	2	26.171	26.188 + 0.017
River Hull	Summer	July	RH86	2	26.189	
River Hull	Summer	July	RH86	2	26.206	
River Hull	Summer	Aug	RH93	1	27.403	27.425 + 0.081
River Hull	Summer	Aug	RH93	1	27.424	
River Hull	Summer	Aug	RH93	1	27.498	
River Hull	Summer	Aug	RH93	1	27.393	
River Hull	Summer	Aug	RH93	1	27.530	
River Hull	Summer	Aug	RH93	1	27.304	
River Hull	Summer	Aug	RH93	2	26.996	27.082 + 0.110
River Hull	Summer	Aug	RH93	2	27.045	
River Hull	Summer	Aug	RH93	2	27.205	
River Hull	Autumn	Sept	RH103	1	32.162	32.170 + 0.135
River Hull	Autumn	Sept	RH103	1	32.315	
River Hull	Autumn	Sept	RH103	1	32.059	
River Hull	Autumn	Sept	RH103	1	32.141	
River Hull	Autumn	Sept	RH103	1	32.340	
River Hull	Autumn	Sept	RH103	1	32.005	
River Hull	Autumn	Sept	RH103	2	32.005	32.265 + 0.291
River Hull	Autumn	Sept	RH103	2	32.211	
River Hull	Autumn	Sept	RH103	2	32.580	

Table S1.2 MIQE checklist (Bustin et al., 2009) for the Gingera et al. (2017) *Cyt* b assay and data from the second chapter.

Item to check	Importance	Details
Experimental design		
Definition of experimental and control groups	E	2 sites sampled in Yorkshire, England, over a period of 12 months Sites: Eccup Reservoir (Leeds; 53.87095, -1.54606) and River Hull (Tickton; 53.85529, -0.39353)
Number within each group	E	10 samples collected at each site and each month n = 120 in total for each site
Assay carried out by the core or investigator's laboratory?	D	Investigator's lab
Acknowledgement of authors' contributions	D	Yes
Sample		
Description	E	eDNA water samples
Volume/mass of sample processed	D	2 L of water were collected and then vacuum-filtered. The volume filtered was recorded for each sample
Microdissection or macrodissection	E	N/A

Processing procedure	E	At each of the 10 sampling locations, 4 subsamples of approximately 500 mL of surface water were collected into a sterile 2 L plastic bottle and samples were transported to the laboratory with ice packs on the same day of collection. Samples were vacuum-filtered within 24h of collection and two 0.45 µm cellulose filters were used for each sample. The volume filtered was recorded for each sample
If frozen, how and how quickly?	E	N/A
If fixed, with what how quickly?	E	N/A
Sample storage conditions and duration (especially for FFPE samples)	E	Filters were stored in 5 mL screw cap Axygen tubes with garnet grit (0.8-1.2 g of both 0.15 mm and 1-1.4 mm diameter sterile beads) and stored at -20°C until DNA extraction
Nucleic acid extraction		
Procedure and/or instrumentation	E	DNA extraction was performed following the water protocol described in Sellers et al. (2018)
Name of kit and details of any modifications	E	
Source of additional reagents used	D	University of Hull
Details of DNase or RNAse treatment	E	N/A
Contamination assessment (DNA or RNA)	E	N/A

Nucleic acid quantification	E	Only samples where inhibition was suspected (due to the colour of the lysate during extractions) were quantified, to assess the purity ratios. This was assessed using a Nanodrop 1000 spectrophotometer following the manufacturer's instructions.
Instrument and method	E	
Purity (A260/A280)	D	
Yield	D	
RNA integrity: method/instrument	E	N/A
RIN/RQI or Cq of 3' and 5' transcripts	E	N/A
Electrophoresis traces	D	N/A
Inhibition testing (Cq dilutions, spike, or other)	E	Samples were tested for inhibition using an exogenous internal positive control. All eDNA samples were tested in duplicate and samples were considered to be inhibited if the average Cq of a sample was higher than the no template reaction by 2 or more cycles.
Reverse Transcription		
Complete reaction conditions	E	N/A
Amount of RNA and reaction volume	E	N/A
Priming oligonucleotide (if using GSP) and concentration	E	N/A

Reverse transcriptase and concentration	E	N/A
Temperature and time	E	N/A
Manufacturer of reagents and catalogue numbers	D	N/A
Cqs with and without reverse transcription	D	N/A
Storage conditions of cDNA	D	N/A
qPCR target information		
Gene symbol	E	*Cyt* b - cytochrome b
Sequence accession number	E	N/A
Location of amplicon	D	N/A
Amplicon length	E	114 bp
In silico specificity screen (BLAST, and so on)	E	N/A
Pseudogenes, retropseudogenes or other homologs?	D	N/A
Sequence alignment	D	N/A

Secondary structure analysis of amplicon	D	N/A
Location of each primer by exon or intron (if applicable)	E	N/A
What splice variants are targeted?	E	N/A
qPCR oligonucleotides		
Primer sequences	E	forward (5’- 3’): CAT TTT CTT ATA CCT TTT ATT TTA TTA GTG CTT TT reverse (5’- 3’): CGG GAC AGT TTG AGT AGA AGT ATC A
RTPrimerDB Identification Number	D	N/A
Probe sequences	D	(5’- 3’): 6FAM-TAG GTT TTC TTC ATA CTA CTG GC-MGBNFQ
Location and identity of any modifications	E	N/A
Manufacturer of oligonucleotides	D	IDT (primers) and Applied Biosystems UK (probe)
Purification method	D	standard desalting (primers) and HPLC (probe)
qPCR protocol		

Complete reaction conditions	E	PCR conditions: 1x TaqMan Environmental Master Mix 2.0 (Fisher Scientific, UK), 0.2 µM of each primer (forward and reverse), 0.1 µM of probe, 4.75 µL of molecular grade water and 2 µL of sample qPCR program: 50°C for 10 min, 95°C for 10 min, followed by 45 cycles of 95°C for 1 min and 60°C for 1 min
Reaction volume and amount of cDNA/DNA	E	Reaction volume = 15 µL amount of DNA = 2 µL
Primer, (probe), Mg^{2+} and dNTP concentrations	E	0.2 µM of each primer (forward and reverse), 0.1 µM of probe
Polymerase identity and concentration	E	TaqMan Environmental Master Mix 2.0 (Fisher Scientific, UK), final concentration of 1x
Buffer/kit identity and manufacturer	E	N/A
Exact chemical composition of the buffer	D	N/A
Additives (SYBR Green I, DMSO, and so forth)	E	N/A
Manufacturer of plates/tubes and catalog number	D	Applied Biosystems™ MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL (12142000, Fisher Scientific, UK)
Complete thermocycling parameters	E	50°C for 10 min; 95°C for 10 min; 45 cycles of 95°C for 1 min and 60°C for 1 min

Reaction setup (manual/robotic)	D	Reactions were made manually
Manufacturer of qPCR instrument	E	StepOne-Plus™ Real-Time PCR system (Fisher Scientific/Thermo Fisher, UK)
qPCR validation		
Evidence of optimisation (from gradients)	D	N/A
Specificity (gel, sequence, melt, or digest)	E	N/A
For SYBR Green I, Cq of the NTC	E	N/A
Calibration curves with slope and y intercept	E	Slope range: -4.06 – -3.67; y-intercept range: 41.51 – 45.45
PCR efficiency calculated from slope	E	76.4 - 87.3%
CIs for PCR efficiency or SE	D	N/A
r2 of standard curve	E	0.981 - 0.999
Linear dynamic range	E	N/A

Cq variation at lower limit	E	N/A
CIs throughout range	D	N/A
Evidence for LOD	E	The lowest standard with at least 95% amplification was 300 copies/µL
If multiplex, efficiency and LOD of each assay	E	N/A
Data analysis		
qPCR analysis program (source, version)	E	StepOne Software version 2.3
Method of Cq determination	E	Performed according to the default setting of the software above
Outlier identification and disposition	E	N/A
Results for NTCs	E	Three wells of no-template negative control were included in all qPCR plates and showed no amplification
Justification of number and choice of reference genes	E	N/A
Description of normalisation method	E	We used standard curve methods
Number and concordance of biological replicates	D	10 samples collected at each site and each month n = 120 in total for each site

Number and stage (reverse transcription or qPCR) of technical replicates	E	Six qPCR replicates for each sample
Repeatability (intraassay variation)	E	A subset of ten samples were run twice separately, which included five samples from each site (Eccup Reservoir and River Hull), from different months and with a range of different DNA concentrations. PCR volumes and thermal cycling conditions were as above. Cq values between both runs were compared and showed to be identical.
Reproducibility (interassay variation, CV)	D	N/A
Power analysis	D	N/A
Statistical methods for result significance	E	Linear mixed-effects models
Software (source, version)	E	R version 4.2.1
Cq or raw data submission using RDML	D	N/A

Table S1.3 Field metadata and average DNA copies/µL plus standard deviation (SD) for all samples collected for the second chapter. Information on sample inhibition is also provided, which corresponds to the difference between the average Cq of each sample and the no template reaction.

Site	Season	Month	Sample code	Temperature (°C)	pH	Turbidity (ppt)	Conductivity (mS)	Calcium (ppm)	Volume (mL)	Water level (m)	Inhibition	Average DNA copies/µL + SD
Eccup Reservoir	Winter	Dec	ER11	6.1	8.64	0.29	0.56	NA	1900	-1.06	0.55	166.10 + 22.52
Eccup Reservoir	Winter	Dec	ER12	5.1	8.54	0.25	0.54	NA	1900	-1.06	0.30	243.64 + 17.29
Eccup Reservoir	Winter	Dec	ER13	5.5	8.50	0.24	0.60	NA	1450	-1.06	0.30	114.40 + 31.82
Eccup Reservoir	Winter	Dec	ER14	6.9	8.45	0.21	0.38	NA	1950	-1.06	0.50	165.27 + 37.57
Eccup Reservoir	Winter	Dec	ER15	4.9	8.33	0.37	0.50	NA	1450	-1.06	0.23	19.21 + 4.73
Eccup Reservoir	Winter	Dec	ER16	7.7	8.44	0.17	0.33	NA	1950	-1.06	0.53	254.48 + 46.58
Eccup Reservoir	Winter	Dec	ER17	7.5	8.25	0.25	0.48	NA	2000	-1.06	0.32	210.58 + 16.56
Eccup Reservoir	Winter	Dec	ER18	6.0	8.45	0.16	0.33	NA	1350	-1.06	0.33	380.84 + 52.30
Eccup Reservoir	Winter	Dec	ER19	7.8	8.39	0.20	0.37	NA	1900	-1.06	0.29	154.32 + 36.41
Eccup Reservoir	Winter	Dec	ER20	8.3	8.43	0.31	0.16	NA	1850	-1.06	0.34	182.64 + 21.46
Eccup Reservoir	Winter	Jan	ER21	5.6	8.08	0.16	0.36	140	1600	-1.8	0.63	826.99 + 120.19

Eccup Reservoir	Winter	Jan	ER22	6.0	8.11	0.16	0.31	100	1550	-1.8	0.29	1529.43 + 146.00
Eccup Reservoir	Winter	Jan	ER23	5.9	7.93	0.22	0.43	110	1850	-1.8	0.25	1222.41 + 120.71
Eccup Reservoir	Winter	Jan	ER24	4.4	7.99	0.16	0.26	110	1850	-1.8	0.44	371.41 + 62.00
Eccup Reservoir	Winter	Jan	ER25	7.1	7.46	0.14	0.28	96	650	-1.8	0.20	105.05 + 75.85
Eccup Reservoir	Winter	Jan	ER26	6.0	7.52	0.16	0.32	130	1400	-1.8	0.35	2247.85 + 77.47
Eccup Reservoir	Winter	Jan	ER27	7.4	7.91	0.14	0.28	110	800	-1.8	0.36	2374.64 + 174.36
Eccup Reservoir	Winter	Jan	ER28	6.0	7.94	0.13	0.26	120	1000	-1.8	0.24	1717.52 + 174.23
Eccup Reservoir	Winter	Jan	ER29	5.6	7.94	0.13	0.27	110	1250	-1.8	0.13	2283.53 + 181.74
Eccup Reservoir	Winter	Jan	ER30	5.8	8.06	0.12	0.24	110	1400	-1.8	0.38	1426.87 + 145.63
Eccup Reservoir	Winter	Feb	ER31	4.4	8.22	NA	NA	92	1250	-1.4	0.66	123.25 + 52.02
Eccup Reservoir	Winter	Feb	ER32	4.7	8.30	NA	NA	89	1200	-1.4	0.28	138.96 + 26.26
Eccup Reservoir	Winter	Feb	ER33	5.6	8.20	NA	NA	86	1050	-1.4	0.29	97.72 + 39.17
Eccup Reservoir	Winter	Feb	ER34	5.6	8.08	NA	NA	94	1150	-1.4	0.41	73.47 + 52.97
Eccup Reservoir	Winter	Feb	ER35	6.5	8.10	NA	NA	96	500	-1.4	0.11	64.49 + 42.42

Eccup Reservoir	Winter	Feb	ER36	6.2	7.76	NA	NA	93	800	-1.4	0.37	133.56 + 50.75
Eccup Reservoir	Winter	Feb	ER37	6.6	8.10	NA	NA	100	500	-1.4	0.16	255.69 + 49.85
Eccup Reservoir	Winter	Feb	ER38	6.1	8.23	NA	NA	98	700	-1.4	0.24	231.89 + 34.86
Eccup Reservoir	Winter	Feb	ER39	5.3	8.27	NA	NA	96	850	-1.4	0.15	59.73 + 31.11
Eccup Reservoir	Winter	Feb	ER40	5.6	8.11	NA	NA	95	750	-1.4	0.37	100.20 + 20.52
Eccup Reservoir	Spring	March	ER41	8.0	8.25	0.17	0.33	39	1300	-0.94	0.61	240.62 + 41.81
Eccup Reservoir	Spring	March	ER42	8.9	9.30	0.22	0.23	51	900	-0.94	0.33	334.77 + 47.15
Eccup Reservoir	Spring	March	ER43	9.3	7.99	0.11	0.22	51	1450	-0.94	0.32	258.68 + 46.70
Eccup Reservoir	Spring	March	ER44	8.3	7.91	0.11	0.23	53	1350	-0.94	0.40	170.15 + 43.32
Eccup Reservoir	Spring	March	ER45	9.2	7.58	0.11	0.24	52	1050	-0.94	0.18	79.82 + 10.24
Eccup Reservoir	Spring	March	ER46	8.2	7.86	0.13	0.26	55	1400	-0.94	0.26	139.65 + 23.51
Eccup Reservoir	Spring	March	ER47	9.5	8.30	0.11	0.22	55	1300	-0.94	0.34	177.64 + 42.53
Eccup Reservoir	Spring	March	ER48	9.5	8.17	0.11	0.23	57	850	-0.94	0.28	176.10 + 36.09
Eccup Reservoir	Spring	March	ER49	9.2	8.39	0.11	0.23	54	1000	-0.94	0.13	149.00 + 22.40

Eccup Reservoir	Spring	March	ER50	9.3	8.38	0.11	0.22	58	700	-0.94	0.44	108.88 + 18.35
Eccup Reservoir	Spring	April	ER51	10.8	8.50	0.16	0.33	52	1400	-1.1	0.52	122.87 + 34.53
Eccup Reservoir	Spring	April	ER52	11.7	8.25	0.14	0.31	69	1900	-1.1	0.19	71.68 + 53.38
Eccup Reservoir	Spring	April	ER53	12.4	8.86	0.13	0.26	97	1550	-1.1	0.18	118.88 + 89.69
Eccup Reservoir	Spring	April	ER54	12.1	8.55	0.13	0.25	87	1600	-1.1	0.34	120.89 + 46.35
Eccup Reservoir	Spring	April	ER55	12.6	8.10	0.13	0.26	95	1700	-1.1	0.12	88.59 + 47.00
Eccup Reservoir	Spring	April	ER56	11.9	8.11	0.13	0.26	99	1700	-1.1	0.41	125.92 + 42.68
Eccup Reservoir	Spring	April	ER57	13.4	9.20	0.13	0.26	87	1800	-1.1	0.18	73.67 + 38.84
Eccup Reservoir	Spring	April	ER58	14.6	9.39	0.13	0.27	95	1600	-1.1	0.19	99.92 + 37.48
Eccup Reservoir	Spring	April	ER59	14.2	8.93	0.13	0.25	90	1700	-1.1	0.11	82.35 + 36.33
Eccup Reservoir	Spring	April	ER60	14.5	8.83	0.13	0.26	93	1900	-1.1	0.26	83.17 + 28.73
Eccup Reservoir	Spring	May	ER61	11.6	8.25	0.18	0.37	84	1900	-0.64	0.44	834.11 + 110.94
Eccup Reservoir	Spring	May	ER62	10.8	8.25	0.15	0.36	88	1950	-0.64	0.15	855.17 + 115.64
Eccup Reservoir	Spring	May	ER63	10.9	8.35	0.16	0.36	99	1950	-0.64	0.19	349.09 + 71.55

