School of Biological, Biomedical and Environmental Sciences

What is an otter's favourite food? – A molecular investigation into the dietary preferences of the Eurasian otter (*Lutra lutra*) across the River Hull Catchment

James Alasdair Macarthur (BSc Zoology)

Supervisors: Dr Bernd Hänfling and Dr Lori Lawson-Handley

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Abstract

Knowledge of top predator diets is fundamental to designing appropriate management strategies which ensure the protection of both predator and prey populations. The Eurasian otter (*Lutra lutra*) has traditionally been described as an opportunist; however, modern studies have demonstrated clear feeding preferences towards slow moving fish species. Prior dietary studies on the Eurasian otter have used morphological analysis of spraints to determine the prey eaten, and electrofishing to inform the fish communities present locally. Traditional morphological analyses are challenging, as it is difficult to identify bones down to the species level. Meanwhile, electrofishing may underestimate rare fish species present in the catchment. This study uses a non-invasive molecular methodology, which provides greater resolution on the available fish community, to understand the dietary preferences of otters along the River Hull. The research questions were: 1) Does otter diet vary spatially in response to local fish communities? 2) Does otter diet vary seasonally in response to changes in fish activity? 3) Is a single DNA extraction sufficient to investigate otter diets? 4) Do otter and mink diets overlap in the River Hull catchment? DNA extracted from otter spraints (n = 81) was sequenced using broad scale vertebrate primers to inform the prey eaten; and compared with eDNA from water samples (n = 48) collected along the River Hull to inform the prey available. Otter diet varied significantly across the upper, middle, and lower River Hull depending on the available fish community, and a consistent selective preference was observed towards the European bullhead (*Cottus gobio*). Overall otter diet did not vary significantly between seasons, however, Eurasian otters fed on significantly more different prey items in spring (Mean= 3.125) than winter (Mean= 2.482). The replicate DNA extraction experiment demonstrated

that a single extraction replicate is sufficient for detecting most prey items. Finally, comparisons between the Eurasian otter and American mink have provided further evidence of niche differentiation between the two species, thus allowing for coexistence with minimal overlap.

1.Introduction

Top predators play an important role in maintaining and regulating ecosystems (Paine, 1969; Mills, Soulé and Doak, 1993; Rio *et al.*, 2001; Ripple and Beschta, 2012). Not only do their impacts control prey populations (Estes and Palmisano, 1974; Henke and Bryant, 1999; Smith, 2006), they also mitigate the effects of potential competitors in the lower trophic levels through intraguild predation (Polis, Myers and Holt, 1989; Ripple *et al.*, 2014) and the loss of top predators can have drastic effects on the wider ecosystem through trophic cascades (Pace *et al.*, 1999; Estes *et al.*, 2011; Ripple *et al.*, 2016).

Top predators are known to exert top down pressure on species in lower trophic levels, through regulating both herbivore and smaller carnivore abundances (Ruiz-Olmo and Jiménez, 2008) and inducing shifts in prey behaviours and habitat use (Nifong, Layman and Silliman, 2015). Many of these top predators are food limited species, where their ecology, breeding and mortality is highly dependent on the available prey (Fowler, 1981; Kruuk and Carss, 1996; Ruiz-Olmo, López-Martín and Palazón, 2001; Ruiz-Olmo and Jiménez, 2008; Ferreira and Funston, 2010; Holser *et al.*, 2021). Therefore, by monitoring predator diets and their prey populations, we can observe how predators will respond to fluctuations in food availability and design appropriate

management strategies to ensure their survival (Elmhagen *et al.*, 2000; Dell'Arte *et al.*, 2007; Terraube and Arroyo, 2011).

Traditionally dietary analyses have been carried out using morphological methods (Olesiuk, 1993), these consist of sieving faecal samples to separate hard parts before identifying prey remains under a microscope (Conroy *et al.*, 1993). However, it is difficult to identify bones down to the species level and morphological methods are also vulnerable to biases dependent on the way in which prey is consumed, species where the whole individual is consumed are often over-represented, meanwhile, species where only soft tissues are eaten may be missed (Symondson, 2002). Finally, in the absence of direct observation it is not always possible to confirm the predator when using traditional methods (Morin *et al.*, 2016).

Recent enhancements in molecular methods have revolutionised the way in which we monitor biodiversity (Lawson Handley, 2015; Deiner *et al.*, 2017; Ruppert, Kline and Rahman, 2019). Modern techniques have enabled researchers to sequence environmental DNA (eDNA), which refers to DNA released by organisms interacting with their environment in the form of shed cells, excreta, gametes or decaying material from water samples (Hänfling et al., 2016). This can be used to monitor whole fish communities from lakes (Hänfling *et al.*, 2016; Lawson Handley *et al.*, 2019; Li *et al.*, 2019), rivers (Shaw *et al.*, 2016; Blackman *et al.*, 2021; Pont *et al.*, 2021), and reservoirs (Blabolil *et al.*, 2021). This method has also been shown to have increased detection sensitivity to rare fish species such as the European eel (*Anguilla anguilla*) which traditional methods may have missed (Griffiths *et al.*, 2020). Molecular metabarcoding uses PCR with broad scale primers in conjunction with high throughput

sequencing to assign DNA sequences for whole communities from environmental samples (Lawson Handley, 2015; Hänfling *et al.*, 2016). Metabarcoding can be used in conjunction with ecological network analysis (ENA) to describe species specific interactions, monitor invasive species and provide novel insights into the stability of an ecosystem to species loss (Roy and Lawson Handley, 2012; Evans *et al.*, 2016; Derocles *et al.*, 2018; Meyer *et al.*, 2020).

Modern studies have used metabarcoding to assign DNA sequences from faecal samples to prey items (Pompanon *et al.*, 2012; Thuo *et al.*, 2019; Harper *et al*., 2020b). This allows improved taxonomic resolution of prey species and, depending on the sensitivity of taxonomic markers most prey items can be assigned to the species level. In addition, molecular methods provide increased sensitivity and may be able to identify species where no hard prey remains are present (Symondson, 2002; Deagle, Kirkwood and Jarman, 2009). Finally, metabarcoding can accurately assign the predator species, removing the potential for human error during field collection which may cause biased results (Harrington *et al.*, 2010; Martínez-Gutiérrez, Palomares and Fernández, 2015; Morin *et al.*, 2016; Harper *et al*., 2020b).

When used in conjunction with data on localised prey abundances, dietary studies provide useful tools to understand how predators respond to fluctuations in prey availability (Janeiro-Otero *et al.*, 2020), changes in prey behaviour (Klare *et al.*, 2010), and changing habitat conditions (Lanszki *et al.*, 2001). This information is key when designing conservation and management strategies to ensure the protection of both predator and prey populations, whilst also mitigating potential human-predator conflicts (Martínez-Gutiérrez, Palomares and Fernández, 2015).

1.1 The Eurasian otter

The Eurasian otter (*Lutra lutra,* Linnaeus, 1758) is a member of the subfamily Lutrinae, family Mustelidae, order Carnivora (Hung and Law, 2016). It is one of seven Mustelid species present in the UK (Dayan and Simberloff, 1994), six of which are native. However, the American mink (*Neogale vison,* formerly *Neovison vison*) is an invasive species which was brought over to Europe by the fur trappers during the 1920s (Macdonald and Harrington, 2003). Otters are the second largest mustelid present in the UK and despite a decline from the 1950s-1970s (Mason and Macdonald, 1986, 1993), they can now be found throughout Scotland and Wales with a complete recolonisation of England predicted by 2030 (Crawford, 2010; Sainsbury *et al.*, 2019). The otter is an elusive, nocturnal, mustelid so visual sightings are rare (Hájková *et al.*, 2009), therefore population studies are heavily reliant on non-invasive sampling methods based around spraint (faecal samples) surveys (Macdonald and Mason, 1983; Kruuk *et al.*, 1986; Remonti *et al.*, 2011), tracking (Erlinge, 1967; Ruiz-Olmo, Saavedra and Jiménez, 2001; Sulkava, 2007) and camera trapping (Leaniz *et al.*, 2006; Bouroş, Ionescu and Hodor, 2019).

Males are larger, with an average weight of 10 kg and a length of around 1.2 mfrom nose to tail, while females weigh 7kg with a length of around 1 m (Kruuk, 2006). Sexual dimorphism is common throughout mustelids with males always being larger (Moors, 1980). There are two main hypotheses behind the evolution of this sexual dimorphism in the mustelids. The first hypothesis is that the sexual dimorphism is a mechanism of niche differentiation to mitigate intersexual competition (Lynch and O'Sullivan, 1993).

The second hypothesis is that the sexual dimorphism in mustelids is a result of the different selection pressures (Ralls, 1977; Moors, 1980). The smaller female body size is an adaptation to reduce energy expenditure, thus allowing females to invest more energy into reproduction (Erlinge, 1979; Moors, 1980). Meanwhile, the larger body size in males is a result of sexual selection for a larger body size to support the

polygynous mating system the otter exhibits (Ralls, 1977; Lynch and O'Sullivan, 1993; Quaglietta *et al.*, 2014).

Figure 1: A photo of a Eurasian otter taken on my camera trap remotely in Dundonnell, Scotland

1.2 Social structure

The typical mustelid social structure characterises individuals into three different groups, residents, temporary residents, and transients (Powell, 1979). Residents stay for extended periods of time, sometimes their entire lives, temporary residents stay for a short period of time, and finally the transients are individuals who are passing

through the territory while looking to establish themselves, these are typically juvenile males or females without cubs (Powell, 1979). Intrasexual territories are established where males are territorial against males, and females are territorial against females, however there is extensive overlap between the sexes (Powell, 1979). The territories of males are said to primarily be of sexual significance with a single males' territory overlapping with numerous females, meanwhile, the territories of females are primarily resource based to ensure her cubs have the best chance of surviving.

The home ranges of the Eurasian otter have been well studied (Erlinge, 1967; Mason and Macdonald, 1986; Kruuk and Moorhouse, 1991; Ó Néill *et al.*, 2009; Quaglietta *et al.*, 2014). The pioneering studies on otter home ranges were carried out by Sam Erlinge in freshwater habitats in southern Sweden (Erlinge, 1967, 1968b). The otter populations in Sweden conformed to the typical mustelid social structure characterised by Roger Powell (1979), where adult dog otters and females with cubs typically have well defined home ranges, meanwhile all other individuals act as transients who move between territories (Erlinge, 1967). There was a strict hierarchy between dog otters which dictates the dispersion. The dominant individuals inhabit the most favourable habitats within a territory meanwhile the subordinates are forced to occupy the sub optimal areas often around the fringes of the territory (Erlinge, 1968b). Similar studies on the home ranges of the Eurasian otter in Shetland demonstrated that females lived in intrasexual group ranges with up to four females in each range (Kruuk and Moorhouse, 1991). Each female had a core area where they spent at least 50% of their time, these areas did not overlap and demonstrated well established boundaries.

The males in this study had larger home ranges which overlapped with at least two female group ranges (Kruuk and Moorhouse, 1991).

1.3 Status in the UK

From the 1950s -1970s there was a large decline in the population of Eurasian otters in the UK (Chanin and Jefferies, 1978). This was primarily attributed to pollution of rivers causing a decline in fish stocks, the loss of riparian habitats and finally casualties due to traffic collisions, fish traps and hunting (Mason and Macdonald, 1986, 1993, 2004; Chanin, 2003). This led to the creation of the National Otter Surveys, the first of which was carried out between 1977-1981 to determine the status of otters throughout Britain (Mason and Macdonald, 1986). The results of the first systematic survey carried out in England between 1977 and 1979 found signs of otters at 170 sites (5.8%) out of the 2940 sites that were visited (Lenton, Chanin and Jefferies, 1980; Mason and Macdonald, 1986; Crawford, 2010). The Eurasian otter was given protected status under the Wildlife and Countryside act (1981) and populations have since recovered, due to mandatory bans on the use of dieldrin since 1981 (Macdonald, 1983), reintroductions to strengthen fragmented populations (Green, 1997), and improvements in water quality to restore fish stocks (Crawford, 2010). During the most recent national survey from 2010, otters were found in 59% of the sites surveyed in England and current estimates predict a full recolonization of the UK by 2030 (Crawford, 2010; Sainsbury *et al.*, 2019).

