Using environmental DNA metabarcoding to monitor fish biodiversity and assess anthropogenic barrier impacts on the river Mersey, UK

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Abstract

Freshwater habitats, despite their limited volume, support almost 10% of global biodiversity and are facing increased anthropogenic threats worldwide. Dams and weirs, for example, create habitat fragmentation, changing water flow and water levels, blocking crucial nutrients passing through river systems, and, above all, preventing crucial movements and migrations of aquatic organisms. Fish passes alleviate the problem, increasing river connectivity, but there is an evident need for a better understanding of their efficiency for fish movements and migration. Monitoring aquatic systems is notoriously difficult and traditional methods such as electrofishing and trapping can be invasive, costly and not always effective. The use of a novel molecular approach, environmental DNA (eDNA) metabarcoding, is now revolutionising biomonitoring. In this thesis, data collected from non-invasive monitoring techniques (camera recordings and eDNA metabarcoding) were combined to monitor Atlantic salmon migration at the Woolston weir fish pass, on the river Mersey, UK. In addition, a fine-scale spatial-temporal sampling approach was employed to investigate changes in fish communities below and above two weirs, from the Mersey estuary to the upper Mersey, in autumn and spring. Atlantic salmon was detected above and below Woolston weir, showing a good degree of connectivity and demonstrating the power of eDNA metabarcoding in detecting even few individuals (as revealed from the camera data). In total, 30 species were detected, including all main UK migratory species except for shad. Temporal variation was found in the detected communities, in line with expectations of different species' migratory patterns. No correlation was found between barriers and community composition along the river, although lower species richness was detected at sites directly above each weir. Highest species richness was found at sampling sites at the geographical border of the lower and upper estuary, possibly due to tidal and river flow combination, suggesting that these types of environments could

be optimal sampling areas to monitor the whole river biodiversity at once. Environmental parameters (pH, oxygen, salinity and temperature) were also integrated in the analyses. For example, the salmonid species showed a positive relationship with oxygen, and are known to require well oxygenated water to spawn. Overall, this work reinforces the potential of eDNA metabarcoding for ongoing and future biomonitoring efforts of fish communities (including migratory species) in a recovering river.

1. Introduction

1.1 Freshwater biodiversity

Anthropogenic impacts have put great stress on the planet's environment, leading to an acceleration in biodiversity loss. The threat of local extinctions and extirpations is altering the processes crucial to sustainability and productivity of the planet's ecosystems (Hooper et al., 2012). Therefore, the first step towards understanding the effects of biodiversity loss is reliably measuring such biodiversity in the first place (Hooper et al., 2012).

Freshwater habitats account for a fraction of all the water on Earth but are one of the most vital resources for life. This environment supports almost 10% of global biodiversity, including approximately 126,000 animal species, making freshwater ecosystems among the most diverse worldwide, despite their relative small volume (Altermatt et al., 2020; Cantonati et al., 2020). Humans rely heavily on freshwater systems for drinking water, food, agriculture and energy (Ahn et al., 2014). As anthropogenic activity increases, the rate of biodiversity loss is growing at unprecedented rates (Rolls et al., 2014). There are over 4,600 freshwater fish species, threatened or endangered globally (IUCN, 2019). Freshwater ecosystems are under threat globally due to climate change, invasive aquatic species, water harvesting, pollution and widespread impoundments. These stressors call for better understanding of their impact on biodiversity and how to counteract them. Although freshwater river systems show large losses of biodiversity, the information we have is taxonomically, temporally and spatially understudied compared to other ecosystems (Altermatt et al., 2020). Rivers are notoriously difficult to study due to the submergence of organisms and the directional movement of water (Isaak et al., 2014), thus requiring efficient and reliable monitoring techniques.

1.2 Anthropogenic obstacles and fish passes

Anthropogenic impacts in rivers and streams include sewage loading, agricultural run-off and other forms of pollution. This can result in reduced oxygen levels which negatively affect biodiversity (Barnes et al., 2014), although recent efforts and investments in wastewater treatment and stricter laws on restricting pollutants have seen improvements in water quality in many of the worlds rivers (Søndergaard & Jeppesen, 2007). One of the main stressors to rivers is the modification of their hydromorphological conditions, as courses are modified or removed for agricultural use, surrounding habitat has been destroyed for canalization and artificial barriers been constructed for water level control and energy creation (Søndergaard & Jeppesen, 2007). The latter has been shown to have a significant impact on the biodiversity distribution along these gradients (Rolls et al., 2014). Ecological barriers are defined as structures that restricts movement of organisms between habitats. They can be natural, from natural blocks, such as waterfalls and rapids, to man-made barriers such as dams and weirs (Silva et al., 2018). Dams are large structures built to block and hold large bodies of water (Mueller et al., 2011) in order to store water for human consumption and create energy (Fu et al., 2010). Weirs are smaller structures that allow water to pass overtop, while controlling water flow and create energy (Mueller et al., 2011). These structures create habitat fragmentation, a major threat to aquatic biodiversity as they block crucial nutrients passing through river systems, change water flow and water levels, and, above all, prevent crucial migration and movements that stunt life history stages such as reproducing and feeding (Pelicice et al., 2015).

Mitigation strategies to increase safe passage for fish are now regarded as one of the most efficient and effective methods associated with solving artificial barrier problems in rivers

(Welsh & Aldinger, 2014). Fish passes are any structure purposely built to enable safe movement of a fish past an obstacle (Silva et al., 2018). The positive effects of fish passes are seen rapidly when put in place (O'Hanley et al., 2013). In Europe, fishways have been constructed since the middle of the 18th century and by the 19th century salmon fishways or ladders had been built and installed into Norwegian rivers with the initial intention to allow salmon to pass over natural waterfalls instead of passes built to navigate over dams or weirs. In 1842, Irish laws stated that weirs are required to have fish passes that allow safe passage for salmon as part of the Fisheries Act (Katopodis & Williams, 2012). In the 19th century the UK followed with the introduction of fish passes on weirs, although in the 1860s it was noticed that most salmon passes were not successful. The ones that did work well (i.e. pool-type fish pass; Fig. 1) were then replicated and the information was passed to weir and dam owners so they could build and/or maintain effective passes (Katopodis & Williams, 2012).

The science of fish passes has developed greatly in recent years, with more recent fish pass construction considering not just safe passage for the target species, but also change in water velocities and flow patterns to make it compatible with certain behaviours and swimming capacities (Silva et al., 2018). Traditionally fish pass constructions did not consider such ecological effects and provided some unknown and unwanted negative effects, such as movement of sediment and changes in depth; this compromised the sustainability of fish populations (Mclaughlin et al., 2013). Some of the main fish passes are 'pool-type fish passes' (Fig. 1), where a series of pools is constructed to divide the height of the pass. This is to ensure dissipation of the waters energy and to give resting areas for fish. Another type of pass is a 'natural-like bypass channel' (Fig. 2), which is a channel created around an obstacle that uses the natural flow of the water that had been previously lost by the obstacle constructed. 'Denil fish passes' (Fig. 3) are mainly used for Atlantic salmon as a flume is developed with baffles

and a steep slope to reduce the velocity of the flow. The resting pools are at greater distances meaning this type of pass is selective to strong adult fish. There are also specially designed passes for eel that use bristles so the fish can use grip to navigate through the pass (Welcomme & Petr, 2003).

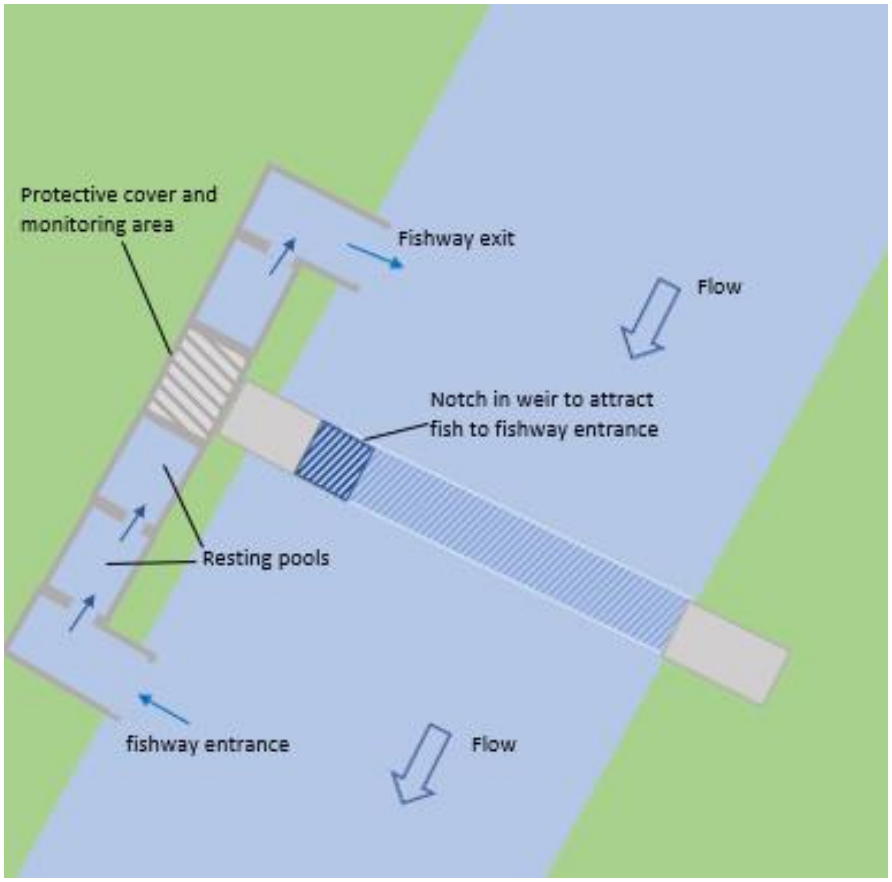


Figure 1: Pool type fish pass

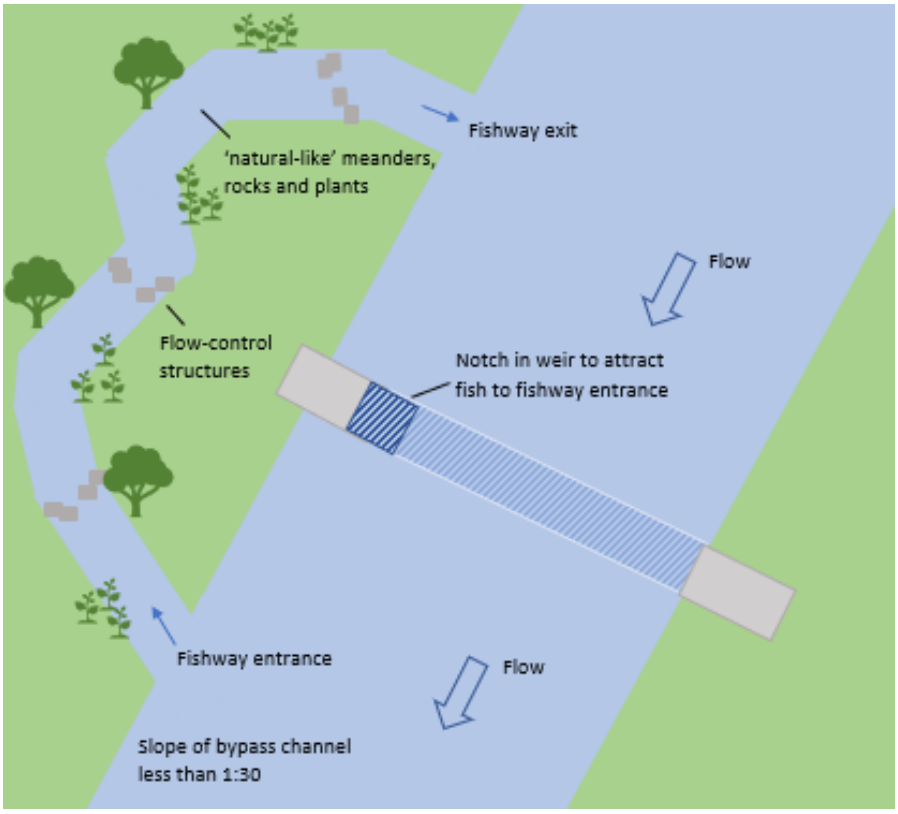


Figure 2: Natural-like bypass channel

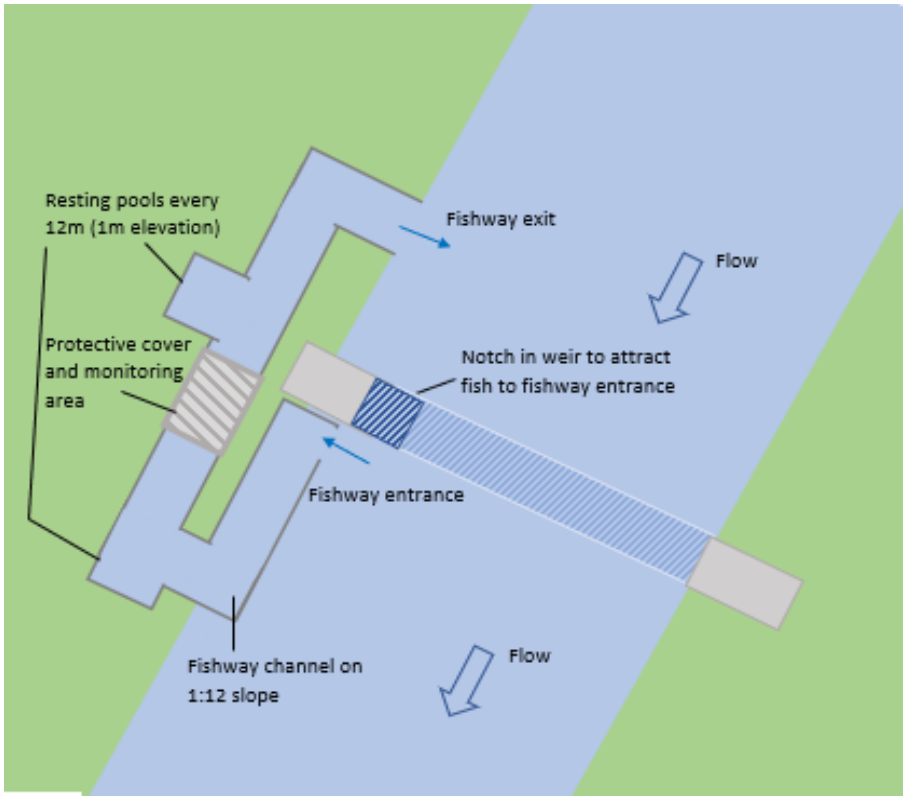


Figure 3: Denil fish pass

‘Pool-type fish passes’ have shown great improvement on migratory species numbers, including Atlantic salmon (*Salmo salar*), whereas Denil fish passes only facilitate salmon. This weir style has shown increases in migrating salmon both entering the river to reproduce, and fish returning to the sea to feed and grow (Nyqvist et al., 2017). Another example of the positive impacts of fish passes is in the river Fre-mur, France, where the critically endangered European eel (*Anguilla anguilla*) resides. Three large dams had blocked longitudinal connectivity of the river and, consequently, eel numbers declined, until the installation of eel passes, after which numbers increased again. This shows how eel passes are crucial to recovering and conserving eel stocks (Laffaille et al., 2005). In the Ebro River, Spain, twaite shad (*Alosa fallax*) were once widespread until the beginning of the 20th century, when their numbers began to decline with the building of dams, weirs and an increase in water pollution. Twaite shad have vulnerable status in Europe due to the declines in populations and in 2004, in the Ebro River small numbers of adult shad were caught up stream from a weir with a fish pass; but not after Xerta weir, which does not have a pass. This highlights the need for fish passes, allowing the species access to over 60 km of spawning habitat (López et al., 2007). The success of a fish pass needs to consider what species can achieve and tolerate the conditions created by the structure. Three hundred and fifty river lampreys (*Lampetra fluviatilis*) were tagged on the River Derwent in the UK to monitor the passage success at Crump weir. Only one individual out of the 350 successfully ascended the weir (0.3% success rate). This pass was modified to reduce flow on the fish pass, consequently out of the 169 individuals tagged, 12 individuals successfully passed, a 7.1% success rate (Tummers et al., 2016). Although there was an increase in passage success, more research is needed to modify and optimise the success of migratory species across weirs to complete their life cycles.

The challenges for fish to bypass man-made obstacles such as dams and weirs have been thoroughly studied in adult anadromous salmonids and clupeids (López et al., 2007). Migration patterns are now well investigated, but far less research has looked at the impact such obstacles have on other life history stages of migratory species, and the effects on resident species (Baras et al., 1994). A study in the Brazilian Paraná River showed how although some adult migratory species can negotiate the passes in place on weirs, some eggs and offspring which are laid above weirs can get washed down stream back through the passes and juvenile stages are unable to get back to the areas in which the adults spawned due to the acceleration in velocity created by the fish pass. It is crucial that these juveniles start their life-cycle in areas in which they were born, as this provides the food and shelter they need (Agostinho et al., 2007). Another issue with fish passes is that many sedentary species who take on fine-scale movements within a river, are unable to pass through these extremely fast flowing passes, risking isolation leading to population decreases and demographic extinctions (McLaughlin et al., 2013). An example of these negative impacts is the common barbel (*Barbus barbus*), which relies on thermal and flow elements in river systems to trigger spawning activity. Adult males and females will move to favourable conditions to maximise survival of their offspring. When weirs and dams are used to modify a river, the change in flow and thermal properties can significantly affect the spawning synchronisation. This is what happened in the River Meuse, Belgium, along with other European rivers, showing that weirs and dams constitute one of the main reasons for the declining populations (Baras et al., 1994). The construction of fish passes can vary, and there are many different types of ladders/passes that will differ in length and structure depending to the size of dam or weir. This can be an important factor for some species; for example common carps (*Cyprinus carpio*) can pass through shorter fish passes but do not have the

capability to negotiate longer ones (Slatick & Basham, 1985). These examples highlight the issues and challenges faced in order to re-connect rivers and allow natural movements of water and species that migrate and move through these waterways.

1.3 Migration

Migrations (predictable movements of species in space and time) are prevalent all over the animal kingdom, as species shift habitats and exploit resources through seasonal variation. Migrations are often linked with reproductive success (Jørgensen at al., 2008). These migrations are crucial in connecting ecosystems as nutrients, carbon and pathogens are carried within organisms and moved into other ecosystems (Childress & Mcintyre, 2015). Many river systems are now going through extreme connectivity diminutions due to dams and weirs significantly effecting longitudinal movements, whilst the effects on flow continuity through the uncertainty of stream discharge are altering temporal connectivity (Chappell et al., 2019). Thus, the results of these constructions greatly reduce connectivity by fragmenting water ways. This reduction of connectivity has been instrumental in the decline of migratory species and has caused local loss of populations, together with pollution (Newton et al., 2018). The interference with sediment and nutrients transport along with altercations of natural migration patterns, has put great stress on species that depend on these waterways to complete essential stages of their life cycles (Garcia De Leaniz, 2008). A challenge which arises from discussing migrations is the definition of the behaviour; although some species migrate over great distances and through many habitats, many species undertake fine scale migrations to perform certain activities within a habitat (Lennox et al., 2019).

Migration is a term used broadly for any species that moves to exploit new environments through the benefits of seasonal change (Jørgensen et al., 2008). Diadromy is the broader definition applied to organisms that move between freshwater and the sea. There are approximately 250 fish species that are defined as diadromous, although this behaviour has been described in freshwater molluscs and crustaceans too (McDowall, 1997). There are three migratory strategies for diadromous migrations: anadromy, catadromy and amphidromy (McDowall, 1999). Anadromy refers to species that spend most of their life at sea, growing and feeding before entering freshwater for reproduction. Catadromy refers to species performing most of the feeding and growing in freshwater, then reproduction is completed by migration into the sea. Finally, amphidromy refers to larval fish migrating into the sea after hatching in freshwater, initially feeding and growth in the sea, followed by small juvenile fish migrating back into freshwater (McDowall, 1997). Being able to study such dynamic populations, in order to protect them is hugely difficult in the case of diadromous species. They are predominantly dependent upon fluent connectivity between marine, estuarine and freshwater habitats to complete their life-history cycles (Tamario et al., 2019). This is due to how the life cycles span over different habitats, and how the dynamics are affected by both anthropogenic and natural forces that apply pressures over time and space (Bevacqua et al., 2015). Although most diadromous fish have great ecological, economic and societal value, most are threatened or close to extinction (Martignac et al., 2015).

1.4 Monitoring techniques

Monitoring is essential to enable adaptive management but is given insufficient attention in conservation management plans (Rees et al., 2020). With more than a third of European freshwater fish species either endangered or threatened, there is an evident need to monitor and understand life in river systems in order to protect them (Sigsgaard et al., 2015). There are many methods that can be utilised to monitor and assess freshwater fish biodiversity. Monitoring aquatic systems is notoriously difficult due to challenges in accessing suitable sampling sites, predicting changes in this highly mobile system and being able to find, identify and correctly sample the species that use, inhabit and transit these dynamic systems.

I. "Traditional" methods

There are several methods that have been traditionally used to collect data about biota in aquatic environments, such as netting, trapping, tagging and electrofishing. These methods require large amounts of effort and man power and can be only deployed in favourable conditions, leading to potentially biased conclusions (Smart et al., 2015). Such trapping methods can be highly intrusive and stressful to the individuals (Glover et al., 2019; McDevitt et al., 2019; Snyder, 2003), thus becoming highly problematic if the target species are in small abundance and part of recovering populations. As well as its invasive nature, these methods can miss the target species because of their rarity or body size. This can produce false results and cause mis management of conservation efforts, as the species not accounted for may need certain conservation strategies for their survival (Smart et al., 2015). Electrofishing is a widely used and seen as an effective way of monitoring fish biodiversity. It is the officially recommended sampling method for river fish by the EU Water Framework Directive (WFD)

(Beier et al., 2007) but requires numerous and well-trained staff, which can be expensive. In larger rivers due to the amount of water, detecting rare species by electrofishing becomes difficult (Pont et al., 2019). Other issues can arise when thresholds of electrical intensity are breached and those can vary depending on the species and life stages. Sudden changes in voltages may damage animals by provoking seizures and leading to spinal injuries as previously reported for several individuals (Snyder, 2003). Apart from the invasive and potentially harmful nature of electrofishing there can be unreliability issues, if electrofishing devices are not calibrated regularly and correctly, so not all fish will respond to the electricity produced, giving biased results (Glover et al., 2019).

II. Remote camera technology

An essential tool that has emerged as a successful monitoring device across many habitats and at different scales is remote camera imagery. Cameras with multi-methods can be used in aquatic environments, providing a new approach and giving new perspectives in understanding rivers (Bicknell et al., 2016). On the Igarapava Dam fish ladder in Brazil, an experiment comparing human observation and video images was conducted to see the most effective way to monitor fish species on a fish ladder. As expected, the experiment showed significant differences between the two approaches and concluded the video technology was the most accurate method due to the reduction of human errors and the possibility of constant monitoring (Bowen et al., 2006). Cameras that are used to count fish provide non-invasive monitoring and enable the ability to acquire free fish movement data; something not achievable with intrusive methods such as trapping, tagging and electrofishing (García-Vega et al., 2017). However, some of the main issues that arise from aquatic camera monitoring, are how multiple fish moving through such fish counters simultaneously, are counted as one

individual; therefore, underrepresenting the population. Also, the minimum fish height registered is capped on some devices, meaning fry, juvenile and small body size fish can be underrepresented (Farrell et al., 1998; García-Vega et al., 2017). Another prevalent issue that comes from using high-tech technology is the cost of the device and its maintenance. As well as the high costs to keep cameras running and maintained, technical problems can arise, resulting in the loss of data. This been said, camera technology is now much more affordable, reliable and is widely used to monitor aquatic species (Bicknell et al., 2016; Martignac et al., 2015; Welsh & Aldinger, 2014)

III. eDNA metabarcoding as a biomonitoring tool

As many aquatic species are rare and populations can be small, they are difficult to detect. This makes it challenging for many monitoring methods to detect and identify many species within the environment (Bracken et al., 2019). Molecular approaches to monitor biodiversity have seen huge advances in recent years, and have revolutionised the field of ecology (Bylemans et al., 2019). Environmental DNA (eDNA) refers to the presence of DNA molecules left in an environment by an organism. This eDNA can be filtered and extracted in order to identify the presence of species (Deiner & Altermatt, 2014). Total eDNA composes of cellular DNA from living cells and extra cellular DNA subsequent from natural cell death and the breakdown of cell structure (Civade et al., 2016). DNA can be found in various environmental media such as water, air, ice or soil and can be shed from skin, mucus, faeces, urine, gametes and other biological substances (Deiner & Altermatt, 2014). DNA retrieved from the environment can therefore be used to detected target species using suitable molecular markers. In order to detect a single species, narrow-target markers are used via Polymerase

Chain Reaction (PCR) or quantitative Polymerase Chain Reaction (qPCR) amplification (Piaggio et al., 2014). To get a broader range of biodiversity within an ecosystem, universal markers and parallel sequencing are used to target whole communities; this is called eDNA metabarcoding (Deiner et al., 2017). Environmental DNA metabarcoding is taxonomically comprehensive, efficient, cost effective and allows the possibility to detect species unknown to an ecosystem (Ji et al., 2013). Barcoding campaigns managed through the Barcode of Life Database (BOLD), have provided a collection of barcoded sequence tags that enable comparisons for eDNA studies (Ji et al., 2013). Previously these methods used cloning vectors which were time consuming and expensive but since its advent, Next Generation Sequencing (NGS) has revolutionised this field, due to its rapid and cost effective sequencing approach (Pawlowski et al., 2014). The analysis of eDNA through NGS applications has greatly improved biodiversity assessments within biological research. Massive amounts of sequencing data have led to better representations of diversity and this development is rapidly growing within eDNA research. The field is still relatively new and a much more understanding on the ecology of eDNA molecules and how best to represent the data is needed to implement in conservation management (Pawlowski et al., 2014; Shokralla et al., 2012).