Eccup Reservoir	Spring	May	ER64	11.1	8.11	0.14	0.29	100	1900	-0.64	0.32	286.71 + 55.81
Eccup Reservoir	Spring	May	ER65	10.7	7.98	0.18	0.40	100	2000	-0.64	0.08	181.46 + 60.99
Eccup Reservoir	Spring	May	ER66	11.3	8.03	0.14	0.28	110	1950	-0.64	0.28	238.73 + 70.72
Eccup Reservoir	Spring	May	ER67	11.2	8.04	0.14	0.28	110	2000	-0.64	0.12	321.66 + 33.60
Eccup Reservoir	Spring	May	ER68	11.2	7.83	0.16	0.31	120	2000	-0.64	0.09	525.60 + 84.49
Eccup Reservoir	Spring	May	ER69	11.1	8.08	0.14	0.28	110	2000	-0.64	0.01	557.15 + 106.39
Eccup Reservoir	Spring	May	ER70	11.6	8.05	0.14	0.27	110	2000	-0.64	0.19	2004.01 + 160.67
Eccup Reservoir	Summer	June	ER71	18.3	8.55	0.17	0.34	97	1900	-0.42	0.46	24438.87 + 486.22
Eccup Reservoir	Summer	June	ER72	19.6	8.27	0.17	0.34	110	2000	-0.42	0.17	16969.67 + 693.92
Eccup Reservoir	Summer	June	ER73	18.8	9.03	0.17	0.33	110	1850	-0.42	0.12	3671.53 + 345.42
Eccup Reservoir	Summer	June	ER74	18.5	8.53	0.17	0.34	110	1950	-0.42	0.22	3924.93 + 348.32
Eccup Reservoir	Summer	June	ER75	19.3	8.40	0.17	0.33	110	1800	-0.42	0.07	1175.37 + 112.87
Eccup Reservoir	Summer	June	ER76	19.3	8.37	0.17	0.34	120	1800	-0.42	0.25	6399.93 + 354.76
Eccup Reservoir	Summer	June	ER77	19.6	8.30	0.17	0.34	120	1750	-0.42	0.09	366.56 + 131.62

Eccup Reservoir	Summer	June	ER78	21.0	7.96	0.18	0.36	120	1650	-0.42	0.16	656.62 + 57.58
Eccup Reservoir	Summer	June	ER79	22.4	8.51	0.17	0.34	120	1800	-0.42	0.00	16760.45 + 1125.29
Eccup Reservoir	Summer	June	ER80	21.3	8.41	0.17	0.35	120	1750	-0.42	0.16	96266.17 + 5650.36
Eccup Reservoir	Summer	July	ER81	21.3	8.75	0.20	0.40	120	1950	-1.02	0.35	24003.20 + 810.93
Eccup Reservoir	Summer	July	ER82	21.0	8.55	0.19	0.39	140	1850	-1.02	0.13	183783.26 + 4425.86
Eccup Reservoir	Summer	July	ER83	21.3	8.95	0.19	0.38	160	1600	-1.02	0.34	33096.12 + 764.87
Eccup Reservoir	Summer	July	ER84	21.1	8.28	0.20	0.39	160	1950	-1.02	0.24	288730.71 + 7287.59
Eccup Reservoir	Summer	July	ER85	21.2	8.30	0.19	0.39	160	1900	-1.02	0.06	49771.15 + 1647.41
Eccup Reservoir	Summer	July	ER86	21.1	8.25	0.20	0.39	170	1950	-1.02	0.21	56781.35 + 349.07
Eccup Reservoir	Summer	July	ER87	19.9	9.87	0.20	0.40	100	2000	-1.02	0.15	7034.37 + 125.92
Eccup Reservoir	Summer	July	ER88	21.2	9.14	0.19	0.37	140	2000	-1.02	0.13	23369.25 + 661.14
Eccup Reservoir	Summer	July	ER89	21.2	8.61	0.19	0.38	160	2000	-1.02	0.05	274827.96 + 13271.10
Eccup Reservoir	Summer	July	ER90	21.7	8.90	0.19	0.38	150	2000	-1.02	0.10	93342.74 + 4410.70
Eccup Reservoir	Summer	Aug	ER91	18.3	8.42	0.23	0.46	48	1850	-0.7	0.50	515647.81 + 32215.12

Eccup Reservoir	Summer	Aug	ER92	18.2	8.10	0.23	0.45	57	1900	-0.7	0.14	101392.14 + 5946.51
Eccup Reservoir	Summer	Aug	ER93	18.6	8.83	0.22	0.44	51	1950	-0.7	0.24	386048.35 + 16016.10
Eccup Reservoir	Summer	Aug	ER94	18.9	8.83	0.22	0.44	50	1700	-0.7	0.24	5856.55 + 285.59
Eccup Reservoir	Summer	Aug	ER95	18.8	8.86	0.22	0.44	45	1700	-0.7	0.05	2760.45 + 199.37
Eccup Reservoir	Summer	Aug	ER96	19.0	8.83	0.22	0.44	47	1650	-0.7	0.42	102.80 + 25.34
Eccup Reservoir	Summer	Aug	ER97	18.7	8.64	0.22	0.44	49	2000	-0.7	0.18	56691.15 + 2132.36
Eccup Reservoir	Summer	Aug	ER98	19.6	9.00	0.21	0.43	46	2000	-0.7	0.24	77170.13 + 2928.42
Eccup Reservoir	Summer	Aug	ER99	19.8	8.59	0.22	0.44	54	2000	-0.7	0.09	41826.01 + 3249.03
Eccup Reservoir	Summer	Aug	ER100	20.6	9.30	0.21	0.43	47	1950	-0.7	0.21	68375.26 + 4116.63
Eccup Reservoir	Autumn	Sept	ER101	16.2	7.67	0.26	0.53	110	1900	-1.98	0.40	61867.40 + 3934.07
Eccup Reservoir	Autumn	Sept	ER102	15.8	7.92	0.23	0.46	110	1900	-1.98	0.19	57043.07 + 3085.11
Eccup Reservoir	Autumn	Sept	ER103	16.3	8.09	0.23	0.47	120	1950	-1.98	0.10	11270.51 + 627.51
Eccup Reservoir	Autumn	Sept	ER104	16.9	7.92	0.23	0.46	130	2000	-1.98	0.33	60357.82 + 3238.76
Eccup Reservoir	Autumn	Sept	ER105	17.8	7.77	0.23	0.47	130	1800	-1.98	0.03	25822.32 + 778.78

Eccup Reservoir	Autumn	Sept	ER106	17.6	7.79	0.23	0.47	140	1950	-1.98	0.16	816.43 + 132.36
Eccup Reservoir	Autumn	Sept	ER107	18.0	8.00	0.23	0.46	130	1400	-1.98	0.07	44954.06 + 2218.86
Eccup Reservoir	Autumn	Sept	ER108	17.8	8.24	0.23	0.45	130	1200	-1.98	0.10	28212.16 + 817.83
Eccup Reservoir	Autumn	Sept	ER109	17.5	8.27	0.23	0.45	140	1400	-1.98	0.00	23252.30 + 2143.84
Eccup Reservoir	Autumn	Sept	ER110	17.6	8.36	0.23	0.45	140	1550	-1.98	0.17	17818.82 + 910.63
Eccup Reservoir	Autumn	Oct	ER111	11.5	7.88	0.21	0.45	120	1950	-0.84	0.44	988.06 + 66.52
Eccup Reservoir	Autumn	Oct	ER112	11.6	7.94	0.21	0.42	130	1900	-0.84	0.16	320.85 + 31.83
Eccup Reservoir	Autumn	Oct	ER113	11.6	8.03	0.21	0.42	130	1900	-0.84	0.13	339.47 + 58.41
Eccup Reservoir	Autumn	Oct	ER114	11.8	7.79	0.21	0.42	140	1950	-0.84	0.21	251.68 + 26.15
Eccup Reservoir	Autumn	Oct	ER115	11.6	8.00	0.21	0.43	140	1950	-0.84	0.00	204.94 + 31.77
Eccup Reservoir	Autumn	Oct	ER116	11.9	7.85	0.21	0.42	140	1900	-0.84	0.25	132.93 + 38.36
Eccup Reservoir	Autumn	Oct	ER117	11.0	7.66	0.23	0.45	120	1850	-0.84	0.08	112.21 + 41.59
Eccup Reservoir	Autumn	Oct	ER118	11.2	7.71	0.21	0.42	140	1600	-0.84	0.15	348.35 + 57.10
Eccup Reservoir	Autumn	Oct	ER119	12.2	8.02	0.21	0.42	140	1650	-0.84	0.01	364.92 + 82.81

Eccup Reservoir	Autumn	Oct	ER120	12.0	7.96	0.21	0.42	140	1800	-0.84	0.07	282.50 + 11.35
Eccup Reservoir	Autumn	Nov	ER121	7.5	8.32	0.20	0.40	110	1700	-1.32	0.40	417.33 + 113.30
Eccup Reservoir	Autumn	Nov	ER122	6.7	7.63	0.19	0.37	140	1950	-1.32	0.21	488.63 + 43.74
Eccup Reservoir	Autumn	Nov	ER123	7.2	7.86	0.19	0.38	140	1950	-1.32	0.21	363.62 + 55.64
Eccup Reservoir	Autumn	Nov	ER124	8.2	7.83	0.19	0.38	140	1950	-1.32	0.19	361.55 + 87.70
Eccup Reservoir	Autumn	Nov	ER125	7.3	7.76	0.19	0.38	150	1900	-1.32	0.08	337.12 + 69.83
Eccup Reservoir	Autumn	Nov	ER126	8.6	7.66	0.19	0.38	150	1950	-1.32	0.23	355.83 + 42.32
Eccup Reservoir	Autumn	Nov	ER127	8.7	7.71	0.23	0.47	120	1950	-1.32	0.21	554.63 + 107.80
Eccup Reservoir	Autumn	Nov	ER128	7.6	8.01	0.20	0.40	160	1900	-1.32	0.17	510.39 + 120.73
Eccup Reservoir	Autumn	Nov	ER129	9.4	8.03	0.19	0.38	150	1900	-1.32	0.02	730.40 + 31.59
Eccup Reservoir	Autumn	Nov	ER130	9.6	8.08	0.19	0.38	150	1900	-1.32	0.23	530.83 + 61.55
River Hull	Winter	Dec	RH11	7.2	8.48	0.36	0.73	NA	1700	2.02	0.50	16.51 + 16.08
River Hull	Winter	Dec	RH12	7.1	8.50	0.35	0.73	NA	1600	2.02	0.15	30.37 + 36.34
River Hull	Winter	Dec	RH13	6.9	8.52	0.35	0.71	NA	1500	2.02	0.31	15.43 + 27.16

River Hull	Winter	Dec	RH14	6.8	8.51	0.35	0.70	NA	1400	2.02	0.24	60.18 + 32.41
River Hull	Winter	Dec	RH15	6.6	8.53	0.35	0.71	NA	1400	2.02	0.21	5.25 + 12.85
River Hull	Winter	Dec	RH16	6.4	8.58	0.35	0.71	NA	1400	2.02	0.44	24.51 + 20.46
River Hull	Winter	Dec	RH17	6.4	8.54	0.36	0.71	NA	1600	2.02	0.15	29.05 + 27.45
River Hull	Winter	Dec	RH18	6.3	8.58	0.35	0.71	NA	1500	2.02	0.19	41.57 + 36.14
River Hull	Winter	Dec	RH19	6.3	8.69	0.35	0.70	NA	1500	2.02	0.00	47.29 + 44.83
River Hull	Winter	Dec	RH20	6.3	8.64	0.35	0.70	NA	1650	2.02	0.24	24.77 + 28.46
River Hull	Winter	Jan	RH21	8.2	8.05	0.32	0.65	370	300	3.49	0.35	0.00 + 0.00
River Hull	Winter	Jan	RH22	8.3	8.08	0.32	0.65	370	400	3.49	0.16	0.00 + 0.00
River Hull	Winter	Jan	RH23	8.2	8.09	0.33	0.65	380	400	3.49	0.17	2.78 + 6.81
River Hull	Winter	Jan	RH24	8.4	8.13	0.32	0.65	370	300	3.49	0.33	0.00 + 0.00
River Hull	Winter	Jan	RH25	8.5	8.15	0.32	0.64	370	350	3.49	0.11	0.00 + 0.00
River Hull	Winter	Jan	RH26	8.5	8.17	0.33	0.65	380	350	3.49	0.24	0.00 + 0.00
River Hull	Winter	Jan	RH27	8.3	8.09	0.32	0.64	380	350	3.49	0.09	0.00 + 0.00

River Hull	Winter	Jan	RH28	8.5	8.10	0.32	0.63	370	350	3.49	0.12	0.00 + 0.00
River Hull	Winter	Jan	RH29	8.2	8.06	0.32	0.65	370	350	3.49	0.04	0.00 + 0.00
River Hull	Winter	Jan	RH30	8.3	8.07	0.32	0.64	370	350	3.49	0.17	3.44 + 8.43
River Hull	Winter	Feb	RH31	10.2	8.55	NA	NA	380	750	3.44	0.56	0.00 + 0.00
River Hull	Winter	Feb	RH32	9.5	8.42	NA	NA	410	850	3.44	0.11	20.11 + 23.54
River Hull	Winter	Feb	RH33	9.4	8.44	NA	NA	380	1000	3.44	0.22	9.73 + 23.84
River Hull	Winter	Feb	RH34	9.7	8.70	NA	NA	380	550	3.44	0.26	4.45 + 10.90
River Hull	Winter	Feb	RH35	9.9	8.85	NA	NA	360	700	3.44	0.11	0.00 + 0.00
River Hull	Winter	Feb	RH36	9.6	8.72	NA	NA	370	600	3.44	0.27	0.00 + 0.00
River Hull	Winter	Feb	RH37	9.4	8.53	NA	NA	400	650	3.44	0.11	5.37 + 13.16
River Hull	Winter	Feb	RH38	8.9	8.35	NA	NA	400	1100	3.44	0.16	0.00 + 0.00
River Hull	Winter	Feb	RH39	9.2	8.39	NA	NA	400	1150	3.44	0.01	0.00 + 0.00
River Hull	Winter	Feb	RH40	9.5	8.52	NA	NA	380	700	3.44	0.13	0.00 + 0.00
River Hull	Spring	March	RH41	10.6	8.13	0.33	0.66	230	1450	2.32	0.45	0.00 + 0.00

River Hull	Spring	March	RH42	11.0	8.08	0.33	0.66	240	1350	2.32	0.09	6.20 + 11.02
River Hull	Spring	March	RH43	10.6	8.14	0.33	0.66	230	1300	2.32	0.19	2.66 + 6.10
River Hull	Spring	March	RH44	10.5	8.12	0.33	0.65	240	1350	2.32	0.26	0.00 + 0.00
River Hull	Spring	March	RH45	10.7	8.12	0.33	0.66	250	1400	2.32	0.01	5.72 + 7.03
River Hull	Spring	March	RH46	10.6	8.12	0.33	0.65	240	1350	2.32	0.29	9.22 + 14.57
River Hull	Spring	March	RH47	10.6	8.15	0.33	0.65	240	1450	2.32	0.15	11.52 + 13.20
River Hull	Spring	March	RH48	10.5	8.15	0.33	0.65	240	1650	2.32	0.07	3.82 + 8.19
River Hull	Spring	March	RH49	10.4	8.19	0.32	0.64	230	1600	2.32	0.06	7.35 + 11.39
River Hull	Spring	March	RH50	10.3	8.16	0.32	0.64	230	1500	2.32	0.13	4.27 + 7.26
River Hull	Spring	April	RH51	13.5	8.05	0.32	0.64	290	1700	1.92	0.45	0.12 + 0.29
River Hull	Spring	April	RH52	13.6	8.12	0.32	0.62	310	1600	1.92	0.14	6.13 + 15.00
River Hull	Spring	April	RH53	12.6	8.08	0.32	0.64	340	1500	1.92	0.20	14.90 + 23.15
River Hull	Spring	April	RH54	13.3	8.10	0.31	0.63	330	1250	1.92	0.25	18.04 + 17.60
River Hull	Spring	April	RH55	12.9	8.10	0.32	0.64	330	1400	1.92	0.13	4.42 + 10.83

River Hull	Spring	April	RH56	12.6	8.13	0.31	0.62	320	1450	1.92	0.48	20.45 + 18.88
River Hull	Spring	April	RH57	12.4	8.12	0.31	0.62	340	1400	1.92	0.20	19.46 + 15.94
River Hull	Spring	April	RH58	12.4	8.13	0.31	0.62	360	1400	1.92	0.14	600.11 + 105.03
River Hull	Spring	April	RH59	12.4	8.13	0.31	0.62	320	1650	1.92	0.05	34.84 + 33.83
River Hull	Spring	April	RH60	12.5	8.17	0.31	0.62	320	1650	1.92	0.14	18.83 + 20.91
River Hull	Spring	May	RH61	12.0	7.79	0.32	0.64	330	450	2.64	0.42	7.36 + 11.46
River Hull	Spring	May	RH62	12.1	7.78	0.32	0.64	350	500	2.64	0.11	18.23 + 23.55
River Hull	Spring	May	RH63	12.2	7.81	0.32	0.64	370	450	2.64	0.09	12.84 + 14.18
River Hull	Spring	May	RH64	12.7	7.88	0.32	0.64	380	450	2.64	0.32	8.84 + 13.70
River Hull	Spring	May	RH65	12.2	7.80	0.32	0.64	370	400	2.64	0.01	9.86 + 19.18
River Hull	Spring	May	RH66	12.2	7.80	0.32	0.64	360	450	2.64	0.23	4.44 + 10.88
River Hull	Spring	May	RH67	12.4	7.84	0.32	0.64	360	450	2.64	0.13	12.04 + 19.82
River Hull	Spring	May	RH68	12.4	7.82	0.32	0.64	370	550	2.64	0.06	36.59 + 18.47
River Hull	Spring	May	RH69	12.4	7.94	0.32	0.64	370	550	2.64	0.02	20.39 + 24.82