While otter populations have recovered, worrying trends have been found from roadkill otters where there has been an increase in the number of otters found with empty

stomachs (Moorhouse-Gann *et al.*, 2020). This study also found evidence that the declines in the European eel (*Anguilla anguilla*), a preferred prey species, were reflected in the poorer body condition of otters. They suggested that alternate prey species such as bullhead (*Cottus gobio*) and three-spined stickleback (*Gasterosteus aculeatus*) were unlikely to compensate for the nutritional disparities.

The Eurasian otter is regularly the subject of human-wildlife conflict in the context of fisheries (Crawford, 2010; Allen and Pemberton, 2019; Allen, Pemberton and Nobajas, 2019). Recreational angling contributes around £1.4 billion to the English economy each year (Environment Agency, 2018; Allen, Pemberton and Nobajas, 2019), and as a predominantly fish based predator (Kruuk, 2006), stillwater fisheries which are heavily stocked provide tempting feeding grounds for otters (Jay *et al.*, 2008). The primary solution to protecting fisheries is otter proof fencing, however, this is expensive and unless the fishery is public access, funding is unlikely (Allen and Pemberton, 2019). More recently, CL36 class licences have been granted by Natural England which allow for live trapping and relocation of problematic otters to prevent further damage to fisheries (Allen, Pemberton and Nobajas, 2019). Allen et al., (2019) highlight the need for increased discussion and integration with stakeholders to ensure protection of fisheries, they discuss the idea of a live system which logs otter sightings to warn fisheries of a potential recolonisation to ensure appropriate measures can be put in place.

1.4 The American mink

The American mink (*Neogale vison*) is an invasive semi-aquatic mustelid that was brought over from America as part of the fur trade (Macdonald and Harrington, 2003). Shortly after their arrival, they began escaping and establishing populations in the wild (Bonesi and Palazon, 2007). Mink farms were banned in the UK in 2003 (Fur Farming (Prohibition) Bill, 2000), however by this point several escapes and deliberate releases had facilitated the creation of wild populations of American mink throughout England (Bonesi and Palazon, 2007) and Scotland, except for the far north (Fraser *et al.*, 2018). The detrimental impacts of mink on water vole (*Arvicola amphibius*) are well known and they were attributed to their near extinction, which subsequently prompted extensive culls to eradicate the mink (Jefferies, Morris and Mulleneux, 1989; Macdonald, Sidorovich and Anisomova, 2002; Macdonald and Harrington, 2003). Previous live trapping studies on the Thames catchment demonstrated a reduction in mink density and range following the reintroduction of Eurasian otters (Bonesi and Macdonald, 2004). Similarly, McDonald *et al*., (2007) described that the continued decline of the invasive American mink is correlated with the recovery of the native Eurasian Otter based on a reduction in field signs. However, a more recent review has questioned the legitimacy of using a decline in faecal samples to indicate a species decline, stating that the mink may instead change their behaviour and marking in the presence of otters to minimise competition (Harrington *et al.*, 2020).

1.5 The River Hull otter population

In the first national otter survey, otters were only found in 4/227 (1.8% of sites) in Yorkshire (Lenton, Chanin and Jefferies, 1980; Crawford, 2010). This led to the

reintroductions of 27 individuals to Yorkshire between 1990-1993 to bolster fragmented populations, particularly around the Derwent section (Green, 1997). In spite of this, a disparity in the sampling design meant that the upper sections of the River Hull were never surveyed (Hampshire, 2003). There is good water quality in this section which provides important spawning habitats for grayling (*Thymallus thymallus*) and brown trout (*Salmo trutta*). Several reports of otter sightings and signs on the River Hull can be found throughout the period when otters declined elsewhere which predate the release programme on the Derwent (Hampshire, 2003; Howes, 2010). In 1995, an otter spraint was found which prompted a survey by the Yorkshire Wildlife Trust (Jay, 1995). This confirmed otter presence in the upper stretches of the River Hull with 4 spraints and 1 footprint (Howes, 2010). This has led to suggestions that the River Hull otter population never went extinct and might in fact be genetically very valuable (Hampshire, 2003).

1.6 Otter diets

The diets of the Eurasian otter have been well studied, initially through morphological analysis of spraints (Mason and Macdonald, 1986; Conroy *et al.*, 1993; Brzeziński, Romanowski and Kopczyński, 2006; Kruuk, 2006; Almeida, Copp and Masson, 2012; Smiroldo, Balestrieri, *et al.*, 2019) and more recently molecular methods (Hong *et al.*, 2019; Buglione *et al.*, 2020b; Drake, 2020; Harper *et al*., 2020b; Martínez-Abraín *et al.*, 2020). Irrespective of the method chosen, otter diets have been shown to comprise primarily of fish across studies (Drake, 2020; Harper *et al*., 2020b), with amphibians (Weber, 1990; Clavero, Prenda and Delibes, 2005; Smiroldo, Villa, *et al.*, 2019) and crayfish (Beja, 1996; Ruiz-Olmo *et al.*, 2002; Britton *et al.*, 2017; Martínez-Abraín *et*

al., 2020; Dettori *et al.*, 2021) acting as secondary, seasonally important prey resources. Previous morphological research on otter spraints in the River Hull has shown invertebrates to account for 21.15% of otter diet (Treddell, 2018). However, it is not possible to distinguish between direct predation or secondary ingestion which may result in an overrepresentation of invertebrate prey in otter diets (Taylor *et al*., 2010). Therefore, in the current study, we only analysed the vertebrate portion of otter diet which Harper *et al*., (2020b) showed primarily consisted of fish. Further comparisons between available prey and prey eaten have highlighted that otters tend to exploit the most abundant prey resources available (Copp and Roche, 2003; Kruuk, 2006; Dettori *et al.*, 2021), with a preference towards slow moving fish species such as the European bullhead (Brzeziński, Romanowski and Kopczyński, 2006; Almeida, Copp and Masson, 2012; Alderton *et al.*, 2015; Grant and Harrington, 2015; Britton *et al.*, 2017; Krpo-Ćetković, Subotić and Skorić, 2019; Drake, 2020; Harper *et al*., 2020b; Moorhouse-Gann *et al.*, 2020). Opportunistic feeding has also been recorded in the Eurasian otter where individuals on Shetland have been recorded feeding on European rabbit (*Oryctolagus cuniculus*) (Kruuk, 2006), bush crickets (*Tettigonidae*) in Greece (Mason and Macdonald, 1986) and birds in Shapwick Heath nature reserve (De la Hey, 2008).

Individual and sex-based differences in the feeding of the Eurasian otter have been demonstrated on the Shetland islands (Kruuk and Moorhouse, 1990). In this study, males and females with cubs fed on the same size fish, however females without cubs fed on significantly smaller prey items. They discuss two yearlings which fed on significantly different prey from other adult otters. They attributed this individual variation to differences in the available prey from each stretch of coast (Kruuk and

Moorhouse, 1990). More recently, research on otter stomach contents found that males were significantly more likely to be found with empty stomachs (Moorhouse-Gann *et al.*, 2020). This research also found that female otters were 10% more likely to prey on bullhead, conversely males were 20% more likely to consume cyprinids than females (Moorhouse-Gann *et al.*, 2020).

Previous molecular research by Harper *et al*., (2020b) used faecal metabarcoding to investigate niche partitioning as a mechanism of coexistence between the Eurasian otter and American mink along the River Hull. They found that otter diets primarily consisted of small slow moving fish species such as three-spined sticklebacks and European bullhead, with amphibians as a secondary prey resource. Meanwhile, mink diets predominantly consisted of mammals and birds. This highlighted the potential of faecal metabarcoding to enhance dietary studies, providing increased taxonomic resolution of prey species and accurate predator identification (Harper *et al*., 2020b).

Traditional dietary preference studies of the Eurasian otter have incorporated morphological data from spraint samples to inform the prey eaten in conjunction with electrofishing and traditional catch methods to inform the prey available (Kruuk, Nolet and French, 1988; Kruuk and Moorhouse, 1990; Taastrøm and Jacobsen, 1999; Lanszki *et al.*, 2001; Copp and Roche, 2003; Krpo-Ćetković, Subotić and Skorić, 2019; Sittenthaler, Koskoff and Pinter, 2019). Morphological methods can be carried out without an advanced laboratory (Pertoldi *et al.*, 2021), and have been shown through captive feeding trials to demonstrate a reasonable picture of the most common prey species (Erlinge, 1968a; Carss and Parkinson, 1996). However, challenges arise when assigning prey remains to species level, and morphological methods can be

biased by how prey is consumed (Carss and Parkinson, 1996; Symondson, 2002), misidentification of prey remains due to poor quality taxonomic keys (Taylor *et al.*, 2010), or mistaken predator identity (Harrington *et al.*, 2010; Monterroso *et al.*, 2013; Martínez-Gutiérrez, Palomares and Fernández, 2015; Morin *et al.*, 2016; Andersen *et al.*, 2021). Faecal metabarcoding can bypass these issues through; accurately assigning the predator (Harper *et al*., 2020b), providing improved taxonomic resolution of prey species (Deagle, Kirkwood and Jarman, 2009; Hardy *et al.*, 2017; Drake, 2020; Quéméré *et al.*, 2021) and increased sensitivity which may be able to identify species where no hard prey remains are present (Berry *et al.*, 2015; Drake, 2020; Traugott *et al.*, 2021). However, when using molecular methods it is not possible to quantify the number or size of individual prey items which could be done using traditional morphological analysis (Pertoldi *et al.*, 2021).

Similarly, previous otter dietary studies have been reliant on electrofishing and traditional catch methods to inform the available fish communities (Erlinge, 1969; Jacobsen, 2005; Remonti *et al.*, 2010; Grant and Harrington, 2015). However, this has often been constrained to small stretches of river (Taastrøm and Jacobsen, 1999), which means catchment wide surveys to study localised differences in the fish community within a river are challenging (Pont *et al.*, 2021). In addition, electrofishing and traditional catch methods have been shown to overlook rare and smaller species which may underestimate their abundances (Hänfling *et al.*, 2016; Li *et al.*, 2019; Griffiths *et al.*, 2020). By contrast, eDNA water sampling allows increased sensitivity for rare species such as eels (Griffiths *et al.*, 2020; Zou *et al.*, 2020). eDNA can also be used to observe spatial variations in community assemblages and map biodiversity hotspots within a river system (Altermatt *et al.*, 2020; Blackman *et al.*, 2021). Thus

providing more detailed information on the localised prey communities available to otters.

1.7 Thesis statement

This study uses a novel molecular methodology for estimating dietary selection, which incorporates faecal metabarcoding of spraints in conjunction with eDNA water sampling of local fish communities to investigate dietary preferences of a top predator within an ecosystem.

1.8 Research questions and aims

The aim of this study was to use molecular methods to provide a better understanding of the factors influencing dietary preferences of otters across the River Hull. The current study aimed to build upon the research from Harper *et al*., (2020b) with an increased number of mink scats which would provide further insight into the niche separation and their detrimental impacts on native fauna. Additionally, otter spraints from Harper *et al*., (2020b) were primarily collected at Tophill Low nature reserve. Therefore, the current study aimed to expand on this with a more comprehensive spatial study of the River Hull to investigate how otter dietary preferences varied in response to local fish communities.

The research questions were as follows:

1. Do otter diets vary spatially in response to local fish communities?
2. Do otter diets vary seasonally in response to changes in fish activity such as spawning seasons?
3. Is a single DNA extraction sufficient to investigate otter diets?