As mentioned, being able to identify species with low abundancy has always been a challenge for monitoring in aquatic environments, but eDNA monitoring has been verified as a powerful tool in detecting the presence of even a few individuals (Ficetola et al., 2008). As eDNA is released from an organism it gets transported, diluted and degraded, giving eDNA a relatively short persistence in the water column (Ostberg et al., 2019). The literature shows that persistence of eDNA in water is usually from one day to two weeks, with a much higher detection rate within the first 72 hours (Barnes et al., 2014). This being said, some show in

the right conditions (i.e. cold and slow moving waters) eDNA can persist for more than a month (Hansen et al., 2018). The persistence of eDNA can also vary due to species-specific shedding rates and differential degradation of DNA in diverse habitats (Calderón-Sanou., 2019). A challenge in applying eDNA monitoring in lotic systems is that an organism's DNA can be transported downstream, thus questioning the occurrence of a species in the sampled location (Deiner & Altermatt, 2014). Studies have shown that there may be species specific transport distances, and that seasonality can also influence the distance in which DNA is detected. This infers chemical processes such as changes in pH and oxygen levels through seasonal change can influence DNA breakdown and must be taken into consideration (Deiner & Altermatt, 2014; Jane et al., 2015). Studies have been conducted evaluating the transportation of eDNA in lotic systems (Cristescu & Hebert, 2018; Deiner & Altermatt, 2014; Jane et al., 2015) however, far less studies have investigated the effects of tidal transportation of eDNA. The literature on the topic is not conclusive: highlights of false positives of species detection, due to tidal transportation (Jeunen et al., 2019) are reported while stability in detection over time and tide, across geographic sites (Kelly et al., 2018) is also described, with both studies suggesting that intertidal eDNA research should focus more on ecological variables such as salinity and temperature (Jeunen et al., 2019; Kelly et al., 2018). Studies focusing on the behaviour of eDNA in lotic and estuarine systems (both river flow or oceanic tides), suggest that influences on eDNA detection come more prevalent from ecological factors such as temperature, pH, salinity and oxygen (Barnes et al., 2014; Deiner & Altermatt, 2014; Kelly et al., 2018; Strickler et al., 2015). An experiment by Strickler et al. (2015) focussed on the effects of temperature and pH levels on the persistence of eDNA in aquatic habitats: found that colder and more alkaline aquatic environments holds eDNA longer than warmer and neutral or acidic habitats, making detection of rare or elusive species harder in warmer

months of the year and highlighting the need for well thought out sampling methods to maximise the chance of detecting such species. Less research has been done on the effects salinity and oxygen levels have on eDNA molecules but multiple studies suggest environmental conditions play an integral role in the fate and state of eDNA once shed from its host (Barnes & Turner, 2016; Jeunen et al., 2019; Strickler et al., 2015). Monitoring and understanding the movements and behaviour of eDNA within the water can provide crucial understanding on spatial and temporal distribution of aquatic species (Ostberg et al., 2019). Environmental DNA approaches are now widely applied but the best processing practices are still debated within the literature (Kumar et al., 2020). It is thus important to understand the behaviours of organisms in aquatic systems, along with the ecology of eDNA before making any incisive conservation decisions (Barnes & Turner, 2016).

A critical step in eDNA research is the collecting process, as the sampling methodology (i.e. location and number of replicates) is crucial to the subsequent data analyses. In addition, samples are taken on a specific day and time and can only be completed once, unlike other steps (PCR, bioinformatics and statistical analyses) which can be repeated (Dickie et al., 2018). Furlan et al. (2016) conducted a study on the sensitivities of eDNA surveys, reporting on the methods used out in the field (e.g. number of samples, volume and locations of sampling). Methods such as replicating samples through the water column were important in detecting species that congregate at different depths. Similarly, the importance of spatial and temporal sampling is highlighted due to congregations of fish in certain locations along with movements over time. Another important finding which is also found among other studies, is that an increase in the number of sample replicates or PCR replicates per sample will increase the probability of species detection (Deiner et al., 2017; Furlan et al., 2016). This though does not

consider time and cost, as an increase of field samples and PCR replicates would cost much more (Furlan et al., 2016).

As previously mentioned, eDNA can degrade at a fast rate. To overcome DNA been lost after the samples have been collected at the field, it is important to quickly filter the DNA and either extract within 5 hours of filtering or preserve in the appropriate way. This is especially important when tracking rare species where DNA copy numbers could be low. If water samples cannot be filtered on site the appropriate storage methods include chilling, freezing or adding preservatives (e.g. lysis buffer), although best practices indicate that water samples are best filtered on site (Kumar et al., 2020; Spens et al., 2017).

A crucial step in the eDNA sampling process is the filtration step. A fine mesh within the filter is attached to syringes (manual filtration) or a filtration machine (automatic filtration) to capture DNA as water passes through (Spens et al., 2017). Many different filters have been utilised to extract DNA and it is still debated what filter is best (dependant on the environment sample; Furlan et al., 2016). A popular filtering method uses syringes (e.g. Sterivex filter); this enables numerous processing of samples independently, which greatly reduces contamination (Lacoursière-Roussel et al., 2018). Pore size within the filters can greatly affect the recovery of eDNA: larger pore size filters can decrease the time taken to filter but reduce the recovery of eDNA, whereas smaller pore size can increase time filtering but recover more eDNA (Deiner et al., 2018). This trade-off is an important consideration when sampling for eDNA. Freezing the filters at -20 °C after DNA filtration is most widely used (two-thirds of published aqueous eDNA surveys) before DNA extraction (Spens et al., 2017). Other preservation methods include liquid preservations or drying in silica beads/gel. Conflicting

results through studies have shown that DNA yield has fluctuated across studies comparing these methods (Furlan et al., 2016; Kumar et al., 2020).

The choice of the metabarcode in any DNA metabarcoding study is critical on the impacts it has on the results. Therefore careful consideration must be taken to ensure the definition of the target taxonomic group, the level of taxonomic resolution, the size of the selected barcode fragments and how distinguished the sequence reference database is for the target taxa (Taberlet et al., 2018). When looking for communities and aiming to identify fish previously not reported in a habitat, it is crucial to choose the correct genetic marker and primer set. Mitochondrial cytochrome c oxidase subunit I (COI) and mitochondrial 12S (12S) primers are the most common used in eDNA studies (Deiner et al., 2017). A study done by Collins et al. (2019) demonstrated that 12S primers outperform COI primers as the latter showed low levels of reproducibility, thus suggesting 12S primers are best suited for metabarcoding studies. MiFish 12S primers (Miya et al., 2015) are used in many studies, especially for fishes and are known to be able to detect all fish that are found in the UK (Handley et al., 2019; McDevitt et al., 2019).

In recent years eDNA analysis has revolutionised the way aquatic monitoring is performed due to its non-invasive, high sensitivity, and cost-effective capabilities. Studies have recently found that eDNA metabarcoding is up to 3.5 times more efficient in detecting species richness in rivers than the electrofishing method (Pont et al., 2019), even though there are still many questions and challenges for eDNA metabarcoding to be overcome, as mentioned throughout this section.

1.5 A recovering river: the river Mersey

The river Mersey forms as the river Goyt and the river Tame meet in Stockport, England and then it flows through two major urban areas, Manchester and Liverpool before finishing its 110 km journey into the Mersey estuary and out into the Irish Sea (Mersey Basin Campaign, 2010). The river Mersey and Mersey estuary once had prolific fisheries, but as the industrial revolution flourished in the mid-1800s the water quality deteriorated and the Mersey was known infamously as one of Europe's most polluted rivers (Jones, 2006). As the rise in industry escalated, the 1820s brought a rapid human population increase. Anecdotal evidence showed that water quality had been clean before the start of the Industrial revolution, but as chemical and cotton industries arose, pollutants were pumped into the river. By the 1950s, this same anecdotal evidence showed there was no fish in the river (Burton, 2003). As new water quality legislations were introduced in the 1970's, there was an improvement in water quality and in 1999 there was video footage of salmonids negotiating weirs on the river Bollin, which is a tributary off the Manchester Ship canal and runs parallel to the river Mersey (Jones, 2006).

Woolston weir stretches across the river Mersey in the town of Woolston, close to Warrington. It was built in the early 1990s to replace a former construction and is key to regulating water levels on the river Mersey and the Manchester Ship canal (Peel Energy, 2012). To assess the population of salmon and other species on the Mersey, in the year 2000, the fish pass that was in place at Woolston weir was adapted to a 'pool-and-notch' type pass. Before this the weir was seen as impassable to salmon except in high flows (Tonks et al., 2009). Only nine species have been identified in 10 years of sampling (2001-2011) at the Woolston weir fish pass (Table 1; Peel Energy, 2012). The sampling periods each year varied in length, but all sampling was conducted in late autumn by the Environment Agency. For

Atlantic salmon to reach their spawning grounds, Woolston weir is an obstacle they must cross and is seen as a key location for monitoring efforts (Peel Energy, 2012).

Table 1: Fish trapping data over a 10-year period at Woolston weir, collected by the Environment Agency (Peel Energy, 2012).

Species	**Common name**	**Year**										
		2001	**2002**	**2003**	**2005**	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**	**Total**
Salmo salar	Atlantic salmon	3	26	1	42	8	35	45	3	30	16	**209**
Salmo trutta/morpha trutta	Brown/ sea trout		3	3	5	2	1	1				**15**
Salmo trutta	Brown trout								1			**1**
Squalius cephalus	Chub				3		12	3	2	5		**25**
Leuciscus leuciscus	Dace		2									**2**
Anguilla anguilla	European eel					15						**15**
Perca fluviatilis	Perch					1			1	2		**4**
Lampetra fluviatilis	River Lamprey		3									**3**
Total		**3**	**34**	**4**	**50**	**26**	**48**	**49**	**7**	**37**	**16**	**274**

This is the only fish data available from this section of the river Mersey, from the fish pass at Woolston weir. This sampling was performed in autumn/winter annually (Energy, 2012), so this data only accounts for species who have the ability to negotiate the pass, and are present in this part of the river in the months of sampling.

1.6 Migratory species in the river Mersey

Diadromous fishes have complex life cycles, relying on different habitats for their survival, leaving them vulnerable to anthropogenic pressures (i.e. pollution, global warming, overfishing and artificial barriers). In spite of their ecological, cultural and economic value, diadromous species have suffered global declines and collapse of populations (Legrand et al., 2020). Across Europe most diadromous fish species are endangered (IUCN, 2020) and the importance of understanding these species and their complexed life cycles is imperative to

install correct conservation techniques (Lassalle et al., 2008). The river Mersey was once a clean and prolific fishing ground with historical records showing an abundance of diadromous fish, before industrial pollution made the river lifeless (Burton, 2003). A steady recovery has seen some key UK freshwater migratory returning to the river and gives hope to the potential of other returning diadromous species.

Atlantic salmon (*Salmo salar*) are anadromous ray-finned fish, in the family Salmonidae (Webb et al., 2007). The species is native to subarctic and temperate areas of the North Atlantic Ocean (Thorstad et al., 2011). The population of wild Atlantic salmon has been declining throughout its native range. There are many reasons for such decline, including dewatering, pollution and the building of dams and weirs which prevents migration to spawning grounds (Parrish et al., 1998). A report by the International Council for the Exploration of the Sea (ICES 2011) showed catching decreases falling 90% within a forty-year period from the start of the 1970s, from approximately 10,000 to 1000 tons. Being anadromous, Atlantic salmon are reliant on favourable conditions in both marine and freshwater environments. Atlantic salmon are also dependant on healthy conditions in the brackish waters of estuaries, as they will use these transition zones to undertake morphological changes, congregate and re-energise before the long migration into the river systems (Nicola et al., 2018). A fundamental part of Atlantic salmon life cycle is their ability to return to spawn in rivers in which they spawned and spent their juvenile lives. This ability is called homing and is still not fully understood (Webb et al., 2007), although chemical imprinting of natal streams is considered integral to this ability. If the rivers they return to are obstructed by damming, and no alternative route is available, there is no possibility for the adult salmons to return to their spawning grounds to reproduce (Webb et al., 2007). When

salmon enter river systems, it becomes easier to monitor them due to the more confined areas in which they migrate to spawn. This brings its own issues, as adult salmon migrating to reproduce expend lots of energy overcoming strong downstream currents, making them even more susceptible to injury caused by invasive monitoring techniques (Garcia De Leaniz, 2008). Most rivers in the England supported Atlantic salmon populations prior to the industrial revolution. The declines were recognised the most on the rivers Trent, Thames, Mersey and Medway (Mawle & Milner, 2003). After years of absence, in 2001, for the first time in decades, the first Atlantic salmon was caught in the river Mersey (Ikediashi et al., 2012), giving promise to the recovering river as salmon are key indicators of good water quality, relying on higher oxygen levels for their spawning behaviour (Webb et al., 2007). As mentioned, Atlantic salmon return to their natal rivers through homing abilities. This cannot be the case with the river Mersey as the fish were absent for many years. A genetic study researching the recolonization of Atlantic salmon on the river Mersey by Ikediashi et al., (2012) showed that adult Atlantic salmon had originated in other rivers around the UK and Europe but strayed (rare occasions when the homing ability does not work and a fish enters a non-natal river), therefore establishing a small population of Atlantic salmon in the river Mersey.

European eel (*Anguilla anguilla*) are catadromous fishes (Daniele Bevacqua et al., 2015). Geographically the European eel is found all over freshwater bodies in Europe and North Africa, but it has undergone drastic declines and is now critically endangered globally, according to the IUCN red list (Bevacqua et al., 2015). Eels move out of freshwater and travel approximately 5000 km to spawning grounds in the Sargasso Sea. Here the mature eels spawn and eel larvae migrate to European waters where the larvae develop into glass eels. Some stay in coastal waters but most enter freshwater for a period of years before this epic

migration starts again (Aarestrup et al., 2009; Kroes., 2020). Details of this epic migration are still poorly understood, with some hypothesising mature eel migrate vertically through the water column, in the Sargasso sea to spawn, making monitoring extremely difficult to an already difficult species to monitor, due to its many life stages and long migration (Aarestrup et al., 2009; Palstra et al., 2020; Tamario et al., 2019). The decline of *A. anguilla* has been attributed to many different causes including overexploitation, as the decline of eel became evident from overfishing in the 1960s, but the first focused restoration plans only began in 2003 (Bevacqua et al.,2009). Oceanic climate change is also having a negative impact on the species, due to the larvae depending on strong currents bringing them across the Atlantic Ocean. These currents are being altered due to climatic pressures (Tamario et al., 2019). In freshwater, poor placement and functionality of eel passes are key contributors to their decline. This is due to the arriving young eels searching for foraging grounds and having to negotiate weirs and dams along with natural barriers such as waterfalls. Eels are strong climbers but poor swimmers, therefore the conventional 'pool-and-notch' type pass is not suitable for eel as it is for strong swimming species, such as Atlantic salmon. This means suitable passes for climbing are required so they can negotiate and pass the obstruction, and although eels have this remarkable climbing ability, only small numbers successfully pass dams and weirs (Tamario et al., 2019).

Lampreys belong to the order Petromyzonidae. They are aquatic, jawless, and largely parasitic vertebrates that comprise 39 different species and are widely distributed in the Northern and Southern hemispheres (Renaud, 2011). There are three species of lamprey that are native to UK waters: the river lamprey *Lampetra fluviatilis,* the brook lamprey *Lampetra planeri* and the sea lamprey *Petromyzon marinus*. The brook lamprey is sedentary and the smallest of the

lampreys, it is found in streams and smaller rivers around the UK. Both the river and sea lamprey are anadromous, as the river lamprey usually migrates from British estuaries and coastal waters to spawning grounds in freshwater rivers. The sea lamprey migrates from the Atlantic ocean to freshwaters to spawn (Maitland, 2000). They are both priority species according to the Joint Nature Conservation Committee, (JNCC) and have seen declines in UK rivers (JNCC, 2019). They are very sensitive species: when the river Clyne saw an improvement in water quality, the Atlantic salmon (also highly sensitive to water quality) recolonized the river; river lampreys started making a return to this river 20 years after this, showing a much delayed recolonization (Maitland, 2000). Similarly, sea lampreys have struggled to maintain their spawning grounds across all UK rivers due to a number of reasons, including their poor ability to climb obstacles such as weirs and dams (JNCC, 2019).

Shad are diadromous fishes in the herring family (Clupedae). In north west Europe there are two main species: allis and twaite shad (*Alosa alosa* and *Alosa fallax*), both of which are classified as rare and are listed in the Bern Convention and Annex and vulnerable on the EC Habitats directive. Also, both species are included in section 9 of the Wildlife and Countryside Act (1981) which makes it an offence to deliberately block, damage or destroy spawning areas (Aprahamian et al., 1998; Coscia et al., 2010). The anadromous *A. fallax* has seen considerable declines since being hugely abundant and widespread across Europe. This is due to anthropogenic disturbances, primarily the building of weirs, due to reduced access to spawning grounds (Bolland et al., 2019). Although there have been no historical reports of *A. fallax* or *A. alosa* in the river Mersey, there has been no studies looking to identify their presence. *Alosa alosa* is not known to spawn in any rivers in UK at present (Maitland & Lyle, 2005) but the occurrence of *Alosa fallax* in the nearby river Severn and the other side of the

Irish sea in Irelands rivers (Aprahamian et al., 2010; Bolland et al., 2019; Coscia et al., 2010) gives promise they could be entering the river Mersey.

The **brown trout** (*Salmo trutta*) belongs to the salmonid family and is a socio-economically important species (Schreiber & Diefenbach, 2005). Sea trout is the name given to the anadromous form of *Salmo trutta*. Brown trout are commonly known as non-migratory, which confuses their classification (Harris & Milner, 2004). This confusion has been recorded on the river Mersey (table 1) as brown and sea trout have been recorded as both the same species and separate species (Energy, 2012). The migratory form of *S. trutta* (sea trout) maintains gene flow in the populations and reduces the effect of genetic drift (Bekkevold et al., 2019). This form of *S. trutta* is also most at risk due anthropogenic pressures, including climate change, overfishing and barriers such as dams and weirs (Bekkevold et al., 2019). Some individuals show partial migration where they enter estuaries to feed and return to freshwater to spawn. Overall, there is a poor understanding of how *S. trutta* use marine and estuarine ecosystems (Honkanen et al., 2020), highlighting the need for more research on the species.

Many of the diadromous species described above have well studied migrations patterns in the Mersey and surrounding rivers (table 2; Coscia et al., 2010; Goodwin et al., 2008; Ikediashi et al., 2012; Jensen et al., 2018; Jones, 2006). As mentioned the *Alosa* species, not reported for the river Mersey but found in the relatively close river Severn (250 km south) (Bolland et al., 2019) and S. *trutta,* due to the still open debate to distinguish anadromous and sedentary forms (Harris & Milner, 2004).

Table 2: Migration table of approximate dates in which some species may enter or leave the river Mersey based on the literature presented within the table. 'Upstream' and 'downstream' represents the direction in which the matching life history stage of the species is migrating within the river.

<table>
<tr><th>Species</th><th>Salmo salar</th><th>Lampetra fluviatilis</th><th>Alosa fallax</th><th>Alosa alosa</th><th>Anguilla anguilla</th></tr>
<tr><td>Common name</td><td>Atlantic salmon</td><td>River lamprey</td><td>Twaite shad</td><td>Allis shad</td><td>European eel</td></tr>
<tr><td>Migration behaviour</td><td>Anadromous</td><td>Anadromous</td><td>Anadromous</td><td>Anadromous</td><td>Catadromous</td></tr>
<tr><td>February</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>March</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>April</td><td rowspan="2">Juvenile/smolt (downstream) (Ikediashi et al., 2012; A. J. Jensen et al., 2012)</td><td rowspan="3">Juvenile/smolt (downstream) (Goodwin et al., 2008; Maitland, 2000)</td><td rowspan="3">Adult (upstream) (Magath et al., 2013; Maitland & Lyle, 2005)</td><td rowspan="3">Adult (upstream) (Maitland & Lyle, 2005)</td><td rowspan="2">Juvenile/glass eel (upstream) (D. Bevacqua et al., 2009; Tamario et al., 2019)</td></tr>
<tr><td>May</td></tr>
<tr><td>June</td><td></td><td></td></tr>
<tr><td>July</td><td></td><td></td><td></td><td></td><td rowspan="2">Adult (downstream) (Daniele Bevacqua et al., 2015; Kroes et al., 2020)</td></tr>
<tr><td>August</td><td></td><td></td><td rowspan="2">Juvenile (downstream) (Breine et al., 2017; Maitland & Lyle, 2005)</td><td rowspan="2">Juvenile (downstream) (Maitland & Lyle, 2005)</td></tr>
<tr><td>September</td><td></td><td></td><td></td></tr>
<tr><td>October</td><td rowspan="4">Adult (upstream) (Ikediashi et al., 2012; Thorstad et al., 2011)</td><td rowspan="4">Adult (upstream) (Maitland, 2000; Tummers et al., 2016)</td><td></td><td></td><td></td></tr>
<tr><td>November</td><td></td><td></td><td></td></tr>
<tr><td>December</td><td></td><td></td><td></td></tr>
<tr><td>January</td><td></td><td></td><td></td></tr>
</table>

1.7 Aims of the project

The current project has the following aims:

1) Apply and compare two non-invasive monitoring methods (camera technology and eDNA metabarcoding) to monitor migratory species and fish passage success at Woolston weir. Monitoring results will also be compared to historical data from previous studies at Woolston weir.
2) Perform a spatial and temporal analysis of the river Mersey's fish community diversity, with a transect sampling protocol from the estuary mouth near Liverpool, up to where the river Mersey meets the Manchester Ship canal near Lymm, Warrington. The sampling method will use samples from the lower Mersey estuary, upper Mersey estuary and the river Mersey and assess if anthropogenic barriers influence fish communities.
3) Compare autumn and spring eDNA results at the area around Woolston weir to detect migratory species, and if there are seasonal changes in fish community diversity.
4) Assess how environmental factors influence diversity and distribution of species along the river Mersey

2 Methods

2.1 Study site

The area of the Mersey catchment covers approximately 4650 km^2 in the North West of England (Preston & Raymundo, 1993). The river Mersey has a total length of 110 km (Fig. 4) and forms at the confluence of the river Tame and the river Goyt in Stockport, Greater Manchester (Mersey Basin Campaign, 2010). The Mersey estuary flows into Liverpool Bay, in the eastern basin of the Irish Sea. It is split into two sections: the lower Mersey estuary (outer estuary) which is defined from the estuary mouth until the Mersey Gateway bridge at Runcorn; and the upper Mersey estuary (inner estuary) which is defined from the Mersey Gateway bridge up to Howley weir in Warrington. After Howley weir everything upstream is considered the river Mersey until it meets the Manchester Ship canal near Latchford locks and the Thelwall viaduct (Fig. 4) (Lallias et al., 2015; Tonks et al., 2009). The Mersey estuary has a large tidal range varying between 4 and 10 metres. At its highest tide the water levels are known to overwhelm Howley weir and push tidal waters into the river Mersey (Wilson et al., 1988). Woolston Weir is located north of Howley weir and to the east of Warrington on the river Mersey. Woolston weir has a pool-type fish pass in which monitoring has taken place in previous years by the Environment Agency (Peel Energy, 2012). Howley weir has no working fish pass but historically had a lock that was used to bypass water around the weir, not currently in use (Falconer & Lin, 2003).

2.2 Vaki River-watcher

The River-watcher by Vaki (fish counting camera manufacturers, Iceland) is a high-spec fish monitoring camera that monitors migratory fish patterns. It is currently installed in over 300 monitoring fish ladders, passes and weirs in different physical conditions. The counter collects data as fish swim through the net of beams produced by the camera, which creates silhouette imagery of the fish and provides data on the fishes length, width and direction of travel, along with a short video clip to identify the species (Vaki River Watcher, 2018). The camera was installed in the fish pass at Woolston weir (Fig. 5) and started collecting data from the 6th of September 2018. Fortnightly data collected were downloaded until the camera was turned off on the 1st of March 2019. The downloaded data was analysed using Winari software (River watcher Manual, 2015). This enabled silhouette imagery and video clips in which each video was manually examined to identify the species of fish moving through the passage. The software enabled monitoring of direction of migration, size of fish, time and date and establishes any patterns over the observation period (Vaki River Watcher, 2018).