River Hull	Spring	May	RH70	12.3	7.90	0.32	0.64	360	550	2.64	0.11	9.95 + 11.19
River Hull	Summer	June	RH71	18.5	8.17	0.30	0.60	310	2000	2.23	0.53	7.88 + 7.38
River Hull	Summer	June	RH72	18.2	8.26	0.30	0.60	300	2000	2.23	0.15	2597.66 + 181.64
River Hull	Summer	June	RH73	17.6	8.27	0.30	0.59	310	1950	2.23	0.05	54.62 + 37.01
River Hull	Summer	June	RH74	17.6	8.26	0.29	0.59	310	1900	2.23	0.09	3413.06 + 359.80
River Hull	Summer	June	RH75	17.6	8.32	0.29	0.59	310	1950	2.23	0.03	27.24 + 23.22
River Hull	Summer	June	RH76	17.5	8.20	0.30	0.60	320	1800	2.23	0.18	11.75 + 18.39
River Hull	Summer	June	RH77	17.9	8.27	0.30	0.59	310	1600	2.23	0.09	42.01 + 17.34
River Hull	Summer	June	RH78	17.7	8.22	0.30	0.59	320	1900	2.23	0.07	3695.97 + 225.08
River Hull	Summer	June	RH79	17.7	8.23	0.30	0.59	320	1950	2.23	0.04	12.73 + 21.78
River Hull	Summer	June	RH80	17.6	8.29	0.29	0.59	330	1900	2.23	0.16	10.06 + 8.82
River Hull	Summer	July	RH81	20.0	8.31	0.28	0.56	340	1750	2.22	0.39	33725.90 + 1284.40
River Hull	Summer	July	RH82	20.3	8.30	0.28	0.56	350	2000	2.22	0.21	18231.03 + 256.90
River Hull	Summer	July	RH83	20.3	8.30	0.28	0.56	350	1950	2.22	0.12	30696.66 + 981.50

River Hull	Summer	July	RH84	20.4	8.24	0.28	0.57	350	1950	2.22	0.21	45522.33 + 1524.88
River Hull	Summer	July	RH85	20.2	8.31	0.28	0.56	340	2000	2.22	0.01	52981.03 + 1988.80
River Hull	Summer	July	RH86	20.0	8.31	0.28	0.56	340	1900	2.22	0.19	51959.28 + 3400.41
River Hull	Summer	July	RH87	19.8	8.30	0.28	0.57	350	1850	2.22	0.12	55960.65 + 1510.23
River Hull	Summer	July	RH88	20.5	8.22	0.28	0.57	360	1950	2.22	0.10	49404.77 + 1785.12
River Hull	Summer	July	RH89	20.6	8.27	0.29	0.58	360	1900	2.22	0.09	40604.50 + 3453.50
River Hull	Summer	July	RH90	20.4	8.32	0.29	0.58	370	1900	2.22	0.13	32382.00 + 1669.54
River Hull	Summer	Aug	RH91	17.5	8.01	0.31	0.62	100	1900	2.00	0.46	16160.47 + 1035.61
River Hull	Summer	Aug	RH92	17.7	7.92	0.31	0.62	110	1950	2.00	0.11	5967.34 + 453.04
River Hull	Summer	Aug	RH93	17.7	7.87	0.31	0.62	110	1900	2.00	0.29	33329.84 + 1678.36
River Hull	Summer	Aug	RH94	17.6	7.91	0.31	0.62	110	1850	2.00	0.38	9272.59 + 732.90
River Hull	Summer	Aug	RH95	17.8	7.88	0.31	0.62	110	1900	2.00	0.13	15613.58 + 560.88
River Hull	Summer	Aug	RH96	17.9	7.95	0.31	0.62	110	1900	2.00	0.57	11507.85 + 974.06
River Hull	Summer	Aug	RH97	17.7	7.89	0.31	0.62	110	1900	2.00	0.17	11408.11 + 702.74

River Hull	Summer	Aug	RH98	17.6	7.91	0.31	0.62	110	1950	2.00	0.26	6240.12 + 503.34
River Hull	Summer	Aug	RH99	17.5	7.96	0.31	0.62	110	1950	2.00	0.07	4321.20 + 504.22
River Hull	Summer	Aug	RH100	17.4	7.93	0.31	0.62	110	1950	2.00	0.24	4448.58 + 134.62
River Hull	Autumn	Sept	RH101	16.9	8.04	0.33	0.66	270	1900	1.84	0.42	27.49 + 15.72
River Hull	Autumn	Sept	RH102	17.0	8.05	0.32	0.64	280	1950	1.84	0.10	2664.73 + 127.17
River Hull	Autumn	Sept	RH103	16.8	7.94	0.32	0.64	270	1950	1.84	0.14	1184.91 + 93.16
River Hull	Autumn	Sept	RH104	16.9	7.97	0.32	0.64	280	1900	1.84	0.34	39.13 + 22.10
River Hull	Autumn	Sept	RH105	16.7	8.00	0.32	0.64	280	1950	1.84	0.02	40.84 + 27.49
River Hull	Autumn	Sept	RH106	16.9	8.04	0.32	0.64	270	1950	1.84	0.26	7.33 + 13.38
River Hull	Autumn	Sept	RH107	16.5	7.99	0.32	0.64	270	1950	1.84	0.09	2208.29 + 210.38
River Hull	Autumn	Sept	RH108	16.6	8.08	0.32	0.63	280	2000	1.84	0.06	3492.79 + 132.62
River Hull	Autumn	Sept	RH109	16.5	8.06	0.32	0.63	270	2000	1.84	0.05	2586.91 + 315.89
River Hull	Autumn	Sept	RH110	16.4	8.09	0.32	0.64	280	2000	1.84	0.19	31.46 + 25.78
River Hull	Autumn	Oct	RH111	11.9	7.83	0.35	0.70	300	1700	1.72	0.54	84.80 + 67.78

River Hull	Autumn	Oct	RH112	11.9	7.85	0.35	0.69	300	1700	1.72	0.06	147.62 + 64.65
River Hull	Autumn	Oct	RH113	11.7	7.92	0.35	0.69	310	1700	1.72	0.08	104.07 + 45.94
River Hull	Autumn	Oct	RH114	11.7	7.99	0.34	0.69	300	1550	1.72	0.12	92.15 + 24.31
River Hull	Autumn	Oct	RH115	11.6	8.05	0.34	0.68	300	1800	1.72	0.01	119.36 + 46.79
River Hull	Autumn	Oct	RH116	11.6	8.03	0.34	0.67	300	1850	1.72	0.21	85.59 + 39.12
River Hull	Autumn	Oct	RH117	11.4	7.98	0.33	0.67	300	2000	1.72	0.02	149.21 + 45.17
River Hull	Autumn	Oct	RH118	11.3	8.01	0.33	0.66	310	2000	1.72	0.13	134.18 + 72.47
River Hull	Autumn	Oct	RH119	11.1	8.06	0.33	0.67	310	1900	1.72	0.07	147.27 + 42.50
River Hull	Autumn	Oct	RH120	11.4	8.03	0.33	0.66	310	2000	1.72	0.19	68.39 + 33.12
River Hull	Autumn	Nov	RH121	6.9	7.88	0.35	0.70	370	1700	1.92	0.43	151.95 + 48.12
River Hull	Autumn	Nov	RH122	6.9	7.77	0.35	0.70	380	1650	1.92	0.14	184.12 + 55.23
River Hull	Autumn	Nov	RH123	6.9	7.78	0.35	0.70	380	1500	1.92	0.14	73.93 + 21.64
River Hull	Autumn	Nov	RH124	7.0	7.86	0.35	0.70	380	1500	1.92	0.17	123.61 + 93.27
River Hull	Autumn	Nov	RH125	6.6	7.68	0.35	0.70	380	1550	1.92	0.02	54.56 + 66.28

River Hull	Autumn	Nov	RH126	6.6	7.73	0.35	0.69	370	1750	1.92	0.17	153.37 + 38.67
River Hull	Autumn	Nov	RH127	6.7	7.77	0.34	0.69	380	1950	1.92	0.02	163.60 + 30.56
River Hull	Autumn	Nov	RH128	6.7	7.82	0.34	0.68	380	1900	1.92	0.08	163.90 + 33.60
River Hull	Autumn	Nov	RH129	6.6	7.78	0.34	0.67	360	1900	1.92	0.05	206.84 + 53.55
River Hull	Autumn	Nov	RH130	6.7	7.77	0.33	0.67	370	1950	1.92	0.19	128.19 + 25.60

Table S1.4 Number of positive qPCR replicates (out of 6) for each of the ten samples collected each month at each site, and total number of positive replicates (n = 60) and samples (n = 10) for each month and each site.

Season	Month	Samples (1-10)										Total replicates	Total samples
Eccup Reservoir													
Winter	Dec	6	6	6	6	6	6	6	6	6	6	60	10
	Jan	6	6	6	6	6	6	6	6	6	6	60	10
	Feb	6	6	6	6	6	6	6	6	6	6	60	10
Spring	March	6	6	6	6	6	6	6	6	6	6	60	10
	April	6	5	6	6	6	6	6	6	6	6	59	10
	May	6	6	6	6	6	6	6	6	6	6	60	10

Summer	June	6	6	6	6	6	6	6	6	6	6	60	10
	July	6	6	6	6	6	6	6	6	6	6	60	10
	Aug	6	6	6	6	6	6	6	6	6	6	60	10
Autumn	Sept	6	6	6	6	6	6	6	6	6	6	60	10
	Oct	6	6	6	6	6	6	6	6	6	6	60	10
	Nov	6	6	6	6	6	6	6	6	6	6	60	10
River Hull													
Winter	Dec	4	4	2	6	1	4	4	4	4	4	37	10
	Jan	0	0	1	0	0	0	0	0	0	1	2	2
	Feb	0	3	1	1	0	0	1	0	0	0	6	4
Spring	March	0	2	2	0	3	2	3	2	2	2	18	8
	April	1	1	2	4	1	4	4	6	4	3	30	10
	May	2	3	3	2	2	1	2	6	3	3	27	10
Summer	June	4	6	6	6	5	3	6	6	2	4	48	10
	July	6	6	6	6	6	6	6	6	6	6	60	10
	Aug	6	6	6	6	6	6	6	6	6	6	60	10
Autumn	Sept	5	6	6	5	5	2	6	6	6	5	52	10
	Oct	4	6	6	6	6	6	6	6	6	6	58	10
	Nov	6	6	6	5	3	6	6	6	6	6	56	10

Appendix 2: Additional information for chapter 3

DNA extraction protocol: DNeasy Blood & Tissue kit (Qiagen)

1. Carefully wipe the outer surfaces of all filter units with 10% bleach and 70% ethanol and let them defrost.

2. Remove the plunger from a 3 mL luer lock syringe, as well as the top cap from the filter, and attach the syringe to the inlet of the filter.

3. Add 130 µL of 2 mg/mL of proteinase K to each filter. Make sure that the liquid is placed just above the filter.

4. Place the plunger back on the syringe and push the proteinase K into the filter. Carefully unscrew the syringe and cap the filter.

5. Incubate the capped filters at 56°C overnight.

6. Using a new 3 mL syringe expel the lysate into a 5 mL low retention microcentrifuge tube.

7. Add equal amounts of AL buffer to lysate in each tube, then vortex and incubate at 56°C for 30 minutes.

8. Add equal amounts of cold 100% ethanol as lysate to each tube and incubate for 10 minutes at room temperature.

9. For each sample transfer 600 µL of lysate to a spin column and centrifuge for 30 seconds at 6,000 x g. Discard flow-through and collection tube.

10. Repeat step 9 until all lysate is used. On the final spin centrifuge for 2 minutes.

11. Transfer spin column to new collection tube and add 500 µL of AW1 to spin column. Centrifuge for 1 minutes at 6,000 x g. Discard flow-through and collection tube.

12. Transfer spin column to new collection tube and add 500 µL of AW2 to spin column. Centrifuge for 3 minutes at 20,000 x g. Discard flow-through and collection tube.

13. Transfer spin column to a 1.5 low retention microcentrifuge tube, add 50 µL of AE buffer and incubate for 1 minute at room temperature. Centrifuge for 1 minute at 6,000 x g.

14. Add another 50 µL of AE buffer to the same tube and incubate for 1 minute at room temperature. Centrifuge for 1 minute at 6,000 x g.

Gingera et al. (2017) assay: repeatability test

To ensure repeatability of the assay, despite the low efficiency, a subset of 12 samples were run twice, separately. This included three samples from each of the four site types (canals, lakes, reservoirs, rivers), and samples amplifying at a range of different DNA concentrations, i.e., amplifying at high and low cycles. PCR volumes and thermal cycler conditions are the same as described in chapter 2.

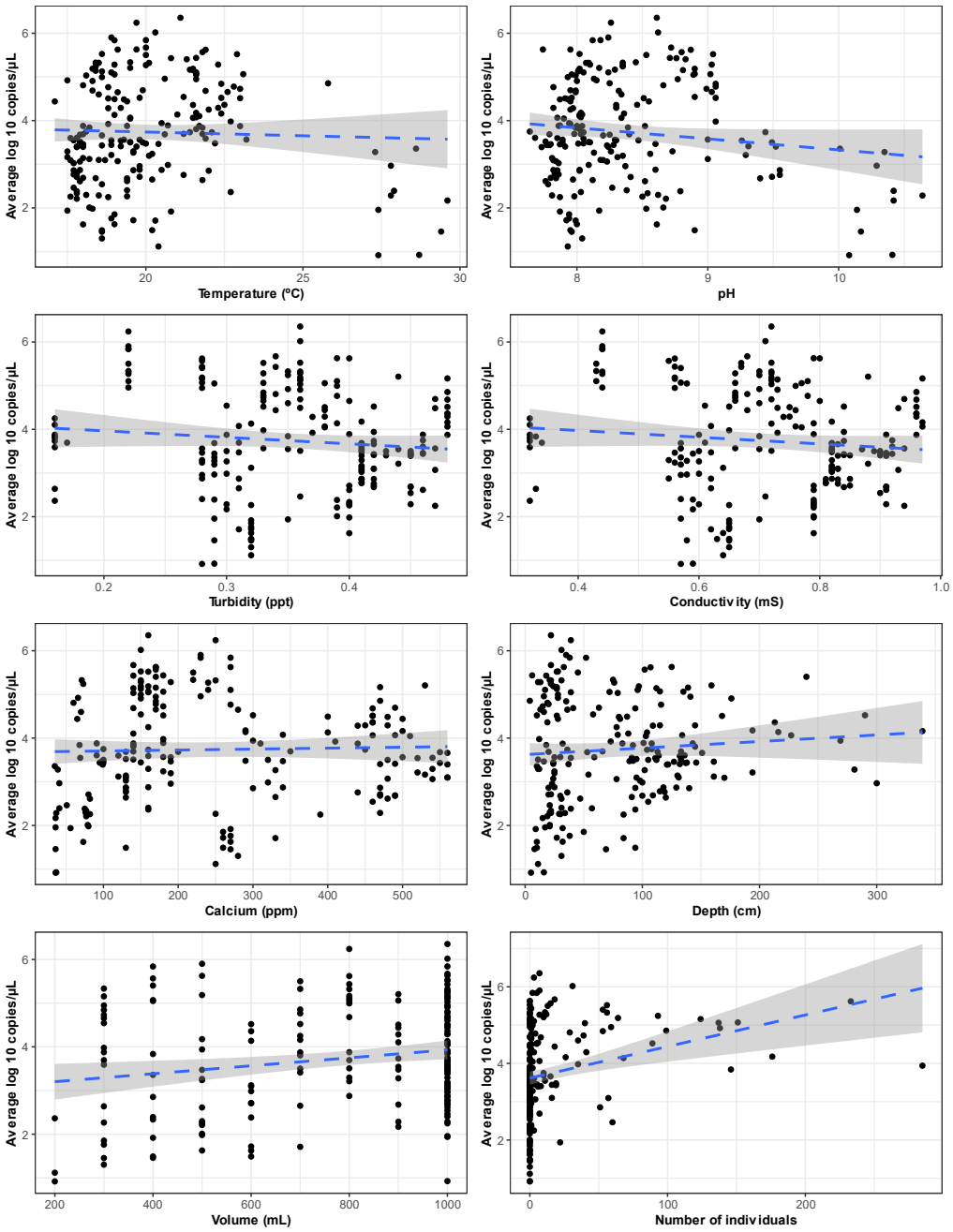

Figure S2.1 Scatterplots showing the effect of the several environmental variables on average log 10 DNA copies/µL. The dashed blue line corresponds to the linear regression line and grey shading represents the confidence intervals. For visual purposes log 10 DNA copies/µL is being used instead of non-transformed data.

Table S2.1 Sample information and field metadata for all samples collected for the third chapter, specifically volume filtered, temperature (temp.), pH, turbidity (turb.) conductivity (cond.), calcium (calc.), substrate (subs.), depth and number of individuals (ind.) Information on inhibition (inhib; the difference between the average Cq values of each sample and the no template reaction) and average DNA copies/µL plus standard deviation (SD) is also provided.