4. Do the otter and mink diets overlap in the River Hull catchment?

2.Methods:

2.1 Study area

The study was carried out in the catchment of the River Hull, East Yorkshire which runs from Driffield where it is a highly productive chalk stream to the Humber Estuary. The headwaters have been designated as a Site of Special Scientific Interest (SSSI) due to its rich invertebrate and aquatic vegetation communities which are characteristic of chalk streams (Rayner and White, 2010). The River Hull was initially partitioned into three sections based on the topography of the river channel and differences in river ecosystem types (Figure 2). The section Lower River Hull runs from Beresford Park until Tickton. This section is very urban and contains both the city of Hull and the town of Beverley. This section is strongly influenced by tidal movements and includes the estuary into the Humber and the fish fauna resembles the typical bream zone (Aarts and Nienhuis, 2003). The banks are steep with large reed beds and there are very few nearby water bodies, or drains flowing into the main river channel. The section Middle River Hull runs from Pulfin bog until Bethells bridge. This section contains two nature reserves, Pulfin bog/High Eske and Tophill Low, both with large reservoirs and a network of smaller ponds and streams. In addition, there are numerous canals, becks and drains which flow into this section such as Leven canal, Scorborough beck and Watton beck. This creates a mosaic of different habitats for fish species and the fish fauna resembles the typical barbel zone (Aarts and Nienhuis, 2003). Thus, the most diverse fish community should be found in the middle section.

The section Upper River Hull runs from Skerne wetlands to Driffield where it forms part of England's most Northern chalk stream system (Driffield trout stream). This section contains, Skerne wetlands nature reserve and Wansford trout farm, the river runs parallel to Driffield navigation canal with multiple tributaries flowing from the main river channel including Frodingham, Skerne, Nafferton and Driffield becks. Previous studies of the Upper River Hull (West Beck) by the Environment Agency and Wild Trout trust have demonstrated this section to be an important habitat for brown trout, European grayling and European river lamprey (*Lampetra fluviatilis*) (Pedley, 2016).

There are two primary barriers to fish migration along the River Hull: Whinhill weir which is used to raise water levels in the upper River Hull, and Hempholme weir which is managed by Yorkshire Water at Tophill Low (Nunn *et al.*, 2007; Rayner and Dennis, 2010). Hempholme weir is the main control for the water levels along the River Hull and the structure is used to abstract water which then feeds the two reservoirs at Tophill Low as well as the water treatment works (Rayner and Dennis, 2010). Construction of a fish pass at Hempholme weir was finished in March 2020, however, this was towards the end of the seasonal water sampling so the impacts of improved fish migration will likely have been missed. Hempholme weir represents the tidal reaches of the River Hull (Rayner and Dennis, 2010), and this study therefore expected to find an increase in marine species such as European flounder (*Platichthys flesus*) and catadromous species like the European eel in the Lower River Hull section.

The river has been stocked in the upper section by the West Beck Preservation Society (WBPS) and the Environment Agency to support the nearby fishery. Historically, Rainbow trout (*Oncorhynchus mykiss*), European grayling, common barbel (*Barbus*

barbus) and common dace (*Leusiscus leuciscus*) have all been stocked in this river. In 2016, stocking primarily consisted of brown trout with 450 large 28-36cm (11 – 14”) individuals being added each year, split across three separate introductions (Pedley, 2016).

2.2 eDNA water sampling and DNA extraction

To explore spatial variation in the fish communities along the River Hull, 12 sites were selected for eDNA water sampling by PhD student Nathan Griffiths andDr Graham Sellers (Figure 2). Sample collection, filtration and extraction followed the methodology described in Griffiths *et al.,* (2020). Briefly, 2 litre water samples were collected using sterile Gosselin HDPE plastic bottles (Fisher Scientific UK Ltd., Loughborough, UK). Each 2L sample consisted of 5 x 400ml subsamples to account for the stochastic distribution of eDNA within the water (Griffiths *et al.*, 2020). To assess seasonal variation in the fish communities, each site was visited 4 times, across the 4 seasons, between August 2019 and May 2020. Field blanks (n = 7) (2 L purified water) were transported alongside samples in insulated coolboxes with ice packs throughout the fieldwork.

Water samples were vacuum filtered within 24 hours of collection following the methodology described in Griffiths *et al*.,(2020). 2 litre samples were filtered through sterile 0.45 µm mixed cellulose ester membrane filters with pads (47 mm diameter; Whatman, GE Healthcare) using Nalgene filtration units. Two filters were used for each sample and up to 30 minutes per filter was allowed for water to pass through. Field blanks (n = 7) were filtered during the last round of filtration. Filters were removed

from pads using sterile tweezers and placed in 5 ml screw-cap tubes (Axygen, Fisher Scientific UK Ltd.) containing 1 g each of 0.15 mm and 1 - 1.4 mm diameter sterile garnet beads and stored at -20°C until DNA extraction.

DNA extractions were carried out following the mu-DNA lysis water protocol described in Sellers *et al*., (2018). Briefly, filters were defrosted at room temperature before 750 µl lysis solution and 250µl water lysis additive was then added. The tube was then placed on a Vortex Genie (Scientific Industries) with Vortex Adapter (MoBio) at maximum speed for five minutes. The mu-DNA water lysis protocol was then followed which is a spin column based extraction consisting of 4 steps: inhibitor removal, silica binding, ethanol wash, and elution (Sellers *et al.*, 2018). An extraction blank (n = 5), consisting only of extraction buffers and sterile garnet beads, was extracted alongside samples. 20µl working aliquots were taken from the stock tube this ensured stock tubes did not undergo continual freeze thawing. DNA extracts were then frozen at -20°C prior to PCR amplification.

The subsequent library preparation stages and bioinformatics for the water samples underwent the same protocols described later in the methods (Section: Library preparation and sequencing). All field and lab work for the water samples was carried out by a PhD student Nathan Griffiths and Dr Graham Sellers. The downstream analysis in R was carried out by myself.

2.3 Spraint sampling and DNA extraction

23 sampling sites were selected across the River Hull catchment (Figure 2), chosen with the help of local wardens based on high levels of otter activity (tracks, spraints and runs). At each site, several locations were identified to prioritise checks for spraints, including under bridges, well known runs and exposed areas or large rocks at the edge of the banks (Kruuk, 2006). A sampling regime was designed which involved each site being visited 3 times at eight-week intervals. This allowed sufficient spraints to build up prior to collection, whilst also ensuring that our presence collecting did not affect the otter's ecology. If multiple spraints were found in a single spot, a maximum of two spraints and one jelly sample were taken to minimise pseudo replication. Spraints of all ages were collected for dietary analysis, in cases where several spraints were available, only the freshest spraints were taken.

Mink and other potential mustelid faecal samples were also opportunistically collected whilst out in the field to build upon the previous research from Harper *et al*., (2020b) where only 19 mink samples were collected. This would allow for a more comprehensive overview of the niche separation between the two mustelids along the River Hull catchment.

To allow additional insight into the otter diets across the wider catchment, 8 sampling sites were identified along nearby tributaries (Kirkburn, Watton, Scorborough, Leven, Brigham, Frodingham), and waterbodies (Kingfisher lakes), these were primarily based under bridges where nearby road access was possible.

DNA was extracted following the mu-DNA lysis otter protocol described in Sellers *et al*., (2018). All weighing boats and equipment were sterilised in between samples using 10% bleach and 70% ethanol prior to weighing. Extractions were carried out in sets of 12 with one extraction blank per day to monitor for contamination.

A 0.25g sample of spraint was mixed with 0.5g of 1-1.4mm diameter sterile garnet beads, 550 µl lysis solution and 200µl soil lysis additive were then added. The mixture was then placed in a TissueLyser II (Qiagen, Manchester, UK) at 30 Hz for 10 minutes to homogenise then centrifuged at 4000 x g for 1 min. The supernatant was then transferred to a new tube and spun again at 10,000 x g for 1 min. The mu-DNA otter spraint protocol was then followed which is a spin column based extraction consisting of 4 steps: inhibitor removal, silica binding, ethanol wash, and elution (Sellers *et al.*, 2018). Problematic samples with lots of hair and feathers had an increased volume of lysis buffer and water lysis additive added to ensure sufficient supernatant could be taken. If the supernatant was still insufficient an additional 550µl lysis solution plus 200µl molecular grade water was added prior to the flocculant step to ensure sufficient supernatant could be taken forward for the subsequent stages. The DNA concentrations for a subset of samples were recorded using the Qubit 3.0 fluorometer high-sensitivity (HS) dsDNA assay (Invitrogen). In addition, DNA purity was measured using the Nanodrop 1000 spectrophotometer (Fisher Scientific UK Ltd., Loughborough, UK). Samples were then frozen at -18℃ prior to PCR and subsequent library preparation.

To assess the reproducibility of our extraction method, a small-scale replicate study was carried out, in which 10 otter spraints were chosen at random. Each spraint was extracted three times prior to being sequenced in triplicate. This allowed us to compare the replicability of our extraction method and understand which prey species may drop out.

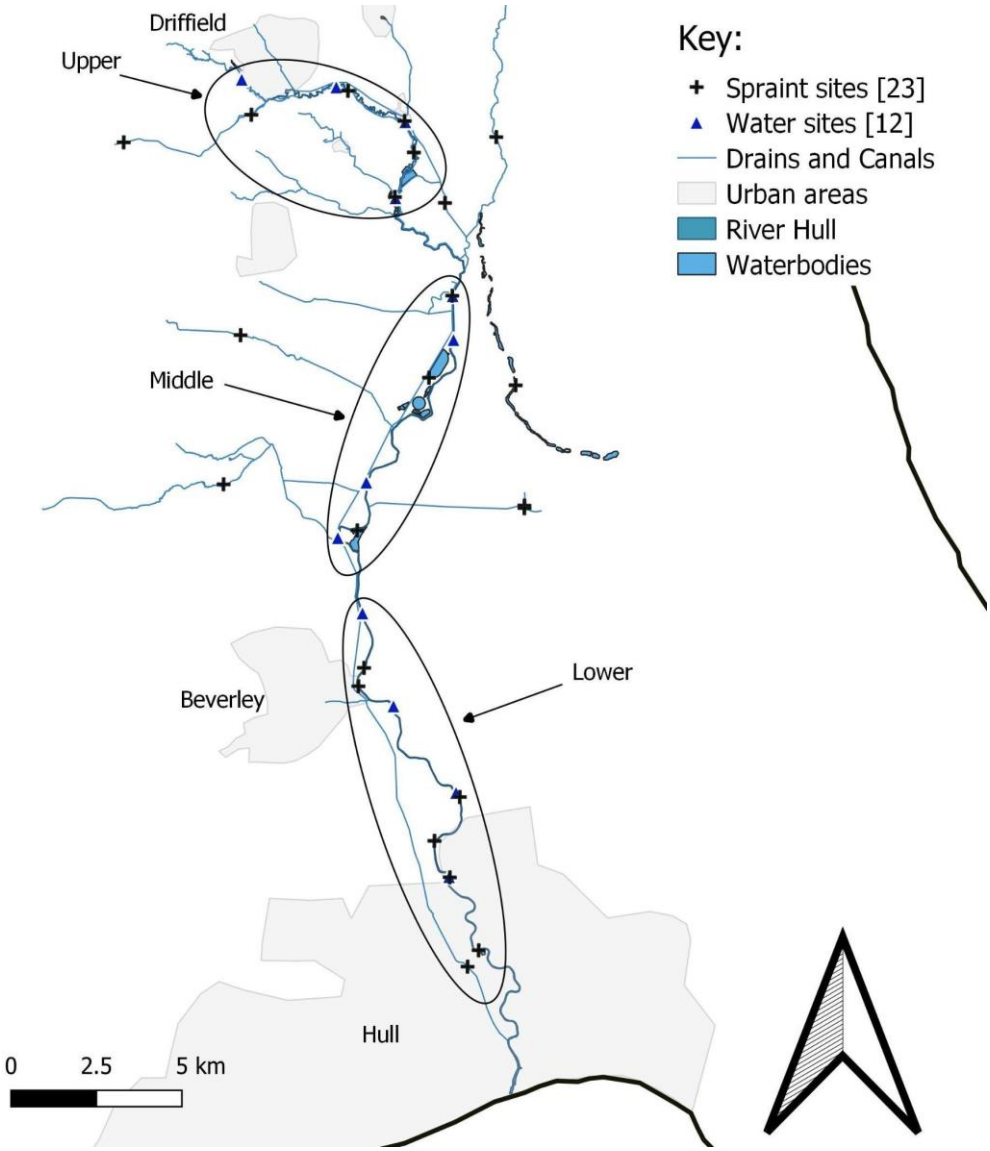


Figure 2: Spraint and water sampling locations across the River Hull catchment. The 23 spraint sites are represented by black crosses. The 12 eDNA water sampling sites are represented by the blue triangles. Ellipses demonstrate the three sections the river was partitioned into for the spatial analysis.