Figure 4: Location of the river Mersey (red dot in map of the United Kingdom in the right corner) and extent of the river Mersey from source to ocean from QGIS (QGIS 3.4.13). The red dots are sampling locations and the weirs are indicated on the top right. A) Lower Mersey estuary); B) Upper Mersey estuary; C) River Mersey. Howley weir (53.384731, -2.577445) is located furthest west on the B/C border and Woolston weir (53.39370, -002.52190) further east in section C.

Figure 5: a) Drone image of Woolston weir (courtesy of Dr Neil Entwistle) with the fish pass where the Vaki River-watcher has been installed (highlighted in red); b) Vaki River-watcher being installed in the weir; c) Installed and working Vaki River-watcher.

2.3 Environmental DNA sampling

Water samples were collected during two different seasons (autumn and spring), five sites sampled during the autumn and 14 sites sampled during the spring. The first sampling was conducted during the autumn season while the Vaki River-watcher was active in order to allow for a comparison between the two methodologies herein tested (River-watcher fish counter and eDNA metabarcoding). This sampling aimed specifically to monitor the salmon population during the upstream migration. The time periods were chosen to run parallel with the camera collecting data, and to sample within the salmon migration period which is between September and late December (Ikediashi et al., 2012). Samples were collected covering a three-week period in three sampling dates: 22nd November 2018, 29th November 2018 and 6th December 2018. In each sample site (N=5) three replicates were collected summing up to 15 samples each week. Additionally, one field blank was taken at the beginning of each sampling day and one at the end of the sampling period. In total, 49 samples were analysed for this season (45 eDNA samples, and four field blanks).

The second sampling period began in the spring and included a total of 14 sampling points (Fig. 7) from the estuary mouth near Liverpool all the way up to where the River Mersey joins the Manchester Ship canal. Water sampling started on the 6th May 2019 with temporal sampling repeated in the same locations on the 21st May and 3rd June 2019. The sampling locations were split into three habitats: A) lower Mersey estuary (sites 1-5); B) upper Mersey estuary (sites 6-9); C) river Mersey (sites 10-14) (Fig. S1). The dates were chosen as a period in which some key migratory species would be most active in the river, giving the best opportunity to record presence data (Table 2). Three replicates were taken at each location,

with three blank samples a day collected to apply control sampling throughout each sampling day. A total of 135 samples were collected in the spring sampling period.

All the water samples were collected using 2 L bottles and handled with nitrile gloves to limit contamination. However, as three of the sample points were elevated 1.9 metres from the river on a bridge, we used a 2.2 metre aluminium pole to lower the sampling bottle in the water. This pole was decontaminated using a 50% bleach solution before and after every sampling event. Filtering was conducted on site at the end of each sampling day, within 2 hours of sampling (Fig. 6). Between 70 ml and 600 ml of water was filtered (Table S1 & S3-S5) from each bottle using Sterivex 0.45 µM filters (Merck; www.merckmillipore.com), due to the difficulty levels of pushing different samples through the filters because of different sediment concentration in the water. The samples were then sealed and stored in a cooler box with ice packs and transferred to the eDNA sample freezer (-20 °C). The transportation time from finished filtering to freezer storage was less than one hour. Several environmental factors were also recorded, including salinity, pH, temperature and oxygen levels (Table S1 & S3-S5). Salinity was measured in parts per thousand (PPT), pH on a standard unit of 0-14, temperature in degrees centigrade and dissolved oxygen levels in milligrams per litre.

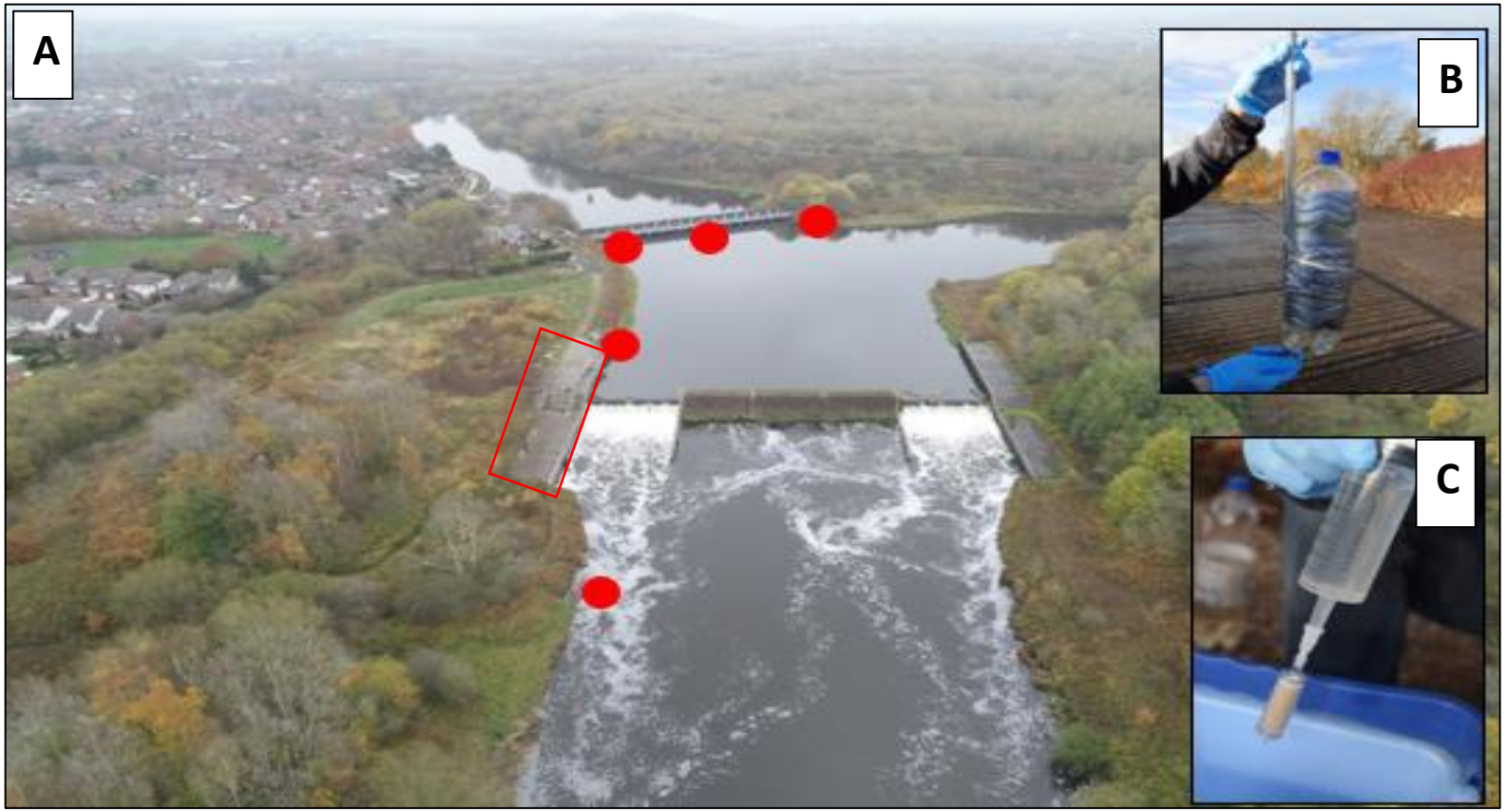


Figure 6: a) Sample locations (red dots) for the autumn sampling period and fish pass (red square) on Woolston weir; b) Water collection method attached to the pole (top right); c) Filtering method with syringe (bottom right).

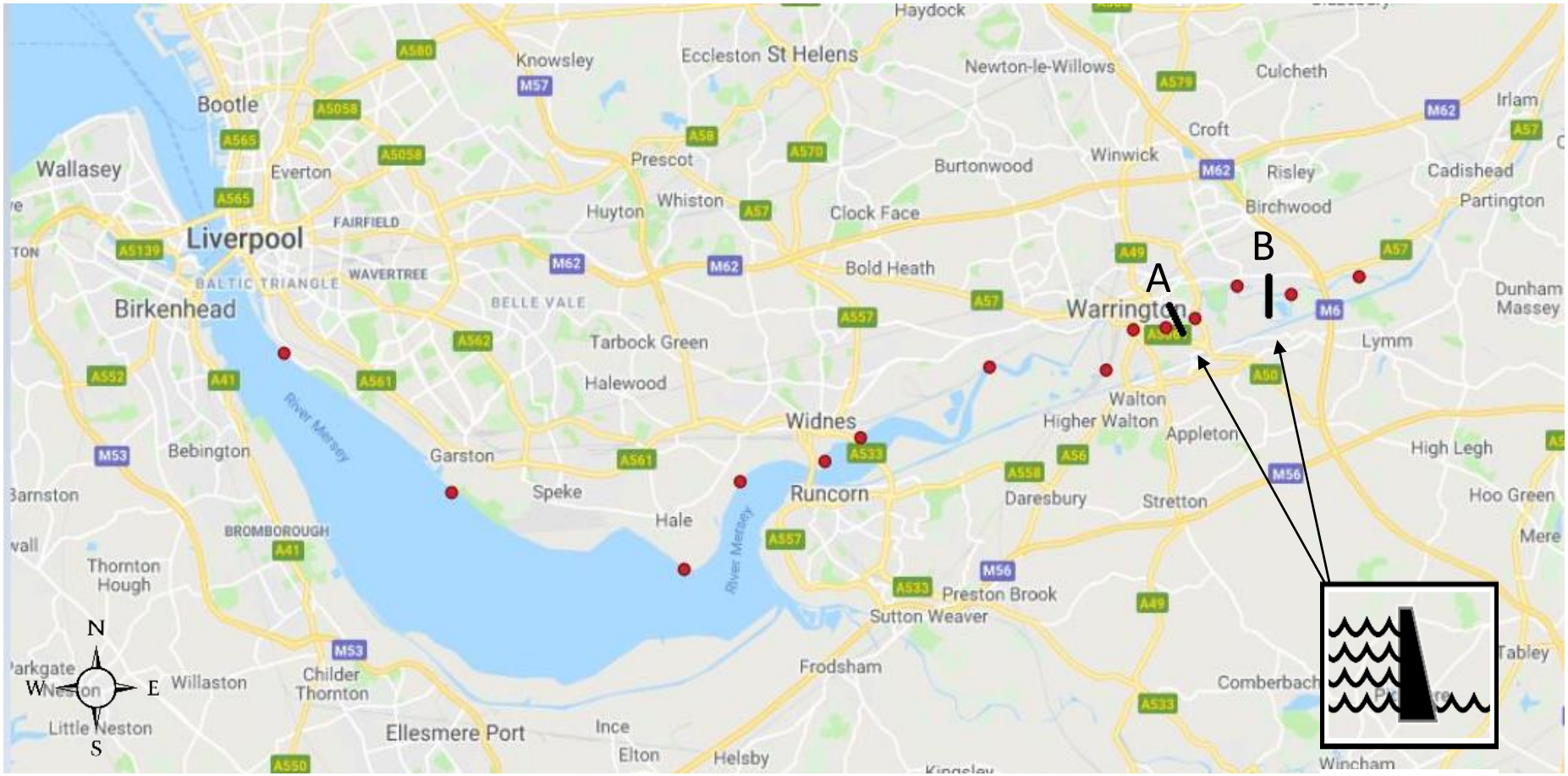


Figure 7: Sampling locations for spring 2019 (red dots), with location of the two weirs considered in this study. A) Howley weir; B) Woolston weir

2.4 DNA extraction and amplification

In a lab dedicated to eDNA research the field blanks were extracted first, for contamination control purposes, followed by the extraction of the samples. The MuDNA protocol (Sellers et al., 2018) was used for DNA extractions. For autumn sampling, three extraction blanks were added to the samples and in spring six extraction blanks were included, in order to identify in which stage, if any, a source of contamination was present (Tables S1 & S3-S5). Samples from both sampling periods were extracted and amplified following the same procedure, with the only difference being the number of samples (n=51 for autumn; n= 141 for spring, including field and extraction blanks). Purified extracts were then assessed using a Qubit fluorometer (Thermo Fisher Scientific) to quantify DNA. The primers used were the MiFish fish-specific 12S primer set (Miya et al., 2015) and were prepared separately under controlled conditions using forward and reverse combinations from a 24 plate primer set to create a 96 combo primer plate, with seven-base sample MIDs and a variable number (2-4) of fully degenerate positions, which increases variability in amplicon sequences (Table S2 & S6) . Amplicons of 169-172 bp from a variable region of the mitochondrial 12S rRNA gene were attained with the MiFish primers (MiFish-U-F,5'- GCCGGTAAAACTCGTGCCAGC-3'; MiFish-U-R,5'ACATTATCATAGTGGGGTATCTAATCCCAGTTTG -3', Miya et al., 2015). For each sample the polymerase chain reaction (PCR) contained a final volume of 20 µL which included 10 µL of Amplitaq, 0.16 µL of BSA, 5.84 µL of ultrapure water, 2 µL of primer, and 2 µL of DNA (Sales et al., 2019). This also included PCR blanks in order to again, be able to identify the stage of contamination, if present. The PCRs were then replicated three times to optimise detection of DNA (Fig. 8). The PCR profile conditions consisted of an initial denaturing step of 95°C for

10 minutes, 40 cycles of 95°C for 30 seconds, 60°C for 45 seconds and 72°C for 30 seconds with a final 5-minute extension step at 72°C (Sales et al., 2020). Visualisation of PCR products were seen with 1.5% agarose gel in an electrophoresis gel before library preparation (Cambridge Bioscience).

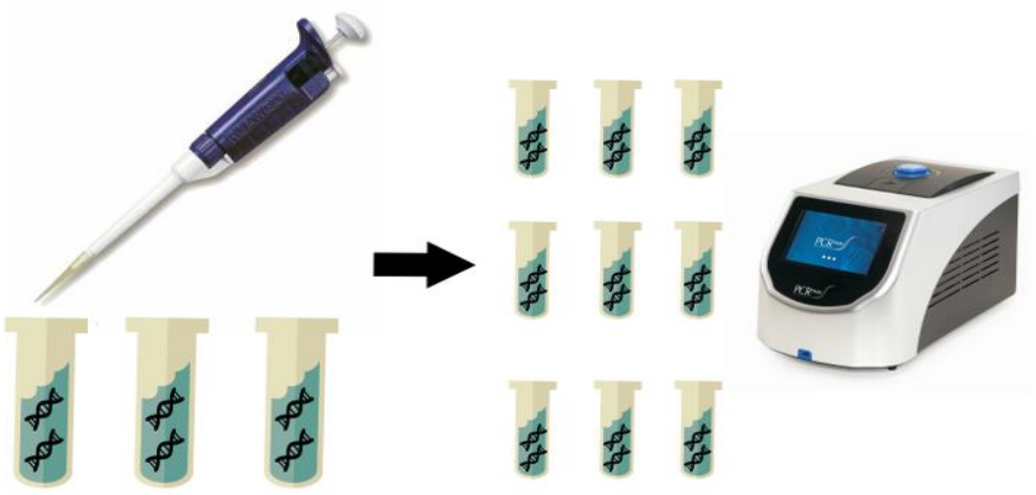


Figure 8: After extraction each sample is replicated 3 times ready for PCR amplification.

2.5 Library preparation and sequencing

The Agencourt AMPure XP (Beckman Coulter, 2013) protocol for left-size selection using a 1:1 ratio and KAPA Hyper Prep Kit for the library preparation (KapaBiosystems) were used for all samples in the two sampling seasons. The autumn samples were multiplexed into one library and had an adapter ligation added, as the sequencing run was to be combined with another project. The spring samples were halved and multiplexed into two libraries and had adapter ligation added. Both used two-plex, dual-index adapters. Qubit and TapeStation analysis were performed before moving from the clean-up stage to the library preparation, this was to re-assure correct DNA concentrations and efficient clean-up process. Using NEBNext qPCR quantification kit (BioLabs) the libraries were quantified and pooled in equimolar

concentrations alongside with 1% PhiX and sequenced using single multiplexed Illumina Miseq (www.illumina.com). The libraries were run at a final molarity of 9 pM on an Illumina MiSeq platform using the 2 x 150 bp v2 chemistry. The autumn library was run with a non-related project and the spring libraries were run as their own project.

2.6 Bioinformatics

After the completion of sequencing the original files were downloaded from BaseSpace (basespace.illumina.com). Raw files were unzipped and paired-end aligned using the *ObiTools* metabarcoding package (Boyer et al., 2016). Two output files were created using 'obiannotate', use only sequencing lengths of over 40 bp. The files were then demultiplexed with '*ngsfilter'* and then filtered by length with no '*N*' using '*obigrep*'. This selected fragments of 140-190 bp and removed any short fragments which remnant from the library preparation, such as primer-dimer. Strictly identical sequencing clusters was completed using *'obiuniq'* and a subsequent chimera removal step was performed within '*vsearch*' (Rognes et al., 2016). A SWARM 2.0 algorithm was used to delaminate the Molecular Operational Taxonomic Unit (MOTU) with a distance value of d=3 (Mahé, et al., 2014); this distance value was chosen as a threshold to avoid mismatching. Taxonomic assignment was performed using '*ecotag*' (Boyer et al., 2016) as it uses a custom reference database which includes all known sequences for vertebrates (Fig. 9) on a 12S fragment. To check any uncertain taxonomic assignments a standard nucleotide BLAST was performed against GenBank nucleotide database (NCBI). To avoid any false positives, sequencing errors or contamination, any reads detected in the negative controls (Table S9) were removed across the samples and any MOTU's containing 5 or less reads were removed from any further analysis (Cilleros et al., 2019; Sales et al., 2019).

To further clean up the data, only MOTUs assigned to species level were used, although MOTUs assigned at family level were put through a standard nucleotide BLAST (NCBI) in order to assign clear identification at species level; only results with 100% of query coverage and 97% similarity were kept (Keskin et al., 2016). MOTUs were assigned to species based on 97% sequence similarity (minimum identity 0.97). This threshold was based on published eDNA metabarcoding studies detecting a great diversity of fish species (Li et al., 2018; Sales et al., 2019). Finally, species that were considered as putative contamination were removed.

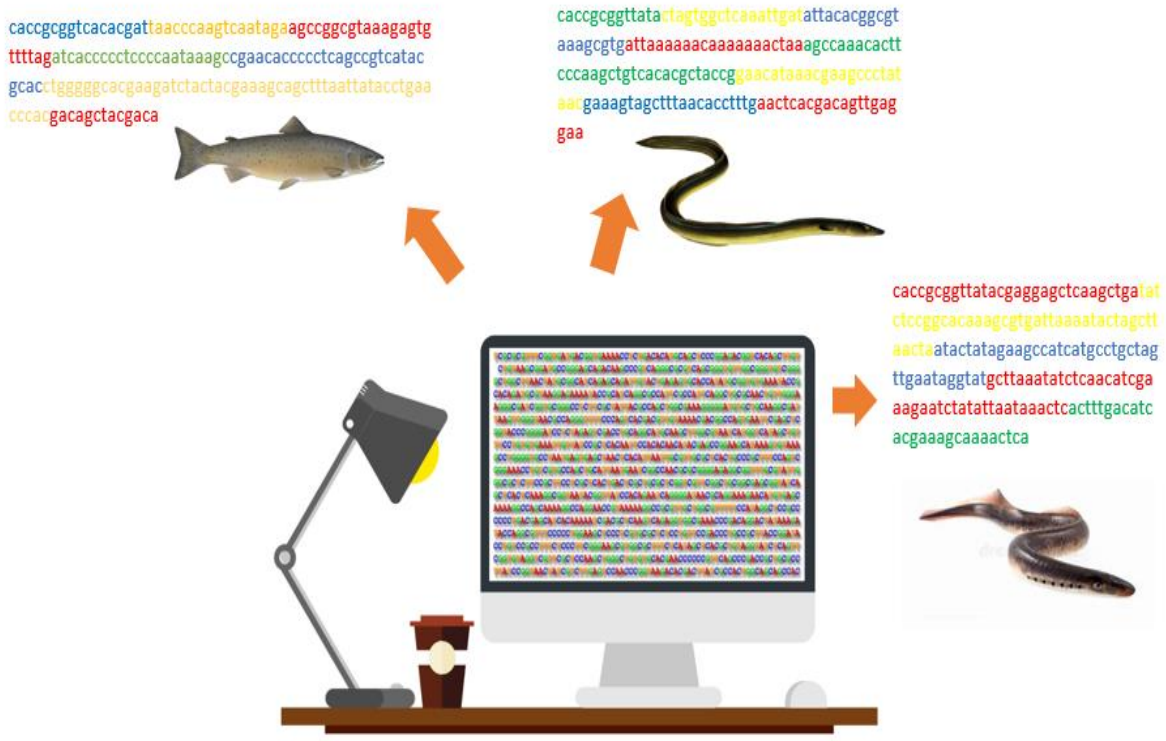


Figure 9: Bioinformatics bridges the results from sequencing into organised and readable data ready for statistical analyses.

2.7 Statistical analyses and visualisation of results

The camera data was uploaded onto the Winari software (Vaki River Watcher, 2019) and visualised as bar plots. For the eDNA analyses, to increase detection rates, replicates were pooled for each location. Autumn samples were analysed according to two sites: above and below Woolston weir, whereas spring samples were combined into 3 sections: 1) below Howley weir; 2) between Howley and Woolston weir; 3) after Woolston weir. For the temporal analyses, data was analysed into the dates in which the samples were collected, and further combined according to the season sampled to compare autumn and spring samples above and below to Woolston weir. All analyses and visualisations were performed in R v3 5.1 (R Cran, 2019). Using 'ggplot' with a 'geom_jitter' function a series of bubble plots were created to look at species detection and relative abundance across dates and sites. A Venn diagram was created in excel to compare trapping and molecular sampling techniques. Alpha-diversity (species richness) was calculated by the total number of species assigned to each sampling location across each sampling period in spring and then a combined richness across the spring sampling period. This was repeated with the autumn results for a seasonal comparison species richness at Woolston weir. The β-diversity was attained by applying the Jaccard distance which is based on presence or absence of species, using the vegan package 2.5-2 with the "vegdist" command (Oksanen et al., 2015). A simple 0 and 1 value were used in the Jaccard distance analysis, with 0 representing both sites sharing the same species and 1 representing sites having no species in common. The relationships were then visualised with Principle Coordinate Analysis (PCoA) plots that used the β-diversity matrix and the "cmdscale" command. This was also repeated to test seasonal differences between autumn and spring. The correlations of β-diversity with longitudinal distance and the presence of weirs, were analysed using the Mantel test, running the "mantel.rtest" function (Li et al., 2018). Distances

were calculated and obtained from google maps following the Mersey way path that follows the banks of the river (Google maps, 2019). The matrix for testing the influences of the weirs was constructed using distance values according to the presences of a weir between sites (i.e., 0 = no weir between sites; 1 = one weir between sites; 2 = two weirs between sites) (Sales et al.,2020). Finally, a Canonical Correlation Analysis (CCA) was performed using PAST (Past3, 2019) to look for correlations with species detected and environmental factors. For each site there was three replicates taken as mentioned in the methods. A score out of three was given for each species detected in each location (i.e., 0 = no presence of species recorded across all replicates; 2 = species present in 2 out of the 3 replicates; 3 = species present in all replicates).

2.8 Historical data

Historical data collected from multiple sources over a 19-year period (2000-2019) was compiled into a table (S11). To compare eDNA as a monitoring method to more traditional approaches, the data acquired from the Environment Agency (Peel Energy, 2012) and the Mersey Gateway Environmental trust was used to create a Venn diagram. This data was used as it monitored species in the same area as the eDNA sampling took place (Woolston weir) across multiple years (Table S12).

3. Results

3.1 Vaki River-watcher

The Vaki River-watcher camera started collecting data from the 10th September 2018 and was intended to run constantly for 168 days. No fish were detected until the 28th December 2018, although, during this time there was a 24-day period (23rd November 2018 - 17th December 2018) in which data was not collected. This was due to the camera frame positioning, which triggered the camera sensors, thus draining the petrol fuelled camera system and preventing any data collection. Five adult salmon were captured migrating upstream on the camera within four days from the 28th and the 31st December 2018; after these dates no more fish were detected on the camera up to the last data collection on the 13th February 2018 (Fig. 10). A 15-second video clip of the fish swimming is automatically filmed allowing for positive identification of Atlantic salmon (Fig. 11). A subsequent silhouette image is created by the Winari software of fish (Fig. 10), with length, swimming depth in the fish pass and swimming speed. These results show a small migration within a four-day period. In total five adult salmon were captured on the camera, with four of these being on the same day.