Type	Site	Sample code	Latitude Longitude	Volume (mL)	Temp. (°C)	pH	Turb. (ppt)	Cond. (mS)	Calc. (ppm)	Subs.	Depth (cm)	Ind.	Inhib.	Average DNA copies/µL + SD
lake	Farnham Lake	FL1	54.03504 -1.46743	300	21.2	8.90	0.30	0.61	150	B	58	6	0.05	34844.21 + 1314.12
lake	Farnham Lake	FL2	54.03483 -1.46713	400	21.6	8.89	0.29	0.58	160	B	39	40	0.09	112111.85 + 3966.70
lake	Farnham Lake	FL3	54.03484 -1.46671	400	21.6	8.90	0.28	0.57	160	SA	115	151	0.08	117371.04 + 3557.15
lake	Farnham Lake	FL4	54.03482 -1.46645	500	21.5	8.90	0.28	0.56	160	S	97	64	0.07	153405.29 + 6463.58
lake	Farnham Lake	FL5	54.03502 -1.46625	400	21.3	8.83	0.28	0.57	170	S	240	53	0.07	253328.98 + 7384.16
lake	Farnham Lake	FL6	54.03520 -1.46626	400	21.8	8.80	0.28	0.55	170	SA	102	15	0.06	369586.46 + 11643.30
lake	Farnham Lake	FL7	54.03538 -1.46611	800	21.6	8.71	0.28	0.56	180	G	6	1	0.05	272484.83 + 12666.98
lake	Farnham Lake	FL8	54.03543 -1.46580	300	21.6	8.77	0.28	0.56	170	S	141	59	0.09	88688.37 + 3917.24

lake	Farnham Lake	FL9	54.03530 -1.46561	300	21.5	8.81	0.28	0.56	170	SA	139	124	0.07	143019.41 + 9764.00
lake	Farnham Lake	FL10	54.03509 -1.46541	800	21.9	8.87	0.28	0.56	170	S	107	233	0.04	417622.52 + 12832.27
lake	Eight Acre Lake	AL1	53.76936 -0.65701	1000	21.5	8.34	0.48	0.97	460	S	227	7	0.16	11567.20 + 227.97
lake	Eight Acre Lake	AL2	53.76968 -0.65715	1000	23.0	8.51	0.48	0.96	460	G	27	5	0.11	32572.90 + 1047.46
lake	Eight Acre Lake	AL3	53.76977 -0.65747	1000	22.5	8.46	0.48	0.96	460	G	19	1	0.01	22946.45 + 859.81
lake	Eight Acre Lake	AL4	53.76976 -0.65793	700	23.0	8.52	0.48	0.96	440	S	32	1	0.08	7475.27 + 188.22
lake	Eight Acre Lake	AL5	53.76969 -0.65902	700	22.4	8.36	0.48	0.97	500	G	339	26	0.00	14483.44 + 695.70
lake	Eight Acre Lake	AL6	53.76960 -0.65970	700	25.8	8.25	0.48	0.96	470	G	4	99	0.00	71543.09 + 1311.36
lake	Eight Acre Lake	AL7	53.76927 -0.65961	1000	22.3	8.30	0.48	0.96	450	G	115	41	0.01	19602.37 + 509.89
lake	Eight Acre Lake	AL8	53.76896 -0.65952	800	21.9	8.32	0.48	0.96	460	G	94	8	0.01	47890.66 + 1628.07
lake	Eight Acre Lake	AL9	53.76867 -0.65931	600	21.5	8.30	0.48	0.96	460	S	213	1	0.06	22800.85 + 1537.18

lake	Eight Acre Lake	AL10	53.76856 -0.65920	800	21.4	8.29	0.48	0.97	470	S	27	1	0.06	146614.66 + 7438.76
canal	Leeds & Liverpool Canal	LL1	53.79679 -1.56658	300	21.8	8.12	0.16	0.33	130	S	120	0	0.01	434.48 + 78.46
canal	Leeds & Liverpool Canal	LL2	53.79749 -1.56754	300	21.8	8.03	0.17	0.34	140	S	133	0	0.11	4951.11 + 302.68
canal	Leeds & Liverpool Canal	LL3	53.79822 -1.56843	400	21.8	7.99	0.16	0.33	140	S	92	0	0.15	6847.54 + 325.43
canal	Leeds & Liverpool Canal	LL4	53.79895 -1.56932	300	21.7	8.00	0.16	0.32	140	S	108	0	0.10	7529.07 + 614.35
canal	Leeds & Liverpool Canal	LL5	53.79955 -1.57052	700	21.6	8.02	0.16	0.32	140	S	145	0	0.05	6372.56 + 532.55
canal	Leeds & Liverpool Canal	LL6	53.80013 -1.57164	900	21.7	8.02	0.16	0.32	140	S	88	0	0.00	12692.41 + 385.04
canal	Leeds & Liverpool Canal	LL7	53.80062 -1.57290	700	22.0	7.95	0.16	0.32	160	S	78	0	0.03	17810.32 + 591.50
canal	Leeds & Liverpool Canal	LL8	53.80111 -1.57414	300	21.9	8.05	0.16	0.32	160	S	132	0	0.10	3896.43 + 217.28
canal	Leeds & Liverpool Canal	LL9	53.80161 -1.57541	900	22.1	8.05	0.16	0.32	160	S	104	0	0.04	5404.81 + 233.54
canal	Leeds & Liverpool Canal	LL10	53.80192 -1.57680	200	22.7	8.03	0.16	0.32	160	S	94	1	0.02	231.08 + 47.44

lake	Pugneys Country park	PC1	53.65976 -1.49995	200	27.4	10.08	0.28	0.57	36	SA	5	0	0.01	8.29 + 12.87
lake	Pugneys Country park	PC2	53.65950 -1.50165	1000	27.4	10.14	0.29	0.57	36	SA	17	0	0.02	90.35 + 24.96
lake	Pugneys Country park	PC3	53.65886 -1.50352	400	28.6	10.01	0.29	0.57	35	SA	10	0	0.08	2278.08 + 180.52
lake	Pugneys Country park	PC4	53.65831 -1.50566	400	29.4	10.17	0.29	0.58	36	SA	8	0	0.06	28.73 + 19.98
lake	Pugneys Country park	PC5	53.65723 -1.50814	900	29.6	10.42	0.30	0.59	36	B	15	0	0.11	147.57 + 61.68
lake	Pugneys Country park	PC6	53.65725 -1.50913	1000	28.7	10.41	0.29	0.59	37	B	16	0	0.03	8.42 + 13.17
lake	Pugneys Country park	PC7	53.65715 -1.50978	400	27.9	10.42	0.29	0.59	41	B	57	1	0.05	246.65 + 77.78
lake	Pugneys Country park	PC8	53.65691 -1.50986	1000	27.8	10.29	0.29	0.58	41	S	300	0	0.08	924.52 + 97.35
lake	Pugneys Country park	PC9	53.65672 -1.51031	900	27.3	10.35	0.29	0.58	39	S	281	2	0.16	1902.49 + 126.84
lake	Pugneys Country park	PC10	53.65662 -1.51107	900	27.8	10.64	0.30	0.60	37	S	34	0	0.04	192.17 + 41.68
reservoir	Ulley Reservoir	UR1	53.38432 -1.31950	1000	22.9	9.04	0.33	0.67	160	B	27	56	0.10	331258.29 + 8045.43

reservoir	Ulley Reservoir	UR2	53.38379 -1.32019	300	22.4	9.06	0.33	0.66	170	B	35	53	0.16	69568.09 + 2835.75
reservoir	Ulley Reservoir	UR3	53.38346 -1.32059	1000	22.8	9.06	0.33	0.66	170	B	20	4	0.16	60032.44 + 3042.83
reservoir	Ulley Reservoir	UR4	53.38314 -1.32086	300	22.6	9.03	0.33	0.66	170	S	13	1	0.04	45196.66 + 1691.96
reservoir	Ulley Reservoir	UR5	53.38302 -1.32046	900	23.1	9.02	0.33	0.66	180	S	135	137	0.17	115316.67 + 3820.58
reservoir	Ulley Reservoir	UR6	53.38284 -1.31968	1000	22.3	9.03	0.33	0.66	190	S	11	12	0.03	191380.69 + 4851.79
reservoir	Ulley Reservoir	UR7	53.38285 -1.31911	1000	23.2	9.00	0.33	0.66	180	B	30	0	0.12	3687.68 + 266.81
reservoir	Ulley Reservoir	UR8	53.38284 -1.31843	300	23.0	8.99	0.33	0.66	180	B	15	39	0.11	53410.58 + 1790.82
reservoir	Ulley Reservoir	UR9	53.38241 -1.31737	300	22.7	9.06	0.33	0.65	180	S	12	35	0.10	9494.50 + 978.74
reservoir	Ulley Reservoir	UR10	53.38200 -1.31714	600	22.4	9.06	0.33	0.65	180	S	10	5	0.00	33100.43 + 1304.40
reservoir	Staunton Harold Reservoir	SH1	52.81516 -1.44220	1000	18.9	8.28	0.29	0.59	150	S	24	1	0.01	2777.94 + 163.75
reservoir	Staunton Harold Reservoir	SH2	52.81477 -1.44163	1000	19.4	8.38	0.28	0.57	160	S	31	0	0.15	253.62 + 45.64

reservoir	Staunton Harold Reservoir	SH3	52.81238 -1.44332	800	19.6	8.73	0.28	0.55	160	S	11	0	0.18	1973.57 + 125.51
reservoir	Staunton Harold Reservoir	SH4	52.81200 -1.44404	800	19.8	8.60	0.28	0.55	160	S	25	0	0.13	750.39 + 120.07
reservoir	Staunton Harold Reservoir	SH5	52.81160 -1.44450	1000	19.8	8.37	0.28	0.57	190	S	23	1	0.12	904.37 + 119.50
reservoir	Staunton Harold Reservoir	SH6	52.81113 -1.44519	1000	19.8	8.29	0.28	0.57	190	S	25	0	0.13	3647.66 + 151.12
reservoir	Staunton Harold Reservoir	SH7	52.81089 -1.44574	500	20.2	8.56	0.28	0.56	180	S	25	0	0.16	1730.96 + 82.48
reservoir	Staunton Harold Reservoir	SH8	52.81072 -1.44655	1000	20.4	8.60	0.28	0.56	190	S	16	0	0.09	2920.01 + 162.21
reservoir	Staunton Harold Reservoir	SH9	52.81052 -1.44719	1000	20.6	8.40	0.29	0.57	200	S	20	10	0.19	4848.16 + 714.82
reservoir	Staunton Harold Reservoir	SH10	52.81049 -1.44797	1000	20.1	8.35	0.29	0.57	190	S	25	0	0.08	1566.23 + 173.61
canal	Gloucester & Sharpness Canal	GS1	51.85453 -2.25864	500	19.1	7.95	0.40	0.80	270	S	33	0	0.01	422847.83 + 58689.75
canal	Gloucester & Sharpness Canal	GS2	51.85487 -2.25848	1000	18.9	8.05	0.39	0.78	270	S	96	3	0.07	126839.53 + 5585.42
canal	Gloucester & Sharpness Canal	GS3	51.85537 -2.25820	700	18.8	7.82	0.39	0.78	270	S	116	1	0.08	58308.97 + 1313.77

canal	Gloucester & Sharpness Canal	GS4	51.85599 -2.25807	1000	19.1	7.99	0.40	0.80	280	S	36	1	0.15	44670.16 + 824.75
canal	Gloucester & Sharpness Canal	GS5	51.85648 -2.25893	700	19.8	7.96	0.42	0.84	300	S	290	89	0.17	33284.12 + 729.95
canal	Gloucester & Sharpness Canal	GS6	51.85691 -2.25866	500	20.5	7.94	0.42	0.84	300	S	269	285	0.03	8717.42 + 412.51
canal	Gloucester & Sharpness Canal	GS7	51.85710 -2.25763	600	21.0	8.04	0.39	0.79	290	S	216	68	0.03	13754.82 + 643.35
canal	Gloucester & Sharpness Canal	GS8	51.85785 -2.25691	500	21.6	8.03	0.41	0.82	290	S	194	176	0.09	15014.98 + 669.80
canal	Gloucester & Sharpness Canal	GS9	51.85838 -2.25637	900	22.2	8.05	0.41	0.82	290	S	92	19	0.15	3017.43 + 199.45
canal	Gloucester & Sharpness Canal	GS10	51.85935 -2.25580	400	22.0	8.02	0.41	0.83	300	S	140	51	0.16	712.48 + 48.41
river	River Severn	RS1	52.05942 -2.20139	300	19.4	7.98	0.32	0.65	250	S	29	0	0.07	184.45 + 61.18
river	River Severn	RS2	52.06028 -2.20206	200	20.4	7.93	0.32	0.64	250	S	11	0	0.14	13.11 + 18.68
river	River Severn	RS3	52.06083 -2.20281	400	20.8	7.87	0.32	0.65	270	S	9	0	0.05	82.69 + 37.25
river	River Severn	RS4	52.06119 -2.20331	400	20.2	7.95	0.32	0.65	260	S	10	0	0.08	30.90 + 20.56

river	River Severn	RS5	52.06211 -2.20458	600	19.6	7.92	0.32	0.65	260	S	27	0	0.07	52.27 + 38.05
river	River Severn	RS6	52.06239 -2.20567	500	19.0	8.00	0.32	0.64	270	S	32	0	0.05	42.33 + 54.57
river	River Severn	RS7	52.06281 -2.20697	300	19.0	7.94	0.32	0.65	260	S	50	0	0.05	71.14 + 34.67
river	River Severn	RS8	52.06297 -2.20794	300	18.9	7.94	0.32	0.65	270	S	38	0	0.07	57.67 + 32.35
river	River Severn	RS9	52.06311 -2.20892	300	18.6	8.04	0.32	0.65	280	S	31	0	0.02	20.05 + 24.86
river	River Severn	RS10	52.06333 -2.21050	300	18.6	7.96	0.32	0.65	270	S	69	0	0.02	28.42 + 15.06
reservoir	Chasewater Reservoir	CR1	52.66789 -1.95655	500	18.9	8.24	0.22	0.44	230	G	35	7	0.11	799461.57 + 12408.98
reservoir	Chasewater Reservoir	CR2	52.66747 -1.95619	400	19.0	8.12	0.22	0.44	230	G	52	5	0.16	691471.49 + 29662.55
reservoir	Chasewater Reservoir	CR3	52.66639 -1.95553	300	19.5	8.45	0.22	0.43	220	G	75	11	0.26	216278.05 + 7711.62
reservoir	Chasewater Reservoir	CR4	52.66539 -1.95519	700	20.0	8.53	0.22	0.43	220	B	45	13	0.18	317580.89 + 11705.80
reservoir	Chasewater Reservoir	CR5	52.66400 -1.95319	1000	20.0	8.35	0.22	0.44	240	B	76	7	0.10	186299.95 + 6571.60

reservoir	Chasewater Reservoir	CR6	52.66189 -1.95089	1000	20.6	8.13	0.22	0.44	230	SA	16	0	0.12	90637.30 + 2700.28
reservoir	Chasewater Reservoir	CR7	52.66228 -1.94950	800	21.6	8.24	0.22	0.43	240	SA	17	2	1.07	127264.59 + 14556.91
reservoir	Chasewater Reservoir	CR8	52.66221 -1.94797	800	20.1	8.07	0.22	0.44	250	G	22	0	0.08	209549.13 + 8031.37
reservoir	Chasewater Reservoir	CR9	52.66211 -1.94669	800	19.7	8.26	0.22	0.44	250	B	39	3	0.18	1744056.66 + 108304.03
reservoir	Chasewater Reservoir	CR10	52.66200 -1.94425	1000	20.0	8.18	0.22	0.44	270	B	38	2	0.19	694862.55 + 18902.14
canal	Worcester & Birmingham Canal	WB1	52.30253 -2.06686	500	19.6	8.13	0.30	0.60	330	S	102	0	0.15	1843.89 + 150.37
canal	Worcester & Birmingham Canal	WB2	52.30289 -2.06550	1000	19.4	8.04	0.30	0.60	310	S	83	0	0.12	7506.24 + 451.92
canal	Worcester & Birmingham Canal	WB3	52.30328 -2.06408	700	19.5	8.10	0.30	0.60	320	S	105	0	0.10	3173.20 + 258.20
canal	Worcester & Birmingham Canal	WB4	52.30358 -2.06261	600	20.7	8.09	0.30	0.60	320	S	100	0	0.12	964.77 + 164.62

canal	Worcester & Birmingham Canal	WB5	52.30415 -2.06139	500	20.0	8.01	0.31	0.62	340	S	89	0	0.02	2969.43 + 137.19
canal	Worcester & Birmingham Canal	WB6	52.30489 -2.06036	1000	20.5	8.02	0.31	0.62	340	S	118	0	0.01	742.35 + 66.39
canal	Worcester & Birmingham Canal	WB7	52.30561 -2.05919	800	21.2	7.98	0.31	0.61	350	S	66	0	0.12	4965.81 + 322.58
canal	Worcester & Birmingham Canal	WB8	52.30642 -2.05858	1000	21.6	8.03	0.31	0.62	340	S	79	0	0.03	11908.82 + 461.82
canal	Worcester & Birmingham Canal	WB9	52.30731 -2.05831	700	20.2	7.97	0.31	0.62	330	S	91	0	0.10	447.53 + 64.41
canal	Worcester & Birmingham Canal	WB10	52.30817 -2.05800	700	20.3	7.99	0.31	0.62	330	S	84	0	0.05	51.31 + 56.33
canal	Grand Union Canal	GU1	52.78369 -1.21750	1000	19.0	8.02	0.32	0.72	400	G	104	0	0.03	13487.68 + 653.54
canal	Grand Union Canal	GU2	52.78289 -1.21717	1000	19.0	7.93	0.36	0.73	400	G	87	0	0.12	31015.08 + 541.52

canal	Grand Union Canal	GU3	52.78209 -1.21677	1000	18.8	7.95	0.37	0.73	410	G	73	0	0.07	8325.99 + 482.71
canal	Grand Union Canal	GU4	52.78127 -1.21653	900	18.8	7.97	0.37	0.74	440	G	109	0	0.08	19239.07 + 517.50
canal	Grand Union Canal	GU5	52.78014 -1.21613	900	18.8	7.91	0.38	0.75	460	G	168	1	0.12	32213.07 + 1340.63
canal	Grand Union Canal	GU6	52.77911 -1.21572	1000	19.2	7.82	0.38	0.75	480	S	145	3	0.02	19544.20 + 201.80
canal	Grand Union Canal	GU7	52.77820 -1.21539	1000	19.4	7.95	0.38	0.76	500	S	81	0	0.12	27545.99 + 663.96
canal	Grand Union Canal	GU8	52.77744 -1.21414	1000	19.5	7.85	0.38	0.76	490	S	134	1	0.09	11646.36 + 210.90
canal	Grand Union Canal	GU9	52.77683 -1.21322	1000	19.5	7.90	0.38	0.77	510	S	103	2	0.05	11125.98 + 416.94
canal	Grand Union Canal	GU10	52.77469 -1.21067	900	19.5	8.17	0.44	0.88	530	S	159	10	0.29	160845.18 + 4126.82
lake	Holme Pierrepont	HP1	52.95000 -1.07361	1000	21.4	9.44	0.42	0.84	450	SA	36	0	0.06	5431.48 + 567.01
lake	Holme Pierrepont	HP2	52.95056 -1.07103	1000	21.2	9.55	0.42	0.83	440	B	36	0	0.15	570.21 + 64.03
lake	Holme Pierrepont	HP3	52.95403 -1.06986	1000	19.9	9.49	0.43	0.89	480	B	112	1	0.49	3166.01 + 159.93