2.4 Library preparation and sequencing

Library preparation and sequencing followed the established 12S protocol at Hull University described in Griffiths *et al*., (2020) and Harper *et al*., (2020b). Briefly, nested metabarcoding was carried out following a two-step PCR approach in which both PCRs used multiplex identification tags to enable sample identification following the protocols described in Kitson et al., (2019) (Griffiths *et al.*, 2020). PCR 1 used 24 individually indexed primer combinations to amplify a 106 bp region of the 12S ribosomal RNA mitochondrial gene (Riaz et al., 2011). Meanwhile PCR 2, bound pre-adapters, indexes, and Illumina adapters to pooled sub-libraries prior to sequencing.

For the first PCR, samples were split across 7 sub-libraries and a typical sub-library consisted of: 20 faecal extractions, 2 extraction blanks, a positive control quantified at 0.05 ng/µl from the non-native exotic cichlid (*Maylandia zebra*) and a negative control of molecular grade water. The first PCR amplified a 106 bp fragment using published 12S ribosomal RNA primers 12S-V5-F (5’ -ACTGGGATTAGATACCCC-3’) and 12S-V5-R (5’ -TAGAACAGGCTCCTCTAG-3’) (Riaz *et al.*, 2011; Kelly *et al.*, 2014). These primers have been validated both in vitro and *in situ* for UK vertebrates (Hänfling *et al.*, 2016; Harper *et al.*, 2019; 2020; 2020). A master mix for 80 reactions was made containing: 1000 µL of Q5 High-fidelity 2 x master mix (New England Biolabs, UK), 40 µl of Bovine Serum Albumin (BSA) (Fisher Scientific UK Ltd., Loughborough, UK) and 560 µL of molecular grade water. The mix was vortexed in a 5 ml Eppendorf before 20 µl was added to each strip tube. Next, 3 µl of 24 individually indexed 12S-V5 primer (1.5 µl of each forward and reverse 10 µM Primer) was pipetted into each tube, subsequently 2 µl of DNA was added to the tube. The reaction was then sealed using

one drop of mineral oil. PCR was performed on an Applied Biosystems® Veriti Thermal Cycler (Life Technologies, CA, USA) with the following conditions: 98°C for 5 min, 35 cycles of 98°C for 10 s, 58°C for 20 s and 72°C for 30 s. Final elongation step was at 72°C for 7 min. To minimise PCR and sequencing bias PCRs were carried out in triplicate (3 x PCR replicates) (Sellers *et al.*, 2018). After the first PCR replicates were then pooled at 20 µl each providing a final volume of 60 µl.

2 µl of each pooled sample was mixed with 1µl of loading dye (R0621, Mass Ruler DNA loading dye (6 x), Fisher Scientific UK Ltd., Loughborough, UK) before samples were visualised via gel electrophoresis on a 2% agarose gel (1.6 g Bioline® Agarose in 80 mL 1 x sodium borate) (Brody and Kern, 2004) using a sodium borate buffer (1 x) with gel red (1000 x) (Cambridge Bioscience, Cambridge, UK) as a stain and 2 µl of EasyLadder I (BIO-33046, EasyLadder I (500 Lanes), Meridian Bioscience, Scientific Laboratory Supplies Ltd, Nottingham, UK) for the ladder. The gel conditions used were 200 V for 20 min and gels were imaged using Image Lab Software (Bio-Rad Laboratories Ltd, Watford, UK). Samples were deemed successful if amplification was seen in the target region of 250 bp.

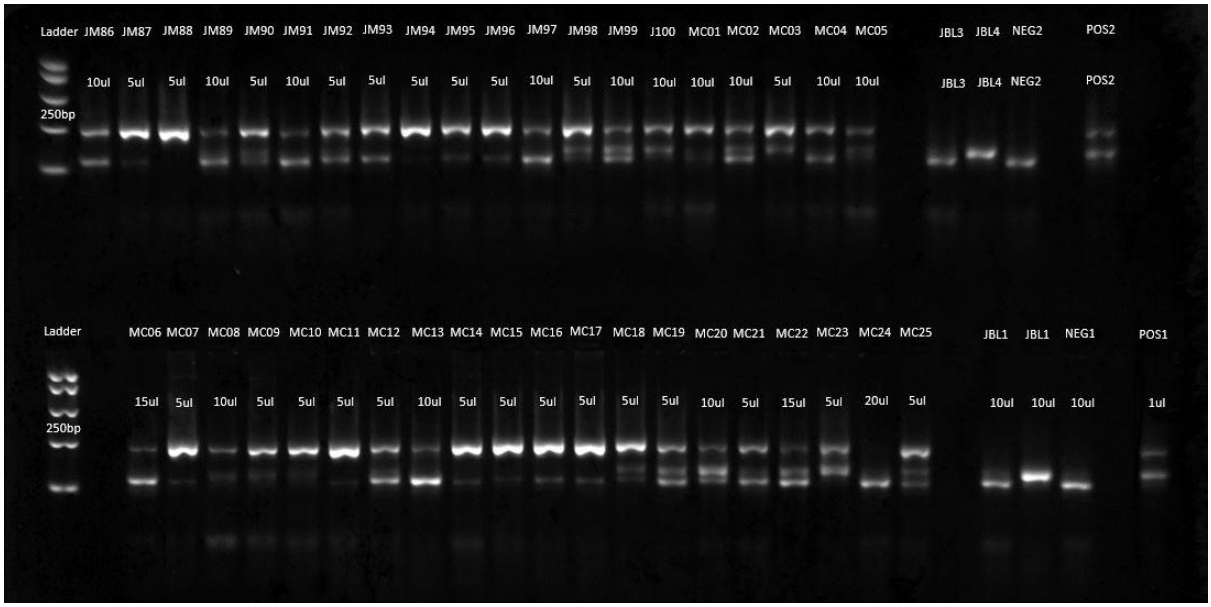


Figure 3: An annotated gel showing the post pcr1 product and the normalisation values used to pool samples within sub libraries prior to the first clean-up. The target band can be seen at 250 bp

Following the first PCR, samples were pooled within sub-libraries with volumes based on band strength as follows: 5 µl - very bright band, 10 µl - bright band, 15 µl - weak band, 20 µl - no band (Alberdi, Aizpurua and Gilbert, 2018) (Figure 4). Subsequently, to remove non-specific amplification around the target band of 250 bp, sub-libraries underwent a double size selection clean-up (Bronner *et al.*, 2014) using MagBIND RxnPure Plus magnetic beads (Omega Bio-tek Inc., Norcross, GA, USA) (Griffiths *et al.*, 2020). Ratios of 0.9 x and 0.15 x magnetic beads to 100 µl of each purified sub-library were used following the protocol described in Harper *et al*., (2020b). The higher ratio of 0.9 x bound non-specific amplification upwards of 300 bp, meanwhile, the second ratio of 0.15 x was used to remove any non-specific amplification below 200 bp (primer dimer). 2 µl of Eluted DNA (23 µl) was then visualised via gel electrophoresis to confirm that the target band (~250 bp) remained. Cleaned product was then stored at 4℃, prior to the second PCR.

A second PCR was carried out to bind pre adapters, indexes, and Illumina adapters to each sub-library. Sub-libraries underwent 2 x PCR replicates (duplicate) each at 50 µl reaction volumes containing: 25 µl Q5 High-fidelity 2 x master mix (New England Biolabs, UK), 15 µl molecular grade water, 6 µl primers (3 x each 10 µM Primer (Integrated DNA Technologies, Belgium) and 4 µl of purified sub-library. The second PCR followed the adapted thermocycling profile described in Griffiths et al., (2020): 95°C for 3 min, 10 cycles of 98°C for 20 s and 72°C for 1 min, followed by a final elongation step at 72°C for 5 min. Replicate PCR products were then pooled and visualised via gel electrophoresis following the conditions described previously. Amplification was deemed successful when a band was present in the expected region of (~300-400 bp) (Harper *et al*., 2020b). A second double size selection clean-up (Bronner *et al.*, 2014) was then carried out to remove primer dimer and nonspecific amplification following the ratios outlined in Harper *et al*., (2020b), these were 0.7 x and 0.15 x magnetic beads to 50 µl of PCR product. The target fragment length for the second PCR was 336 bp and the higher ratio of 0.7 x bound non-specific amplification upwards of 400 bp, meanwhile, the second ratio of 0.15 x was used to remove any non-specific amplification below 200 bp (primer dimer at 100 bp). Cleaned products were once again visualised via gel electrophoresis using the previously described conditions to ensure DNA from the target region of 336 bp remained.

Cleaned sub-libraries were quantified using the Qubit 3.0 fluorometer high-sensitivity (HS) dsDNA assay (Invitrogen) and pooled proportionally according to sample number and concentration (Harper *et al*., 2020b). The pooled library was visualised once more, before undergoing a final double size selection clean-up (Bronner *et al.*, 2014) using the same ratios of 0.7 and 0.15 x magnetic beads to 50 µl of library as the previous

clean-up. The library was then diluted to 4 nM before being quantified via qPCR using the NEBNext Library Quant Kit for Illumina (New England Biolabs Inc., Ipswich, MA, USA) (Griffiths *et al.*, 2020). Once the desired quantification was confirmed, the final library was denatured and sequenced at 15 pM with 10% PhiX Control on an Illumina MiSeq using a MiSeq Reagent Kit v3 (600 cycle) (Illumina Inc., San Diego, CA, USA).

2.5 Bioinformatics

Illumina Miseq sequencing data were analysed using an in-house bioinformatics pipeline at the University of Hull. Raw sequence output was demultiplexed following a custom python script, then subsequently, sequences were processed using metaBEAT v0.97.13 (https://github.com/HullUni-bioinformatics/metaBEAT). Raw reads were quality trimmed from the read ends with a 5 bp sliding window (per base phred score Q30) using Trimmomatic v0.32 (Bolger, Lohse and Usadel, 2014). Primers were removed by trimming the first 18 bp of remaining reads. Reads were tail cropped to a maximum length of 110 bp and reads shorter than 90 bp were discarded. Sequence read pairs were merged into single reads using FLASH v1.2.11 (Magoč and Salzberg, 2011), provided there was a minimum overlap of 10 bp and no more than 10% mismatch between pairs. Only forward reads were kept from read pairs that failed to be merged. A final length filter (106 bp ± 20%) was applied to ensure sequence lengths approximated the expected fragment size (106 bp). Retained sequences were screened for chimeric sequences using the uchime algorithm (Edgar *et al.*, 2011), as implemented in vsearch v1.1 (--uchime_ref) (Rognes *et al.*, 2016). Redundant sequences were removed by clustering at 100% identity (--cluster_fast) in vsearch v1.1. Clusters represented by less than three sequences were omitted from further

processing. Non-redundant sets of query sequences were then compared against our custom curated reference database using BLAST (Zhang *et al.*, 2000); faecal samples against a UK vertebrate reference database (Harper *et al.*, 2020a) and water samples against a UK fish reference database (Hänfling *et al.*, 2016). Taxonomic identity was assigned using a lowest common ancestor (LCA) approach based on the top 10% bit-score BLAST hits with at least 90% query coverage and a minimum identity of 98%. Remaining unassigned sequences underwent a separate BLAST search against the complete NCBI nucleotide (nt) database at 98% identity (Harper *et al*., 2020b).