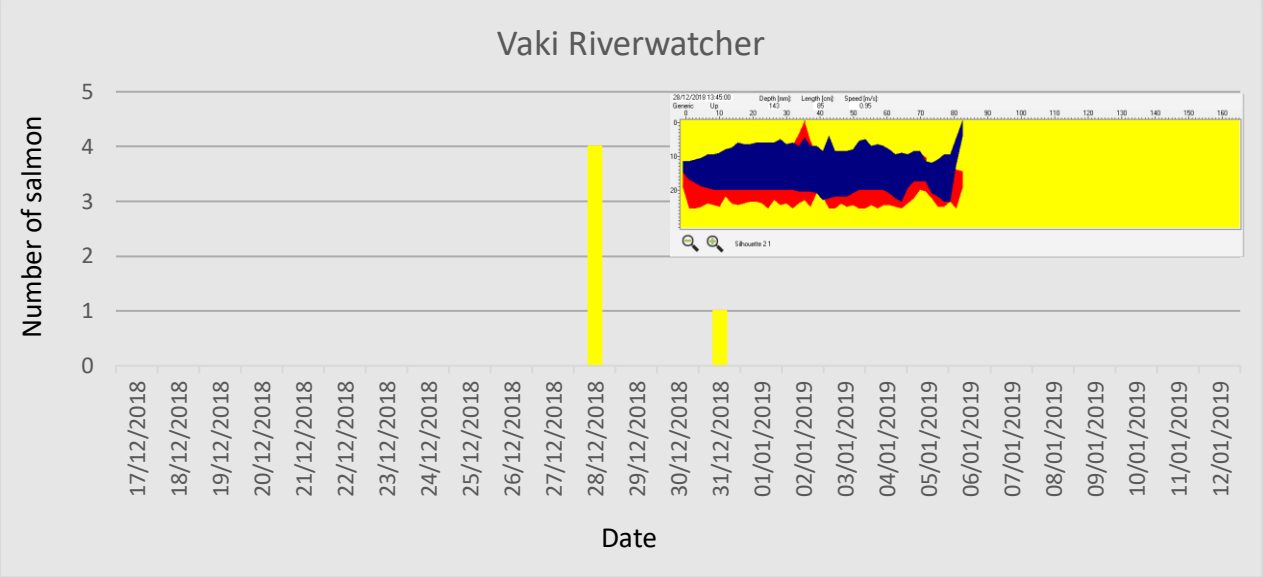


Figure 10: Five salmons were detected over a 27-day period. Four were detected within a single day and one 3 days after

Figure 11: Screenshot of an adult Atlantic salmon migrating through the fish pass at Woolston weir.

3.2 Environmental DNA

A total of 14,025,451 raw reads were retrieved from one Illumina MiSeq run on the autumn sampling. This data included 6,307,285 reads for this project (library 1) and a further 7,718,166 reads for another nonrelated project (library 2). After the quality filtering and applying removal of chimaera steps there was a total of 4,357,127 reads retained. MOTUs representing the same species were combined, MOTUs with 5 or less reads were removed, as well as MOTUs with an identity of 97% or less (see methods). Finally, only reads belonging to the order Actinopterygii and Agnatha were retained, and all reads found in the blank samples were removed. This gave a final data set for statistical analyses of 2,646,263 reads over 20 MOTUs which could be assigned to 20 different species (Table 3).

For spring sampling, a total of 12,706,647 raw reads was obtained from the Illumina MiSeq run across library 1 and 2. After the quality filtering and applying removal of chimaera steps there was a total of 5,230,274 reads kept. Data organisation followed the same protocol described for the autumn sampling leaving a final data set for statistical analyses comprising of 4,365,441 reads over 23 MOTUs which could be assigned to 23 species (Table 3).

The compiled species list across both sampling periods resulted in 30 species (Table 3 & S5). A total of 13 species were detected in both seasons, 7 were detected only in autumn and 9 only in spring.

A total of 20 species were detected in the autumn sampling period (Table 3; It is important to note that because the autumn and spring sampling periods had different sampling locations, this table is not a direct comparison), 19 being from the class Actinopterygii and one (*Lampetra fluviatilis*) belonging to Agnatha. A comparison of biodiversity above and below Woolston weir in a temporal framework (Fig. 12) revealed that some species dominated the

accumulated read count and were found on all dates in both locations. The species found both above and below the weir across all sampling dates were *Salmo trutta, Rutilus rutilus, Gasterosteus aculeatus, Esox lucius, Barbatula barbatula* and *Anguilla anguilla.* The common roach *R. rutilus* and three-spine stickleback *G. aculeatus* accounted for approximately 85% (2,227,013) of the total reads. Some species had less reads detected but were detected across multiple dates and locations; for example, *Abramis brama* was detected above and below the weir on the 29th November and on the 6th December 2018 but was absent on the 22nd November 2018. The only anadromous species that was detected just one side of the weir was the river lamprey *L. fluviatilis,* which reads were only found on 22nd November 2018 above the weir. The Atlantic salmon *S. salar* was the only species detected above and below the weir on a singular sampling date (6th December 2018). Numerous marine species were also detected including the European sea bass *Dicentrarchus labrax*, Atlantic mackerel *Scomber scombrus* and the European pilchard *Sardina pilchardus* (Fig. 12). The common sole *Solea solea*, Atlantic mackerel *Scomber scombus*, European plaice *Pleuronectus platessa* and the pollock *Pollachius pollachius* were detected only above the weir on the final sampling date (06/12/2018)

For the spring samples the results were accumulated into three areas: before Howley weir which is tidal and ranges from the estuary mouth to the weir, **between** Howley and Woolston weir and **after** Woolston weir, up to where the Manchester Ship canal and the river Mersey join (Fig. 7). A comparison of biodiversity with a temporal and spatial view (Fig. 13) was created using the same protocol described in the autumn sampling. A total of 23 species were detected in the spring sampling period (table 3), 21 being from the class Actinopterygii and two (*L. fluviatilis* and *Petromyzon marinus*) belonging to Agnatha. The species found both above, between and below the weir across all sampling dates were *R. rutilus, G. aculeatus, E.*

lucius and *A. anguilla.* The common roach *R. rutilus* and European eel *A. anguilla* accounted for approximately 90% (3,899,501) of the total reads. Some species like the Atlantic salmon *S. salar,* river lamprey *L. fluviatilis*, sea lamprey *P. marinus* and grayling *T. thymallus* were detected on a single sampling date and only before Howley weir. Out of the 23 detected species, 21 had an eDNA signal recognised below Howley weir. Sixteen were detected also in between the two weirs and only 12 were detected above Woolston weir. Three out of the five migratory species described (Table 2) were detected across both seasons, with the only species not detected being the shad *Alosa sp.*

Table 3: Species detected within both sampling periods (autumn and spring).

Species	Common name	Habitat	Autumn	Spring
Gadus morhua	Atlantic cod	Marine		x
Solea solea	Common sole	Marine	x	x
Dicentrarchus labrax	European bass	Marine	x	x
Chelon labrosus	Thicklip grey mullet	Marine		x
Platichthys stellatus	Starry flounder*	Marine		x
Clupea harengus	Atlantic herring	Marine		x
Pomatoschistus minutus	Sand goby	Marine		x
Sardina pilchardus	Pilchard	Marine	x	x
Scomber scombus	Atlantic mackrel	Marine	x	
Oreochromis aureus	Blue tilapia	Marine	x	
Pleuronectes platessa	European plaice	Marine	x	
Pollachius pollachius	Pollock	Marine	x	
Ammodytes marinus	Sand eel	Marine		x
Barbus barbus	Barbel	Freshwater	x	
Cottus gobio	Bulhead	Freshwater	x	x
Abramis brama	Common bream	Freshwater	x	
Cyprinus carpio	Common carp	Freshwater	x	x
Phoxinus phoxinus	Common minnow	Freshwater		x
Perca fluviatilis	European perch	Freshwater	x	
Thymallus thymallus	Grayling	Freshwater		x
Esox lucius	Northern pike	Freshwater	x	x
Rutilus rutilus	Roach	Freshwater	x	x
Gymnocephalus cernua	Ruffe	Freshwater		x
Barbutula barbutula	Stone loach	Freshwater	x	x
Gasterosteus aculeatus	Three spined stickleback	Freshwater	x	x
Salmo trutta	Brown trout	Anadromous	x	x
Salmo salar	Atlantic salmon	Anadromous	x	x
Anguilla anguilla	European eel	Anadromous	x	x
Lampetra fluviatilis	River lamprey	Anadromous	x	x
Petromyzon marinus	Sea lamprey	Anadromous		x
Total			**20**	**23**

Figure 12: Spatial and temporal diversity plot above and below Woolston weir during the autumn sampling. The number of reads is represented by the size of the circle and the dates are represented by the different coloured circles. The arrows represent the direction of water flow along the river Mersey.

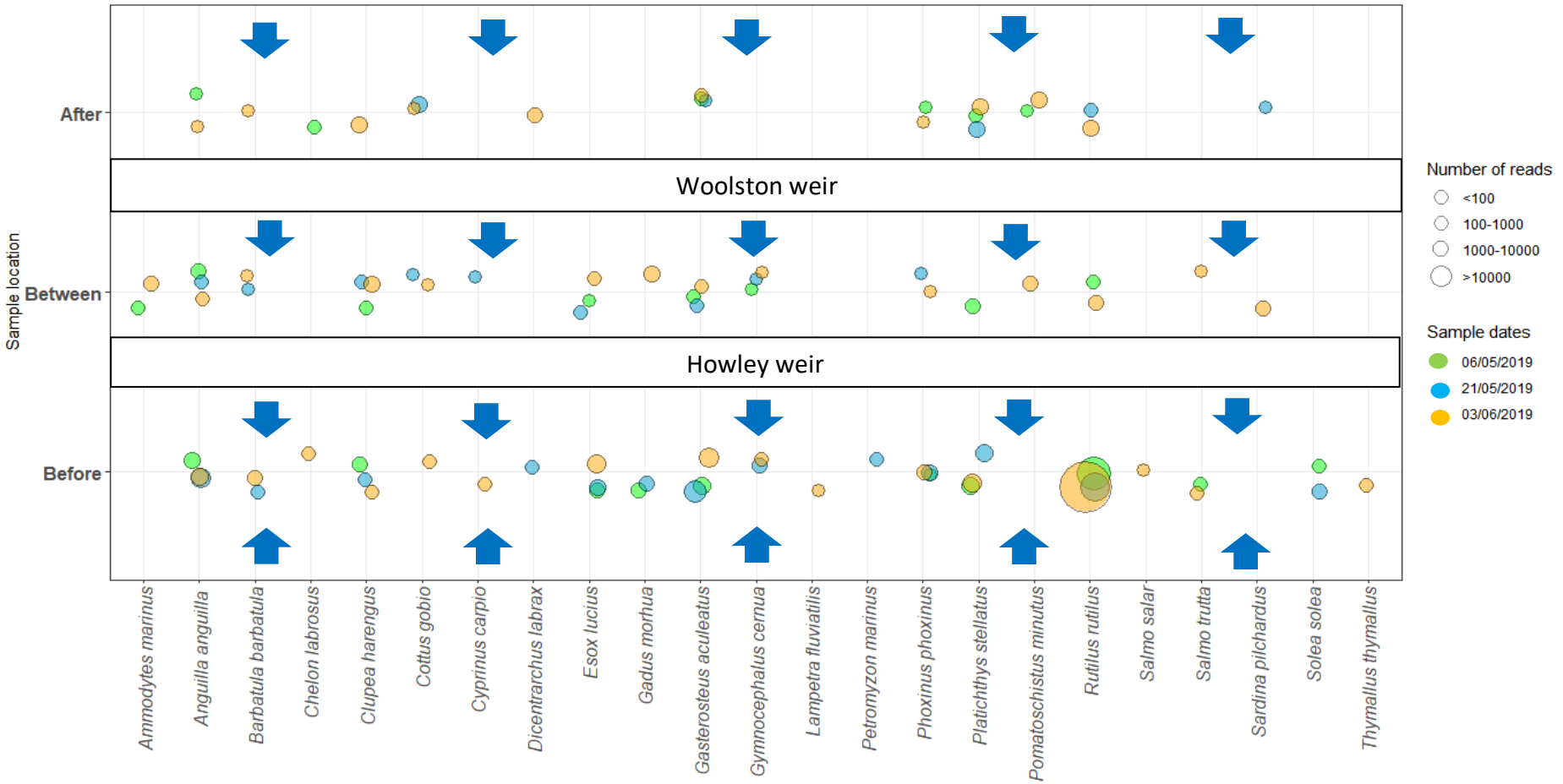
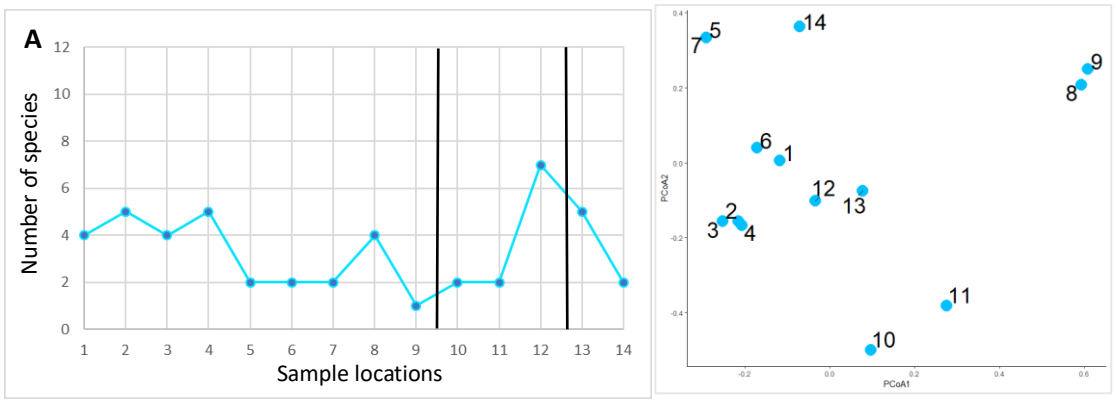
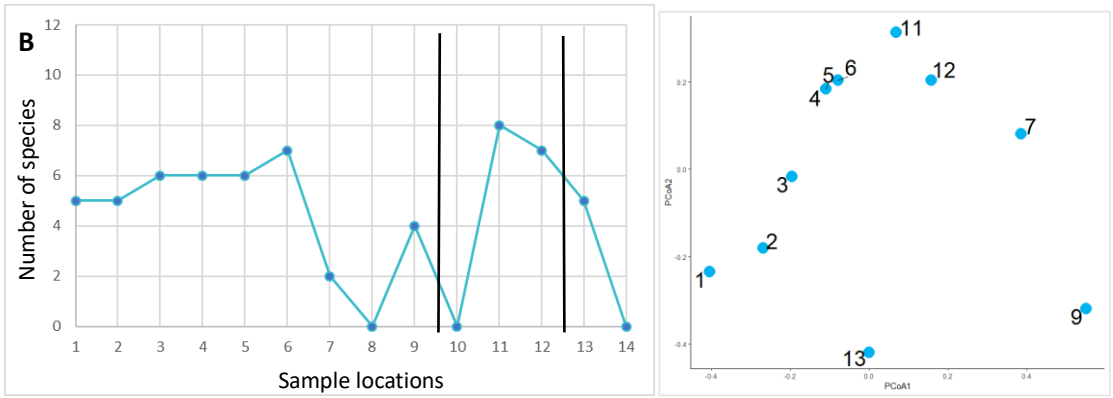
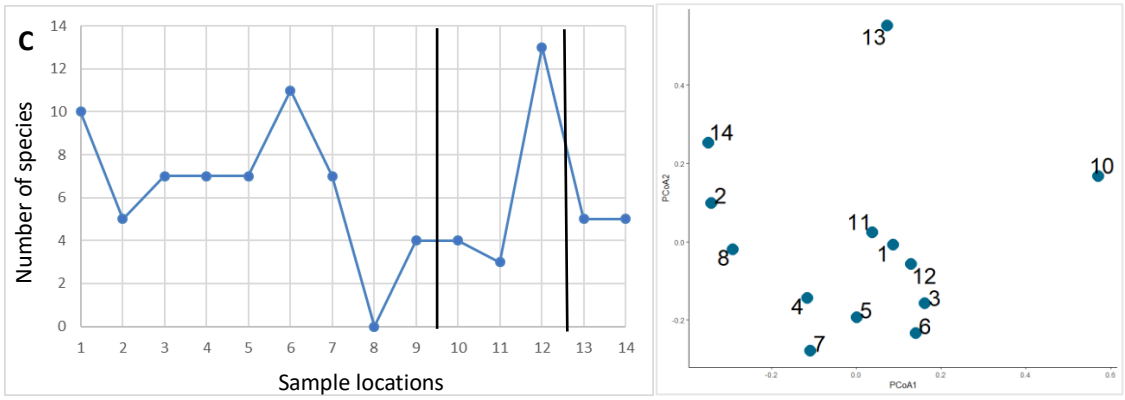


Figure 13: Spatial and temporal diversity plot before, between and after weirs along the river Mersey during the spring sampling. The number of reads is represented by the size of the circle and the dates are represented by the different coloured circles. The downward arrows represent the flow of the river and the upwards arrows represent the direction of the tide.

Sites 1 to 9 are all in the Mersey estuary (1 to 5 in the lower Mersey estuary, more influenced by saltwater; 6 to 9 in the upper Mersey estuary; Jones, 2006). Sites 10 to 14 are in the river Mersey, with Howley weir located between sites 9 and 10, and Woolston weir located between sites 12 and 13 (Fig. 14). During the Spring there were 3 sampling dates; species richness on the 6th May 2019 (Fig. 14A) was highest in the estuarine sampling sites (sites 1, 2, 3,4 and 8) and in one site between the two weirs on the river Mersey (site 12). The least amount of species was recovered from sample site 9 and the β-diversity was alike in sites 8 and 9. The species richness on the 24th May 2019 (Fig. 14B) shows sample sites 1 to 6 with similar species richness. Sample site 11 showed the highest richness of species, whereas sites 8, 10 and 14 had no detection of species. The β-diversity showed sample sites 4,5 and 6 to be most similar and sample sites 9 and 13 to be most distinct. The final sampling event (3rd June 2019; Fig. 14C) was two weeks after the second sampling period and showed an overall increase in species richness. Species richness fluctuated the most in this sampling period with sample site 12 having the highest (14) amount of species and sample site 8 having the lowest (0). Sample site 12 and 6 had the highest richness and sites 10 and 13 showed the most distinct fish assemblage. The final plots (Fig. 14D) show a combination of the temporal sampling over Spring. Overall site 6 and 12 showed highest species richness and the two lowest sites in terms of species richness were located directly before and after Howley weir (site 9 and 10). Sample site 10 showed an overall distinct assemblage compared to other sampling sites. Longitudinal distance and presence of weirs did not have a significant effect on β-diversity amongst sample sites and across time (Table 4).


A
Number of species
Sample locations
PCoA1
PCoA2


B
Number of species
Sample locations
PCoA1
PCoA2


C
Number of species
Sample locations
PCoA1
PCoA2

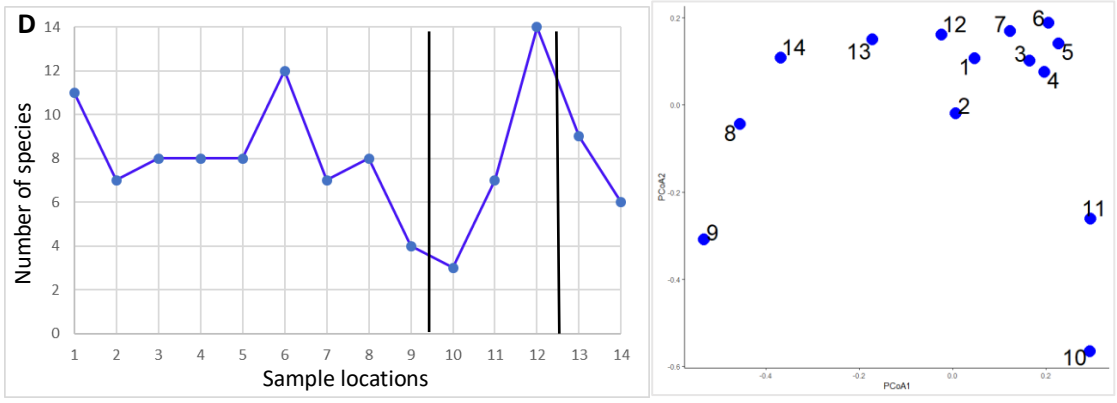

D
Number of species
Sample locations
PCoA1
PCoA2

Figure 14: The distribution of species richness in the spring period is shown along the River Mersey, along with a Principal Coordinate Analysis (PCoA) of β-diversity of sampling locations (Jaccard distance). **A)** Samples obtained 06/05/2019. **B)** Samples obtained 21/05/2019. **C)** Samples obtained 03/06/2019. **D)** Samples combined over the three dates in A, B and C.

Table 4: Mantel *r* and *p*-values of β-diversity in spring, across sampling dates, combined dates, geographic distance and presence of barriers (weirs).

Spring 2019					
	Longitudinal distance				Barrier distance
	06/05/2019	21/05/2019	03/06/2019	Combined dates	Presence of weirs
Observation	**-0.35**	**-0.596**	**-0.38**	**-0.413**	**-0.188**
p-Value	**0.988**	**1**	**0.996**	**0.998**	**0.924**
Standard obs	-2.604	-5.516	-2.611	-3.096	-1.505
Expectation	-0.002	-0.005	-0.002	-0.005	0.004
Variance	0.017	0.011	0.02	0.018	0.015

3.3 Woolston weir- Seasonal comparison

Comparing diversity at Woolston weir across the two sampling periods (Fig. 15), a total of 26 species were detected; 13 species were detected only in autumn, 6 only in spring and 8 were detected across both seasons. *Anguilla anguilla, G. aculeatus, Cottus gobio and R. rutilus* were detected in both seasons and both below and above Woolston weir. Some species such as the common bream *Abramis brama,* the common carp *Cyprinus carpio*, the barbel *Barbus barbus,* river lamprey *Lampetra fluviatilis* and the Atlantic salmon *Salmo salar* were solely detected in autumn. On the contrary, some species were detected only in the spring sampling, such as the common ruffe *Gymnocephalus cernua,* blue tilapia *Oreochromis aureus* and the common minnow *Phoxinus phoxinus.* Several marine species were detected across both seasons around Woolston weir, including the Atlantic cod *Gadus morhua*, Atlantic herring *Clupea harengus* and the Atlantic mackerel *Scomber scombus*, although only one marine species was detected across both seasons: the European pilchard *Sardina pilchardus*.

Overall, in autumn there was a higher species richness than in spring at the Woolston weir area of the river Mersey, particularly above the weir (Fig. 16). The β-diversity showed distinct assemblages across both seasons and from within seasons (above/below the weir; Fig. 16).

Figure 15: Number of reads per species (indicated by the size of the circles), by season (green = autumn sampling; blue = spring sampling) above and below Woolston weir.

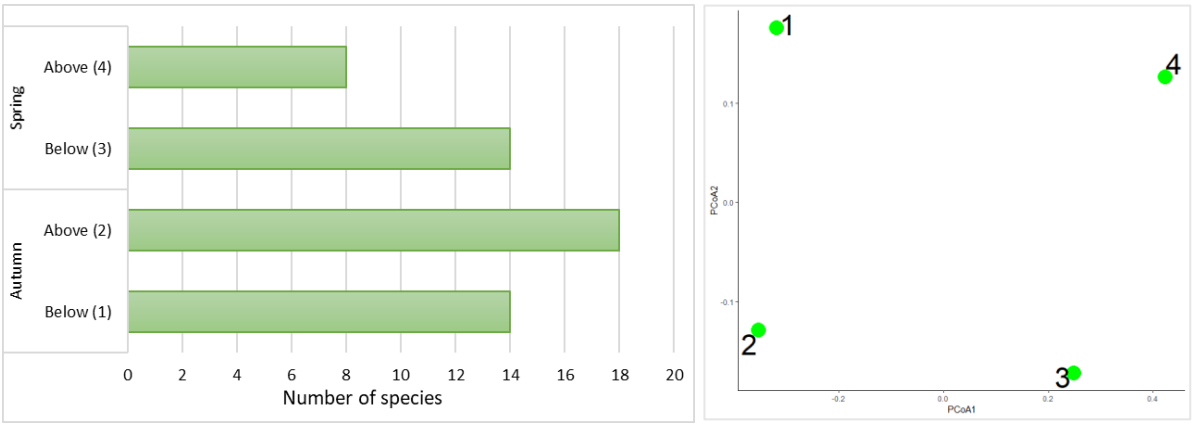


Figure 16: Distribution of species richness at Woolston weir for both autumn and spring sampling, providing a comparison of seasonal and temporal species richness in regards to an anthropogenic barrier. A Principal Coordinate Analysis (PCoA) of β-diversity of sampling locations (Jaccard distance) is also illustated. The sites (1-4) represented on the bar plot correspond to the sites on the PCoA.

3.4 Woolston weir – historical comparison

Historical data has been collected by trapping fish at Woolston weir fish pass, along with trawling and angling around Woolston weir, with 16 years of data within a 19-year period (2000-2019) by the environment agency (Energy, 2012) and the Mersey Gateway Environmental Trust. This was compared with data obtained from the eDNA samples from Woolston weir in autumn 2018 and Spring 2019. A total of 32 species were detected, with six species only detected by traditional monitoring methods; seventeen species detected only in eDNA samples, and nine detected by both methods. The tilapia *Oreochromis aureus** is

highlighted as although eDNA was detected the species would not be found in this river system, therefore not a direct monitoring method comparison.