lake	Holme Pierrepont	HP4	52.95161 -1.07667	1000	19.7	9.52	0.43	0.85	470	SA	37	0	0.18	2536.00 + 92.10
lake	Holme Pierrepont	HP5	52.94931 -1.08008	1000	19.6	9.55	0.42	0.85	470	SA	20	0	0.15	731.30 + 79.90
lake	Holme Pierrepont	HP6	52.94836 -1.08194	600	19.8	9.49	0.42	0.85	470	SA	22	0	0.12	516.41 + 17.62
lake	Holme Pierrepont	HP7	52.94692 -1.08483	900	19.4	9.40	0.42	0.84	490	SA	30	0	0.05	475.52 + 78.26
lake	Holme Pierrepont	HP8	52.94547 -1.08767	700	19.2	9.31	0.42	0.84	490	SA	24	0	0.05	2585.28 + 321.79
lake	Holme Pierrepont	HP9	52.94203 -1.09361	900	19.2	9.26	0.44	0.87	520	SA	111	0	0.06	3485.76 + 220.17
lake	Holme Pierrepont	HP10	52.94089 -1.09475	800	18.9	9.29	0.44	0.88	520	SA	194	0	0.01	1633.28 + 133.93
river	River Weaver	RW1	53.22497 -2.53517	1000	18.8	7.87	0.47	0.94	390	S	84	3	0.09	177.39 + 43.96
river	River Weaver	RW2	53.22422 -2.53411	1000	18.6	7.78	0.45	0.90	460	S	105	0	0.11	348.48 + 92.96
river	River Weaver	RW3	53.22358 -2.53294	1000	18.4	7.84	0.45	0.91	470	S	100	0	0.09	479.97 + 69.56
river	River Weaver	RW4	53.22292 -2.53217	1000	18.0	7.80	0.45	0.91	490	S	109	7	0.12	488.46 + 24.21

river	River Weaver	RW5	53.22211 -2.53136	1000	18.5	7.81	0.45	0.91	470	S	22	0	0.02	193.17 + 70.47
river	River Weaver	RW6	53.22017 -2.52961	1000	19.4	7.76	0.46	0.91	480	S	47	0	0.07	411.40 + 42.16
river	River Weaver	RW7	53.21942 -2.52886	1000	19.1	7.84	0.47	0.93	480	S	24	0	0.03	1176.65 + 154.93
river	River Weaver	RW8	53.21822 -2.52766	1000	19.3	7.89	0.47	0.94	500	S	59	0	0.09	3652.33 + 180.40
river	River Weaver	RW9	53.21733 -2.52689	1000	19.3	7.87	0.47	0.93	480	S	80	1	0.02	30538.68 + 1927.23
river	River Weaver	RW10	53.21650 -2.52606	1000	19.9	7.95	0.47	0.94	490	S	63	0	0.04	49548.06 + 1739.22
canal	Shropshire Union Canal	SU1	52.98706 -2.50958	1000	17.5	8.26	0.41	0.82	540	S	12	1	0.12	1966.76 + 128.53
canal	Shropshire Union Canal	SU2	52.98603 -2.50942	1000	17.5	8.31	0.41	0.82	530	S	54	0	0.08	1457.76 + 79.52
canal	Shropshire Union Canal	SU3	52.98483 -2.50897	1000	17.9	8.25	0.41	0.82	550	S	53	0	0.01	4762.06 + 266.11
canal	Shropshire Union Canal	SU4	52.98378 -2.50903	1000	17.7	8.30	0.41	0.82	540	S	91	1	0.26	3547.88 + 170.84
canal	Shropshire Union Canal	SU5	52.98283 -2.50914	1000	17.9	8.23	0.41	0.82	540	S	78	4	0.02	1151.27 + 46.69

canal	Shropshire Union Canal	SU6	52.98172 -2.50922	1000	18.0	8.24	0.41	0.83	560	S	122	0	0.03	2495.49 + 301.44
canal	Shropshire Union Canal	SU7	52.98064 -2.50919	1000	18.5	8.18	0.42	0.83	560	S	131	16	0.06	1257.53 + 89.09
canal	Shropshire Union Canal	SU8	52.97950 -2.50936	1000	18.6	8.20	0.42	0.83	560	S	151	15	0.16	4580.03 + 119.69
canal	Shropshire Union Canal	SU9	52.97831 -2.50925	1000	18.6	8.23	0.42	0.84	550	S	137	19	0.05	2696.44 + 229.45
canal	Shropshire Union Canal	SU10	52.97719 -2.50939	600	18.7	8.20	0.42	0.83	560	S	95	57	0.32	1246.56 + 56.35
reservoir	Rutland Water	RU1	52.64375 -0.65544	1000	17.7	8.27	0.36	0.71	51	B	21	60	0.08	290.02 + 64.59
reservoir	Rutland Water	RU2	52.64328 -0.65222	1000	17.5	8.17	0.35	0.70	56	B	32	22	0.28	86.76 + 55.50
reservoir	Rutland Water	RU3	52.64211 -0.64867	1000	17.5	8.15	0.35	0.70	66	G	28	138	0.13	83390.42 + 2864.57
reservoir	Rutland Water	RU4	52.64038 -0.64875	1000	18.0	8.66	0.34	0.68	60	S	19	29	0.15	64299.37 + 3409.94
reservoir	Rutland Water	RU5	52.63992 -0.64544	900	17.1	8.55	0.34	0.68	65	B	28	18	0.11	27443.73 + 1044.52
reservoir	Rutland Water	RU6	52.63925 -0.63717	700	18.4	8.35	0.35	0.70	71	S	21	56	0.08	211957.81 + 11924.31

reservoir	Rutland Water	RU7	52.64111 -0.63328	1000	18.4	8.35	0.35	0.70	73	B	22	93	0.09	174347.41 + 5399.10
reservoir	Rutland Water	RU8	52.64161 -0.62908	1000	18.2	8.39	0.35	0.70	68	G	18	146	0.13	6939.34 + 257.72
reservoir	Rutland Water	RU9	52.64346 -0.62659	1000	18.5	8.34	0.35	0.70	70	G	16	35	0.15	39705.11 + 2475.94
reservoir	Rutland Water	RU10	52.64441 -0.62401	1000	18.9	8.47	0.35	0.70	69	G	39	11	0.09	3531.37 + 237.19
river	River Nene	RN1	52.56586 -0.22081	1000	17.8	7.68	0.46	0.92	91	G	136	3	0.05	4028.75 + 240.10
river	River Nene	RN2	52.56586 -0.22233	800	18.0	7.86	0.46	0.91	91	G	143	2	0.07	7552.54 + 354.86
river	River Nene	RN3	52.56581 -0.22411	1000	18.0	7.80	0.46	0.91	98	G	161	5	0.11	2881.12 + 146.15
river	River Nene	RN4	52.56578 -0.22572	1000	17.9	7.77	0.46	0.92	100	G	146	16	0.19	2750.36 + 43.59
river	River Nene	RN5	52.56572 -0.22747	1000	17.9	7.79	0.45	0.91	95	G	138	5	0.07	2741.37 + 145.84
river	River Nene	RN6	52.56575 -0.22917	1000	17.8	7.73	0.45	0.91	99	G	121	6	0.12	2471.64 + 152.79
river	River Nene	RN7	52.56578 -0.23075	1000	18.1	7.64	0.46	0.92	100	G	108	10	0.06	5605.38 + 252.13

river	River Nene	RN8	52.56631 -0.23381	1000	17.9	7.87	0.45	0.90	97	G	127	3	0.09	2861.83 + 176.09
river	River Nene	RN9	52.56672 -0.23500	1000	17.8	7.91	0.45	0.90	99	G	132	8	0.10	2543.34 + 208.03
river	River Nene	RN10	52.56717 -0.23633	1000	17.9	7.89	0.45	0.90	100	G	133	2	0.16	3182.30 + 302.34
river	Little Ouse River	LO1	52.50003 0.36703	1000	17.9	7.82	0.41	0.82	130	S	129	0	0.04	737.72 + 55.82
river	Little Ouse River	LO2	52.49908 0.36731	1000	17.8	7.83	0.41	0.81	130	S	115	0	0.15	605.65 + 116.51
river	Little Ouse River	LO3	52.49778 0.36706	1000	17.7	7.85	0.41	0.81	130	S	94	0	0.05	725.48 + 131.62
river	Little Ouse River	LO4	52.49661 0.36733	1000	17.7	7.86	0.41	0.81	130	S	119	0	0.08	584.10 + 62.24
river	Little Ouse River	LO5	52.49542 0.36761	1000	17.6	7.85	0.41	0.82	120	S	114	0	0.11	3976.50 + 200.71
river	Little Ouse River	LO6	52.49442 0.36881	1000	17.6	7.82	0.41	0.82	130	S	170	0	0.04	1233.96 + 154.02
river	Little Ouse River	LO7	52.49369 0.36942	1000	17.7	7.86	0.41	0.82	130	S	98	0	0.04	1060.60 + 147.66
river	Little Ouse River	LO8	52.49289 0.36989	1000	17.9	7.76	0.41	0.82	130	S	95	0	0.09	3314.48 + 268.27

river	Little Ouse River	LO9	52.49214 0.37061	1000	18.0	7.79	0.41	0.82	130	S	113	0	0.05	4856.49 + 836.12
river	Little Ouse River	LO10	52.49169 0.37106	1000	18.0	7.81	0.41	0.82	130	S	130	0	0.14	1387.71 + 140.58
lake	Willen Lake (south)	WL1	52.05412 -0.72267	600	18.0	8.61	0.40	0.79	73	S	10	0	0.30	41.89 + 23.53
lake	Willen Lake (south)	WL2	52.05422 -0.71783	600	17.8	8.79	0.39	0.79	75	S	39	0	0.06	241.58 + 30.10
lake	Willen Lake (south)	WL3	52.05478 -0.71545	600	17.8	8.41	0.40	0.79	80	B	20	0	0.05	511.13 + 48.45
lake	Willen Lake (south)	WL4	52.05358 -0.71586	500	17.8	8.68	0.39	0.79	77	B	19	0	0.04	162.43 + 32.58
lake	Willen Lake (south)	WL5	52.05250 -0.71547	500	18.1	8.62	0.40	0.79	76	B	21	0	0.06	198.40 + 43.99
lake	Willen Lake (south)	WL6	52.05169 -0.71486	500	17.9	8.52	0.40	0.79	82	B	31	0	0.04	408.81 + 48.73
lake	Willen Lake (south)	WL7	52.05089 -0.71392	400	17.8	8.54	0.40	0.79	77	B	22	0	0.03	216.72 + 55.48
lake	Willen Lake (south)	WL8	52.04925 -0.71389	500	18.3	8.53	0.40	0.79	80	B	21	0	0.02	95.79 + 17.92
lake	Willen Lake (south)	WL9	52.04778 -0.71536	500	17.6	8.50	0.40	0.79	82	S	30	0	0.08	181.35 + 32.54

lake	Willen Lake (south)	WL10	52.04753 -0.71819	500	18.2	8.66	0.39	0.79	79	S	21	0	0.06	102.40 + 33.03
river	River Thames	RT1	51.56283 -0.70869	1000	18.4	8.01	0.36	0.73	140	S	112	0	0.14	138564.90 + 6382.90
river	River Thames	RT2	51.56350 -0.71017	1000	18.3	8.00	0.36	0.73	150	S	176	1	0.00	80835.27 + 6662.39
river	River Thames	RT3	51.56461 -0.71225	1000	18.1	7.99	0.36	0.72	140	S	79	0	0.03	107811.01 + 5498.58
river	River Thames	RT4	51.56575 -0.71286	1000	18.3	8.02	0.36	0.72	150	S	24	0	0.06	154086.90 + 10181.01
river	River Thames	RT5	51.56661 -0.71267	1000	18.3	7.99	0.36	0.72	140	S	41	0	0.03	48662.72 + 2497.10
river	River Thames	RT6	51.56775 -0.71239	1000	18.6	8.01	0.36	0.72	150	SA	27	0	0.04	133736.11 + 9220.34
river	River Thames	RT7	51.56853 -0.71228	1000	18.6	8.02	0.36	0.72	150	SA	28	0	0.02	335817.03 + 20385.69
river	River Thames	RT8	51.56939 -0.71231	1000	18.5	8.09	0.36	0.72	150	SA	31	0	0.02	210288.13 + 14218.61
river	River Thames	RT9	51.57033 -0.71242	800	19.5	8.09	0.36	0.72	140	SA	11	0	2.08	3223.68 + 469.06
river	River Thames	RT10	51.57136 -0.71242	1000	19.0	8.10	0.36	0.72	150	G	13	0	0.18	191096.42 + 16963.15

reservoir	Walthamstow Reservoirs	WR1	51.58439 -0.05322	600	18.2	9.00	0.32	0.64	120	S	161	0	0.09	1316.69 + 134.37
reservoir	Walthamstow Reservoirs	WR2	51.58342 -0.05133	800	18.6	7.93	0.39	0.76	150	S	28	2	0.04	98954.55 + 2288.53
reservoir	Walthamstow Reservoirs	WR3	51.58261 -0.05156	600	18.6	8.90	0.32	0.63	130	S	94	0	0.11	30.82 + 39.41
reservoir	Walthamstow Reservoirs	WR4	51.58117 -0.05178	800	19.0	7.96	0.38	0.77	160	S	72	0	0.25	113886.02 + 3024.99
reservoir	Walthamstow Reservoirs	WR5	51.57953 -0.05069	1000	20.0	8.72	0.34	0.68	140	S	22	18	0.05	469704.39 + 11541.63
reservoir	Walthamstow Reservoirs	WR6	51.58047 -0.05058	1000	20.7	7.87	0.40	0.79	180	S	101	1	0.17	7741.99 + 357.13
reservoir	Walthamstow Reservoirs	WR7	51.58197 -0.04869	1000	20.8	8.76	0.34	0.67	140	S	18	0	0.09	268841.27 + 8311.62
reservoir	Walthamstow Reservoirs	WR8	51.58214 -0.04844	1000	21.1	8.61	0.36	0.72	160	S	22	7	0.12	2261074.89 + 75169.45
reservoir	Walthamstow Reservoirs	WR9	51.58392 -0.04897	1000	19.7	7.74	0.39	0.79	170	S	125	0	0.11	425265.60 + 21493.08
reservoir	Walthamstow Reservoirs	WR10	51.58536 -0.05086	1000	20.3	8.62	0.36	0.71	150	S	31	31	0.05	1044426.67 + 60660.13

Table S2.2 Individual and average Cq values obtained for each replicate and sample, respectively, for each run of the repeatability test. The standard deviation (SD) for each sample is also provided.

Type	Site	Sample code	Run	Cq	Average Cq + SD
canal	Gloucester & Sharpness Canal	GS3	1	24.545	24.540 + 0.035
canal	Gloucester & Sharpness Canal	GS3	1	24.551	
canal	Gloucester & Sharpness Canal	GS3	1	24.477	
canal	Gloucester & Sharpness Canal	GS3	1	24.560	
canal	Gloucester & Sharpness Canal	GS3	1	24.577	
canal	Gloucester & Sharpness Canal	GS3	1	24.532	
canal	Gloucester & Sharpness Canal	GS3	2	24.565	24.530 + 0.037
canal	Gloucester & Sharpness Canal	GS3	2	24.491	
canal	Gloucester & Sharpness Canal	GS3	2	24.533	
canal	Grand Union Canal	GU5	1	27.135	27.120 + 0.071
canal	Grand Union Canal	GU5	1	27.130	
canal	Grand Union Canal	GU5	1	27.231	
canal	Grand Union Canal	GU5	1	27.018	
canal	Grand Union Canal	GU5	1	27.073	
canal	Grand Union Canal	GU5	1	27.135	
canal	Grand Union Canal	GU5	2	27.275	27.245 + 0.069
canal	Grand Union Canal	GU5	2	27.293	
canal	Grand Union Canal	GU5	2	27.166	
canal	Worcester & Birmingham Canal	WB1	1	31.919	31.771 + 0.133
canal	Worcester & Birmingham Canal	WB1	1	31.584	
canal	Worcester & Birmingham Canal	WB1	1	31.791	
canal	Worcester & Birmingham Canal	WB1	1	31.652	
canal	Worcester & Birmingham Canal	WB1	1	31.777	
canal	Worcester & Birmingham Canal	WB1	1	31.902	
canal	Worcester & Birmingham Canal	WB1	2	32.147	31.975 + 0.253
canal	Worcester & Birmingham Canal	WB1	2	32.093	
canal	Worcester & Birmingham Canal	WB1	2	31.685	
lake	Eight Acre Lake	AL9	1	26.730	

lake	Eight Acre Lake	AL9	1	26.543	26.661 + 0.113
lake	Eight Acre Lake	AL9	1	26.847	
lake	Eight Acre Lake	AL9	1	26.625	
lake	Eight Acre Lake	AL9	1	26.657	
lake	Eight Acre Lake	AL9	1	26.565	
lake	Eight Acre Lake	AL9	2	26.529	26.450 + 0.136
lake	Eight Acre Lake	AL9	2	26.527	
lake	Eight Acre Lake	AL9	2	26.292	
lake	Farnham Lake	FL3	1	23.730	23.809 + 0.052
lake	Farnham Lake	FL3	1	23.889	
lake	Farnham Lake	FL3	1	23.806	
lake	Farnham Lake	FL3	1	23.807	
lake	Farnham Lake	FL3	1	23.827	
lake	Farnham Lake	FL3	1	23.793	
lake	Farnham Lake	FL3	2	23.679	23.551 + 0.120
lake	Farnham Lake	FL3	2	23.533	
lake	Farnham Lake	FL3	2	23.442	
lake	Pugneys Country park	PC8	1	32.311	32.227 + 0.173
lake	Pugneys Country park	PC8	1	32.238	
lake	Pugneys Country park	PC8	1	31.982	
lake	Pugneys Country park	PC8	1	32.463	
lake	Pugneys Country park	PC8	1	32.288	
lake	Pugneys Country park	PC8	1	32.078	
lake	Pugneys Country park	PC8	2	32.124	32.134 + 0.181
lake	Pugneys Country park	PC8	2	32.320	
lake	Pugneys Country park	PC8	2	31.958	
reservoir	Chasewater Reservoir	CR1	1	21.518	21.531 + 0.027
reservoir	Chasewater Reservoir	CR1	1	21.572	
reservoir	Chasewater Reservoir	CR1	1	21.517	
reservoir	Chasewater Reservoir	CR1	1	21.501	
reservoir	Chasewater Reservoir	CR1	1	21.524	