All statistical analyses and data visualisation was carried out using the statistical programming environment R v.3.6.3 (R Core Team, 2020). Following the taxonomic assignment of DNA sequences against a curated vertebrate library (Harper *et al.*, 2020a), a false positive threshold of 1% was applied to remove any reads below 1% of the total number of reads, as was previously applied in faecal metabarcoding research using this 12S marker (Harper *et al*., 2020b). For the eDNA water samples, a false positive threshold of 0.1% was applied as was previously applied in water sample metabarcoding research using this 12S marker(Hänfling *et al.*, 2016; Griffiths *et al.*, 2020). For the following groups it was not possible to accurately assign sequences to a single species, therefore, reads were pooled at the higher taxonomic level; Family: ducks [*Anatidae*]*,* pigeons and doves [*Columbidae*]*,* perch and zander [*Percidae*]; Genus level: Voles [*Microtus*]*,* brook or river lamprey [*Lampetra*]. Sequences assigned to human (*Homo sapiens*) and domestic animals' cat [*Felis catus*], cow [*Bos taurus*], dog [*Canis lupis familiaris*] and pig [*Sus scrofa domesticus*] were regarded as environmental or lab contaminants and therefore omitted prior to downstream analyses.

2.6 Predator assignment

In order to confirm that the analysed spraints were correctly identified as otter or mink during collection in the field a predator assignment analysis was carried out. Predator assignment was carried out in R using the dplyr package (Wickham *et al.*, 2021). First sequence reads for all samples were filtered to maintain only the two target species Eurasian otter, American mink, and other potential predator species, i.e., other mustelids and European red fox (*Vulpes vulpes*). To assign the predator as otter, samples were filtered to remove any sample which contained 0 reads of Eurasian otter. Subsequently, reads for the remaining samples were converted into frequencies, and any sample which contained less than 0.9 (90%) of reads assigned to the otter was removed. The same methodology was then repeated to assign mink scats. Following this, remaining faecal samples belonged solely to either the Eurasian otter or American mink. In addition, samples with less than 100 reads assigned to any predator were omitted prior to downstream analyses.

2.7 Statistical analysis

Statistical approaches were based upon methodology established by Harper *et al*., (2020b). For figure creation, the dietary and water sample data for each section was summarised as the percentage occurrence of taxa in spraints/water samples. This was calculated as follows:

% of occurrences = (number of detections of a single taxon in a section/total number of detections for all taxa in this section) x 100

This calculation was then repeated following the removal of non-fish vertebrates from spraint samples for the selectivity calculations to avoid bias from non-fish species. This was chosen based on previous metabarcoding studies using otter spraints and mink scats carried out by Harper *et al*., (2020b) which demonstrated that proportional read counts and occurrence data were broadly comparable. It is typically impossible to quantify how many individuals have been eaten from molecular dietary studies (Ruppert, Kline and Rahman, 2019), since primer bias, most recent species fed on and the nature of our extraction method can all influence prey detection. Therefore, the Jaccard index was chosen as a conservative approach which takes presence/absence to indicate the occurrence of a species within a spraint.

Samples collected from tributaries (n=20) were omitted from the spatial analyses as this was not a clear geographic group and instead a combination of spraints collected from sites outside of the main River Hull channel. In addition, we did not have existing water sample data from tributaries to inform prey communities for the selectivity analysis. A further 6 samples were omitted from the data set as they were collected outside of the sampling window. The seasonal analysis included the data from tributaries; however, 7 samples were omitted from the data set as they were collected outside of the sampling window.

Alpha diversity was calculated in R as the average number of prey species per faecal sample, this was subsequently used to investigate spatial and seasonal variation in otter diets as well as differences between otter and mink diets. In all three cases, the data was not normally distributed (spatial: Shapiro-Wilk normality test: W = 0.919, P= <0.002; seasonal: Shapiro-Wilk normality test: W= 0.914, P= <0.001; predator:

Shapiro-Wilk normality test: W= 0.931, P= <0.001) and sample sizes were unbalanced. Therefore, nonparametric Kruskal-Wallis tests and Dunn's tests from the packages stats v3.6.3 and FSA v0.8.32 (Ogle, Wheeler and Dinno, 2021) were used to compare alpha diversity of otter diets between sections (Harper *et al*., 2020b). Nonparametric Mann-Whitney U Tests were chosen to compare seasonal differences in the alpha diversity of otter spraints, and between otter and mink spraints.

The DECOSTAND function in the package vegan v2.5-7 (Oksanen *et al.*, 2020) was used to convert the read counts into presence/absence for all statistical analyses of beta diversity. To assess spatial variation in otter diets across the three sections, Jaccard dissimilarity matrices were created using the VEGDIST function and visualised via Non-Metric multidimensional scaling (NMDS) using the METAMDS function (Harper *et al*., 2020b). A series of PERMANOVAs were then carried out to statistically test for differences in the prey community across the three sections of the River Hull. The "strata" command was used throughout to constrain permutations to within season, thus ensuring variation in diet was a result of the section. Subsequent analyses to assess seasonal variation in otter diets, differences in the vertebrate prey communities eaten by otter and mink, and variation in community composition between replicates were analysed in the same way as the spatial analysis.

It is difficult to quantify abundance from water samples, particularly in flowing rivers, since primer bias, downstream transportation of eDNA and the nature of our extraction method can all influence species read counts (Pawluczyk *et al.*, 2015; Roussel *et al.*, 2015). Therefore, the Jaccard index was chosen as a conservative approach which

takes presence/absence to indicate the occurrence of a fish species within a water sample.

Alpha diversity was calculated in R as the average number of fish taxa per water sample, this was subsequently used to investigate spatial and seasonal variation in fish communities. To assess spatial variation, the data was not normally distributed (Shapiro-Wilk normality test: $W = 0.951$, $P < 0.05$), therefore, nonparametric Kruskal-Wallis tests and Dunn's tests from the packages stats v3.6.3 and FSA v0.8.32 (Ogle, Wheeler and Dinno, 2021) were used to compare alpha diversity of fish communities between sections (Harper *et al*., 2020b). A paired t-test was chosen to compare alpha diversity of fish communities between winter and spring. To test differences in the beta diversity of fish communities across the three sections and between seasons, data was analysed in the same way as the faecal spatial analysis.

Alongside all PERMANOVAs, the homogeneity of multivariate dispersions (MVDISP) was calculated using the BETADISPER function in vegan v2.5-7 and statistically tested using ANOVA. This was used to verify whether a significant result was due to a difference in the mean community composition as opposed to a difference in dispersion between samples. All PERMANOVA were performed with a Jaccard distance matrix and 999 permutations, using the function "adonis" in the Vegan package (Oksanen *et al.*, 2020).

2.8 Dietary preference

To assess dietary preference, the Ivlev's electivity index was calculated in R following the approach described in Lanszki *et al*., (2001), where the percentage occurrence of fish species in spraint samples was used to inform prey eaten, and the percentage occurrence of fish species in water samples was used to inform prey available. Due to the sporadic nature of non-fish vertebrate interactions with rivers the Ivlev's electivity index was only applied to fish species. The calculation was done for fish species across each section of the River Hull as follows:

Electivity index = (% occurrences of fish species in spraints - % occurrences of fish in eDNA water samples)/ (% occurrences of fish species in spraints + % occurrences of fish in eDNA water samples)

This gave a value of between -1 and 1 to indicate the level of dietary preference, with 1 indicating a strong preference and -1 avoidance of prey species. All figures were produced in R using the package ggplot2 v3.3.5 (Wickham, 2016).

3.Results

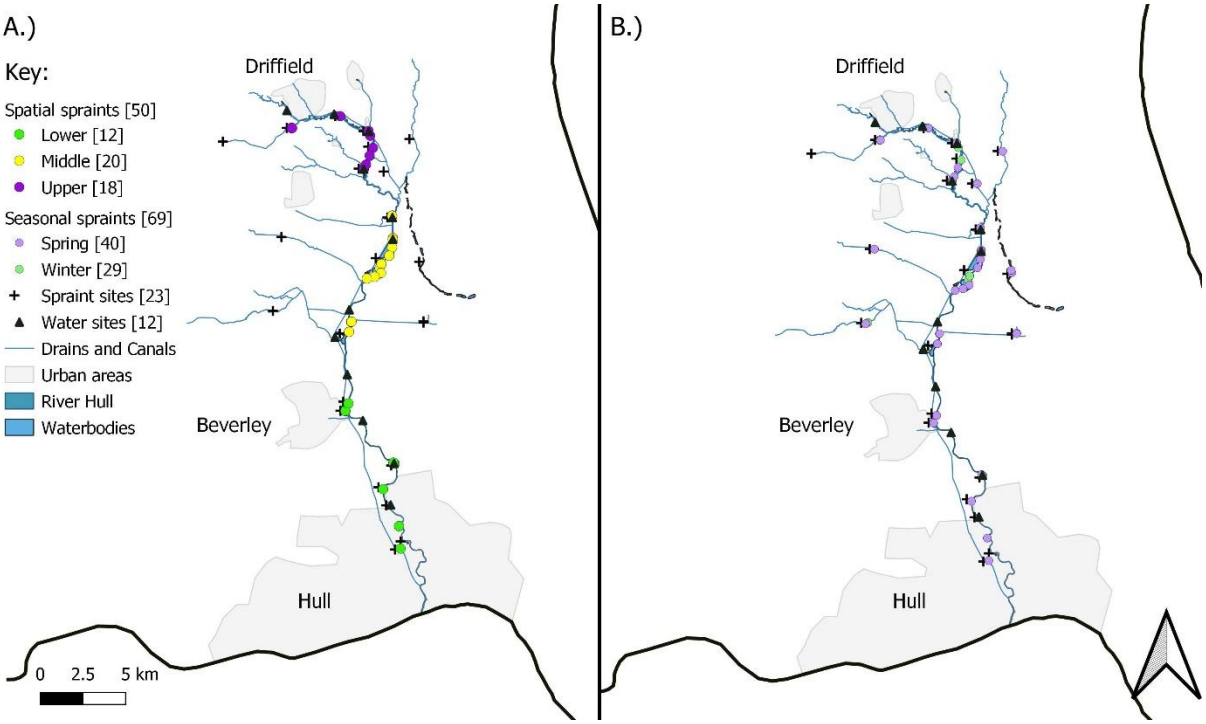


Figure 4: Locations of confirmed otter spraint samples collected for the spatial (A.) and temporal (B.) analysis of otter diets across the River Hull catchment.

88 faecal samples were collected across the three sampling periods (including 2 opportunistically collected samples in March). An additional 11 faecal samples were collected during a separate sampling trip in May, however, only 3 sampling sites were visited during this trip, so they were later omitted from the spatial and seasonal analyses. The three one week long sampling periods were carried out in November (N=29), February (N=12) and April (N=45), thus allowing us to explore the seasonal variation in otter diet between winter and spring. During the February sampling campaign, flooding and snow made sampling challenging with 21/23 sites being either fully or partially inaccessible, additionally existing spraint samples were washed away or covered by snowfall. As a result, only 12 faecal samples were collected. Therefore,

samples from this period were merged with samples from the November campaign to allow sufficient data for the seasonal analyses. In addition to the spraints, soil from 4 track samples was opportunistically taken to observe what DNA we could detect.

The average read count across faecal samples before threshold filtering was 91,512 reads per sample. Prior to threshold application, we detected 104 taxa across 141 samples (137 faecal samples and 4 track samples). This consisted of 7 amphibian taxa, 24 bird taxa, 37 fish taxa and 36 mammal taxa. Following threshold application (1%), 48 taxa remained across the 137 faecal samples, containing 2 amphibian taxa, 12 bird taxa, 19 fish taxa and 15 mammal taxa.

Contamination from different sources was observed in the extraction blanks and PCR controls (Appendix 3), in addition, up to 65 reads of cichlid DNA was detected in faecal samples. Despite this, read counts in extraction blanks were below the levels found in faecal samples with the exception of (*Cyprinus carpio*) which was detected at 1606 reads in JBL9. This species has remained in the dataset as it is a genuine prey species, however, it should be interpreted with caution. Following the application of our 1% threshold, no cichlid DNA remained in faecal samples (Appendix 4).

Following the removal of replicate samples (n=20), one sample below 500 reads and one sample collected outside of the study area, 115 faecal samples remained. Initially, all samples without reads of otters were removed and 84 faecal samples remained. One sample was omitted due to uncertain predator assignment (<90% of reads assigned to a single predator), two samples with mixed predator detections were

assigned to the American mink and one sample with mixed predator assignment for otter and European red fox, was assigned to otter.