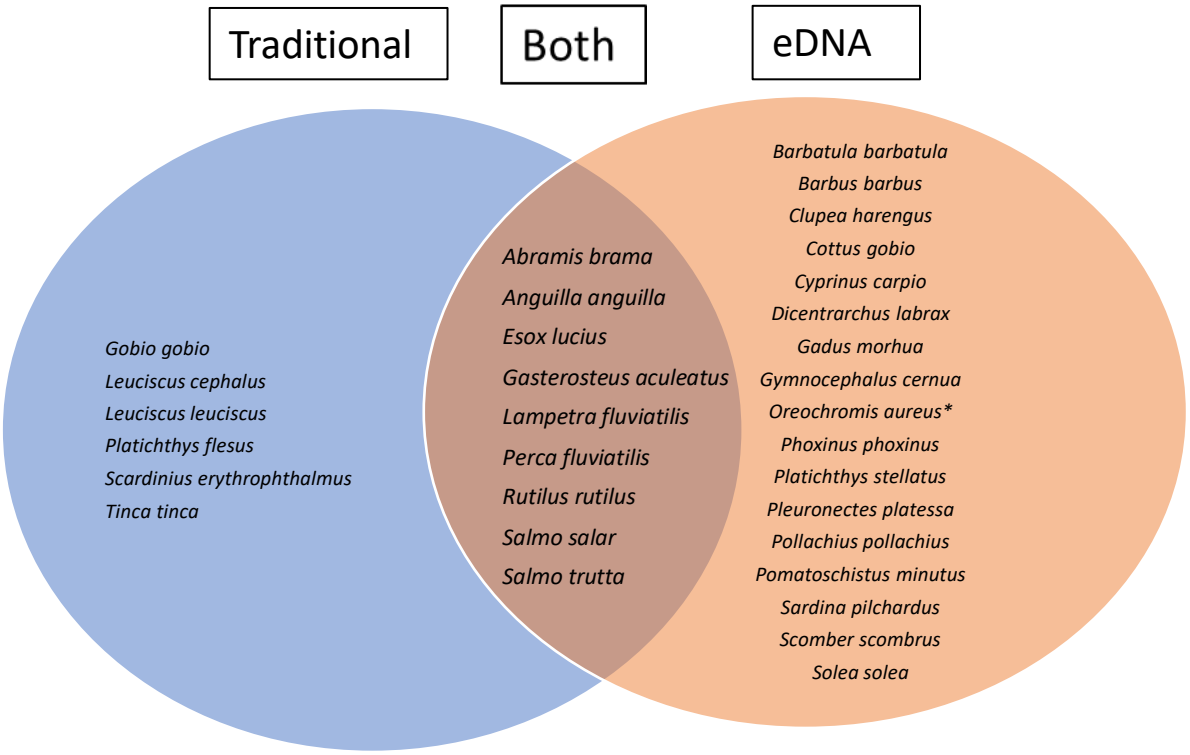


Figure 17: Venn diagram comparing data collected at the Woolston weir area of the river Mersey over a nineteen-year period by the Environment Agency and MGET in blue, against the combined season eDNA sampling (autumn and spring) at Woolston weir in 2018 and 2019 in orange. Species detected in both methods lay in the intersection. The tilapia *Oreochromis aureus** was detected using eDNA metabarcoding, but the species would not be found residing in this river system.

3.5 Environmental influences

A canonical correlation analysis (CCA) was used to show if there are any abiotic factors that influence the species detected over the spring sampling period (Fig. 18; Table S10). Environmental DNA from the European sea bass *Dicentrarchus labrax* was correlated with sites characterized with higher temperature. The Atlantic salmon *Salmo salar* and sea lamprey *Petromyzon marinus* were detected mostly in sites with high oxygen levels. The presence of eDNA from grayling, *Thymallus thymallus* was somewhat linked with increased salinity, pH and oxygen levels. The presence of the eDNA of the rest of the species did not show a strong dependence of any environmental parameters considered in the analysis.

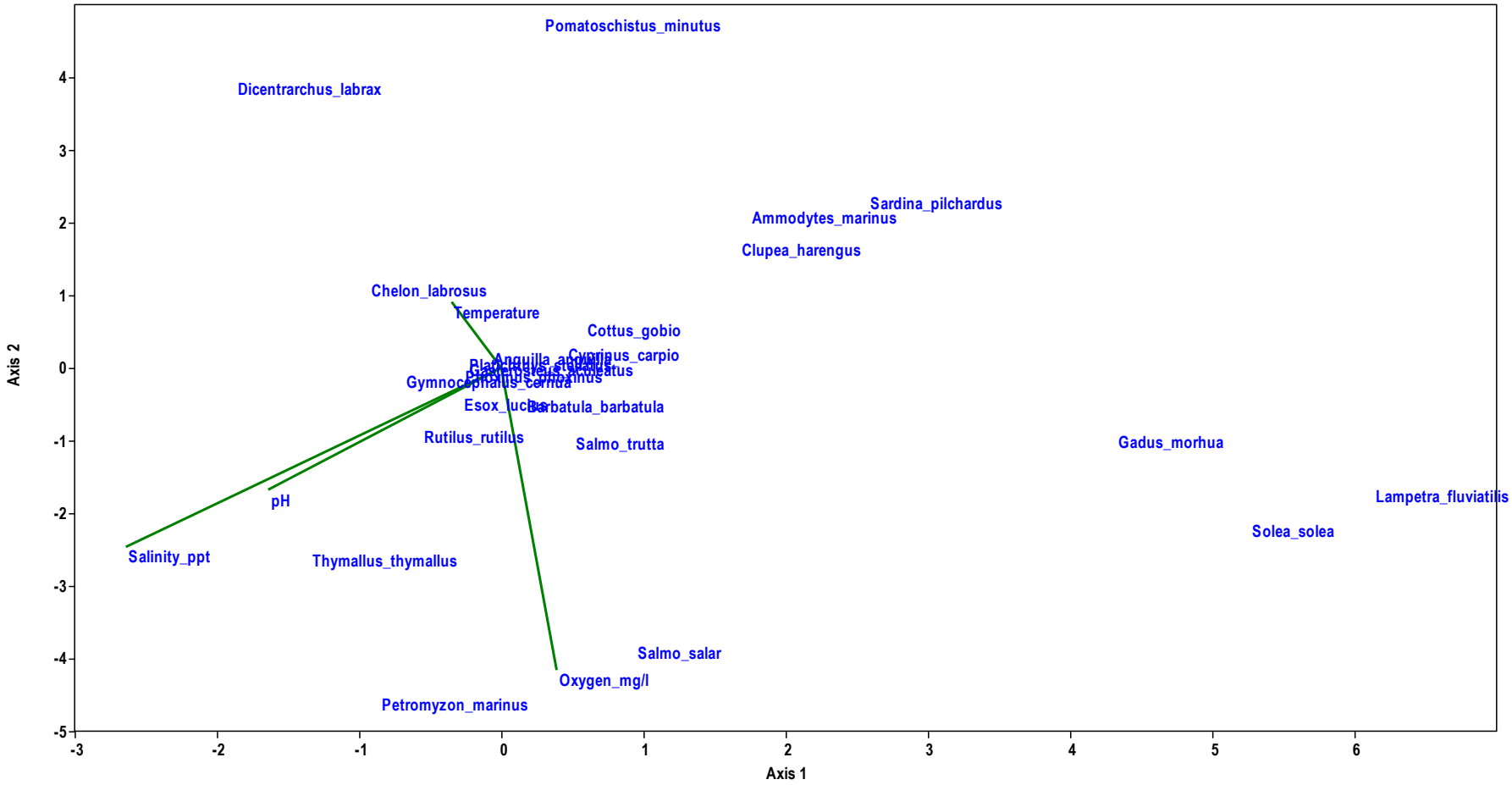


Figure 18: CCA plot of all species against the environmental factors.

4 Discussion

In order to apply correct conservation strategies and management schemes it is imperative to understand the temporal and spatial distribution of species and community structure (Lawson Handley et al., 2019). Most methods to date have not been able to do this in a rapid and non-invasive way and over a spatio-temporal scale (Civade et al., 2016). This is especially important in a recovering river, with low diversity and a known small recovering population of important migratory species, such as the river Mersey (Ikediashi et al., 2012). We used eDNA metabarcoding and camera technology to first assess biodiversity and fish passage success above and below Woolston weir. The aim was to understand fish community composition, and the effects of anthropogenic barriers along the river Mersey, using a spatial and temporal sampling approach.

4.1 Non-invasive monitoring at Woolston weir

Multiple studies have investigated the need for temporal and spatial sampling to assess biodiversity and understand community composition in aquatic systems (Beentjes et al., 2019; Lawson Handley et al., 2019; Sales et al., 2020). The need for this type of sampling is particularly relevant in a flowing river system that is subject to regular natural changes, along with anthropogenic manipulations. Comparative studies have shown the importance in understanding how seasonal changes can affect biodiversity in aquatic systems (Handley et al., 2019), but less have focused on finer scaled sampling approach.

In the river Mersey there is a small recovering population of Atlantic salmon that uses Woolston weir fish pass to migrate to spawning grounds (Ikediashi et al., 2012). Their numbers and exact migration pattern are not fully understood. A promising non-destructive tool that has been developed for monitoring fishes is remote camera technology. It enables

continuous monitoring of individuals, thus allowing to monitor populations and communities (Bicknell et al., 2016). Camera technology is particularly promising on fish passes where migratory species move through a small area, where cameras can collect a multitude of data (i.e. species, size, direction; Martignac et al., 2015). For this study we used a River-watcher camera (Vaki) which was installed by the Mersey Gateway Environmental Trust on Woolston weir. Unfortunately, in the initial pilot study for the camera, we ran into technical difficulties which caused the loss of fuel and subsequently the camera became non-operative for a three-week period. This coincided with the expected salmon migration period and highlights the issue of reliability with such technology. When the camera started working again five adult salmons were detected travelling upstream through the fish pass, consistently with migration to their spawning grounds, further up the river Mersey (Ikediashi et al., 2012). We also used a state-of-the-art molecular approach, eDNA metabarcoding, to monitor above and below Woolston weir. Overall, a twelve-week period of monitoring was covered by both camera and eDNA samples as this was aimed to cover the salmon migration period. The eDNA samples were taken in the key salmon migration period (November/December) over three sampling times and showed the importance of temporal sampling. Due to the technical issues resulting in missing data from the camera, monitoring salmon migrations using this method alone would have been of little use. On the other hand, based on the three sampling dates, eDNA alone would not have given a full representation of the entire migration period, as salmon were detected much later in December (Fig. 10). Even though we were unable to get a full dataset from the camera to monitor the salmon migration, by combining both methods we were able to monitor the migration period for Atlantic salmon on the river Mersey; eDNA signals for salmon were first detected on the 5th of December 2018 and the final camera detection was recorded on the 31st of December 2018. Even in small numbers and in a short

period of time, these two non-invasive monitoring techniques provided useful migratory information for the vulnerable Atlantic salmon population on the Mersey.

By sampling in weekly intervals our fine-scale eDNA sampling approach showed no detection of salmon on the first and second sampling dates (22/11/2018 and 29/11/2018) but detection of salmon eDNA on the final sampling date (05/12/2018), both below and above the weir (Fig. 12). Increased water flow and lower temperatures are known to influence the spawning migration of salmon. With warmer temperatures and reduced precipitation, freshwaters are known to increase in toxicity and contaminant levels, which can lower disease resistance of salmon due to higher stress levels (Moore et al., 2012). 2018 was one of the driest and warmers summers on record across the UK (Kendon., 2019); this could explain the later migration signal picked up by the metabarcoding method along with the small number of individuals caught on camera (Fig. 10). Another key point to this research is that as seasonality becomes unsettled and varies due to climate change, so do the patterns and behaviours of the organisms. This means sampling methods must take this into consideration and adapt to the unpredictable and ever-changing climate. Similarly, river lamprey was detected on one date, but unlike the salmon, it was only detected above the weir. This could be consistent with the end of the migration period, as the river lamprey make their way from the ocean up into rivers for spawning (Docker, 2015).

The amount of DNA that an organism releases into the environment is dependent on the specific species and such behaviours such as spawning, feeding, migration or movement and the health of the individual (Barnes et al., 2014; Jane et al., 2015). Spawning fish will release gametes which would contribute more DNA into the water, whereas, a fish passing through an area would be releasing a lot less DNA into the environment (Tillotson et al., 2018). The

European eel *Anguilla anguilla* is critically endangered (Redlist, 2019), it lives within the river and then migrates to the ocean to spawn. We expect to recover more DNA over time and space due to the species persisting in the environment for most of its life (Bevacqua et al., 2015). As expected, eel DNA reads were high and found on all dates, both above and below Woolston weir. This highlights the power of using molecular monitoring approaches to detect both critically endangered species that are more prevalent in the area along with migratory species passing through an ecosystem.

Along with being able to detect migratory species and to show how eDNA metabarcoding can monitor the effectiveness of fish passes, we were able to compare this method with more traditional approaches. The data we collected has given an insight into what species are present around the Woolston area of the river Mersey in autumn 2018 and spring 2019. Historical data was collected over a 16-year period at the Woolston weir fish pass combined with angling and netting monitoring methods (Energy, 2012). This was then and compared with our eDNA data (Fig. 17). This is not a direct comparison of methods, as sampling dates differ. Nevertheless, there is still a clear advantage of using eDNA metabarcoding to traditional methods, as our results show much higher diversity of species detected in eDNA samples compared to trapping. The eDNA results were also obtained in a short sampling period of six separate days in November/December 2018 and May/June 2019, compared to multiple trapping events over a 16-year period. Also, other studies comparing eDNA to traditional methods such as electrofishing have showed clear advantages of a direct comparison in how effective eDNA is to monitor fish communities. It is thus important to recognise that traditional methods are still an important method to investigate certain information such as size, age and abundance (McDevitt et al., 2019; Smart et al., 2015). Although the camera did not provide us with the amount of data we expected, we did detect

Atlantic salmon and were able to conclude that these were large adult salmon migrating upstream, which coincides with migratory behaviours (Ikediashi et al., 2012). Such detailed information is not possible to be obtained with molecular methods. Some studies have started to research the relationship between eDNA reads and relative abundance and have found positive correlations (Lacoursière-Roussel., 2016). This highlights the need for more studies into how accurate eDNA can be when considering relative abundance.

4.2 Spatial and temporal assessment of fish diversity in the river Mersey

The negative effects of dams and weirs on fish biodiversity is well known (Garcia De Leaniz, 2008; Welsh & Aldinger, 2014). This is particularly true for dams, as these large structures completely block the flow of water (Mueller et al., 2011), unlike weirs, where water breaches the top of the structure and allows flow to continue down the river (although with changed flow; Falconer & Lin,2003). Fish passes are a well-used connectivity solution but only select species can use such structures. Therefore, the effects weirs have on fish diversity and community structure need other methods to assess their effects. Our eDNA approach showed that longitudinal distance and weir presence did not have any significant effects on fish community distribution along the Mersey (Fig. 14 and Table 4), but by sampling temporally and combining the data, the two sites with the lowest species richness were those directly either side of Howley weir (Fig. 14D), suggesting an impact. Environmental conditions in a river can be modified by weirs through water flow change, water quality and nutrient dynamics, which can greatly reduce the diversity of fish (Pelicice et al., 2015).

The site across all three dates that showed the highest species richness was site 12, located below Woolston weir. This is surprising due to the known negative effects barriers have on fish diversity (Rolls et al., 2014). An explanation could be the force of the water commencing

over Woolston weir might push water deeper into the river which is churning eDNA that is either lower in the water column or mixing sediment into the shallower waters. This could be causing more eDNA to be accumulated where the samples are taken in the higher water column. This could be important to note when creating a sampling protocol and crucial if eDNA metabarcoding is used in conservation plans on lotic systems, as at first glance species richness in between weirs looks high (Fig. 14). Another explanation is that there are multiple marine species detected in this area, driving the diversity up. These species are common in eateries for human consumption, which could explain the eDNA detection. Warrington is located around sample site 12 which is a largely populated town. The detection of blue tilapia *Oreochromis aureus*, a common species used for human consumption and is farmed at large quantities globally, helps to confirm this theory (Little et al., 2008).

Every species we identified in the spring period was found before Howley weir (Fig.13) and the location that shows the second highest richness of species is site 6 (Fig. 14D) which is the official border between the upper and lower Mersey estuary (Mersey Basin Campaign, 2010). Here it is important to establish that although we find a high richness of both salt and freshwater fish species, we cannot necessarily conclude they all are present at this site. Sample site 5 has a strong influence from both tidal movement and river flow movement. We hypothesise that sample site 5 is a potentially optimal eDNA sampling location for presence of species both upstream, as eDNA is transported down towards this site and downstream towards the ocean mouth, while tides bring eDNA towards this site from the opposite direction. This could be valuable for conservation management, as multiple sampling within an optimal area would reduce the need for temporal sampling, therefore providing a cheaper and more efficient eDNA sampling method for assessing diversity on a single river.

Another explanation for site 6 and 12 showing the highest species richness is their location in less anthropogenic impacted areas. Site 6 was sampled from Widnes Warth, which is a protected salt marsh (Fox et al., 2001) and site 12 is located at the SSSI nature reserve, Woolston Eyes (Martin & Smith, 2007). Similar high species richness results, in low anthropogenic impact areas, were found in a Brazilian river with a similar temporal and spatial sampling method (Sales et., 2020). Woolston eyes is a large bird nesting area, that has migrations of many species of sea birds (Martin & Smith, 2007) that could also be bringing marine species into this freshwater system to feed their young or through faecal deposits.

On average the higher diversity occurred in sites below site 6, as this is the lower Mersey estuary, subject to strong tidal movements, with marine/estuarine and river species all detected in these reaches of the river Mersey. The Mersey estuary supports over 2 million people along its 8 km of shoreline as it extends for approximately 50 km. The Mersey has had extensive clean-up campaigns to combat its polluted history (Collings et al., 1996). Due to its recent recovery (Burton, 2003), it is important to detect and identify what species use this waterbody in order to protect and nurture recovering populations. The sea lamprey *Petromyzon marinus* was detected on just one date (21/05/2019) in spring within the estuary but not at all above the weirs (Fig. 13). *Petromyzon marinus* is anadromous and has seen declines all around Europe (Bracken et al., 2019). Although there was no longitudinal or weir influence significantly effecting species communities on the river Mersey, it is known that one of the main contributors to these declines is the construction of anthropogenic barriers (weirs and dams), which can block migration upstream to suitable spawning grounds (Maitland, 2000). There a have been no previous reports of *P. marinus* entering the river Mersey, making this an important and interesting detection. There is a well-established population of *P. marinus* in the neighbouring River Severn (Bird et al., 1994). This shows it is possible the

species has entered the Mersey and has been unable to negotiate the weirs to find spawning grounds, therefore leaving to enter more suitable rivers. Another possibility is *P. marinus* uses the Manchester Ship canal to migrate upstream to spawning grounds and bypass the obstructing weirs (Fig 19). As detection was on one sampling day, another possibility for the detection of *P. marinus*, is that the DNA can been transported in from coastal waters by tidal movements into the estuary. There are conflicting studies with some reporting that tides had little effect on eDNA transportation (Kelly et al., 2018), while others reporting false-positive results due to tidal movements (Jeunen et al., 2019), but both agreeing more studies are required on the abiotic influences of DNA persistence within the environment. The Atlantic cod *Gadus morhua* has a vulnerable conservation status due to declines in populations all over its native range (IUCN Redlist, 2019). Increased water temperatures due to climatic change and overfishing are thought to the main contributors to the decline (Dinesen et al., 2018). Reports from the mid-1900s using angler surveys tell us that once the Mersey estuary became cleaner and a healthier water, *G. morhua* have been migrating into the Mersey estuary to spawn (Collings et al., 1996). We detected *G. morhua* on two dates over the spring period (Fig. 13). Also, we have detection of the species above the first weir, but as above the weir is freshwater, we know that *G. morhua* cannot tolerate such waters and would be unable to negotiate the weir at Howley. The reason for eDNA detection above this weir could be down to the high tides breaching the weir and either releasing water with DNA into the reaches of this section of the river or the species itself.

The European bullhead *Cottus gobio* is a freshwater species adapted to conditions of a narrow range making them a stenotopic species, meaning they are particularly vulnerable to increasing temperatures and low oxygen levels (Adamczyk et al., 2019). Well-established bullhead populations indicate good longitudinal connectivity (Adamczyk et al., 2019): our

results show *C. gobio* is detected both above and below Woolston weir on most sampling dates in autumn (Fig. 12) and is found less so in spring but still eDNA presence was detected (Fig. 13). The Eurasian ruffe *Gymnocephalus cernua* is invasive within the UK, its native region is the Ponto-Caspian region and the North Sea basin (Zhang et al., 2019). It has been an extremely successful invader in British waters due to its ability to tolerate many variations of salinity, depths and temperatures and has high fecundity (Volta et al., 2013). Studies have found correlations with ruffe and perch *Perca fluviatilis* biomass due to having positive association through dynamic congruence. Though it is known that the invasive ruffe can compete with the native perch for resources, both are known prey for pike *Esox lucius*, which may weaken the predator pressure on perch (Öğlü et al., 2019). Perch and ruffe are detected in the same locations and pike are also found in all these locations, and on the same dates (Fig. 13). These eDNA results may be showing both predator prey and dynamic congruence interaction.

Our study has been able to show how by using eDNA to monitor fish we can find that rare and endangered species are entering the river Mersey. Although there are no significant effects regarding longitudinal and obstacle distances, there are drops in species richness above the weirs. We have demonstrated that weirs with good connectivity (fish passes) may contribute to positive connectivity. By looking at species individually and understanding their behaviours and movement patterns, we can better understand community structure in the river Mersey. Also, we need better understand the behaviours of eDNA molecules, in terms of how abiotic and anthropogenic factors influence the results we find.

4.3 Seasonal patterns and influences

The rate of production of DNA within the environment has a major influence on the concentrations of eDNA at any one point. These levels are influenced by several things including behaviours such as spawning, feeding activity and migration through the habitat, and changes within the abiotic environment such as reduced/increased flow that can affect eDNA degradation. Therefore, the amount of eDNA will differ through seasons and in response to behaviours and changes in both biotic and abiotic factors (Barnes et al., 2014).

Although the sampling was conducted within the same area of river between autumn and spring, there is a clear change in community composition (Fig. 15 and Fig. 16). Only eight of the thirteen species detected across both sampling periods were detected around Woolston weir in both autumn and spring. The presence and absence of some species are much more easily explainable due to known behaviours and life history traits. Atlantic salmon and river lamprey are anadromous migratory species which use the pass at Woolston weir to reach spawning grounds in the autumn period (Ikediashi et al., 2012; Tummers et al., 2018). In spring the individuals ready to enter the ocean should have made their way into estuarine and oceanic waters (Docker, 2015; Wolter, 2015). Both the lamprey and salmon were absent in the Woolston weir section of the river Mersey in all spring samples. Both were though detected within the Mersey estuary on the last sampling period of spring (Fig. 13), demonstrating the importance of spatial and temporal sampling, as we can illustrate behavioural patterns through eDNA reads. This is critical for conservation management plans, due to the decline of both these migratory species; Atlantic salmon are of vulnerable status across Europe (IUCN), and the river lamprey are priority species status, due to their declines throughout the UK (JNCC).

The presence/absence of other species from season to season are not as easily explained as the two diadromous species just mentioned. This is due to *S. salar* and *L. fluviatilis* having well-studied long-distance migration patterns (Legrand et al., 2020; Nicola et al., 2018; Slatick & Basham, 1985; Tummers et al., 2018). The freshwater bream *Abramis brama* is a species that has a wide geographical range with a multitude of habitats, from ponds and lakes to river systems (Brodersen et al., 2019). Although the bream is thought to be most active within the spring and summer periods due to feeding and spawning habits (Yurchenko & Morozov, 2018), our results show no detection within spring but detection throughout the autumn period. This can be explained due to the extreme weather in 2018, as the UK experienced one of the hottest summers on record, which pushed warm temperatures in rivers and lack of rains into the latter end of 2018 (Kendon et al., 2019). The extremely dry summer in 2018 is what has been hypothesised as the reason for the late salmon migration and could be the reason bream are detected in autumn and not spring. Bream need temperatures between 8°C and 12°C to spawn and will become more active feeders at this time as they display circadian vertical migration (Yurchenko & Morozov, 2018). Temperatures in the spring sampling period were much higher than in the autumn sampling period (Table S1; S2-S4), which could mean the temperature was too warm in spring, but the previous autumn bream were active longer into the year due to the unusually warm autumn waters. Here lies an issue with our eDNA sampling method, as the samples obtained were only from surface water, meaning that if in the spring sampling period the bream had migrated lower into the rivers water column, they may have been missed in the sampling. Indeed, even if samples were taken at different depths, due to the lack of activity of the species there could still be a chance of little to non-detection. Another species that was present in autumn around Woolston weir was the European barbel *Barbus barbus*, which was not detected in spring at all along the

river Mersey (Fig. 15). An explanation for this might be that the species has cyclical migration behaviours throughout the year, moving downstream through the autumn period, thus shedding DNA through the river, whereas in the spring, the species migrates upstream in association with spawning events (Roberts et al., 2019). To explain the absence of detection on the river Mersey throughout spring, there are two weirs in which *B. barbus* will find difficult to negotiate due to the nature of the powerfully flowing fish pass (Baras et al., 1994). The Manchester Ship canal connects the upper Mersey estuary below the first weir (Howley weir) and the river Mersey after the second weir (Woolston weir). This gives a potential route in which *B. barbus* can migrate upstream, before entering back into the river Mersey (Figure 19). *B. barbus* have been caught by anglers in canal systems attached to river systems in the East of the UK (Canal and River Trust). Although there has never been reports of the species in the river Mersey, there has been introductions for recreational anglers on rivers such as the river Severn, which like the river Mersey, is also in the West of the UK (Roberts et al., 2019), giving potential for native invasion. Thus, this shows how eDNA metabarcoding could provide a possible early detection of a species not thought to be within the river system.