reservoir	Chasewater Reservoir	CR1	1	21.556	
reservoir	Chasewater Reservoir	CR1	2	22.327	22.200 + 0.121
reservoir	Chasewater Reservoir	CR1	2	22.189	
reservoir	Chasewater Reservoir	CR1	2	22.085	
reservoir	Rutland Water	RU3	1	25.440	25.440 + 0.060
reservoir	Rutland Water	RU3	1	25.430	
reservoir	Rutland Water	RU3	1	25.361	
reservoir	Rutland Water	RU3	1	25.543	
reservoir	Rutland Water	RU3	1	25.412	
reservoir	Rutland Water	RU3	1	25.454	
reservoir	Rutland Water	RU3	2	25.267	25.227 + 0.064
reservoir	Rutland Water	RU3	2	25.260	
reservoir	Rutland Water	RU3	2	25.154	
reservoir	Staunton Harold Reservoir	SH7	1	31.889	31.907 + 0.075
reservoir	Staunton Harold Reservoir	SH7	1	31.962	
reservoir	Staunton Harold Reservoir	SH7	1	31.981	
reservoir	Staunton Harold Reservoir	SH7	1	31.940	
reservoir	Staunton Harold Reservoir	SH7	1	31.771	
reservoir	Staunton Harold Reservoir	SH7	1	31.900	
reservoir	Staunton Harold Reservoir	SH7	2	31.816	31.819 + 0.162
reservoir	Staunton Harold Reservoir	SH7	2	31.982	
reservoir	Staunton Harold Reservoir	SH7	2	31.658	
river	Little Ouse River	LO5	1	29.727	29.693 + 0.087
river	Little Ouse River	LO5	1	29.832	
river	Little Ouse River	LO5	1	29.659	
river	Little Ouse River	LO5	1	29.686	
river	Little Ouse River	LO5	1	29.564	
river	Little Ouse River	LO5	1	29.688	
river	Little Ouse River	LO5	2	29.749	29.932 + 0.159
river	Little Ouse River	LO5	2	30.016	
river	Little Ouse River	LO5	2	30.031	

river	River Nene	RN6	1	31.188	31.165 + 0.110
river	River Nene	RN6	1	31.340	
river	River Nene	RN6	1	31.038	
river	River Nene	RN6	1	31.083	
river	River Nene	RN6	1	31.113	
river	River Nene	RN6	1	31.225	
river	River Nene	RN6	2	30.838	30.864 + 0.195
river	River Nene	RN6	2	30.683	
river	River Nene	RN6	2	31.070	
river	River Weaver	RW9	1	26.758	26.756 + 0.115
river	River Weaver	RW9	1	26.705	
river	River Weaver	RW9	1	26.975	
river	River Weaver	RW9	1	26.761	
river	River Weaver	RW9	1	26.683	
river	River Weaver	RW9	1	26.656	
river	River Weaver	RW9	2	26.410	26.437 + 0.056
river	River Weaver	RW9	2	26.400	
river	River Weaver	RW9	2	26.502	

Table S2.3 MIQE checklist (Bustin et al., 2009) for the Gingera et al. (2017) *Cyt* b assay and data from the third chapter.

Item to check	Importance	Details
Experimental design		
Definition of experimental and control groups	E	20 sites (5 canals, 5 lakes, 5 reservoirs and 5 rivers) spread throughout England
Number within each group	E	10 samples were collected at each site
Assay carried out by the core or investigator's laboratory?	D	Investigator's lab
Acknowledgement of authors' contributions	D	Yes
Sample		
Description	E	eDNA water samples
Volume/mass of sample processed	D	The volume filtered for each sample is described in Table S3
Microdissection or macrodissection	E	N/A

Processing procedure	E	Water samples were filtered on site with NatureMetrics filters. Water was pushed through the filter as many times as possible until the filter clogged, air was passed through the filter to dry it and 1 mL of Longmire's buffer was added to each filter to preserve the sample.
If frozen, how and how quickly?	E	N/A
If fixed, with what how quickly?	E	N/A
Sample storage conditions and duration (especially for FFPE samples)	E	Samples were stored at room temperature while on fieldwork and stored at -20°C upon return to the lab (maximum 1 week).
Nucleic acid extraction		
Procedure and/or instrumentation	E	We used a modified version of the DNeasy Blood & Tissue Kit (Qiagen, UK), available in the supplementary material
Name of kit and details of any modifications	E	
Source of additional reagents used	D	University of Hull
Details of DNase or RNAse treatment	E	N/A
Contamination assessment (DNA or RNA)	E	N/A

Nucleic acid quantification	E	Assessed using a Nanodrop 1000 spectrophotometer following manufacturer's instructions.
Instrument and method	E	
Purity (A260/A280)	D	
Yield	D	
RNA integrity: method/instrument	E	N/A
RIN/RQI or Cq of 3' and 5' transcripts	E	N/A
Electrophoresis traces	D	N/A
Inhibition testing (Cq dilutions, spike, or other)	E	Samples were tested for inhibition using an exogenous internal positive control. All eDNA samples were tested in duplicate and samples were considered to be inhibited if the average Cq of a sample was higher than the no template reaction by more than 2 cycles.
Reverse Transcription		
Complete reaction conditions	E	N/A
Amount of RNA and reaction volume	E	N/A
Priming oligonucleotide (if using GSP) and concentration	E	N/A

Reverse transcriptase and concentration	E	N/A
Temperature and time	E	N/A
Manufacturer of reagents and catalogue numbers	D	N/A
Cqs with and without reverse transcription	D	N/A
Storage conditions of cDNA	D	N/A
qPCR target information		
Gene symbol	E	*Cyt* b - cytochrome b
Sequence accession number	E	N/A
Location of amplicon	D	N/A
Amplicon length	E	114 bp
In silico specificity screen (BLAST, and so on)	E	N/A
Pseudogenes, retropseudogenes or other homologs?	D	N/A
Sequence alignment	D	N/A

Secondary structure analysis of amplicon	D	N/A
Location of each primer by exon or intron (if applicable)	E	N/A
What splice variants are targeted?	E	N/A
qPCR oligonucleotides		
Primer sequences	E	forward (5’- 3’): CAT TTT CTT ATA CCT TTT ATT TTA TTA GTG CTT TT reverse (5’- 3’): CGG GAC AGT TTG AGT AGA AGT ATC A
RTPrimerDB Identification Number	D	N/A
Probe sequences	D	(5’- 3’): 6FAM-TAG GTT TTC TTC ATA CTA CTG GC-MGBNFQ
Location and identity of any modifications	E	N/A
Manufacturer of oligonucleotides	D	IDT (primers) and Applied Biosystems UK (probe)
Purification method	D	standard desalting (primers) and HPLC (probe)
qPCR protocol		

Complete reaction conditions	E	PCR conditions: 1x TaqMan Environmental Master Mix 2.0 (Fisher Scientific, UK), 0.2 µM of each primer (forward and reverse), 0.1 µM of probe, 4.75 µl of molecular grade water and 2 µl of sample qPCR program: 50°C for 10 min, 95°C for 10 min, followed by 45 cycles of 95°C for 1 min and 60°C for 1 min
Reaction volume and amount of cDNA/DNA	E	Reaction volume = 15 µL amount of DNA = 2 µL
Primer, (probe), Mg^{2+} and dNTP concentrations	E	0.2 µM of each primer (forward and reverse), 0.1 µM of probe
Polymerase identity and concentration	E	TaqMan Environmental Master Mix 2.0 (Fisher Scientific, UK), final concentration of 1x
Buffer/kit identity and manufacturer	E	N/A
Exact chemical composition of the buffer	D	N/A
Additives (SYBR Green I, DMSO, and so forth)	E	N/A
Manufacturer of plates/tubes and catalog number	D	Applied Biosystems™ MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL (12142000, Fisher Scientific, UK)
Complete thermocycling parameters	E	50°C for 10 min; 95°C for 10 min; 45 cycles of 95°C for 1 min and 60°C for 1 min

Reaction setup (manual/robotic)	D	Reactions were made manually
Manufacturer of qPCR instrument	E	StepOne-Plus™ Real-Time PCR system (Fisher Scientific/Thermo Fisher, UK)
qPCR validation		
Evidence of optimisation (from gradients)	D	N/A
Specificity (gel, sequence, melt, or digest)	E	N/A
For SYBR Green I, Cq of the NTC	E	N/A
Calibration curves with slope and y intercept	E	Slope range: -4.04 – -3.57; y-intercept range: 41.56 – 45.03
PCR efficiency calculated from slope	E	76.9 - 90.6%
CIs for PCR efficiency or SE	D	N/A
r2 of standard curve	E	0.988-0.999
Linear dynamic range	E	N/A

Cq variation at lower limit	E	N/A
CIs throughout range	D	N/A
Evidence for LOD	E	The lowest standard with at least 95% amplification was 300 copies/µL.
If multiplex, efficiency and LOD of each assay	E	N/A
Data analysis		
qPCR analysis program (source, version)	E	StepOne Software version 2.3
Method of Cq determination	E	Performed according to the default setting of the software above
Outlier identification and disposition	E	N/A
Results for NTCs	E	Three wells of no-template negative controls were included in all qPCR plates and showed no amplification
Justification of number and choice of reference genes	E	N/A
Description of normalisation method	E	We used standard curve methods
Number and concordance of biological replicates	D	10 samples were collected at each site

Number and stage (reverse transcription or qPCR) of technical replicates	E	Six qPCR replicates for each sample
Repeatability (intraassay variation)	E	A subset of twelve samples were run twice separately, which included three samples from each of the four site types (canals, lakes, reservoirs, rivers), and with a range of different DNA concentrations. PCR volumes and thermal cycling conditions were as above. Cq values between both runs were compared and showed to be identical.
Reproducibility (interassay variation, CV)	D	N/A
Power analysis	D	N/A
Statistical methods for result significance	E	Artemis package; Kruskal Wallis test
Software (source, version)	E	R version 4.2.1
Cq or raw data submission using RDML	D	N/A

Table S2.4 Number of positive qPCR replicates (n = 6) for each of the ten samples collected at each site. Replicates that were lost due to contamination are not included. The number of positive qPCR replicates for field (n = 6), extraction (n = 6) and PCR (n = 3) negative controls is also provided.

Type	Site	Samples										Negative controls		
		1	2	3	4	5	6	7	8	9	10	Field	Extraction	PCR
canal	Leeds & Liverpool Canal	6	6	6	6	6	6	6	6	6	6	0	0	0
	Gloucester & Sharpness Canal	6	6	6	6	6	6	6	6	6	6	0	0	0
	Worcester & Birmingham Canal	6	6	6	6	6	6	6	6	6	4	4	0	0
	Grand Union Canal	6	6	6	6	6	6	6	6	6	6	0	0	0
	Shropshire Union Canal	6	6	6	6	6	6	6	6	6	6	0	0	0
lake	Farnham Lake	6	6	6	6	6	6	6	6	6	6	0	0	0
	Eight Acre Lake	6	6	6	6	6	6	6	6	6	6	0	0	0
	Pugneys Country park	2	6	6	5	6	2	6	6	6	6	0	0	0
	Holme Pierrepont	6	6	6	6	6	6	6	6	6	6	1	0	0
	Willen Lake (south)	6	6	6	6	6	6	6	6	6	6	2	0	0
reservoir	Ulley Reservoir	6	6	6	6	6	6	6	6	6	6	0	0	0
	Staunton Harold Reservoir	6	6	6	6	6	6	6	6	6	6	0	0	0
	Chasewater Reservoir	6	6	6	6	6	6	6	6	6	6	3	0	0
	Rutland Water	6	5	6	6	6	6	6	6	6	6	0	0	0
	Walthamstow Reservoirs	6	6	4	6	6	6	6	6	6	6	1	0	0

river	River Severn	6	3	6	6	6	6	6	5	4	5	2	0	0
	River Weaver	6	6	6	6	6	6	6	6	6	6	0	0	0
	River Nene	6	6	6	6	6	6	6	6	6	6	0	0	0
	Little Ouse River	6	6	6	6	6	6	6	6	6	6	0	0	0
	River Thames	6	6	6	6	6	6	6	6	6	6	0	0	0

Table S2.5 Model comparison conducted with the loo R package, ranked from the best (top) to the worst (bottom) model. Model 1 contains all explanatory variables except conductivity, and for each subsequent model a different variable was removed. Parameters provided correspond to the leave-one out information criteria (looic), the effective number of parameters (p_loo), the difference in expected log predictive density (elpd_diff) and the respective standard error (sd_diff). Lower values of looic indicate better model performance. The parameter elpd_diff is 0 for the best model, and the remaining models are compared against it (hence the negative values).

Model	Variables	looic	p_loo	elpd_diff	se_diff
3	volume, calcium, individuals, pH, turbidity	-359.7	198.9	0.0	0.0
1	volume, calcium, depth, individuals, pH, temperature, turbidity	-358.8	198.9	-0.4	2.5
6	volume, calcium	-357.1	201.3	-1.3	2.0
4	volume, calcium, individuals, pH	-356.8	202.8	-1.5	2.6
5	volume, calcium, pH	-355.2	202.1	-2.3	1.9
7	volume	-354.6	202.7	-2.6	2.1
2	volume, calcium, depth, individuals, pH, turbidity	-347.4	206.3	-6.1	2.2

Table S2.6 Model estimates (mean and 95% credible interval) provided by the artemis models for the probability of detecting zebra mussels depending on the number of replicates and volume filtered. The data in this table corresponds to Figure 3.5d.

Volume	Replicate	Model estimates	Volume	Replicate	Model estimates
250 mL	1	0.88 (0.00, 0.92)	750 mL	1	0.92 (0.92, 0.92)
	2	0.95 (0.00, 0.99)		2	0.99 (0.99, 0.99)
	3	0.96 (0.00, 1.00)		3	1.00 (1.00, 1.00)
	4	0.96 (0.00, 1.00)		4	1.00 (1.00, 1.00)
	5	0.96 (0.00, 1.00)		5	1.00 (1.00, 1.00)
	6	0.96 (0.00, 1.00)		6	1.00 (1.00, 1.00)
	7	0.96 (0.00, 1.00)		7	1.00 (1.00, 1.00)
	8	0.96 (0.00, 1.00)		8	1.00 (1.00, 1.00)
	9	0.96 (0.00, 1.00)		9	1.00 (1.00, 1.00)
	10	0.96 (0.00, 1.00)		10	1.00 (1.00, 1.00)

500 mL	1	0.91 (0.92, 0.92)	1000 mL	1	0.92 (0.92, 0.92)
	2	0.98 (0.99, 0.99)		2	0.99 (0.99, 0.99)
	3	0.99 (1.00, 1.00)		3	1.00 (1.00, 1.00)
	4	0.99 (1.00, 1.00)		4	1.00 (1.00, 1.00)
	5	0.99 (1.00, 1.00)		5	1.00 (1.00, 1.00)
	6	0.99 (1.00, 1.00)		6	1.00 (1.00, 1.00)
	7	0.99 (1.00, 1.00)		7	1.00 (1.00, 1.00)
	8	0.99 (1.00, 1.00)		8	1.00 (1.00, 1.00)
	9	0.99 (1.00, 1.00)		9	1.00 (1.00, 1.00)
	10	0.99 (1.00, 1.00)		10	1.00 (1.00, 1.00)

Table S2.7 Contingency table displaying the frequency each substrate (boulders - B, gravel - G, silt - S, sand – SA) was recorded in each waterbody type (canals, lakes, reservoirs, rivers), and the corresponding sum of counts.

	B	**G**	**S**	**SA**	
canal	0	5	45	0	**50**
lake	13	7	15	15	**50**
reservoir	13	8	27	2	**50**
river	0	11	35	4	**50**
	26	**31**	**122**	**21**	

Appendix 3: Additional information for chapter 4

DNA extraction: modified version of the Mu-DNA protocol

Reagents needed: All reagents used in this protocol are from the Mu-DNA protocol (Sellers et al., 2018). Please refer to it for indications on how to make the solutions.

Preparation:

Prepare 1.5 mL tubes with 300 µL Flocculant Solution and place in the fridge until required.

Prepare 5 mL tubes with 2000 µL of Binding Solution and place in the oven at 55°C until required.

Place Elution Buffer in the oven at 55°C until required.

Protocol:

1. Carefully wipe the outer surfaces of all filter units with 10% bleach and 70% ethanol and let them defrost.

2. Attach a 3 mL luer lock syringe to the inlet of the filter and push the Longmire's buffer from the filter unit into a 2 mL sterile tube. Make sure to remove as much buffer as possible from the filter.

3. Spin the 2 mL tube with the buffer at 6,000 x g for 30 mins at room temperature. Discard the supernatant and dissolve the pellet (might not be visible) in 60 µL of Lysis Solution**.** Vortex for 15 s and transfer the liquid into the filter unit.

4. Keeping the outlet end closed, carefully add 660 µL of Lysis Solution, 180 µL of Tissue Lysis Additive (6% SDS) and 60 µL of 10 mg/mL proteinase K to the filter (using either a luer lock syringe or a 1000 µL pipet). Close the inlet, seal with parafilm and handshake vigorously for a few seconds. Do not vortex as this could dislodge the luer lock caps.

5. Incubate the samples overnight at 55°C on a rotating platform. The filter units should lay horizontally and be allowed to roll back and forth.

6. Handshake the filter unit vigorously. Attach a luer lock syringe to the inlet of the filter and pull (do not push) the lysate into the syringe. Transfer the lysate into a 2 mL tube and discard the filter.

7. Spin down for 15 s at 10,000 x g to remove any foam. Transfer as much liquid as possible (~900 µL) to the prepared tube with 300 µL (0.3x volume) of Flocculant Solution**.** Vortex to mix.