Following predator assignment, 81 confirmed otter spraints remained. Two samples (JM94 and MC19) were omitted as there were no prey reads detected, and three additional samples were omitted as they were collected outside of the 2020/2021 sampling period. This gave a final dataset of 76 otter spraints which following taxonomic refinement and removal of domestic species contained 23 prey taxa (20 assigned to species level), consisting of 2 amphibian taxa, 4 bird taxa, 16 fish taxa and 1 mammal taxon.

To assess niche differentiation with the American mink, samples collected in 2019-2021 were merged with faecal samples collected by previous projects dating back to 2015 (Harper *et al*., 2020b). The combined dataset consisted of 335 samples (including controls) across 142 taxa. Following threshold application (1%), removal of controls and environmental species 53 taxa remained across the 291 faecal samples, containing 4 amphibian taxa, 15 bird taxa, 20 fish taxa and 14 mammal taxa.

3.1 Spatiotemporal variation in the Eurasian otter diet

Across the 76 confirmed otter spraints collected from the River Hull catchment, 23 vertebrate prey species were detected in spraints, 16 fish species, 4 bird species, 2 amphibian species and 1 mammal species. The vertebrate diet primarily consisted of fish accounting for 92.34% of prey occurrences, meanwhile birds and amphibians

accounted for 3.6% of prey occurrences each with the remaining 0.46% assigned to mammals.

Otter diet varied significantly between sections of the River Hull (PERMANOVA, R2=0.16849, DF= 2, P=<0.001, Figure 5). Multivariate dispersions were homogeneous between sections (ANOVA, F=1.8372, DF= 2, P=0.17). In the Lower River Hull, otters fed on 9 prey species: European bullhead, European eels and European flounder were the most common prey species accounting for 30%, 27% and 12% of prey occurrences respectively (Figure 7). In the Middle section, otters fed on 11 prey species. The primary prey species in this section were: the European bullhead, three-spined stickleback, perch and stone loach (*Barbatula barbatula*) accounting for 22.2%, 17.46%, 12.69% and 12.69% of prey occurrences respectively. In the Upper River Hull otters fed on 11 prey species: European bullhead, European minnow (*Phoxinus phoxinus*), three-spined stickleback and Rainbow trout accounting for 31.4%, 17.6%, 13.7% and 11.8% of prey occurrences respectively. Alpha diversity was highest in the Middle section where otter spraints contained 3.15 prey species per spraint, compared with 2.83 and 2.75 in the Upper and Lower sections respectively. However, these differences were not significant (Kruskal-Wallis, X2 = 0.65767, DF=2, P=0.7198, Figure 9).

Otter diet did not vary significantly between seasons (PERMANOVA, R2=0.02654, DF=1, P=0.172, Figure 6). Multivariate dispersions were homogeneous between seasons (ANOVA, F=1.3549, DF=1, P=0.249). In winter, otters fed on 17 prey species, European bullhead, three-spined stickleback and rainbow trout were the most common prey species accounting for 29.17%, 13.89% and 6.9% of prey occurrences

respectively (Figure 8). In spring, otters fed on 15 vertebrate prey species, European bullhead, three-spined stickleback and European eels were the most common prey species accounting for 24.8%, 14.4% and 9.6% of prey occurrences respectively. Season influenced alpha diversity of vertebrate prey in otter spraints (Mann-Whitney U Test, W=757, N=69, p-value=<0.026, Figure 10), and the number of species per spraint was significantly higher in spring (Mean= 3.125) than winter (Mean= 2.482) (Figure 10).

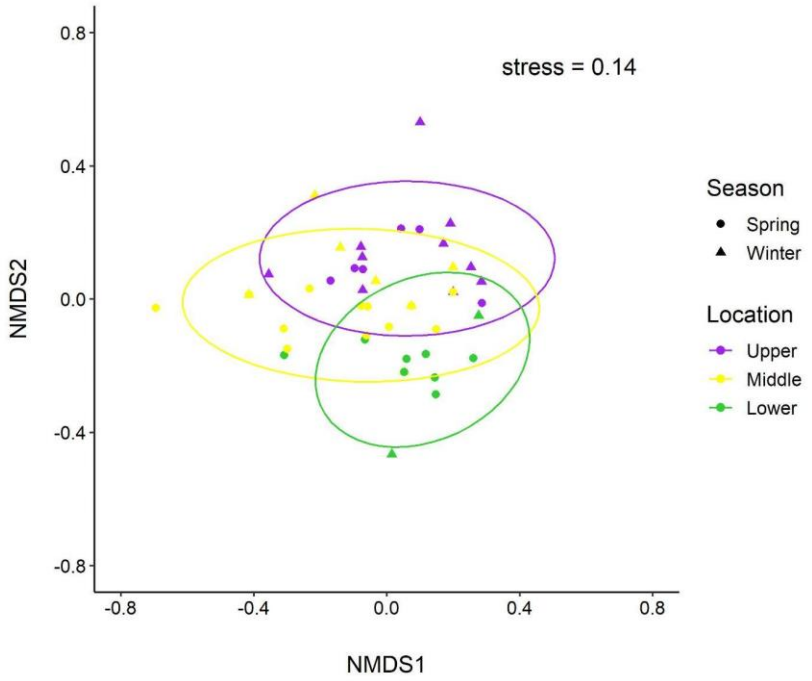


Figure 5: Non-metric Multidimensional Scaling (NMDS) plot of prey communities from otter spraints across different sections of the River Hull. Points and ellipses are coloured by section (Upper: purple, Middle: yellow, Lower: green). The shape of points shows the season they were collected (circles: spring, Triangles: winter) (PERMANOVA, R2=0.16849, DF= 2, P=<0.001).

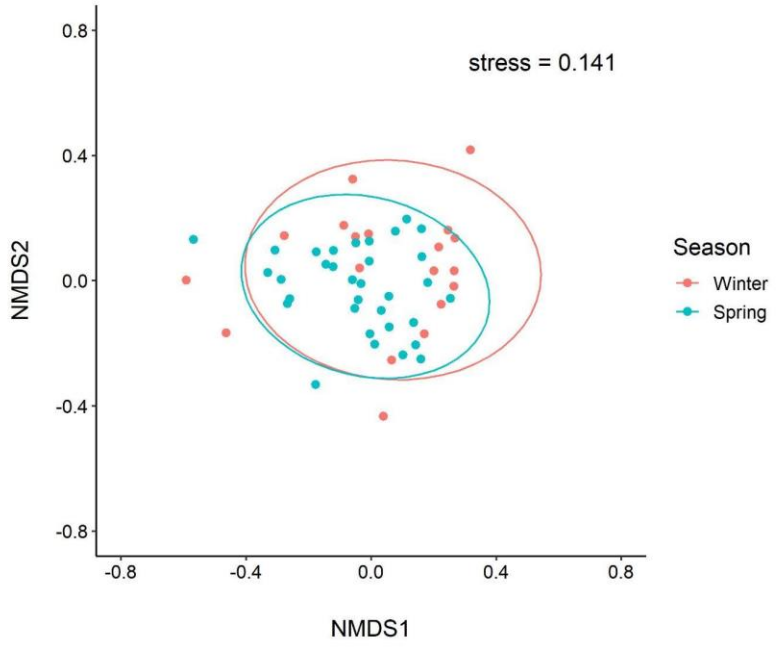


Figure 6: Non-metric Multidimensional Scaling (NMDS) plots of prey communities from otter spraints between winter and spring. Points and ellipses are coloured by season (winter: red, spring: blue), (PERMANOVA, R2=0.02654, DF=1, P=0.172).

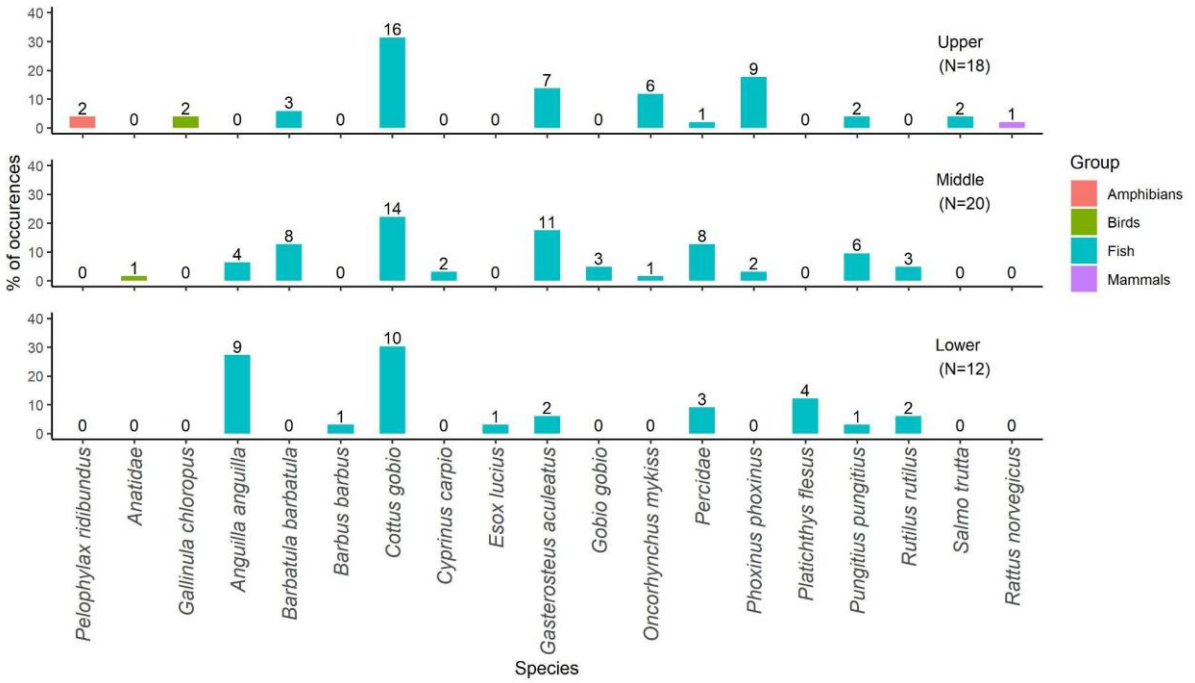


Figure 7: The percentage occurrence of prey taxa from otter spraints within each section of the River Hull. Species detected are coloured dependent on their vertebrate group and the numbers above the bars indicate the number of occurrences for each species.

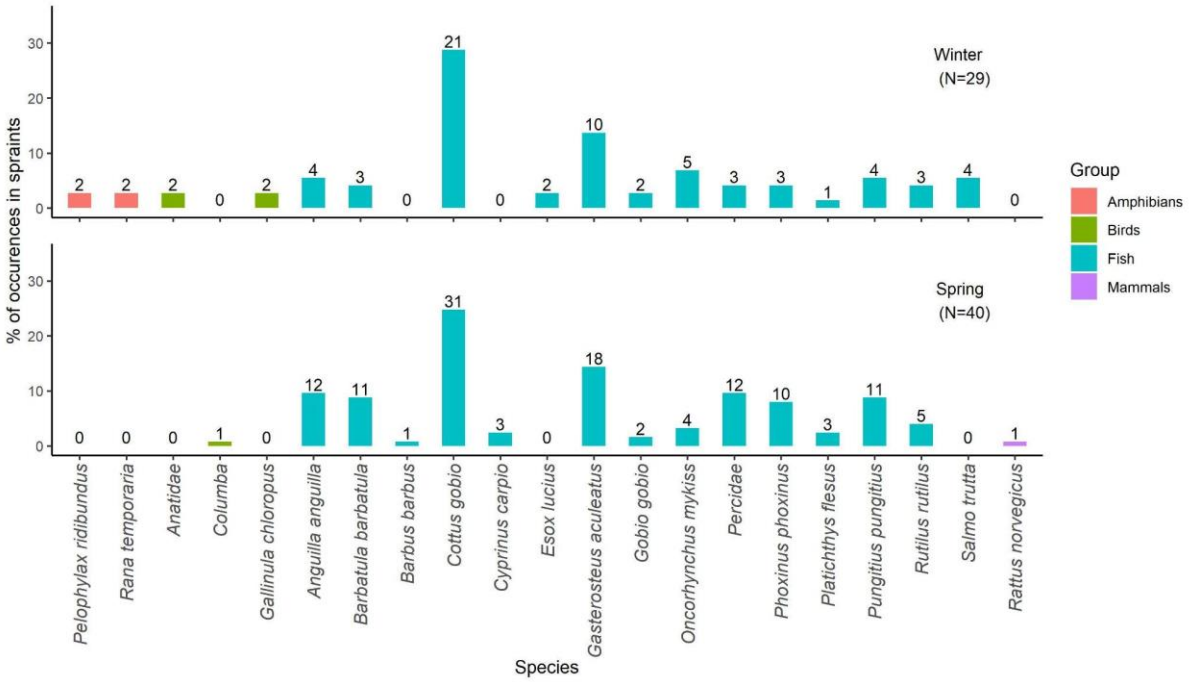


Figure 8: The percentage occurrence of prey taxa from otter spraints within seasons across the River Hull. Species detected are coloured dependent on their vertebrate group and the numbers above the bars indicate the number of occurrences for each species.