Figure 19: The Manchester Ship canal shown in red running alongside the river Mersey. A) Green arrows: indicates fish can enter the canal from the Mersey; B) Yellow arrow: indicates where the Manchester Ship canal meets back with the river Mersey; C) Howley weir (53.384731, -2.577445); D) Woolston weir (53.39370, -2.52190).

The common minnow *Phoxinus phoxinus* was detected in spring but not at all in autumn and this could be explained by the change in behaviour over a finer scale along with seasonal change. A study from the river Frome, UK, showed how minnow move into shallow areas in the warmer part of the day and then retreat to deeper areas throughout the colder period (Garner et al., 1998). This could then relate to seasonal change, as in warmer spring months minnow will be more active in shallower waters for longer periods which enhance the opportunity for food but also enhance the opportunity to become prey. In the colder autumn and winter months food is more scarce so they may stay in deeper waters as the opportunities for food in shallow waters are not plenty enough to warrant going into shallower waters; thus making them more vulnerable to predation (Garner et al., 1998). This trade-off could explain why the minnow is detected within the spring months and not the autumn period and yet again asks the questions about sampling within different depths within the river system to detect species which move vertically in the water column. Our results show a shift in community structure around the Woolston weir area of the river Mersey and how the ß-diversity from autumn to spring differs (Fig. 16). This provides valuable knowledge on how eDNA methods can help us understand seasonal changes within river systems.

4.4 Environmental influences on diversity

Environmental variables are what characterise aquatic environments and are what fish communities respond to. It is crucial to understand how these parameters shape fish community structures over space and time, in order to manage and conserve fisheries efficiently (Araújo et al., 2009). Although species richness is higher in our estuarine sampling

locations, there are multiple freshwater species detected here. It could be that some species are able to tolerate increased salinity levels, but it could also be that the eDNA has been transported from further up river, thus overestimating species richness (Deiner et al., 2016). We were unable to measure flow rates in this study, but many have looked at how far eDNA can be transported before detection is lost (Barnes & Turner, 2016; Jane et al., 2015; Jeunen et al., 2019). The area is still understudied, but studies show that there may be species specific transport distances and other environmental factors that can all influence the transportation detection of eDNA (Deiner & Altermatt, 2014). Here we looked at the correlations with four environmental factors (temperature, salinity, oxygen and pH) measured during the spring sampling period (Fig. 18), assessing why certain parameters have a relationship with species we detected along the river Mersey.

The Atlantic *Salmo salar*, brown trout *Salmo trutta* and grayling *Thymallus thymallus* are all from the Salmonidae family. The Atlantic salmon is anadromous and relies on high levels of dissolved oxygen (DO) to spawn. Although some brown trout and grayling stay within the river system they also heavily rely on well oxygenated waters at spawning periods, as this increases egg survival (Einum et al., 2002). There is a relationship with the three Salmonid species towards oxygen and pH, strongest for *T. thymallus* and *S. salar*.

Adult European sea bass *Dicentrarchus labrax* will migrate to coastal areas to spawn, as their larvae develops, they migrate back into estuaries where they spend the early years of their lives in sheltered areas to grow (Vinagre et al., 2012). Studies looking at juvenile *D. labrax* have monitored how activity and migration into spawning grounds coincides with increasing temperatures. Juveniles will only enter estuaries in late May and early June, when estuary temperatures increase, thus increasing feeding activity and sheltering in warmer estuarine

nurseries (Cabral & Costa, 2001). There are strong correlations with temperature and *D. labrax* presence, as there was no detection on the 6th of May 2019 but detection on the 21st of May and 3rd of June 2019. The average temperatures in the Mersey estuary from our study were 11.6 °C (06/05/2019), 16.4 °C (21/05/2019) and 16.4 °C (03/06/2019). Our results relate exactly to the hypothesis that *D. labrax* respond to temperature change. Most studies have used invasive and time-consuming methods (Cabral et al., 2001; Legrand et al., 2020), whereas this study provided similar conclusions, using a non-invasive and rapid eDNA metabarcoding technique along with some simple collection of environmental factors. This highlights the reliability of eDNA metabarcoding to monitor species and implement in future conservation management plans.

It is important to note that whilst eDNA metabarcoding is fast becoming a driver in revolutionising the field of ecology, there are still many elements to the field that are not completely understood. One key issue is how environmental factors influence persistence and degradation of DNA (Barnes et al., 2014). Our study has not looked directly into these influences but noticed the need for better understanding of DNA's behaviour in the environment, before conclusions on the behaviour of the corresponding species. In saying this, our study has still been able to correlate temporal and spatial sampling, environmental factors and relevant literature to give strong evidence that eDNA works and is powerful in understanding fish community structures and can be applied to practically manage river systems.

5 Conclusions

The well documented rate of populations declines globally is becoming increasingly worrying. The river Mersey was one of Europe's most polluted rivers and now after huge conservation efforts is seeing healthier waters and returning migratory species (Ikediashi et al., 2012), along with the Mersey estuary being an important habitat for many oceanic species that enter to spawn and nurse their young (Jones, 2006). Due to the rivers recovering populations, it is crucial we apply non-invasive, rapid and efficient methods to efficiently monitor this fragile ecosystem. We have demonstrated the power of eDNA metabarcoding to identify migratory and rare species and understand patterns in movements through the river, showing a retention in connectivity through weirs. We have also demonstrated that eDNA sampling can identify species that were not previously thought to be entering the Mersey. Crucially we have shown the importance of spatial and temporal eDNA sampling and the challenges in understanding eDNA molecules within the environment. In doing so we have been able to compare traditional methods with our eDNA approach, which will be hugely beneficial in contributing to future biomonitoring. These fish eDNA approaches are fast becoming important, especially in large rivers due the difficulties that come with traditional methods (Pont et al., 2019). However, much more understanding on eDNA in the environment with regards to factors such as tide and flow, is needed to further grow the field of eDNA metabarcoding so it can reach its full potential.

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Supplementary material

Table S1: Field log data from Autumn sampling period

Site	Replicate	date	start time	finish time	GPS	Oxygen mg/l	Temp	pH	ml (filter)
Field Blank	N/A	22/11/2018	10:14		SJ- 65358. BNG- 88726	N/A	N/A	N/A	
RHB bridge	1	22/11/2018			SJ- 65358. BNG- 88726	6.3	8.6	7.5	300
RHB bridge	2	22/11/2018			SJ- 65358. BNG- 88726	6.3	8.6	7.5	320
RHB bridge	3	22/11/2018			SJ- 65358. BNG- 88726	6.3	8.6	7.5	300
Middle bridge	1	22/11/2018			SJ- 65386. BNG- 88708	6.7	8.3	7.1	360
Middle bridge	2	22/11/2018			SJ- 65386. BNG- 88708	6.7	8.3	7.1	300
Middle bridge	3	22/11/2018			SJ- 65386. BNG- 88708	6.7	8.3	7.1	320
LHB bridge	1	22/11/2018			N-53.39370. W-002.52190	6.2	8.5	7.5	300
LHB bridge	2	22/11/2018			N-53.39370. W-002.52190	6.2	8.5	7.5	320
LHB bridge	3	22/11/2018			N-53.39370. W-002.52190	6.2	8.5	7.5	300
Above weir	1	22/11/2018			N-53.39402. W-002.52431	6.2	8.8	7.43	400
Above weir	2	22/11/2018			N-53.39402. W-002.52431	6.2	8.8	7.43	380
Above weir	3	22/11/2018			N-53.39402. W-002.52431	6.2	8.8	7.43	400
Below weir	1	22/11/2018			N-53.39405. W-002.52424	5.9	8.8	7.35	300
Below weir	2	22/11/2018			N-53.39405. W-002.52424	5.9	8.8	7.35	360
Below weir	3	22/11/2018			N-53.39405. W-002.52424	5.9	8.8	7.35	320
Blank	N/A	29/11/2018	11:20		SJ- 65358. BNG- 88726	N/A	N/A	N/A	
RHB bridge	1	29/11/2018			SJ- 65358. BNG- 88726	6.7	8.9	7.4	300
RHB bridge	2	29/11/2018			SJ- 65358. BNG- 88726	6.7	8.9	7.4	300
RHB bridge	3	29/11/2018			SJ- 65358. BNG- 88726	6.7	8.9	7.4	300
Middle bridge	1	29/11/2018			SJ- 65386. BNG- 88708	6.9	8.8	7.2	300
Middle bridge	2	29/11/2018			SJ- 65386. BNG- 88708	6.9	8.8	7.2	300
Middle bridge	3	29/11/2018			SJ- 65386. BNG- 88708	6.9	8.8	7.2	350
LHB bridge	1	29/11/2018			N-53.39370. W-002.52190	6.7	9	7.3	350
LHB bridge	2	29/11/2018			N-53.39370. W-002.52190	6.7	9	7.3	300
LHB bridge	3	29/11/2018			N-53.39370. W-002.52190	6.7	9	7.3	300
Above weir	1	29/11/2018			N-53.39402. W-002.52431	6.6	8.9	7.26	325
Above weir	2	29/11/2018			N-53.39402. W-002.52431	6.6	8.9	7.26	300
Above weir	3	29/11/2018			N-53.39402. W-002.52431	6.6	8.9	7.26	300
Below weir	1	29/11/2018			N-53.39405. W-002.52424	6.2	8.7	7.42	260
Below weir	2	29/11/2018			N-53.39405. W-002.52424	6.2	8.7	7.42	250
Below weir	3	29/11/2018		14:35	N-53.39405. W-002.52424	6.2	8.7	7.42	320
Blank	N/A	06/12/2018	08:00		SJ- 65358. BNG- 88726	N/A	N/A	N/A	
RHB bridge	1	06/12/2018			SJ- 65358. BNG- 88726	7.8	7.52	8.8	130
RHB bridge	2	06/12/2018			SJ- 65358. BNG- 88726	7.8	7.52	8.8	120
RHB bridge	3	06/12/2018			SJ- 65358. BNG- 88726	7.8	7.52	8.8	100
Middle bridge	1	06/12/2018			SJ- 65386. BNG- 88708	7.8	7.3	8.7	150
Middle bridge	2	06/12/2018			SJ- 65386. BNG- 88708	7.8	7.3	8.7	240
Middle bridge	3	06/12/2018			SJ- 65386. BNG- 88708	7.8	7.3	8.7	600
LHB bridge	1	06/12/2018			N-53.39370. W-002.52190	7.9	7.42	8.8	160
LHB bridge	2	06/12/2018			N-53.39370. W-002.52190	7.9	7.42	8.8	300
LHB bridge	3	06/12/2018			N-53.39370. W-002.52190	7.9	7.42	8.8	150
Above weir	1	06/12/2018			N-53.39402. W-002.52431	7.7	7.42	8.7	140
Above weir	2	06/12/2018			N-53.39402. W-002.52431	7.7	7.42	8.7	180
Above weir	3	06/12/2018			N-53.39402. W-002.52431	7.7	7.42	8.7	160
Below weir	1	06/12/2018			N-53.39405. W-002.52424	8.6	7.84	8.69	130
Below weir	2	06/12/2018			N-53.39405. W-002.52424	8.6	7.84	8.69	120
Below weir	3	06/12/2018			N-53.39405. W-002.52424	8.6	7.84	8.69	110
Blank (end of sampling	N/A	06/12/2018		10:30	SJ- 65358. BNG- 88726	N/A	N/A	N/A	

Table S2: Autumn field data combined with lab data and primer combination

Site	Replicate	field collection date	Extraction date	lab no.	Sample/Primer	MiFish Primer combination
LHB bridge	1	22/11/2018	04/03/2019	1	1A	F01-R01
LHB bridge	2	22/11/2018	04/03/2019	2	1B	F02-R02
LHB bridge	3	22/11/2018	04/03/2019	3	1C	F03-R03
Middle bridge	1	22/11/2018	04/03/2019	4	1D	F04-R04
Middle bridge	2	22/11/2018	04/03/2019	5	1E	F05-R05
Middle bridge	3	22/11/2018	04/03/2019	6	1F	F06-R06
RHB bridge	1	22/11/2018	04/03/2019	7	1G	F07-R07
RHB bridge	2	22/11/2018	04/03/2019	8	1H	F08-R08
RHB bridge	3	22/11/2018	04/03/2019	9	2A	F09-R09
Above weir	1	22/11/2018	04/03/2019	10	2B	F10-R10
Above weir	2	22/11/2018	04/03/2019	11	2C	F11-R11
Above weir	3	22/11/2018	04/03/2019	12	2D	F12-R12
Below weir	1	22/11/2018	04/03/2019	13	2E	F13-R13
Below weir	2	22/11/2018	04/03/2019	14	2F	F14-R14
Below weir	3	22/11/2018	04/03/2019	15	2G	F15-R15
Field blank	N/A	22/11/2018	04/03/2019	16	2H	F16-R16
Extraction blank	N/A	N/A	04/03/2019	17	3A	F17-R17
LHB bridge	1	29/11/2018	06/03/2019	1	3B	F18-R18
LHB bridge	2	29/11/2018	06/03/2019	2	3C	F19-R19
LHB bridge	3	29/11/2018	06/03/2019	3	3D	F20-R20
Middle bridge	1	29/11/2018	06/03/2019	4	3E	F21-R21
Middle bridge	2	29/11/2018	06/03/2019	5	3F	F22-R22
Middle bridge	3	29/11/2018	06/03/2019	6	3G	F23-R23
RHB bridge	1	29/11/2018	06/03/2019	7	3H	F24-R24
RHB bridge	2	29/11/2018	06/03/2019	8	4A	F01-R04
RHB bridge	3	29/11/2018	06/03/2019	9	4B	F02-R05
Above weir	1	29/11/2018	06/03/2019	10	4C	F03-R06
Above weir	2	29/11/2018	06/03/2019	11	4D	F04-R07
Above weir	3	29/11/2018	06/03/2019	12	4E	F05-R08
Below weir	1	29/11/2018	06/03/2019	13	4F	F06-R09
Below weir	2	29/11/2018	06/03/2019	14	4G	F07-R10
Below weir	3	29/11/2018	06/03/2019	15	4H	F08-R11
Field Blank	N/A	29/11/2018	06/03/2019	16	5A	F09-R12
Extraction blank	N/A	N/A	06/03/2019	17	5B	F10-R13
LHB bridge	1	06/12/2018	07/03/2019	1	5C	F11-R14
LHB bridge	2	06/12/2018	07/03/2019	2	5D	F12-R15
LHB bridge	3	06/12/2018	07/03/2019	3	5E	F13-R16
Middle bridge	1	06/12/2018	07/03/2019	4	5F	F14-R17
Middle bridge	2	06/12/2018	07/03/2019	5	5G	F15-R18
Middle bridge	3	06/12/2018	07/03/2019	6	5H	F16-R19
RHB bridge	1	06/12/2018	07/03/2019	7	6A	F17-R20
RHB bridge	2	06/12/2018	07/03/2019	8	6B	F18-R21
RHB bridge	3	06/12/2018	07/03/2019	9	6C	F19-R22
Above weir	1	06/12/2018	07/03/2019	10	6D	F20-R23
Above weir	2	06/12/2018	07/03/2019	11	6E	F21-R24
Above weir	3	06/12/2018	07/03/2019	12	6F	F22-R01
Below weir	1	06/12/2018	07/03/2019	13	6G	F23-R02
Below weir	2	06/12/2018	07/03/2019	14	6H	F24-R03
Below weir	3	06/12/2018	07/03/2019	15	7A	F01-R07
Field blank	N/A	06/12/2018	07/03/2019	16	7B	F02-R08
Extraction blank	N/A	N/A	07/03/2019	17	7C	F03-R09
Field Blank	N/A	12/11/2018	07/03/2019	22	7H	F08-R14
PCR Blank	N/A				8A	F09-R15
PCR Blank	N/A				8B	F10-R16
PCR Blank	N/A				8C	F11-R17
PCR Blank	N/A				8D	F12-R18
PCR Blank	N/A				8E	F13-R19
PCR Blank	N/A				8F	F14-R20
PCR Blank	N/A				8G	F15-R21
PCR Blank	N/A				8H	F16-R22

Table S3: Field log data from Spring sampling period (6th May 2019)

Zone	Site	GPS	Site no.	Replicate	sample date	Oxygen mg/l	Salinity ppt	pH	Temp	ml (filter)
Estuary	Liverpool (shung ku)	53.378637, -2.973815	Blank	1	6.5.19					1000
Upper Estuary	Widnes salt marsh	53.356643, -2.7153	Blank	2	6.5.19					1000
River	Howley weir (above)	53.385295, -2.578389	Blank	3	6.5.19					1000
Lab Extraction			Blank	N/A						
Estuary	Liverpool (shung ku)	53.378637, -2.973815	1	A	6.5.19	8.8	23.2	8.03	11.1	160
		53.378637, -2.973815		B	6.5.19	8.8	23.2	8.03	11.1	150
		53.378637, -2.973815		C	6.5.19	8.8	23.2	8.03	11.1	180
Estuary	Speke coastal reserve	53.342356, -2.898703	2	A	6.5.19	8.4	21.3	8.03	11	180
		53.342356, -2.898703		B	6.5.19	8.4	21.3	8.03	11	120
		53.342356, -2.898703		C	6.5.19	8.4	21.3	8.03	11	120
Estuary	Lighthouse Road (Hale)	53.322335, -2794428	3	A	6.5.19	8.5	19.1	8.02	11.6	120
		53.322335, -2794428		B	6.5.19	8.5	19.1	8.02	11.6	120
		53.322335, -2794428		C	6.5.19	8.5	19.1	8.02	11.6	120
Estuary	Pickering pasture	53.345195, -2.769289	4	A	6.5.19	8.1	19.2	7.95	11.7	270
		53.345195, -2.769289		B	6.5.19	8.1	19.2	7.95	11.7	180
		53.345195, -2.769289		C	6.5.19	8.1	19.2	7.95	11.7	120
Estuary	Catalyst museum	53.35048, -2.731319	5	A	6.5.19	7.9	17.5	7.9	11.4	110
		53.35048, -2.731319		B	6.5.19	7.9	17.5	7.9	11.4	120
		53.35048, -2.731319		C	6.5.19	7.9	17.5	7.9	11.4	110
Upper Estuary	Widnes salt marsh	53.356643, -2.7153	6	A	6.5.19	7.9	19.8	7.91	11.8	100
		53.356643, -2.7153		B	6.5.19	7.9	19.8	7.91	11.8	120
		53.356643, -2.7153		C	6.5.19	7.9	19.8	7.91	11.8	180
Upper Estuary	Fiddler ferry	53.375017, -2.657484	7	A	6.5.19	7.9	0.35	7.54	12.8	300
		53.375017, -2.657484		B	6.5.19	7.9	0.35	7.54	12.8	250
		53.375017, -2.657484		C	6.5.19	7.9	0.35	7.54	12.8	260
Lab extraction			Blank	N/A						
Upper Estuary	Eastford road (Warrington)	53.374244, -2.60519	8	A	6.5.19	8	0.32	7.75	12.8	180
		53.374244, -2.60519		B	6.5.19	8	0.32	7.75	12.8	130
		53.374244, -2.60519		C	6.5.19	8	0.32	7.75	12.8	120
Upper Estuary	Arpley rd (Warrington)	53.384742, -2.592994	9	A	6.5.19	7.6	0.38	7.54	12.1	70
		53.384742, -2.592994		B	6.5.19	7.6	0.38	7.54	12.1	70
		53.384742, -2.592994		C	6.5.19	7.6	0.38	7.54	12.1	70
River	Howley weir (above)	53.385295, -2.578389	10	A	6.5.19	6.6	0.26	7.52	12.1	360
		53.385295, -2.578389		B	6.5.19	6.6	0.26	7.52	12.1	360
		53.385295, -2.578389		C	6.5.19	6.6	0.26	7.52	12.1	360
River	Kingsway st (bridge)	53.387683, -2.565306	11	A	6.5.19	6.7	0.26	7.57	11.9	370
		53.387683, -2.565307		B	6.5.19	6.7	0.26	7.57	11.9	400
		53.387683, -2.565308		C	6.5.19	6.7	0.26	7.57	11.9	370
River	Paddington meadows	53.396108, -2.546427	12	A	6.5.19	5.9	0.25	7.45	11.8	400
		53.396108, -2.546427		B	6.5.19	5.9	0.25	7.45	11.8	370
		53.396108, -2.546427		C	6.5.19	5.9	0.25	7.45	11.8	290
River	Woolston weir	53.393941, -2.522169	13	A	6.5.19	4.8	0.25	7.48	12	350
		53.393941, -2.522169		B	6.5.19	4.8	0.25	7.48	12	300
		53.393941, -2.522169		C	6.5.19	4.8	0.25	7.48	12	300
River	Swithen hill wood	53.398541, -2.491708	14	A	6.5.19	5.2	0.25	7.5	12.1	340
		53.398541, -2.491708		B	6.5.19	5.2	0.25	7.5	12.1	300
		53.398541, -2.491708		C	6.5.19	5.2	0.25	7.5	12.1	340

Table S4: Field log data from Spring sampling period (21st May 2019)

Zone	Site	GPS	Site no.	Replicate	sample date	Oxygen mg/l	Salinity ppt	pH	Temp	ml (filter)
Estuary	Liverpool (shung ku)	53.378637, -2.973815	Blank	1	20.5.19					1000
Upper Estuary	Widnes salt marsh	53.356643, -2.7153	Blank	2	20.5.19					1000
River	Howley weir (above)	53.385295, -2.578389	Blank	3	20.5.19					1000
Lab Extraction			Blank	N/A						
Estuary	Liverpool (shung ku)	53.378637, -2.973815	1	A	20.5.19	8.7	25.2	8.04	15.3	160
		53.378637, -2.973815		B	20.5.19	8.7	25.2	8.04	15.3	180
		53.378637, -2.973815		C	20.5.19	8.7	25.2	8.04	15.3	180
Estuary	Speke coastal reserve	53.342356, -2.898703	2	A	20.5.19	8.4	24.3	8.04	17.4	200
		53.342356, -2.898703		B	20.5.19	8.4	24.3	8.04	17.4	240
		53.342356, -2.898703		C	20.5.19	8.4	24.3	8.04	17.4	300
Estuary	Lighthouse Road (Hale)	53.322335, -2794428	3	A	20.5.19	7.9	20.6	7.99	17	250
		53.322335, -2794428		B	20.5.19	7.9	20.6	7.99	17	300
		53.322335, -2794428		C	20.5.19	7.9	20.6	7.99	17	480
Estuary	Pickering pasture	53.345195, -2.769289	4	A	20.5.19	7.2	21.1	7.92	15.8	240
		53.345195, -2.769289		B	20.5.19	7.2	21.1	7.92	15.8	240
		53.345195, -2.769289		C	20.5.19	7.2	21.1	7.92	15.8	240
Estuary	Catalyst museum	53.35048, -2.731319	5	A	20.5.19	7	20.5	7.86	16.4	360
		53.35048, -2.731319		B	20.5.19	7	20.5	7.86	16.4	300
		53.35048, -2.731319		C	20.5.19	7	20.5	7.86	16.4	330
Upper Estuary	Widnes salt marsh	53.356643, -2.7153	6	A	20.5.19	7	22.5	7.91	16	300
		53.356643, -2.7153		B	20.5.19	7	22.5	7.91	16	360
		53.356643, -2.7153		C	20.5.19	7	22.5	7.91	16	360
Upper Estuary	Fiddler ferry	53.375017, -2.657484	7	A	20.5.19	6.6	10.5	7.7	17.1	240
		53.375017, -2.657484		B	20.5.19	6.6	10.5	7.7	17.1	300
		53.375017, -2.657484		C	20.5.19	6.6	10.5	7.7	17.1	300
Lab extraction			Blank	N/A						
Upper Estuary	Eastford road (Warrington)	53.374244, -2.60519	8	A	20.5.19	6.8	0.43	8.03	16.8	180
		53.374244, -2.60519		B	20.5.19	6.8	0.43	8.03	16.8	180
		53.374244, -2.60519		C	20.5.19	6.8	0.43	8.03	16.8	200
Upper Estuary	Arpley rd (Warrington)	53.384742, -2.592994	9	A	20.5.19	8.4	0.43	7.88	16.5	180
		53.384742, -2.592994		B	20.5.19	8.4	0.43	7.88	16.5	200
		53.384742, -2.592994		C	20.5.19	8.4	0.43	7.88	16.5	180
River	Howley weir (above)	53.385295, -2.578389	10	A	20.5.19	6.1	0.3	7.6	15.2	480
		53.385295, -2.578389		B	20.5.19	6.1	0.3	7.6	15.2	450
		53.385295, -2.578389		C	20.5.19	6.1	0.3	7.6	15.2	420
River	Kingsway st (bridge)	53.387683, -2.565306	11	A	20.5.19	6.9	0.3	7.58	15.4	600
		53.387683, -2.565307		B	20.5.19	6.9	0.3	7.58	15.4	480
		53.387683, -2.565308		C	20.5.19	6.9	0.3	7.58	15.4	540
River	Paddington meadows	53.396108, -2.546427	12	A	20.5.19	6.1	0.3	7.78	14.7	400
		53.396108, -2.546427		B	20.5.19	6.1	0.3	7.78	14.7	360
		53.396108, -2.546427		C	20.5.19	6.1	0.3	7.78	14.7	360
River	Woolston weir	53.393941, -2.522169	13	A	20.5.19	6.1	0.29	7.54	15.4	440
		53.393941, -2.522169		B	20.5.19	6.1	0.29	7.54	15.4	440
		53.393941, -2.522169		C	20.5.19	6.1	0.29	7.54	15.4	420
River	Swithen hill wood	53.398541, -2.491708	14	A	20.5.19	6.3	0.36	7.7	19.4	420
		53.398541, -2.491708		B	20.5.19	6.3	0.36	7.7	19.4	480
		53.398541, -2.491708		C	20.5.19	6.3	0.36	7.7	19.4	420