8. Incubate in the fridge for at least 10 min.

9. Centrifuge tubes at >10,000 x g for 1 min. Rotate the tubes by 180° (facing the opposite direction) in the centrifuge and centrifuge again for 1 min to make sure all the particles are pelleted.

10. Transfer 1000 µL of supernatant to the prepared tube with 2000 µL (2x volume) of Binding Solution, vortex to mix and centrifuge at >10,000 x g for 5 sec.

11. Transfer 670 µL of mixture to spin column and centrifuge at >10,000 x g for 10 sec, discard flow through. Repeat this until all mixture has passed through the spin column.

12. Add 500 µL of Wash Solution and centrifuge at >10,000 x g for 10 sec, discard flow through. Repeat this a second time.

13. Centrifuge spin columns at >10,000 x g for 2 min. Discard collection tube and contents.

14. Transfer spin column to a 1.5 mL labelled tube. Add 100 µL of Elution Buffer to spin column membrane, incubate at room temp for 1 min.

15. Centrifuge at >10,000 x g for 1 min.

Table S3.1 Details of samples collected in the fourth chapter, namely site name and sample code, coordinates, volume filtered and the TaqMan master mix used for both species-specific and inhibition qPCRs. Information on sample inhibition is provided in "Cq difference before" and "Cq difference after" columns, which indicate the difference between the average Cq of each sample and the no template reaction before and after 10x dilution, respectively.

Site name	Sample code	Latitude	Longitude	Volume filtered (mL)	TaqMan	Cq difference before	10X dilution	Cq difference after
Alton	ALT 1	51.979	1.157	720	Universal	7.557	Yes	0.471
Alton	ALT 2	51.979	1.157	540	Universal	26.828	Yes	0.512
Alton	ALT 3	51.979	1.158	660	Universal	26.828	Yes	0.149
Alton	ALT 4	51.978	1.158	200	Universal	3.142	Yes	0.141
Alton	ALT 5	51.978	1.157	420	Universal	0.501	No	NA
Ardleigh	ARD 1	51.893	0.952	420	Universal	1.773	No	NA
Ardleigh	ARD 2	51.893	0.952	480	Universal	1.701	No	NA
Ardleigh	ARD 3	51.891	0.952	660	Universal	26.131	Yes	0.034
Bedford WTW direct intake	BED 1	52.158	-0.491	420	Universal	1.314	No	NA
Bedford WTW direct intake	BED 2	52.158	-0.491	420	Universal	0.163	No	NA
Bedford WTW direct intake	BED 3	52.165	-0.529	300	Universal	1.340	No	NA
Bedford WTW direct intake	BED 4	52.165	-0.526	360	Universal	3.191	Yes	0.057
Bedford WTW direct intake	BED 5	52.165	-0.526	420	Universal	0.368	No	NA
Bishopbridge	BIS 1	53.406	-0.450	300	Universal	0.664	No	NA
Bishopbridge	BIS 2	53.406	-0.450	300	Universal	0.910	No	NA
Bishopbridge	BIS 3	53.406	-0.450	300	Universal	8.093	Yes	0.095
Bishopbridge	BIS 4	53.406	-0.450	210	Universal	1.312	No	NA

Bishopbridge	BIS 5	53.406	-0.450	120	Universal	3.403	Yes	0.050
Bottisham Lock	BOT 1	52.269	0.209	360	Universal	3.982	Yes	0.451
Bottisham Lock	BOT 2	52.269	0.209	360	Universal	0.488	No	NA
Bottisham Lock	BOT 3	52.269	0.209	360	Universal	2.527	Yes	0.032
Bottisham Lock	BOT 4	52.269	0.209	360	Universal	0.269	No	NA
Bottisham Lock	BOT 5	52.269	0.209	360	Universal	0.611	No	NA
Costessey Pits	COS 1	52.679	1.191	360	Universal	0.860	No	NA
Costessey Pits	COS 2	52.679	1.191	360	Universal	0.376	No	NA
Costessey Pits	COS 3	52.678	1.191	360	Universal	0.436	No	NA
Costessey Pits	COS 4	52.678	1.191	360	Universal	0.165	No	NA
Costessey Pits	COS 5	52.678	1.193	360	Universal	0.366	No	NA
Covenham	COV 1	53.449	0.030	240	Universal	0.004	No	NA
Covenham	COV 2	53.449	0.029	240	Universal	0.207	No	NA
Covenham	COV 3	53.448	0.030	300	Universal	0.133	No	NA
Covenham	COV 4	53.449	0.029	300	Universal	0.052	No	NA
Covenham	COV 5	53.448	0.030	300	Universal	0.010	No	NA
Elsham	ELS 1	53.513	-0.492	115	Universal	26.828	Yes	0.692
Elsham	ELS 2	53.513	-0.492	44	Universal	2.597	Yes	0.152
Elsham	ELS 3	53.513	-0.492	102	Universal	26.828	Yes	0.338
Elsham	ELS 4	53.513	-0.492	33	Universal	8.608	Yes	0.098
Elsham	ELS 5	53.513	-0.492	109	Universal	3.412	Yes	0.030

Ely Waterfront/Marina	ELY 1	52.394	0.268	360	Universal	0.766	No	NA
Ely Waterfront/Marina	ELY 2	52.394	0.268	360	Universal	3.830	Yes	0.089
Ely Waterfront/Marina	ELY 3	52.394	0.268	360	Universal	0.484	No	NA
Ely Waterfront/Marina	ELY 4	52.394	0.268	360	Universal	0.422	No	NA
Ely Waterfront/Marina	ELY 5	52.395	0.269	360	Universal	0.003	No	NA
Grafham	GRA 1	52.280	-0.222	480	Universal	10.418	Yes	0.122
Grafham	GRA 2	52.279	-0.222	360	Universal	26.131	Yes	0.003
Grafham	GRA 3	52.279	-0.222	360	Universal	26.131	Yes	0.768
Grafham	GRA 4	52.281	-0.222	360	Universal	0.852	No	NA
Grafham	GRA 5	52.278	-0.222	360	Universal	5.703	Yes	0.128
Grand Union Canal	GU 1	52.784	-1.218	1000	Environmental	0.030	No	NA
Grand Union Canal	GU 3	52.782	-1.217	1000	Environmental	0.069	No	NA
Grand Union Canal	GU 5	52.780	-1.216	900	Environmental	0.125	No	NA
Grand Union Canal	GU 7	52.778	-1.215	1000	Environmental	0.120	No	NA
Grand Union Canal	GU 9	52.777	-1.213	1000	Environmental	0.053	No	NA
Holme Pierrepont park	HP 10	52.941	-1.095	800	Environmental	0.013	No	NA
Holme Pierrepont park	HP 2	52.951	-1.071	1000	Environmental	0.154	No	NA
Holme Pierrepont park	HP 4	52.952	-1.077	1000	Environmental	0.176	No	NA
Holme Pierrepont park	HP 6	52.948	-1.082	600	Environmental	0.119	No	NA
Holme Pierrepont park	HP 8	52.945	-1.088	700	Environmental	0.046	No	NA
Isleham Marina	ISL 1	52.355	0.420	360	Universal	1.158	No	NA

Isleham Marina	ISL 2	52.355	0.420	360	Universal	0.596	No	NA
Isleham Marina	ISL 3	52.354	0.420	360	Universal	0.129	No	NA
Isleham Marina	ISL 4	52.354	0.420	360	Universal	3.401	Yes	0.411
Isleham Marina	ISL 5	52.355	0.420	360	Universal	0.279	No	NA
Little Ouse River	LO 1	52.500	0.367	1000	Environmental	0.035	No	NA
Little Ouse River	LO 3	52.498	0.367	1000	Environmental	0.046	No	NA
Little Ouse River	LO 5	52.495	0.368	1000	Environmental	0.113	No	NA
Little Ouse River	LO 7	52.494	0.369	1000	Environmental	0.038	No	NA
Little Ouse River	LO 9	52.492	0.371	1000	Environmental	0.051	No	NA
Marham	MAR 1	52.678	0.548	360	Universal	0.188	No	NA
Marham	MAR 2	52.678	0.549	360	Universal	0.027	No	NA
Marham	MAR 3	52.679	0.549	360	Universal	0.398	No	NA
Marham	MAR 4	52.679	0.549	360	Universal	0.098	No	NA
Marham	MAR 5	52.678	0.548	360	Universal	0.162	No	NA
River Nene	RN 1	52.566	-0.221	1000	Environmental	0.047	No	NA
River Nene	RN 3	52.566	-0.224	1000	Environmental	0.113	No	NA
River Nene	RN 5	52.566	-0.227	1000	Environmental	0.074	No	NA
River Nene	RN 7	52.566	-0.231	1000	Environmental	0.064	No	NA
River Nene	RN 9	52.567	-0.235	1000	Environmental	0.099	No	NA
River Thames	RT 1	51.563	-0.709	1000	Environmental	0.135	No	NA
River Thames	RT 3	51.565	-0.712	1000	Environmental	0.026	No	NA

River Thames	RT 5	51.567	-0.713	1000	Environmental	0.032	No	NA
River Thames	RT 7	51.569	-0.712	1000	Environmental	0.021	No	NA
River Thames	RT 9	51.570	-0.712	800	Environmental	2.077	No	NA
Rutland - Tinwell	RUT 1	52.642	-0.500	300	Universal	1.341	No	NA
Rutland - Tinwell	RUT 2	52.642	-0.499	300	Universal	0.175	No	NA
Rutland - Tinwell	RUT 3	52.642	-0.497	300	Universal	0.905	No	NA
Rutland - Tinwell	RUT 4	52.642	-0.496	300	Universal	0.229	No	NA
Rutland - Tinwell	RUT 5	52.643	-0.494	300	Universal	0.440	No	NA
Rutland Water	RU 1	52.644	-0.655	1000	Environmental	0.082	No	NA
Rutland Water	RU 3	52.642	-0.649	1000	Environmental	0.129	No	NA
Rutland Water	RU 5	52.640	-0.645	900	Environmental	0.113	No	NA
Rutland Water	RU 7	52.641	-0.633	1000	Environmental	0.087	No	NA
Rutland Water	RU 9	52.643	-0.627	1000	Environmental	0.149	No	NA
St Ives	STI 1	52.322	-0.076	360	Universal	0.465	No	NA
St Ives	STI 2	52.322	-0.076	360	Universal	0.319	No	NA
St Ives	STI 3	52.322	-0.075	420	Universal	0.181	No	NA
St Ives	STI 4	52.322	-0.075	360	Universal	0.204	No	NA
St Ives	STI 5	52.322	-0.075	360	Universal	0.163	No	NA
Staunton Harold Reservoir	SH 1	52.815	-1.442	1000	Environmental	0.012	No	NA
Staunton Harold Reservoir	SH 3	52.812	-1.443	800	Environmental	0.182	No	NA
Staunton Harold Reservoir	SH 5	52.812	-1.444	1000	Environmental	0.115	No	NA

Staunton Harold Reservoir	SH 7	52.811	-1.446	500	Environmental	0.162	No	NA
Staunton Harold Reservoir	SH 9	52.811	-1.447	1000	Environmental	0.186	No	NA
Toft Newton Reservoir	TOF 1	53.373	-0.448	145	Universal	1.049	No	NA
Toft Newton Reservoir	TOF 2	53.374	-0.448	110	Universal	0.865	No	NA
Toft Newton Reservoir	TOF 3	53.374	-0.448	165	Universal	0.465	No	NA
Toft Newton Reservoir	TOF 4	53.373	-0.449	175	Universal	0.332	No	NA
Toft Newton Reservoir	TOF 5	53.372	-0.448	88	Universal	0.089	No	NA
Walthamstow Reservoirs	WR 1	51.584	-0.053	600	Environmental	0.091	No	NA
Walthamstow Reservoirs	WR 3	51.583	-0.052	600	Environmental	0.113	No	NA
Walthamstow Reservoirs	WR 5	51.580	-0.051	1000	Environmental	0.054	No	NA
Walthamstow Reservoirs	WR 7	51.582	-0.049	1000	Environmental	0.095	No	NA
Walthamstow Reservoirs	WR 9	51.584	-0.049	1000	Environmental	0.107	No	NA
Willen Lake	WL 1	52.054	-0.723	600	Environmental	0.304	No	NA
Willen Lake	WL 3	52.055	-0.715	600	Environmental	0.055	No	NA
Willen Lake	WL 5	52.053	-0.715	500	Environmental	0.059	No	NA
Willen Lake	WL 7	52.051	-0.714	400	Environmental	0.032	No	NA
Willen Lake	WL 9	52.048	-0.715	500	Environmental	0.075	No	NA
Wraysbury Gardens	WG_1	51.437	-0.515	500	Universal	0.237	No	NA
Wraysbury Gardens	WG_2	51.437	-0.515	500	Universal	0.716	No	NA
Wraysbury Gardens	WG_3	51.437	-0.515	500	Universal	0.351	No	NA
Wraysbury Bridge	WB_1	51.448	-0.524	500	Universal	0.041	No	NA

Wraysbury Bridge	WB_2	51.448	-0.524	500	Universal	0.040	No	NA
Wraysbury Bridge	WB_3	51.448	-0.524	500	Universal	2.770	Yes	0.453
Wraysbury Weir	WW_1	51.452	-0.521	500	Universal	0.820	No	NA
Wraysbury Weir	WW_2	51.452	-0.521	500	Universal	0.711	No	NA
Wraysbury Weir	WW_3	51.452	-0.521	500	Universal	0.336	No	NA

Table S3.2 Individual and average Cq values (plus standard deviation, SD) obtained for each replicate and sample, respectively, for all nine samples tested with both pDRB1 and dDRB1 assays. The average DNA copies/µL for each sample is also provided.

Site name	Sample code	Assay	Cq	Average Cq + SD	Average DNA copies/µL
Wraysbury Gardens	WG_1	dDRB1	25.138	25.165 + 0.063	7916.842
Wraysbury Gardens	WG_1	dDRB1	25.237		
Wraysbury Gardens	WG_1	dDRB1	25.120		
Wraysbury Gardens	WG_1	pDRB1	27.854	27.545 + 0.158	16334.305
Wraysbury Gardens	WG_1	pDRB1	27.471		
Wraysbury Gardens	WG_1	pDRB1	27.535		
Wraysbury Gardens	WG_1	pDRB1	27.404		
Wraysbury Gardens	WG_1	pDRB1	27.516		
Wraysbury Gardens	WG_1	pDRB1	27.490		
Wraysbury Gardens	WG_2	dDRB1	26.570	26.166 + 0.377	4240.421
Wraysbury Gardens	WG_2	dDRB1	25.823		
Wraysbury Gardens	WG_2	dDRB1	26.105		
Wraysbury Gardens	WG_2	pDRB1	28.592	28.369 + 0.123	9420.177
Wraysbury Gardens	WG_2	pDRB1	28.355		
Wraysbury Gardens	WG_2	pDRB1	28.377		
Wraysbury Gardens	WG_2	pDRB1	28.216		
Wraysbury Gardens	WG_2	pDRB1	28.352		
Wraysbury Gardens	WG_2	pDRB1	28.319		
Wraysbury Gardens	WG_3	dDRB1	27.968	27.929 + 0.065	1411.283
Wraysbury Gardens	WG_3	dDRB1	27.965		
Wraysbury Gardens	WG_3	dDRB1	27.854		
Wraysbury Gardens	WG_3	pDRB1	30.042	29.972 + 0.096	3234.392
Wraysbury Gardens	WG_3	pDRB1	29.997		
Wraysbury Gardens	WG_3	pDRB1	29.862		
Wraysbury Gardens	WG_3	pDRB1	29.868		
Wraysbury Gardens	WG_3	pDRB1	30.104		
Wraysbury Gardens	WG_3	pDRB1	29.956		

Wraysbury Bridge	WB_1	dDRB1	22.275	22.378 + 0.091	20937.380
Wraysbury Bridge	WB_1	dDRB1	22.408		
Wraysbury Bridge	WB_1	dDRB1	22.450		
Wraysbury Bridge	WB_1	pDRB1	26.223	25.986 + 0.135	46097.418
Wraysbury Bridge	WB_1	pDRB1	25.984		
Wraysbury Bridge	WB_1	pDRB1	26.005		
Wraysbury Bridge	WB_1	pDRB1	25.985		
Wraysbury Bridge	WB_1	pDRB1	25.826		
Wraysbury Bridge	WB_1	pDRB1	25.892		
Wraysbury Bridge	WB_2	dDRB1	24.552	24.393 + 0.166	5118.847
Wraysbury Bridge	WB_2	dDRB1	24.220		
Wraysbury Bridge	WB_2	dDRB1	24.409		
Wraysbury Bridge	WB_2	pDRB1	28.060	27.981 + 0.061	12169.778
Wraysbury Bridge	WB_2	pDRB1	28.048		
Wraysbury Bridge	WB_2	pDRB1	27.953		
Wraysbury Bridge	WB_2	pDRB1	27.951		
Wraysbury Bridge	WB_2	pDRB1	27.901		
Wraysbury Bridge	WB_2	pDRB1	27.974		
Wraysbury Bridge	WB_3	dDRB1	18.603	18.644 + 0.082	462984.451
Wraysbury Bridge	WB_3	dDRB1	18.590		
Wraysbury Bridge	WB_3	dDRB1	18.738		
Wraysbury Bridge	WB_3	pDRB1	19.928	19.782 + 0.084	2865306.355
Wraysbury Bridge	WB_3	pDRB1	19.763		
Wraysbury Bridge	WB_3	pDRB1	19.770		
Wraysbury Bridge	WB_3	pDRB1	19.819		
Wraysbury Bridge	WB_3	pDRB1	19.700		
Wraysbury Bridge	WB_3	pDRB1	19.710		
Wraysbury Weir	WW_1	dDRB1	16.067	16.057 + 0.074	1733209.249
Wraysbury Weir	WW_1	dDRB1	15.979		
Wraysbury Weir	WW_1	dDRB1	16.126		
Wraysbury Weir	WW_1	pDRB1	20.717	20.304 + 0.224	2041309.125

Wraysbury Weir	WW_1	pDRB1	20.352		
Wraysbury Weir	WW_1	pDRB1	20.292		
Wraysbury Weir	WW_1	pDRB1	20.237		
Wraysbury Weir	WW_1	pDRB1	20.117		
Wraysbury Weir	WW_1	pDRB1	20.111		
Wraysbury Weir	WW_2	dDRB1	17.146	17.116 + 0.102	827369.116
Wraysbury Weir	WW_2	dDRB1	17.002		
Wraysbury Weir	WW_2	dDRB1	17.199		
Wraysbury Weir	WW_2	pDRB1	21.046	20.918 + 0.114	1347928.375
Wraysbury Weir	WW_2	pDRB1	21.038		
Wraysbury Weir	WW_2	pDRB1	20.959		
Wraysbury Weir	WW_2	pDRB1	20.865		
Wraysbury Weir	WW_2	pDRB1	20.817		
Wraysbury Weir	WW_2	pDRB1	20.780		
Wraysbury Weir	WW_3	dDRB1	20.630	20.694 + 0.110	420085.720
Wraysbury Weir	WW_3	dDRB1	20.632		
Wraysbury Weir	WW_3	dDRB1	20.821		
Wraysbury Weir	WW_3	pDRB1	21.856	21.712 + 0.110	793622.250
Wraysbury Weir	WW_3	pDRB1	21.832		
Wraysbury Weir	WW_3	pDRB1	21.705		
Wraysbury Weir	WW_3	pDRB1	21.626		
Wraysbury Weir	WW_3	pDRB1	21.589		
Wraysbury Weir	WW_3	pDRB1	21.666		

Table S3.3 MIQE checklist (Bustin et al., 2009) for the newly developed pDRB1 assay.