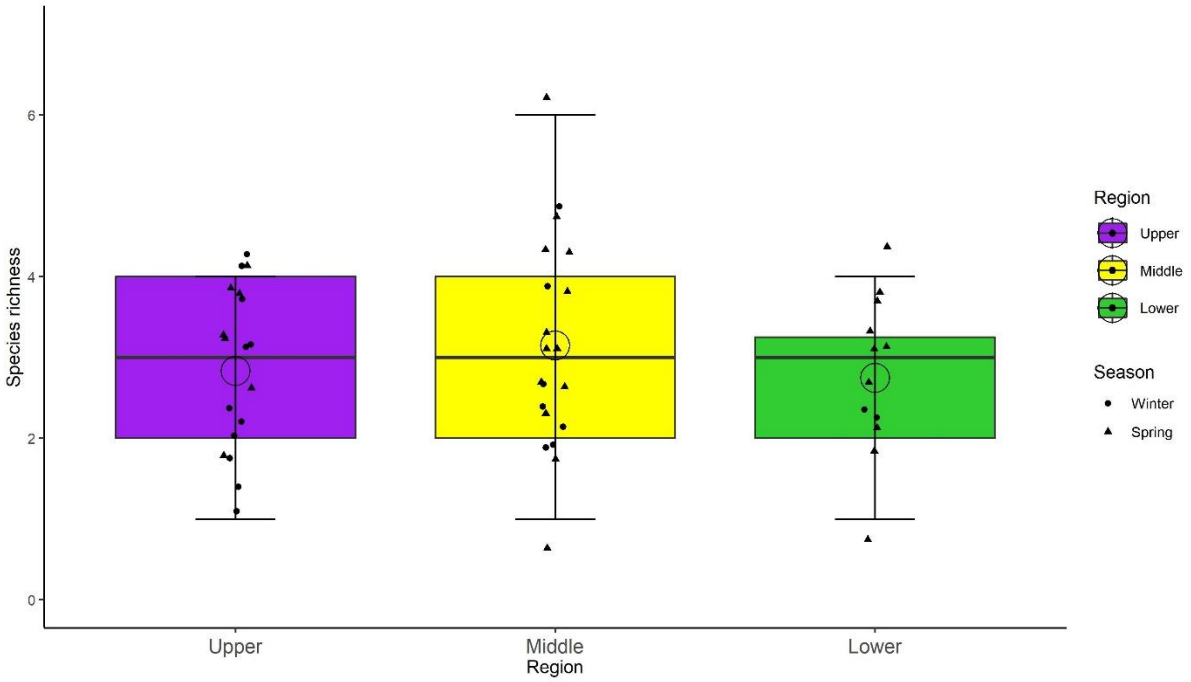


Figure 9: Alpha diversity of spraints across the River Hull. Boxes are coloured by section (Upper: purple, Middle: yellow, Lower: green). The shape of points shows the season they were collected (circles: spring, Triangles: winter) (Kruskal-Wallis, X2 = 0.65767, DF=2, P=0.7198).

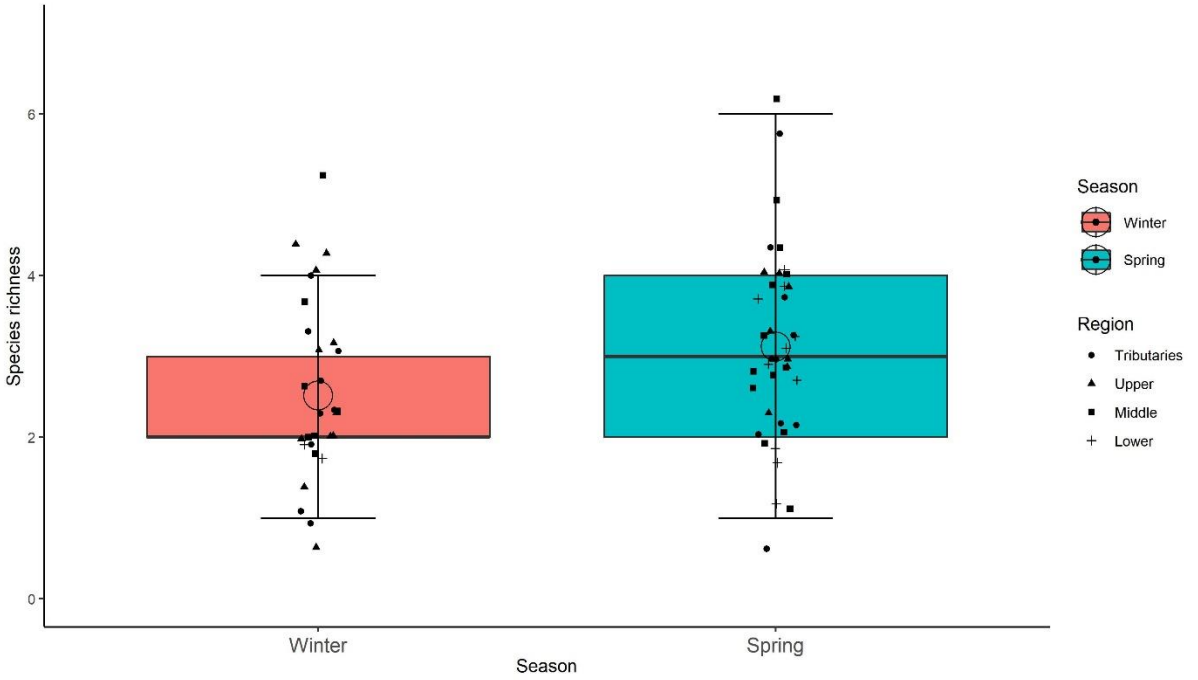


Figure 10: Alpha diversity of spraints across the River Hull. Boxes are coloured by season (winter: red, spring: blue). The shape of points shows the section they were collected (circles: Tributaries, triangles: Upper, square: Middle, cross: Lower). The clear circles show the mean number of species per spraint (winter= 2.48, spring= 3.125) (Mann-Whitney U Test, W=757, N=69, p-value=<0.026).

3.2 Spatiotemporal variation in the River Hull fish communities

Overall, 23 fish species were detected across the 12 sites along the River Hull (Figure 13A + 13B). Species richness was highest in the middle region where 21 fish species were detected. 16 species were detected in the Upper section (Mean=7.38), brown trout, European bullhead and three spined stickleback were widespread throughout this section (16/16 positive detections). In addition, European grayling and lamprey were detected in 10/16 samples from this section. Read counts in the Upper River Hull were dominated by rainbow trout (49.97%), three spined stickleback (20.43%) and European minnow (11.71%) suggesting these species are abundant throughout this section.

Twenty one species were detected in the Middle section (Mean=13.38), European minnow, European bullhead and three-spined stickleback were widespread (16/16 positive detections). Ninespine stickleback, northern pike (*Esox lucius*) and roach (*Rutilus rutilus*) were also widespread in this section (>13/16 positive detections). Read counts were dominated by rainbow trout (27.99%), three-spined stickleback (26.24%) and roach (17.65%).

In the Lower River Hull, 20 fish species were detected (Mean=9.88), European bullhead, three-spined stickleback, roach and common bream (*Abramis brama*) were widespread throughout this section (>14/16 positive detections). European eels and European flounder were also common (>50% of positive detections). Read counts across the section were dominated by roach (57.69%) and perch (14.63%).

Fish communities varied significantly between the Upper, Middle and Lower sections of the River Hull across all four seasons (Figure 11, Table 1). Multivariate dispersions were homogeneous between sections for all four seasons (Table 1). Species richness varied significantly between sections (Kruskal-Wallis, X2 = 23.22, DF=2, P=<0.05, Figure 12), and Dunn's test indicated that species richness was significantly different between all pairwise comparisons (Dunn's test, Lower-Middle: Z = -2.72, P=<0.05; Lower-Upper: Z = 2.089, P=<0.05; Middle-Upper: Z = 4.80, P=<0.05, Figure 12).

Fish communities along the River Hull varied significantly between winter and spring (PERMANOVA, R2=0.10931, DF=1, P=0.001, Figure 14). Multivariate dispersions were homogeneous between seasons (ANOVA, F=0.2519, DF=1, P=0.6207). Species richness was significantly higher in spring (Mean = 12.333) than winter (Mean = 8.416) (paired *t*-test test, t = -5.6731 df = 11, P=0.0001, Figure 16). 19 fish taxa were detected

in winter in comparison to 23 fish taxa in spring, species absent in winter were European eels, common barbel, silver bream (*Blicca bjoerkna*) and sea lamprey (*Petromyzon marinus*) (Figure 15). 17 of the 23 (73.91%) species detected had a higher occupancy in spring than winter.

	Homogeneity of multivariate dispersions (ANOVA)				Community similarity (PERMANOVA)			
	Mean distance to centroid + SE	**df**	**F**	**P**	**df**	**F**	**R2**	**P**
Spring		2	1.011	0.402	2	5.424	0.547	0.001
Upper	0.267 ± 0.026							
Middle	0.158 ± 0.012							
Lower	0.265 ± 0.009							
Summer		2	0.180	0.839	2	7.847	0.636	0.002
Upper	0.247 ± 0.037							
Middle	0.225 ± 0.004							
Lower	0.195 ± 0.006							
Autumn		2	0.161	0.854	2	3.094	0.407	0.002
Upper	0.251 ± 0.019							
Middle	0.259 ± 0.014							
Lower	0.305 ± 0.030							
Winter		2	2.812	0.113	2	4.853	0.519	0.001
Upper	0.290 ± 0.007							
Middle	0.153 ± 0.012							
Lower	0.276 ± 0.005							

Table 1: Summary of statistical analysis used to compare fish communities between sections of the River Hull. Homogeneity of multivariate dispersions between fish communities were compared using (ANOVA), and variation in fish community composition across sections (PERMANOVA).