Table S5: Field log data from Spring sampling period (3rd June 2019)

Zone	Site	GPS	Site no.	Replicate	sample date	Oxygen mg/l	Salinity ppt	pH	Temp	ml (filter)
Estuary	Liverpool (shung ku)	53.378637, -2.973815	Blank	1	03/06/2019					1000
Upper Estuary	Widnes salt marsh	53.356643, -2.7153	Blank	2	03/06/2019					1000
River	Howley weir (above)	53.385295, -2.578389	Blank	3	03/06/2019					1000
Lab Extraction			Blank	N/A	03/06/2019					
Estuary	Liverpool (shung ku)	53.378637, -2.973815	1	A	03/06/2019	8.6	25	8.14	15.8	200
		53.378637, -2.973815		B	03/06/2019	8.6	25	8.14	15.8	240
		53.378637, -2.973815		C	03/06/2019	8.6	25	8.14	15.8	210
Estuary	Speke coastal reserve	53.342356, -2.898703	2	A	03/06/2019	8.5	23.9	8.13	16	200
		53.342356, -2.898703		B	03/06/2019	8.5	23.9	8.13	16	215
		53.342356, -2.898703		C	03/06/2019	8.5	23.9	8.13	16	240
Estuary	Lighthouse Road (Hale)	53.322335, -2794428	3	A	03/06/2019	8.6	21.6	8.12	16.9	300
		53.322335, -2794428		B	03/06/2019	8.6	21.6	8.12	16.9	360
		53.322335, -2794428		C	03/06/2019	8.6	21.6	8.12	16.9	310
Estuary	Pickering pasture	53.345195, -2.769289	4	A	03/06/2019	8.3	22.6	8.7	16.2	300
		53.345195, -2.769289		B	03/06/2019	8.3	22.6	8.7	16.2	240
		53.345195, -2.769289		C	03/06/2019	8.3	22.6	8.7	16.2	300
Estuary	Catalyst museum	53.35048, -2.731319	5	A	03/06/2019	8.6	18.8	8.04	16.8	240
		53.35048, -2.731319		B	03/06/2019	8.6	18.8	8.04	16.8	300
		53.35048, -2.731319		C	03/06/2019	8.6	18.8	8.04	16.8	300
Upper Estuary	Widnes salt marsh	53.356643, -2.7153	6	A	03/06/2019	9.1	21.3	8.07	16.4	320
		53.356643, -2.7153		B	03/06/2019	9.1	21.3	8.07	16.4	360
		53.356643, -2.7153		C	03/06/2019	9.1	21.3	8.07	16.4	300
Upper Estuary	Fiddler ferry	53.375017, -2.657484	7	A	03/06/2019	7.2	12.9	7.95	16.7	240
		53.375017, -2.657484		B	03/06/2019	7.2	12.9	7.95	16.7	240
		53.375017, -2.657484		C	03/06/2019	7.2	12.9	7.95	16.7	240
Lab extraction			Blank	N/A	03/06/2019					
Upper Estuary	Eastford road (Warrington)	53.374244, -2.60519	8	A	03/06/2019	7.8	0.36	7.1	17.9	210
		53.374244, -2.60519		B	03/06/2019	7.8	0.36	7.1	17.9	210
		53.374244, -2.60519		C	03/06/2019	7.8	0.36	7.1	17.9	200
Upper Estuary	Arpley rd (Warrington)	53.384742, -2.592994	9	A	03/06/2019	8.1	0.35	7.4	17	240
		53.384742, -2.592994		B	03/06/2019	8.1	0.35	7.4	17	340
		53.384742, -2.592994		C	03/06/2019	8.1	0.35	7.4	17	300
River	Howley weir (above)	53.385295, -2.578389	10	A	03/06/2019	5.9	0.29	7.52	15.9	300
		53.385295, -2.578389		B	03/06/2019	5.9	0.29	7.52	15.9	330
		53.385295, -2.578389		C	03/06/2019	5.9	0.29	7.52	15.9	410
River	Kingsway st (bridge)	53.387683, -2.565306	11	A	03/06/2019	5.7	0.3	7.49	16.1	600
		53.387683, -2.565307		B	03/06/2019	5.7	0.3	7.49	16.1	400
		53.387683, -2.565308		C	03/06/2019	5.7	0.3	7.49	16.1	480
River	Paddington meadows	53.396108, -2.546427	12	A	03/06/2019	6.4	0.3	7.4	16.7	360
		53.396108, -2.546427		B	03/06/2019	6.4	0.3	7.4	16.7	340
		53.396108, -2.546427		C	03/06/2019	6.4	0.3	7.4	16.7	340
River	Woolston weir	53.393941, -2.522169	13	A	03/06/2019	5.2	0.31	7.7	18.1	440
		53.393941, -2.522169		B	03/06/2019	5.2	0.31	7.7	18.1	360
		53.393941, -2.522169		C	03/06/2019	5.2	0.31	7.7	18.1	360
River	Swithen hill wood	53.398541, -2.491708	14	A	03/06/2019	5.3	0.3	7.62	17.5	420
		53.398541, -2.491708		B	03/06/2019	5.3	0.3	7.62	17.5	420
		53.398541, -2.491708		C	03/06/2019	5.3	0.3	7.62	17.5	360

Table S6: Spring field data combined with lab data and primer combination (controls are highlighted in red)

Site	Replicate	field collection date	lab no.	Sample	MiFish Primer combination
Field blank	N/A	06/05/2109	1	1A	F01-R01
Field blank	N/A	06/05/2019	2	1B	F02-R02
Field blank	N/A	06/05/1929	3	1C	F03-R03
Extraction blank	N/A	06/05/2019	4	1D	F04-R04
Liverpool (shung ku)	A	06/05/2109	5	1E	F05-R05
Liverpool (shung ku)	B	06/05/2019	6	1F	F06-R06
Liverpool (shung ku)	C	06/05/2109	7	1G	F07-R07
Speke coastal reserve	A	06/05/2019	8	1H	F08-R08
Speke coastal reserve	B	06/05/2109	9	2A	F09-R09
Speke coastal reserve	C	06/05/2019	10	2B	F10-R10
Lighthouse Road (Hale)	A	06/05/2109	11	2C	F11-R11
Lighthouse Road (Hale)	B	06/05/2019	12	2D	F12-R12
Lighthouse Road (Hale)	C	06/05/2109	13	2E	F13-R13
Pickering pasture	A	06/05/2019	14	2F	F14-R14
Pickering pasture	B	06/05/2109	15	2G	F15-R15
Pickering pasture	C	06/05/2019	16	2H	F16-R16
Catalyst museum	A	06/05/2109	17	3A	F17-R17
Catalyst museum	B	06/05/2019	18	3B	F18-R18
Catalyst museum	C	06/05/2109	19	3C	F19-R19
Widnes salt marsh	A	06/05/2019	20	3D	F20-R20
Widnes salt marsh	B	06/05/2109	21	3E	F21-R21
Widnes salt marsh	C	06/05/2019	22	3F	F22-R22
Fiddler ferry	A	06/05/2109	23	3G	F23-R23
Fiddler ferry	B	06/05/2019	24	3H	F24-R24
Fiddler ferry	C	06/05/2109	25	4A	F01-R04
Extraction blank	N/A	06/05/2019	26	4B	F02-R05
Eastford road (Warrington)	A	06/05/2109	27	4C	F03-R06
Eastford road (Warrington)	B	06/05/2019	28	4D	F04-R07
Eastford road (Warrington)	C	06/05/2109	29	4E	F05-R08
Arpley rd (Warrington)	A	06/05/2019	30	4F	F06-R09
Arpley rd (Warrington)	B	06/05/2109	31	4G	F07-R10
Arpley rd (Warrington)	C	06/05/2019	32	4H	F08-R11
Howley weir (above)	A	06/05/1929	33	5A	F09-R12
Howley weir (above)	B	06/05/2019	34	5B	F10-R13
Howley weir (above)	C	06/05/2109	35	5C	F11-R14
Kingsway st (bridge)	A	06/05/2019	36	5D	F12-R15
Kingsway st (bridge)	B	06/05/2109	37	5E	F13-R16
Kingsway st (bridge)	C	06/05/2019	38	5F	F14-R17
Paddington meadows	A	06/05/2109	39	5G	F15-R18
Paddington meadows	B	06/05/2019	40	5H	F16-R19

Paddington meadows	C	06/05/2109	41	6A	F17-R20
Woolston weir	A	06/05/2019	42	6B	F18-R21
Woolston weir	B	06/05/2109	43	6C	F19-R22
Woolston weir	C	06/05/2019	44	6D	F20-R23
Swithen hill wood	A	06/05/2109	45	6E	F21-R24
Swithen hill wood	B	06/05/2019	46	6F	F22-R01
Swithen hill wood	C	06/05/2109	47	6G	F23-R02
Field blank	N/A	21/05/2019	48	6H	F24-R03
Field blank	N/A	21/05/2019	49	7A	F01-R07
Field blank	N/A	21/05/2019	50	7B	F02-R08
Extraction blank	N/A	21/05/2019	51	7C	F03-R09
Liverpool (shung ku)	A	21/05/2019	52	7D	F04-R10
Liverpool (shung ku)	B	21/05/2019	53	7E	F05-R11
Liverpool (shung ku)	C	21/05/2019	54	7F	F06-R12
Speke coastal reserve	A	21/05/2019	55	7G	F07-R13
Speke coastal reserve	B	21/05/2019	56	7H	F08-R14
Speke coastal reserve	C	21/05/2019	57	8A	F09-R15
Lighthouse Road (Hale)	A	21/05/2019	58	8B	F10-R16
Lighthouse Road (Hale)	B	21/05/2019	59	8C	F11-R17
Lighthouse Road (Hale)	C	21/05/2019	60	8D	F12-R18
Pickering pasture	A	21/05/2019	61	8E	F13-R19
Pickering pasture	B	21/05/2019	62	8F	F14-R20
Pickering pasture	C	21/05/2019	63	8G	F15-R21
Catalyst museum	A	21/05/2019	64	8H	F16-R22
Catalyst museum	B	21/05/2019	65	9A	F17-R23
Catalyst museum	C	21/05/2019	66	9B	F18-R24
Widnes salt marsh	A	21/05/2019	67	9C	F19-R01
Widnes salt marsh	B	21/05/2019	68	9D	F20-R02
Widnes salt marsh	C	21/05/2019	69	9E	F21-R03
Fiddler ferry	A	21/05/2019	70	9F	F22-R04
Fiddler ferry	B	21/05/2019	71	9G	F23-R05
PCR blank	N/A	21/05/2019	72	9H	F24-R06
PCR blank	N/A	21/05/2019	73	10A	F01-R10
PCR blank	N/A	21/05/2019	74	10B	F02-R11
PCR blank	N/A	21/05/2019	75	10C	F03-R12
Fiddler ferry	C	21/05/2019	76	1A	F01-R01
Extraction blank	N/A	21/05/2019	77	1B	F02-R02
Eastford road (Warrington)	A	21/05/2019	78	1C	F03-R03
Eastford road (Warrington)	B	21/05/2019	79	1D	F04-R04
Eastford road (Warrington)	C	21/05/2019	80	1E	F05-R05
Arpley rd (Warrington)	A	21/05/2019	81	1F	F06-R06
Arpley rd (Warrington)	B	21/05/2019	82	1G	F07-R07
Arpley rd (Warrington)	C	21/05/2019	83	1H	F08-R08
Howley weir (above)	A	21/05/2019	84	2A	F09-R09
Howley weir (above)	B	21/05/2019	85	2B	F10-R10

Howley weir (above)	C	21/05/2019	86	2C	F11-R11
Kingsway st (bridge)	A	21/05/2019	87	2D	F12-R12
Kingsway st (bridge)	B	21/05/2019	88	2E	F13-R13
Kingsway st (bridge)	C	21/05/2019	89	2F	F14-R14
Paddington meadows	A	21/05/2019	90	2G	F15-R15
Paddington meadows	B	21/05/2019	91	2H	F16-R16
Paddington meadows	C	21/05/2019	92	3A	F17-R17
Woolston weir	A	21/05/2019	93	3B	F18-R18
Woolston weir	B	21/05/2019	94	3C	F19-R19
Woolston weir	C	21/05/2019	95	3D	F20-R20
Swithen hill wood	A	21/05/2019	96	3E	F21-R21
Swithen hill wood	B	21/05/2019	97	3F	F22-R22
Swithen hill wood	C	21/05/2019	98	3G	F23-R23
Field blank	N/A	03/06/2019	99	3H	F24-R24
Field blank	N/A	03/06/2019	100	4A	F01-R04
Field blank	N/A	03/06/2019	101	4B	F02-R05
Extraction blank	N/A	03/06/2019	102	4C	F03-R06
Liverpool (shung ku)	A	03/06/2019	103	4D	F04-R07
Liverpool (shung ku)	B	03/06/2019	104	4E	F05-R08
Liverpool (shung ku)	C	03/06/2019	105	4F	F06-R09
Speke coastal reserve	A	03/06/2019	106	4G	F07-R10
Speke coastal reserve	B	03/06/2019	107	4H	F08-R11
Speke coastal reserve	C	03/06/2019	108	5A	F09-R12
Lighthouse Road (Hale)	A	03/06/2019	109	5B	F10-R13
Lighthouse Road (Hale)	B	03/06/2019	110	5C	F11-R14
Lighthouse Road (Hale)	C	03/06/2019	111	5D	F12-R15
Pickering pasture	A	03/06/2019	112	5E	F13-R16
Pickering pasture	B	03/06/2019	113	5F	F14-R17
Pickering pasture	C	03/06/2019	114	5G	F15-R18
Catalyst museum	A	03/06/2019	115	5H	F16-R19
Catalyst museum	B	03/06/2019	116	6A	F17-R20
Catalyst museum	C	03/06/2019	117	6B	F18-R21
Widnes salt marsh	A	03/06/2019	118	6C	F19-R22
Widnes salt marsh	B	03/06/2019	119	6D	F20-R23
Widnes salt marsh	C	03/06/2019	120	6E	F21-R24
Fiddler ferry	A	03/06/2019	121	6F	F22-R01
Fiddler ferry	B	03/06/2019	122	6G	F23-R02
Fiddler ferry	C	03/06/2019	123	6H	F24-R03
Extraction blank	N/A	03/06/2019	124	7A	F01-R07
Eastford road (Warrington)	A	03/06/2019	125	7B	F02-R08
Eastford road (Warrington)	B	03/06/2019	126	7C	F03-R09
Eastford road (Warrington)	C	03/06/2019	127	7D	F04-R10
Arpley rd (Warrington)	A	03/06/2019	128	7E	F05-R11
Arpley rd (Warrington)	B	03/06/2019	129	7F	F06-R12
Arpley rd (Warrington)	C	03/06/2019	130	7G	F07-R13

Howley weir (above)	A	03/06/2019	131	7H	F08-R14
Howley weir (above)	B	03/06/2019	132	8A	F09-R15
Howley weir (above)	C	03/06/2019	133	8B	F10-R16
Kingsway st (bridge)	A	03/06/2019	134	8C	F11-R17
Kingsway st (bridge)	B	03/06/2019	135	8D	F12-R18
Kingsway st (bridge)	C	03/06/2019	136	8E	F13-R19
Paddington meadows	A	03/06/2019	137	8F	F14-R20
Paddington meadows	B	03/06/2019	138	8G	F15-R21
Paddington meadows	C	03/06/2019	139	8H	F16-R22
Woolston weir	A	03/06/2019	140	9A	F17-R23
Woolston weir	B	03/06/2019	141	9B	F18-R24
Woolston weir	C	03/06/2019	142	9C	F19-R01
Swithen hill wood	A	03/06/2019	143	9D	F20-R02
Swithen hill wood	B	03/06/2019	144	9E	F21-R03
Swithen hill wood	C	03/06/2019	145	9F	F22-R04
PCR blank	N/A	03/06/2019	146	9G	F23-R05
PCR blank	N/A	03/06/2019	147	9H	F24-R06
PCR blank	N/A	03/06/2019	148	10A	F01-R10
PCR blank	N/A	03/06/2019	149	10B	F02-R11

Table S7: Autumn eDNA results: the number of eDNA reads across different sampling sites by date

Species	Common name	Date	Below weir	Above weir	Left bridge	Middle bridge	Right bridge
Rutilus rutilus	Roach	22/11/2018	220193	95854	36265	112581	147235
Gasterosteus aculeatus	Three spined stickleback	22/11/2018	72892	40132	19058	40611	68472
Salmo trutta	Brown trout	22/11/2018	4897	2872	753	8666	936
Barbatula barbatula	Stone loach	22/11/2018	11651	14894	2231	3394	7571
Cottus gobio	Bull head	22/11/2018	2853	998	1511	5450	6059
Anguilla anguilla	European eel	22/11/2018	3377	4449	894	71	2628
Esox lucius	Northern pike	22/11/2018	2480	1962	586	3	1203
Cyprinus carpio	Common carp	22/11/2018	0	0	0	0	5
Lampetra fluviatilis	River lamprey	22/11/2018	0	0	0	0	179
Barbus barbus	Barbel	22/11/2018	0	0	0	8	0
Abramis brama	Common bream	22/11/2018	0	0	0	0	0
Salmo salar	Atlantic Salmon	22/11/2018	0	0	0	0	0
Perca fluviatilis	European perch	22/11/2018	0	0	0	0	0
Gobio gobio	Gudgen	22/11/2018	0	0	0	0	0
Dicentrarchus labrax	European sea bass	22/11/2018	30	0	20	0	0
Sardina pilchardus	European pilchard	22/11/2018	0	0	0	0	3410
Scomber scombrus	Atlantic mackerel	22/11/2018	0	0	0	0	0
Solea solea	Common sole	22/11/2018	0	0	0	0	0
Oreochromis aureus	Blue tilapia	22/11/2018	0	0	0	0	0
Pleuronectes platessa	European plaice	22/11/2018	0	0	0	0	0
Pollachius pollachius	Pollock	22/11/2018	0	0	0	0	0
Rutilus rutilus	Roach	29/11/2018	93390	135356	191508	72040	118329
Gasterosteus aculeatus	Three spined stickleback	29/11/2018	51818	111038	29824	38801	56699
Salmo trutta	Brown trout	29/11/2018	6684	23544	3112	2621	9882
Barbatula barbatula	Stone loach	29/11/2018	2920	12661	294	6036	4891
Cottus gobio	Bull head	29/11/2018	0	2379	24	6494	5107
Anguilla anguilla	European eel	29/11/2018	10007	5658	3874	3056	4583
Esox lucius	Northern pike	29/11/2018	4352	2525	4817	0	851
Cyprinus carpio	Common carp	29/11/2018	0	9	0	0	0
Lampetra fluviatilis	River lamprey	29/11/2018	0	0	0	0	0
Barbus barbus	Barbel	29/11/2018	0	0	0	0	4
Abramis brama	Common bream	29/11/2018	5	7	8	0	0
Salmo salar	Atlantic Salmon	29/11/2018	0	0	0	0	0
Perca fluviatilis	European perch	29/11/2018	4	0	0	0	0
Gobio gobio	Gudgen	29/11/2018	3	0	0	0	0
Dicentrarchus labrax	European sea bass	29/11/2018	0	12	7	30	1395
Sardina pilchardus	European pilchard	29/11/2018	0	0	0	0	0
Scomber scombrus	Atlantic mackerel	29/11/2018	0	0	0	0	0
Solea solea	Common sole	29/11/2018	0	0	0	0	0
Oreochromis aureus	Blue tilapia	29/11/2018	0	0	0	0	0
Pleuronectes platessa	European plaice	29/11/2018	0	0	0	0	0
Pollachius pollachius	Pollock	29/11/2018	0	0	0	0	0
Rutilus rutilus	Roach	06/12/2018	69623	39797	59928	46899	94523
Gasterosteus aculeatus	Three spined stickleback	06/12/2018	39756	12563	38136	20810	52982
Salmo trutta	Brown trout	06/12/2018	12642	9878	14465	14045	30788
Barbatula barbatula	Stone loach	06/12/2018	14240	5170	9051	5104	16784
Cottus gobio	Bull head	06/12/2018	4263	3363	2478	3767	6770
Anguilla anguilla	European eel	06/12/2018	1361	0	9	486	1062
Esox lucius	Northern pike	06/12/2018	1935	281	1723	915	2609
Cyprinus carpio	Common carp	06/12/2018	917	0	0	0	0
Lampetra fluviatilis	River lamprey	06/12/2018	0	0	0	0	0
Barbus barbus	Barbel	06/12/2018	11	0	0	6	0
Abramis brama	Common bream	06/12/2018	7	0	2	2	2
Salmo salar	Atlantic Salmon	06/12/2018	8	5	5	5	5
Perca fluviatilis	European perch	06/12/2018	0	0	0	0	0
Gobio gobio	Gudgen	06/12/2018	0	0	0	0	0
Dicentrarchus labrax	European sea bass	06/12/2018	5580	4677	4595	3274	9807
Sardina pilchardus	European pilchard	06/12/2018	0	172	0	0	0
Scomber scombrus	Atlantic mackerel	06/12/2018	0	445	420	0	66
Solea solea	Common sole	06/12/2018	0	0	817	0	0
Oreochromis aureus	Blue tilapia	06/12/2018	776	0	0	0	0
Pleuronectes platessa	European plaice	06/12/2018	0	416	0	0	0
Pollachius pollachius	Pollock	06/12/2018	0	0	0	0	44