Item to check	Importance	Details
Experimental design		
Definition of experimental and control groups	E	24 sites (including 1 canal, 2 lakes, 5 reservoirs and 16 rivers) spread throughout England and 3 sites on the River Wraysbury from Blackman et al. (2020b)
Number within each group	E	From this study: for 23 sites n = 5 and for 1 site n = 3 Samples from Blackman et al., (2020b), n = 3
Assay carried out by the core or investigator's laboratory?	D	Investigator's lab
Acknowledgement of authors' contributions	D	Yes
Sample		
Description	E	eDNA water samples
Volume/mass of sample processed	D	N/A
Microdissection or macrodissection	E	N/A

Processing procedure	E	Samples from this study: Water samples were filtered on site with either NatureMetrics or Sterivex filters. Water was pushed through the filter as many times as possible until the filter clogged, air was passed through the filter to dry it and 1 mL of Longmire's buffer was added to each filter to preserve the sample. Samples from Blackman et al. (2020b): 3 x 500 mL water samples were collected at each sampling point and vacuum filtered through sterile 47 mm diameter 0.45 µm cellulose nitrate membrane filters with pads (Whatman, GE Healthcare, UK) immediately after collection, using Nalgene filtration units (Thermo Fisher Scientific) in combination with a vacuum pump (15~20 in. Hg, Pall Corporation).
If frozen, how and how quickly?	E	N/A
If fixed, with what how quickly?	E	N/A
Sample storage conditions and duration (especially for FFPE samples)	E	Samples from this study: Filters were stored at room temperature while on fieldwork and stored at -20°C upon return to the lab (maximum 1 week). Samples from Blackman et al. (2020b): All samples were stored in petri dishes at -20 °C until DNA extraction
Nucleic acid extraction		
Procedure and/or instrumentation	E	Samples from this study: we used a modified version of the DNeasy Blood & Tissue Kit (Qiagen, UK) and a modified version of the Mu-DNA protocol (Sellers et al., 2018; Di Muri et al., 2020) for NatureMetrics and Sterivex filters, respectively. Both protocols are available in the supplementary material. Samples from Blackman et al. (2020b): used a modified version of the Bolaski et al. (2008) protocol
Name of kit and details of any modifications	E	

Source of additional reagents used	D	University of Hull
Details of DNase or RNAse treatment	E	N/A
Contamination assessment (DNA or RNA)	E	N/A
Nucleic acid quantification	E	Samples from this study: assessed using a Nanodrop 1000 spectrophotometer following the manufacturer's instructions. Samples from Blackman et al. (2020b): assessed using a Qubit 2.0 following the manufacturer's instructions.
Instrument and method	E	
Purity (A260/A280)	D	
Yield	D	
RNA integrity: method/instrument	E	N/A
RIN/RQI or Cq of 3' and 5' transcripts	E	N/A
Electrophoresis traces	D	N/A
Inhibition testing (Cq dilutions, spike, or other)	E	Samples were tested for inhibition using the Applied Biosystems TaqMan Exogenous Internal Positive Control Reagents (Fisher Scientific, UK). All eDNA samples were tested in duplicate and samples were considered to be inhibited if the average Cq of a sample was higher than the no template reaction by 2 or more cycles. Samples that showed inhibition were diluted 10x and re-run

Reverse Transcription		
Complete reaction conditions	E	N/A
Amount of RNA and reaction volume	E	N/A
Priming oligonucleotide (if using GSP) and concentration	E	N/A
Reverse transcriptase and concentration	E	N/A
Temperature and time	E	N/A
Manufacturer of reagents and catalogue numbers	D	N/A
Cqs with and without reverse transcription	D	N/A
Storage conditions of cDNA	D	N/A
qPCR target information		
Gene symbol	E	COI - cytochrome oxidase I
Sequence accession number	E	N/A
Location of amplicon	D	amplicon location: 196 – 384

Amplicon length	E	188 bp including primers
In silico specificity screen (BLAST, and so on)	E	N/A
Pseudogenes, retropseudogenes or other homologs?	D	N/A
Sequence alignment	D	N/A
Secondary structure analysis of amplicon	D	N/A
Location of each primer by exon or intron (if applicable)	E	N/A
What splice variants are targeted?	E	N/A
qPCR oligonucleotides		
Primer sequences	E	forward (5'- 3'): GGA AAC TGG TTG GTC CCG AT reverse (5'- 3'): GGC CCT GAA TGC CCC ATA AT
RTPrimerDB Identification Number	D	N/A
Probe sequences	D	(5'- 3'): 6FAM - TCG GCG TTT AGT GAG GGC GGA TTT - QSY
Location and identity of any modifications	E	N/A

Manufacturer of oligonucleotides	D	IDT (primers) and ThermoFisher/Fisher Scientific (probe)
Purification method	D	HPLC
qPCR protocol		
Complete reaction conditions	E	Volumes: 12.5 µL of TaqMan Environmental or Universal Master Mix 2.0, 1.6 µM of primers (forward and reverse combined), 0.05 µM of probe (Table 1), 0.64 mg/mL of BSA, 5.45 µL of water and 2 µL of sample. qPCR program: 50°C for 10 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 seconds and 62°C for 1 min
Reaction volume and amount of cDNA/DNA	E	Reaction volume = 25 µL amount of DNA = 2 µL
Primer, (probe), Mg^{2+} and dNTP concentrations	E	1.6 µM of primers (forward and reverse combined) and 0.05 µm of probe
Polymerase identity and concentration	E	TaqMan Environmental Master Mix 2.0 (Fisher Scientific, UK) TaqMan Universal Master Mix 2.0 (Fisher Scientific, UK) Both at a final concentration of 1x
Buffer/kit identity and manufacturer	E	N/A
Exact chemical composition of the buffer	D	N/A

Additives (SYBR Green I, DMSO, and so forth)	E	0.64 mg/mL of BSA
Manufacturer of plates/tubes and catalog number	D	Applied Biosystems™ MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL (12142000, Fisher Scientific, UK)
Complete thermocycling parameters	E	50°C for 10 min; 95°C for 10 min; 45 cycles of 95°C for 15 seconds and 62°C for 1 min
Reaction setup (manual/robotic)	D	Reactions were made manually
Manufacturer of qPCR instrument	E	StepOne-Plus™ Real-Time PCR system (Fisher Scientific/Thermo Fisher, UK)
qPCR validation		
Evidence of optimisation (from gradients)	D	Three annealing temperatures (60, 62 and 63°C) were tested and all showed species-specific amplification of quagga mussel with no cross-amplification of the other species tested. Efficiency values were 102.9, 99.1 and 63.4% for the three annealing temperatures, respectively
Specificity (gel, sequence, melt, or digest)	E	N/A
For SYBR Green I, Cq of the NTC	E	N/A
Calibration curves with slope and y intercept	E	Slope range: -3.21 – -3.53; y-intercept range: 41.31–42.83

PCR efficiency calculated from slope	E	91.8 – 104.8%
CIs for PCR efficiency or SE	D	N/A
r2 of standard curve	E	0.984-0.998
Linear dynamic range	E	N/A
Cq variation at lower limit	E	The lowest standard (10 copies/µL) amplified at 93% of replicates
CIs throughout range	D	N/A
Evidence for LOD	E	The LOD, i.e. the lowest standard with at least 95% amplification, was 100 copies/µL
If multiplex, efficiency and LOD of each assay	E	N/A
Data analysis		
qPCR analysis program (source, version)	E	StepOne Software version 2.3
Method of Cq determination	E	Performed according to the default setting of the software above
Outlier identification and disposition	E	N/A

Results for NTCs	E	Eight wells of no-template negative control were included in all qPCR plates and showed no amplification
Justification of number and choice of reference genes	E	N/A
Description of normalisation method	E	We used standard curve methods
Number and concordance of biological replicates	D	From this study: for 23 sites n = 5 and for the remaining site n = 3 Samples from Blackman et al. (2020b), n = 3
Number and stage (reverse transcription or qPCR) of technical replicates	E	Six qPCR replicates for each sample
Repeatability (intraassay variation)	E	N/A
Reproducibility (interassay variation, CV)	D	N/A
Power analysis	D	N/A
Statistical methods for result significance	E	N/A
Software (source, version)	E	N/A
Cq or raw data submission using RDML	D	N/A

Table S3.4 Cq values and DNA copies/µL obtained for all samples and replicates that showed amplification.

Site name	Sample code	Cq	DNA copies/µL
Bottisham Lock	BOT 2	41.894	0.880
Bottisham Lock	BOT 2	39.438	4.806
Bottisham Lock	BOT 4	38.263	10.829
Bottisham Lock	BOT 4	39.281	5.358
Ely Waterfront/Marina	ELY 1	38.938	8.752
Ely Waterfront/Marina	ELY 4	39.036	8.184
Ely Waterfront/Marina	ELY 4	37.721	20.222
Ely Waterfront/Marina	ELY 4	40.912	2.249
Ely Waterfront/Marina	ELY 5	40.139	3.830
St Ives	STI 1	39.410	4.899
St Ives	STI 1	37.340	20.501
St Ives	STI 2	37.022	25.545
St Ives	STI 2	38.191	11.383
St Ives	STI 2	37.116	23.923
St Ives	STI 2	37.507	18.259
St Ives	STI 2	37.464	18.817
Grand Union Canal	GU 1	38.936	8.958
Grand Union Canal	GU 1	38.901	9.168
Grand Union Canal	GU 3	39.696	5.420
Grand Union Canal	GU 5	39.103	8.019
Grand Union Canal	GU 5	37.933	17.367
Grand Union Canal	GU 7	38.948	8.887
Grand Union Canal	GU 7	39.843	4.919
Grand Union Canal	GU 7	37.763	19.443
Grand Union Canal	GU 7	39.181	7.617
Grand Union Canal	GU 9	39.488	6.219
Grand Union Canal	GU 9	36.924	33.825
Grand Union Canal	GU 9	37.713	20.091
Grand Union Canal	GU 9	36.796	36.808
Grand Union Canal	GU 9	37.403	24.655
Grand Union Canal	GU 9	37.648	20.964
Holme Pierrepont park	HP 10	39.271	4.884
Holme Pierrepont park	HP 10	38.450	8.588
Holme Pierrepont park	HP 4	38.653	7.468

Holme Pierrepont park	HP 8	39.581	3.946
Holme Pierrepont park	HP 8	39.664	3.728
Holme Pierrepont park	HP 8	39.441	4.344
Holme Pierrepont park	HP 8	38.645	7.511
Little Ouse River	LO 1	38.775	5.892
Little Ouse River	LO 1	38.596	6.674
Little Ouse River	LO 3	39.561	3.401
Little Ouse River	LO 5	23.417	270573.375
Little Ouse River	LO 5	23.328	287887.188
Little Ouse River	LO 5	23.353	282857.813
Little Ouse River	LO 5	23.397	274389.750
Little Ouse River	LO 5	23.372	279107.406
Little Ouse River	LO 5	23.269	300035.063
Little Ouse River	LO 7	39.943	2.604
Little Ouse River	LO 9	36.928	21.428
Little Ouse River	LO 9	38.644	6.457
Little Ouse River	LO 9	39.643	3.212
Little Ouse River	LO 9	37.953	10.467
River Nene	RN 1	39.856	3.329
River Nene	RN 5	38.088	11.349
River Nene	RN 5	39.082	5.695
River Nene	RN 9	39.078	5.714
River Thames	RT 1	21.980	655017.688
River Thames	RT 1	21.983	653662.438
River Thames	RT 1	21.884	698016.250
River Thames	RT 1	21.926	678640.500
River Thames	RT 1	21.923	680257.500
River Thames	RT 1	21.897	691911.750
River Thames	RT 3	26.256	38878.109
River Thames	RT 3	26.298	37812.781
River Thames	RT 3	26.279	38279.215
River Thames	RT 3	26.363	36213.402
River Thames	RT 3	26.551	31988.484
River Thames	RT 3	26.401	35326.191
River Thames	RT 5	24.981	90228.523
River Thames	RT 5	24.911	94509.758

River Thames	RT 5	24.864	97508.852
River Thames	RT 5	24.874	96831.609
River Thames	RT 5	24.833	99522.734
River Thames	RT 5	24.953	91915.500
River Thames	RT 7	23.964	176681.609
River Thames	RT 7	23.972	175673.672
River Thames	RT 7	23.984	174388.281
River Thames	RT 7	24.000	172454.875
River Thames	RT 7	23.966	176422.688
River Thames	RT 7	24.005	171957.594
River Thames	RT 9	28.127	11293.130
River Thames	RT 9	28.169	10985.740
River Thames	RT 9	28.259	10351.801
River Thames	RT 9	28.431	9240.614
River Thames	RT 9	28.278	10226.050
River Thames	RT 9	28.285	10173.094
Rutland Water	RU 1	31.411	1086.496
Rutland Water	RU 1	31.017	1424.257
Rutland Water	RU 1	31.017	1424.446
Rutland Water	RU 1	31.159	1292.223
Rutland Water	RU 1	31.000	1441.373
Rutland Water	RU 1	31.020	1421.807
Rutland Water	RU 3	19.950	2875904.000
Rutland Water	RU 3	19.939	2898461.750
Rutland Water	RU 3	19.943	2889469.750
Rutland Water	RU 3	20.089	2614143.000
Rutland Water	RU 3	19.947	2882463.500
Rutland Water	RU 3	19.984	2808869.750
Rutland Water	RU 5	24.784	103570.047
Rutland Water	RU 5	24.714	108648.539
Rutland Water	RU 5	24.727	107699.516
Rutland Water	RU 5	24.798	102568.609
Rutland Water	RU 5	24.754	105707.938
Rutland Water	RU 5	24.707	109173.500
Rutland Water	RU 7	21.105	1299377.625
Rutland Water	RU 7	21.081	1321098.750

Rutland Water	RU 7	21.024	1374016.000
Rutland Water	RU 7	21.487	999127.375
Rutland Water	RU 7	21.043	1355820.625
Rutland Water	RU 7	21.034	1364297.375
Rutland Water	RU 9	23.153	317896.813
Rutland Water	RU 9	23.140	320734.656
Rutland Water	RU 9	23.236	300169.625
Rutland Water	RU 9	23.242	298885.406
Rutland Water	RU 9	23.147	319043.000
Rutland Water	RU 9	23.237	300093.281
Walthamstow Reservoirs	WR 1	33.537	250.721
Walthamstow Reservoirs	WR 1	32.760	430.098
Walthamstow Reservoirs	WR 1	34.642	116.378
Walthamstow Reservoirs	WR 1	33.351	285.365
Walthamstow Reservoirs	WR 1	32.841	406.596
Walthamstow Reservoirs	WR 1	32.475	524.356
Walthamstow Reservoirs	WR 3	34.510	127.594
Walthamstow Reservoirs	WR 3	34.966	92.902
Walthamstow Reservoirs	WR 3	34.389	138.765
Walthamstow Reservoirs	WR 3	35.154	81.566
Walthamstow Reservoirs	WR 3	34.766	106.738
Walthamstow Reservoirs	WR 3	34.494	128.996
Walthamstow Reservoirs	WR 5	22.940	395061.063
Walthamstow Reservoirs	WR 5	22.889	409266.094
Walthamstow Reservoirs	WR 5	22.732	456379.750
Walthamstow Reservoirs	WR 5	22.967	387471.094
Walthamstow Reservoirs	WR 5	22.840	423232.000
Walthamstow Reservoirs	WR 5	22.728	457459.281
Walthamstow Reservoirs	WR 7	23.398	287246.188
Walthamstow Reservoirs	WR 7	23.258	316678.719
Walthamstow Reservoirs	WR 7	23.151	340970.719
Walthamstow Reservoirs	WR 7	23.714	230634.797
Walthamstow Reservoirs	WR 7	23.189	332248.125
Walthamstow Reservoirs	WR 7	23.153	340631.563
Walthamstow Reservoirs	WR 9	26.580	31492.461
Walthamstow Reservoirs	WR 9	26.414	35344.160

Walthamstow Reservoirs	WR 9	26.529	32623.395
Walthamstow Reservoirs	WR 9	26.518	32884.672
Walthamstow Reservoirs	WR 9	26.462	34197.934
Walthamstow Reservoirs	WR 9	26.425	35090.160
Willen Lake	WL 3	40.399	2.285