Table S8: Spring eDNA results: the number of eDNA reads across different sampling sites by date

scientific_name	common_name	Habitat	Date	Liverpool	Speke	Lighthouse	Pickering	Catalyst	Widnes	Fiddler	Eastford	Arpley	Howley	Kingsway	Paddington	Woolston	Swithen
Chelon labrosus	Thicklip grey mullet	Marine	06/05/2019	0	0	0	0	0	0	0	0	0	0	0	0	0	2400
Dicentrarchus labrax	European bass	Marine	06/05/2019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gadus morhua	Atlantic cod	Marine	06/05/2019	0	0	0	0	0	0	0	1631	2921	0	0	0	0	0
Solea solea	Common sole	Marine	06/05/2019	0	0	0	0	0	0	0	369	0	0	0	0	0	0
Platichthys stellatus	Starry flounder	Marine	06/05/2019	13844	0	0	0	0	0	0	13606	0	0	0	8137	515	0
Clupea harengus	Atlantic herring	Marine	06/05/2019	0	0	0	0	0	0	0	4824	0	0	0	494	0	0
Pomatoschistus minutus	Sand goby	Marine	06/05/2019	0	0	0	0	0	0	0	0	0	0	0	0	19	0
Sardina pilchardus	Pilchard	Marine	06/05/2019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ammodytes marinus	Sand eel	Marine	06/05/2019	0	0	0	0	0	0	0	0	0	0	578	0	0	0
Barbatula barbatula	Stone loach	Freshwater	06/05/2019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cottus gobio	Bullhead	Freshwater	06/05/2019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cyprinus carpio	Common carp	Freshwater	06/05/2019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Esox lucius	Northern pike	Freshwater	06/05/2019	0	202	5785	1731	0	4	0	0	0	19	0	21	0	0
Gasterosteus aculeatus	Three-spined stickleback	Freshwater	06/05/2019	7409	438	5406	4377	2123	24	9647	0	0	0	0	100	82	32
Gymnocephalus cernua	Ruffe	Freshwater	06/05/2019	0	0	0	0	0	0	0	0	0	0	0	7	0	0
Phoxinus phoxinus	Common minnow	Freshwater	06/05/2019	0	0	0	11	0	0	0	0	0	0	0	0	11	0
Rutilus rutilus	Common roach	Freshwater	06/05/2019	68690	9345	230334	167759	89759	0	89469	0	0	0	0	1209	0	0
Thymallus thymallus	Grayling	Freshwater	06/05/2019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Anguilla anguilla	European Eel	Anadromous	06/05/2019	7391	477	3459	2564	0	0	0	0	0	69	481	4472	8	0
Lampetra fluviatilis	River Lamprey	Anadromous	06/05/2019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Petromyzon marinus	Sea lamprey	Anadromous	06/05/2019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Salmo salar	Atlantic salmon	Anadromous	06/05/2019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Salmo trutta	Brown trout	Anadromous	06/05/2019	0	76	0	0	0	0	0	0	0	0	0	0	0	0
Chelon labrosus	Thicklip grey mullet	Marine	21/05/2019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dicentrarchus labrax	European bass	Marine	21/05/2019	0	0	0	0	0	804	0	0	0	0	0	0	0	0
Gadus morhua	Atlantic cod	Marine	21/05/2019	0	0	0	0	0	0	0	0	7714	0	0	0	0	0
Solea solea	Common sole	Marine	21/05/2019	0	0	0	0	0	0	0	0	8152	0	0	0	0	0
Platichthys stellatus	Starry flounder	Marine	21/05/2019	3544	29823	1398	0	0	0	0	0	0	0	0	0	10403	0
Clupea harengus	Atlantic herring	Marine	21/05/2019	0	0	0	0	0	0	0	0	2179	0	0	112	0	0
Pomatoschistus minutus	Sand goby	Marine	21/05/2019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sardina pilchardus	Pilchard	Marine	21/05/2019	0	0	0	0	0	0	0	0	0	0	0	0	3	0
Ammodytes marinus	Sand eel	Marine	21/05/2019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Barbatula barbatula	Stone loach	Freshwater	21/05/2019	0	0	0	0	0	199	0	0	0	0	12	17	0	0
Cottus gobio	Bullhead	Freshwater	21/05/2019	0	0	0	0	0	0	0	0	0	0	3	12	10490	0
Cyprinus carpio	Common carp	Freshwater	21/05/2019	0	0	0	0	0	0	0	0	0	0	5	0	0	0
Esox lucius	Northern pike	Freshwater	21/05/2019	6857	1228	807	6936	4815	1124	0	0	0	0	133	246	0	0
Gasterosteus aculeatus	Three-spined stickleback	Freshwater	21/05/2019	0	20293	1241	37224	30391	51265	10	0	3976	0	126	134	43	0
Gymnocephalus cernua	Ruffe	Freshwater	21/05/2019	0	0	0	4650	868	0	0	0	0	0	3	0	0	0
Phoxinus phoxinus	Common minnow	Freshwater	21/05/2019	0	0	77	1856	1290	7689	2	0	0	0	5	17	0	0
Rutilus rutilus	Common roach	Freshwater	21/05/2019	26110	17813	7235	105797	113636	129674	0	0	0	0	0	0	1870	0
Thymallus thymallus	Grayling	Freshwater	21/05/2019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Anguilla anguilla	European Eel	Anadromous	21/05/2019	3098	3439	4751	19316	48204	1207	0	0	0	0	567	124	0	0
Lampetra fluviatilis	River Lamprey	Anadromous	21/05/2019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Petromyzon marinus	Sea lamprey	Anadromous	21/05/2019	503	0	0	0	0	0	0	0	0	0	0	0	0	0
Salmo salar	Atlantic salmon	Anadromous	21/05/2019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Salmo trutta	Brown trout	Anadromous	21/05/2019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chelon labrosus	Thicklip grey mullet	Marine	03/06/2019	0	1888	0	0	0	0	0	0	0	0	0	0	0	0
Dicentrarchus labrax	European bass	Marine	03/06/2019	0	0	0	0	0	0	0	0	0	0	0	0	6659	0
Gadus morhua	Atlantic cod	Marine	03/06/2019	0	0	0	0	0	0	0	0	0	0	0	11082	0	0
Solea solea	Common sole	Marine	03/06/2019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Platichthys stellatus	Starry flounder	Marine	03/06/2019	22446	16641	0	0	0	0	0	0	0	0	0	0	0	18698
Clupea harengus	Atlantic herring	Marine	03/06/2019	446	0	0	0	0	0	0	0	0	0	0	19818	12052	5704
Pomatoschistus minutus	Sand goby	Marine	03/06/2019	0	0	0	0	0	0	0	0	0	0	0	4748	12703	0
Sardina pilchardus	Pilchard	Marine	03/06/2019	0	0	0	0	0	0	0	0	0	0	0	9857	0	0
Ammodytes marinus	Sand eel	Marine	03/06/2019	0	0	0	0	0	0	0	0	0	3953	0	0	0	0
Barbatula barbatula	Stone loach	Freshwater	03/06/2019	1345	0	686	0	628	325	890	0	0	3	0	16	0	0
Cottus gobio	Bullhead	Freshwater	03/06/2019	0	0	0	1270	196	660	87	0	0	0	0	21	0	7
Cyprinus carpio	Common carp	Freshwater	03/06/2019	0	0	0	1271	0	0	0	0	0	0	0	0	0	0
Esox lucius	Northern pike	Freshwater	03/06/2019	3641	1209	1965	24547	6949	320	836	0	0	0	106	162	0	0
Gasterosteus aculeatus	Three-spined stickleback	Freshwater	03/06/2019	7543	1160	3998	16092	15854	2866	15919	3304	0	0	21	443	0	99
Gymnocephalus cernua	Ruffe	Freshwater	03/06/2019	585	0	317	0	0	675	0	0	0	2	0	9	0	0
Phoxinus phoxinus	Common minnow	Freshwater	03/06/2019	111	0	320	1058	1832	46	186	226	0	0	0	31	0	0
Rutilus rutilus	Common roach	Freshwater	03/06/2019	217249	203873	73901	284436	486411	275936	626017	383323	0	0	0	3414	4579	9939
Thymallus thymallus	Grayling	Freshwater	03/06/2019	132	0	0	0	0	4	0	0	0	0	0	0	0	0
Anguilla anguilla	European Eel	Anadromous	03/06/2019	4843	0	3605	7238	6257	2940	0	0	0	11	54	379	10	0
Lampetra fluviatilis	River Lamprey	Anadromous	03/06/2019	0	0	0	0	0	0	0	7	0	0	0	0	0	0
Petromyzon marinus	Sea lamprey	Anadromous	03/06/2019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Salmo salar	Atlantic salmon	Anadromous	03/06/2019	0	0	0	0	0	5	0	0	0	0	0	0	0	0
Salmo trutta	Brown trout	Anadromous	03/06/2019	0	0	0	0	0	466	37	0	0	0	0	5	0	0

Figure S1: The 14 Spring sample locations split into 3 sampling areas: A) lower Mersey estuary; B) upper Mersey estuary; C) river Mersey

Table S9: Number of reads and percentages compared to total reads. These numbers were subtracted of the read number used in the results to control for contamination and/or tag switching.

Scientific name	Common name	Total reads	Blank reads			%Blank reads
Autumn			**Field**	**Extraction**	**PCR**	
Rutilus rutilus	Roach	1533521	40	20	1379	0.09%
Gasterosteus aculeatus	Three spined stickleback	693592	15	19	417	0.06%
Salmo trutta	Brown trout	145785	3	3	226	0.15%
Barbatula barbatula	Stone loach	116892	2	2	139	0.12%
Cottus gobio	Bullhead	51517	1	0	72	0.14%
Anguilla anguilla	European eel	41515	0	2	94	0.23%
Esox lucius	Northern pike	26243	0	2	57	0.22%
Cyprinus carpio	Common carp	931	0	0	0	0.00%
Lampetra fluviatilis	River lamprey	179	0	0	0	0.00%
Barbus barbus	Barbel	29	0	0	0	0.00%
Abramis brama	Common bream	43	0	0	0	0.00%
Salmo salar	Atlantic Salmon	21	0	0	0	0.00%
Spring						
Gadus morhua	Atlantic cod	23355	0	0	0	0%
Hippoglossus hippoglossus	Atlantic halibut	113	0	0	0	0%
Salmo salar	Atlantic salmon	5	0	0	0	0%
Salmo trutta	Brown trout	584	0	0	0	0%
Cottus gobio	Bullhead	12747	0	0	0	0%
Cyprinus carpio	Common carp	1276	0	0	0	0%
Limanda limanda	Common dab	45	0	0	0	0%
Phoxinus phoxinus	Common minnow	14701	0	0	0	0%
Rutilus rutilus	Common roach	3781701	4265	107	33	0.12%
Solea solea	Common sole	11059	833	0	0	7.50%
Dicentrarchus labrax	European bass	7463	0	0	0	0%
Anguilla anguilla	European Eel	122170	0	0	0	0%
Perca fluviatlis	European perch	64	0	0	0	0%
Thymallus thymallus	Grayling	138	0	0	0	0%
Esox lucius	Northern pike	63005	7	0	0	0.01%
Lampetra fluviatilis	River Lamprey	7	0	0	0	0%
Gymnocephalus cernua	Ruffe	7119	0	0	0	0%
Petromyzon marinus	Sea lamprey	503	0	0	0	0%
Barbatula barbatula	Stone loach	4128	0	0	0	0%
Chelon labrosus	Thicklip grey mullet	4288	0	0	0	0%
Gasterosteus aculeatus	Three-spined stickleback	242418	0	0	0	0%

Table S10: CCA eigenvalues and permutation test

0	Axis 1	Axis 2	Axis 3	Axis 4
Gadus_morhua	3.79055	0.269161	-0.35959	-0.19431
Hippoglossus_hippoglossus	-1.92388	1.06381	-1.42893	0.017422
Salmo_salar	-0.678988	4.16384	0.175086	-0.11869
Salmo_trutta	0.080536	1.07463	-0.43123	1.37998
Cottus_gobio	0.044206	0.861002	2.25379	-2.20137
Cyprinus_carpio	0.835887	2.31381	0.929191	1.27533
Limanda_limanda	-2.0243	2.0386	-3.5562	-0.70522
Phoxinus_phoxinus	-1.48807	0.29917	0.864034	-0.38625
Rutilus_rutilus	0.258954	-0.636483	-0.17419	-5.14E-05
Solea_solea	5.64385	4.74361	-1.21816	-1.02521
Dicentrarchus_labrax	0.336208	2.68089	2.19774	9.06595
Anguilla	0.589407	-0.456373	-0.29435	0.25099
Thymallus_thymallus	-1.63538	3.25271	-0.83735	-1.11036
Esox_lucius	-0.0391632	0.406818	-0.69515	-0.32796
Lampetra_fluviatilis	-1.01706	0.0363823	2.56626	-3.89277
Gymnocephalus_cernua	-0.837331	0.399233	1.77956	1.76592
Petromyzon_marinus	-2.28401	2.79037	-2.70494	2.35542
Barbatula_barbatula	0.0981435	1.50546	2.54975	-0.76035
Chelon_labrosus	-2.43789	2.56597	-2.27736	0.126692
Gasterosteus_aculeatus	-0.168993	-0.668311	0.02081	0.107788
Liverpool_1	-0.217196	-0.0718097	-0.83562	-0.06931
Speke_1	0.163282	-0.1527	-0.29138	0.235114
Hale_1	0.0987146	-0.321761	-0.28449	-0.02707
Pickering_1	-0.232486	-0.194727	-0.2395	0.002296
Catalyst_1	0.0449804	-0.652397	-0.07669	0.053868
Widnes_1	0.0666187	-0.473209	-0.19602	-0.01876
Fiddler_1	0.0449804	-0.652397	-0.07669	0.053868
Eastford_1	2.10834	0.656685	-0.4441	-0.19373
Arpley_1	1.54631	-0.274565	-0.27604	0.018875
Howley_1	0.207057	-0.291484	-0.32951	-0.00661
Kingsway_1	0.589407	-0.456373	-0.29435	0.25099
Paddington_1	0.082355	-0.37253	0.001641	0.239485
Woolston_1	-0.0785971	-0.508948	0.013694	0.023494
Swithens_1	0.0877751	-0.649214	-0.09619	0.043084
Liverpool_2	-0.872286	0.862942	-1.38305	0.258237
Speke_2	-0.280746	-0.257601	0.053977	-0.07925
Hale_2	-0.0394254	-0.191023	0.127337	0.359337
Pickering_2	-0.506838	-0.0111707	0.050249	0.151601
Catalyst_2	-0.263582	-0.12027	0.45959	-0.1333
Widnes_2	-0.0331732	-0.0983875	0.161215	0.892594
Fiddler_2	0.258954	-0.636483	-0.17419	-5.14E-05
Eastford_2	1.29351	-0.345211	-0.17099	-0.02886
Arpley_2	3.84888	2.95024	-0.87017	-0.68349
Howley_2	0.42418	-0.546428	-0.23427	0.125469
Kingsway_2	-0.0240259	0.22926	0.503375	-0.21052
Paddington_2	0.207057	-0.291484	-0.32951	-0.00661
Woolston_2	0.153213	-0.405921	0.211214	-0.24785
Swithens_2	0.0640407	-0.0841794	0.427924	0.061872
Liverpool_3	-0.839241	0.858327	-1.26934	-0.29049
Speke_3	-0.451419	0.361015	0.061784	-0.00689
Hale_3	-0.299068	0.197983	0.198715	-0.14024
Pickering_3	0.0161372	-0.056629	0.267304	-0.27387
Catalyst_3	-0.245445	0.677648	0.441069	-0.2548
Widnes_3	-0.543834	0.328411	0.189389	-0.2249
Fiddler_3	-0.0566801	-0.159257	0.488485	-0.56234
Arpley_3	0.195283	0.265309	0.210975	1.81934
Howley_3	0.160051	-0.338587	-0.28572	0.007691
Kingsway_3	0.645817	-0.341426	-0.23655	-0.00797
Paddington_3	-0.0927827	0.0703229	0.462123	-0.0053
Woolston_3	-0.0519472	-0.317784	0.292816	-0.26515
Swithens_3	0.258954	-0.636483	-0.17419	-5.14E-05
Oxygen_mg/l	-0.0559116	0.487389	-0.25322	-0.0196
Salinity_ppt	-0.506049	0.150364	-0.11678	-0.03679
pH	-0.20006	0.286969	-0.11546	-0.28518
Temperature	-0.0673319	0.253713	0.358553	-0.03837

Eigenvalues		
Axis	Eigenvalue	%
1	0.1389	47.57
2	0.10456	35.81
3	0.048508	16.61
4	8.33E-07	0.000285

Permutation test		
Axis	Eigenval	p
1	0.1389	0.0396
2	0.1046	0.009901
3	0.04851	0.0495
4	8.33E-07	0.7525

Table S11: Combined historical fish monitoring data from the Mersey Estuary. The colours represent presence of the corresponding species; the blank boxes represent monitoring but no presence of species; and the # represents no monitoring taken place.

SPECIES_NAME	LATIN_NAME	SOURCE	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
Flounder	*Platichthys flesus*	Mersey Gateway	#	#	#	#					#	#	#	#	#	#	#	#	#	#	#	#
Goby	*Pomatoschistus microps*	Mersey Gateway	#	#	#	#					#	#	#	#	#	#	#	#	#	#	#	#
Herring	*Clupea harengus*	Mersey Gateway	#	#	#	#					#	#	#	#	#	#	#	#	#	#	#	#
Pipefish	*Syngnathus rostellatus*	Mersey Gateway	#	#	#	#					#	#	#	#	#	#	#	#	#	#	#	#
Plaice	*Pleuronectes platessa*	Mersey Gateway	#	#	#	#					#	#	#	#	#	#	#	#	#	#	#	#
Sprat	*Sprattus sprattus*	Mersey Gateway	#	#	#	#					#	#	#	#	#	#	#	#	#	#	#	#
Whiting	*Merlangius merlangus*	Mersey Gateway	#	#	#	#					#	#	#	#	#	#	#	#	#	#	#	#
Cod	*Gadus morhua*	Mersey Gateway	#	#	#	#					#	#	#	#	#	#	#	#	#	#	#	#
Lamprey	*Lampetra fluviatilis*	Mersey Gateway	#	#	#	#					#	#	#	#	#	#	#	#	#	#	#	#
Eel	*Anguilla anguilla*	Mersey Gateway	#	#	#	#					#	#	#	#	#	#	#	#	#	#	#	#
Sand eel	*Hyperoplus lanceolatus*	Mersey Gateway	#	#	#	#					#	#	#	#	#	#	#	#	#	#	#	#
Flounder	*Platichthys flesus*	Mersey Gateway	#	#							#	#	#	#	#	#	#	#	#	#	#	#
Goby	*Pomatoschistus microps*	Mersey Gateway	#	#							#	#	#	#	#	#	#	#	#	#	#	#
Herring	*Clupea harengus*	Mersey Gateway	#	#							#	#	#	#	#	#	#	#	#	#	#	#
Pipefish	*Syngnathus rostellatus*	Mersey Gateway	#	#							#	#	#	#	#	#	#	#	#	#	#	#
Plaice	*Pleuronectes platessa*	Mersey Gateway	#	#							#	#	#	#	#	#	#	#	#	#	#	#
Sprat	*Sprattus sprattus*	Mersey Gateway	#	#							#	#	#	#	#	#	#	#	#	#	#	#
Whiting	*Merlangius merlangus*	Mersey Gateway	#	#							#	#	#	#	#	#	#	#	#	#	#	#
Dab	*Limanda limanda*	Mersey Gateway	#	#							#	#	#	#	#	#	#	#	#	#	#	#
Stickleback	*Gasterosteus aculeatus*	Mersey Gateway	#	#							#	#	#	#	#	#	#	#	#	#	#	#
Bream	*Abramis brama*	Mersey Gateway	#	#							#	#	#	#	#	#	#	#	#	#	#	#
Eel	*Anguilla anguilla*	Mersey Gateway	#	#							#	#	#	#	#	#	#	#	#	#	#	#
Hooknose bullhead	*Agonus cataphractus*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
Lesser sand eel	*Ammodytes tobianus*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
European eel	*Anguilla anguilla*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
Transaprent goby	*Aphia minuta*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
Atlantic herring	*Clupea harengus*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
Crystal goby	*Crystallogobius linearis*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
Sea bass	*Dicentrarchus labrax*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
Atlantic cod	*Gadhus morhua*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
Goby sp.	*Gobiidae*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
Dab	*Limanda limanda*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
Whiting	*Merlangius merlangius*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
Smelt	*Osmerus eperlanus*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
Rock gunnel	*Pholis gunnellus*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
Flounder	*Platichthys flesus*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
Plaice	*Pleuronectes platessa*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
Pollack	*Pollachius sp.*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
Common goby	*Pomatoschistus microps*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
Sand goby	*Pomatoschistus minutus*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
Painted goby	*Pomatoschistus pictus*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
Pilchard	*Sardina pilchardus*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
Sole	*Solea solea*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
Sprat	*Sprattus sprattus*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
Pipefish	*Syngnathus rostellatus*	Aecom	#	#	#	#	#	#	#	#	#	#	#	#								
Flounder	*Platichthys flesus*	Mersey Gateway	#	#					#	#	#	#	#	#	#	#	#	#	#	#	#	#
Goby	*Pomatoschistus microps*	Mersey Gateway	#	#					#	#	#	#	#	#	#	#	#	#	#	#	#	#
Herring	*Clupea harengus*	Mersey Gateway	#	#					#	#	#	#	#	#	#	#	#	#	#	#	#	#
Plaice	*Pleuronectes platessa*	Mersey Gateway	#	#					#	#	#	#	#	#	#	#	#	#	#	#	#	#
Sprat	*Sprattus sprattus*	Mersey Gateway	#	#					#	#	#	#	#	#	#	#	#	#	#	#	#	#
Whiting	*Merlangius merlangus*	Mersey Gateway	#	#					#	#	#	#	#	#	#	#	#	#	#	#	#	#
Stickleback	*Gasterosteus aculeatus*	Mersey Gateway	#	#					#	#	#	#	#	#	#	#	#	#	#	#	#	#
Mullet	*Chelon labrosus*	Mersey Gateway	#	#					#	#	#	#	#	#	#	#	#	#	#	#	#	#
Chub	*Leuciscus cephalus*	Mersey Gateway	#	#					#	#	#	#	#	#	#	#	#	#	#	#	#	#
Trout	*Salmo trutta*	Mersey Gateway	#	#					#	#	#	#	#	#	#	#	#	#	#	#	#	#
Roach	*Rutilus rutilus*	Mersey Gateway	#	#					#	#	#	#	#	#	#	#	#	#	#	#	#	#
Bream	*Abramis brama*	Mersey Gateway	#	#					#	#	#	#	#	#	#	#	#	#	#	#	#	#
Eel	*Anguilla anguilla*	Mersey Gateway	#	#					#	#	#	#	#	#	#	#	#	#	#	#	#	#
Bass	*Dicentrarchus labrax*	Mersey Gateway	#	#					#	#	#	#	#	#	#	#	#	#	#	#	#	#

Table S12: Combined historical fish monitoring data from the from the Woolston area, River Mersey (Woolston area)

Roach	*Rutilus rutilus*	Environment Agency	#	#	#				#						#	#	#	#	#	#	#	#	#
Perch	*Perca fluviatilis*	Environment Agency	#	#	#				#						#	#	#	#	#	#	#	#	#
Gudgeon	*Gobio gobio*	Environment Agency	#	#	#				#						#	#	#	#	#	#	#	#	#
Common bream	*Abramis brama*	Environment Agency	#	#	#				#						#	#	#	#	#	#	#	#	#
Flounder	*Platichthys flesus*	Environment Agency	#	#	#				#						#	#	#	#	#	#	#	#	#
European eels > elvers	*Anguilla anguilla*	Environment Agency	#	#	#				#						#	#	#	#	#	#	#	#	#
Dace	*Leuciscus leuciscus*	Environment Agency	#	#	#				#						#	#	#	#	#	#	#	#	#
Chub	*Leuciscus cephalus*	Environment Agency	#	#	#				#						#	#	#	#	#	#	#	#	#
Rudd varieties	*Scardinius erythrophthalmus*	Environment Agency	#	#	#				#						#	#	#	#	#	#	#	#	#
Roach x common bream hybr	*Rutilus rutilus x Abramis brama*	Environment Agency	#	#	#				#						#	#	#	#	#	#	#	#	#
Atlantic salmon	*Salmo salar*	Environment Agency	#			#	#									#	#	#	#	#	#	#	#
Brown / sea trout	*Salmo trutta*	Environment Agency	#			#	#									#	#	#	#	#	#	#	#
Dace	*Leuciscus leuciscus*	Environment Agency	#			#	#									#	#	#	#	#	#	#	#
River lamprey	*Lampetra fluviatilis*	Environment Agency	#			#	#									#	#	#	#	#	#	#	#
European elvers	*Anguilla anguilla*	Environment Agency	#			#	#									#	#	#	#	#	#	#	#
Perch	*Perca fluviatilis*	Environment Agency	#			#	#									#	#	#	#	#	#	#	#
Chub	*Leuciscus cephalus*	Environment Agency	#			#	#									#	#	#	#	#	#	#	#
Atlantic salmon	*Salmo salar*	Mersey Gateway	#	#	#	#	#	#	#	#	#	#	#	#						#	#	#	#
Brown / sea trout	*Salmo trutta*	Mersey Gateway	#	#	#	#	#	#	#	#	#	#	#	#						#	#	#	#
3-spined stickleback	*Gasterosteus aculeatus*	Environment Agency		#	#	#	#	#	#	#				#	#	#	#	#	#	#	#	#	#
Tench	*Tinca tinca*	Environment Agency		#	#	#	#	#	#	#				#	#	#	#	#	#	#	#	#	#
Perch	*Perca fluviatilis*	Environment Agency		#	#	#	#	#	#	#				#	#	#	#	#	#	#	#	#	#
Pike	*Esox lucius*	Environment Agency		#	#	#	#	#	#	#				#	#	#	#	#	#	#	#	#	#
Gudgeon	*Gobio gobio*	Environment Agency		#	#	#	#	#	#	#				#	#	#	#	#	#	#	#	#	#
Roach	*Rutilus rutilus*	Environment Agency		#	#	#	#	#	#	#				#	#	#	#	#	#	#	#	#	#
Dace	*Leuciscus leuciscus*	Environment Agency		#	#	#	#	#	#	#				#	#	#	#	#	#	#	#	#	#
European eels > elvers	*Anguilla anguilla*	Environment Agency		#	#	#	#	#	#	#				#	#	#	#	#	#	#	#	#	#
Brown / sea trout	*Salmo trutta*	Environment Agency		#	#	#	#	#	#	#				#	#	#	#	#	#	#	#	#	#
Roach x chub hybrid	*Rutilus rutilus x Leuciscus cephalus*	Environment Agency		#	#	#	#	#	#	#				#	#	#	#	#	#	#	#	#	#
Common bream	*Abramis brama*	Environment Agency		#	#	#	#	#	#	#				#	#	#	#	#	#	#	#	#	#