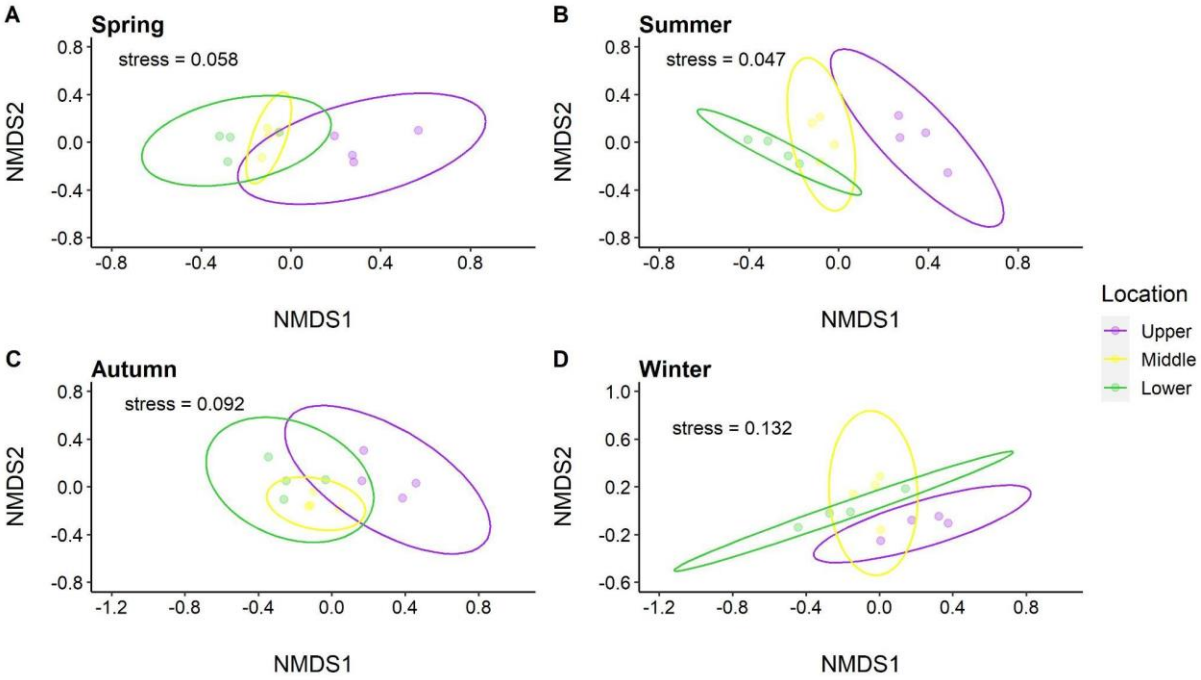


Figure 11: Non-metric Multidimensional Scaling (NMDS) plots of fish communities from water samples across all four seasons. Points and ellipses are coloured by section (Upper: purple, Middle: yellow, Lower: green).

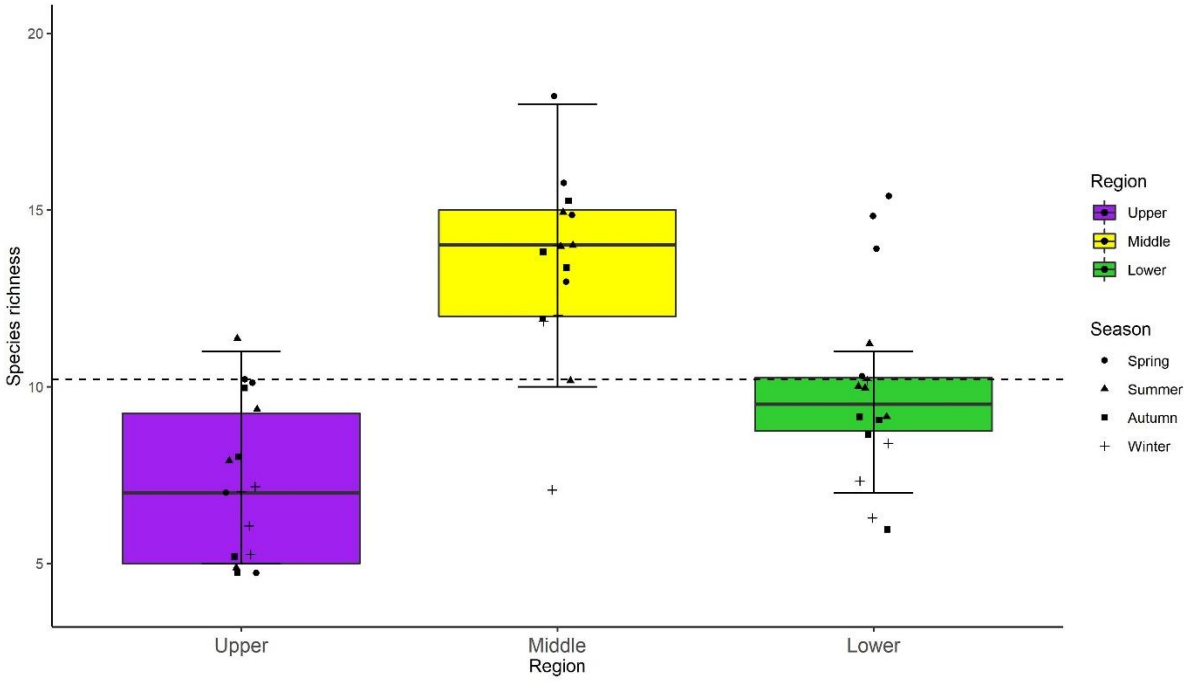
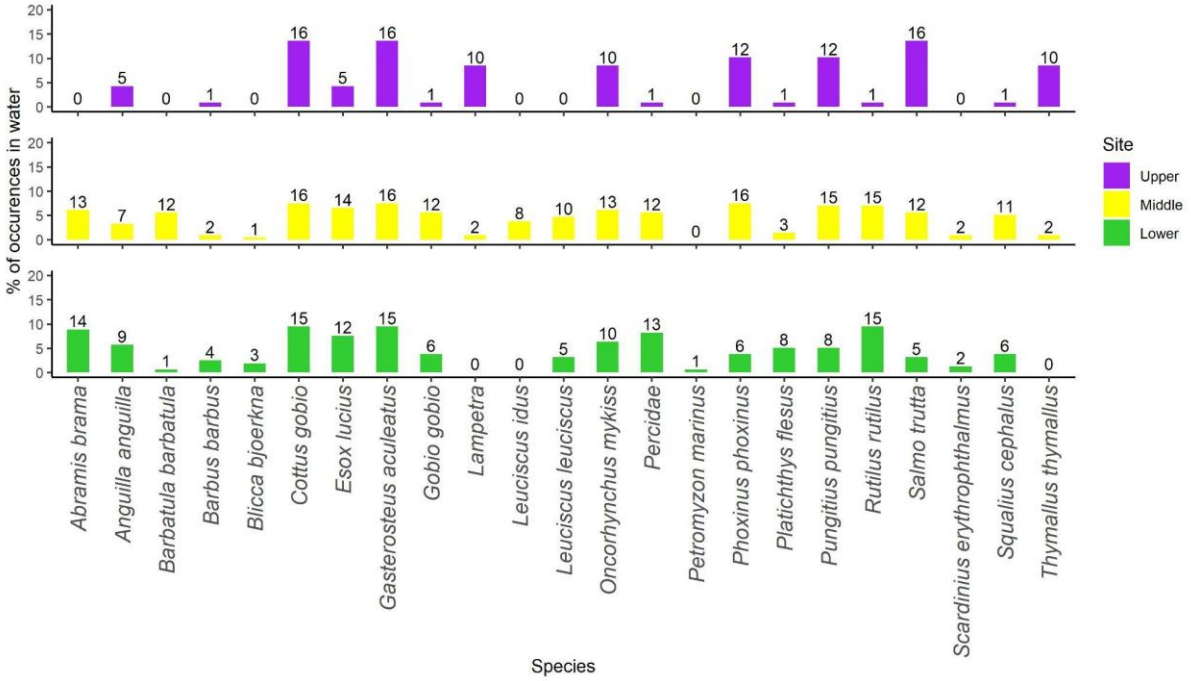


Figure 12: Boxplots of species richness across the three sections of the River Hull. Boxes are coloured by section (Upper: purple, Middle: yellow, Lower: green). The shape of points shows the season they were collected (circles: spring, triangles: Summer, square: Autumn, cross: winter). The mean species richness across all 48 samples (10.208) is represented by the dashed line (Kruskal-Wallis, X2 = 23.22, DF=2, P=<0.05).

A.



B.

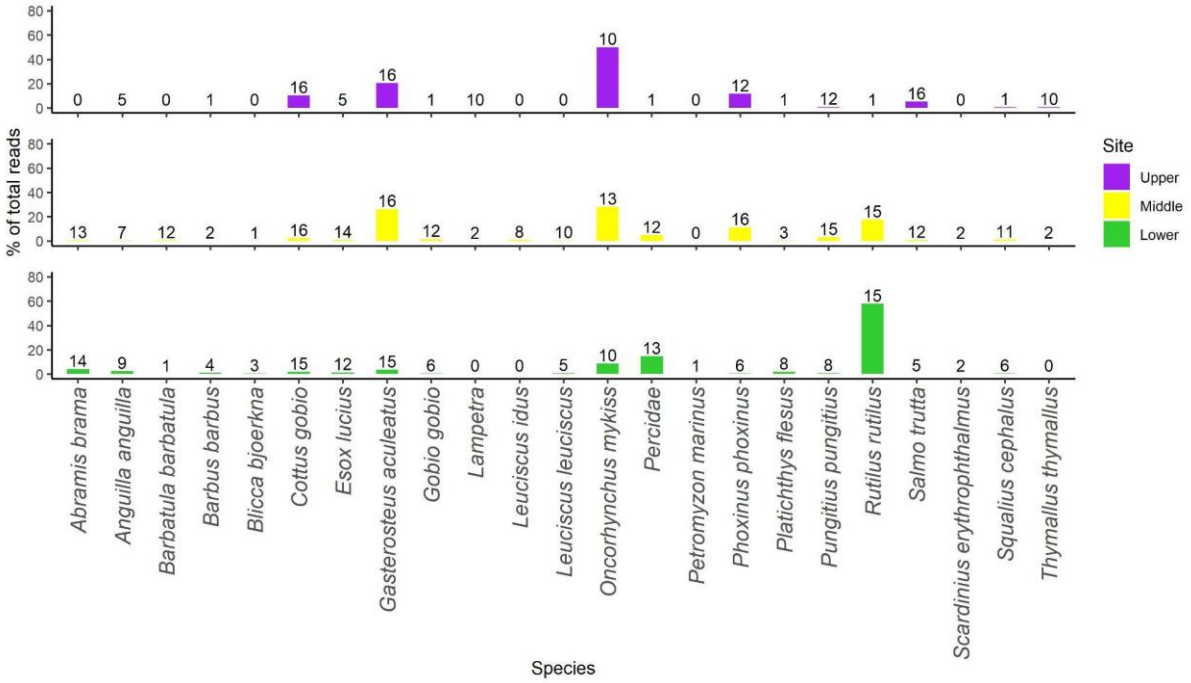


Figure 13A + 13B: The fish communities detected across the River Hull, represented as percentage of occurrences (A) and the percentage of fish reads (B) assigned to each fish taxa from water samples collected across the three sections. Barplots are coloured by location (Upper: purple, Middle: yellow, Lower: green), the numbers above the bars indicate the number of positive detections for each species.

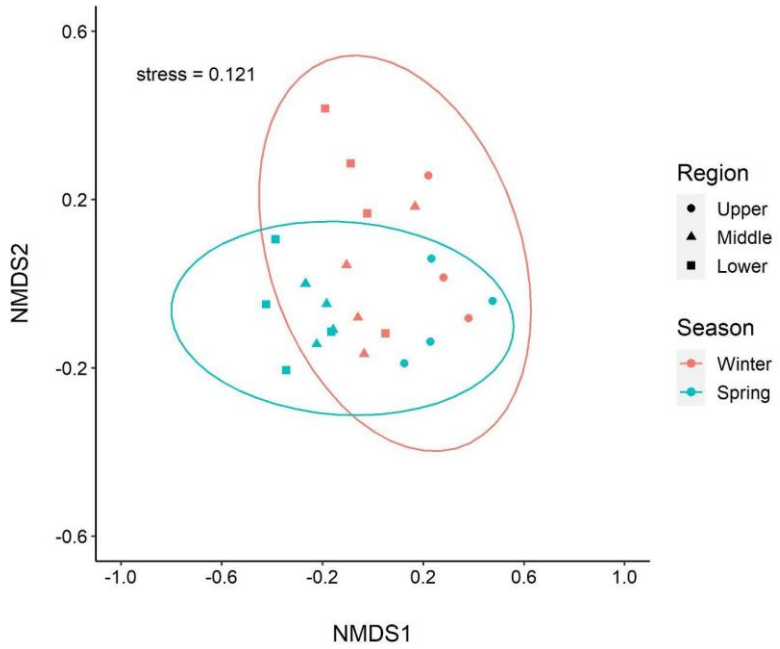


Figure 14: Non-metric Multidimensional Scaling (NMDS) plots of fish communities from water samples between winter and spring. Points and ellipses are coloured by season (winter: red, spring: blue). The shape of points shows the section they were collected (circles: Upper, triangles: Middle, squares: Lower) (PERMANOVA, R2=0.10931, DF=1, P=0.001).

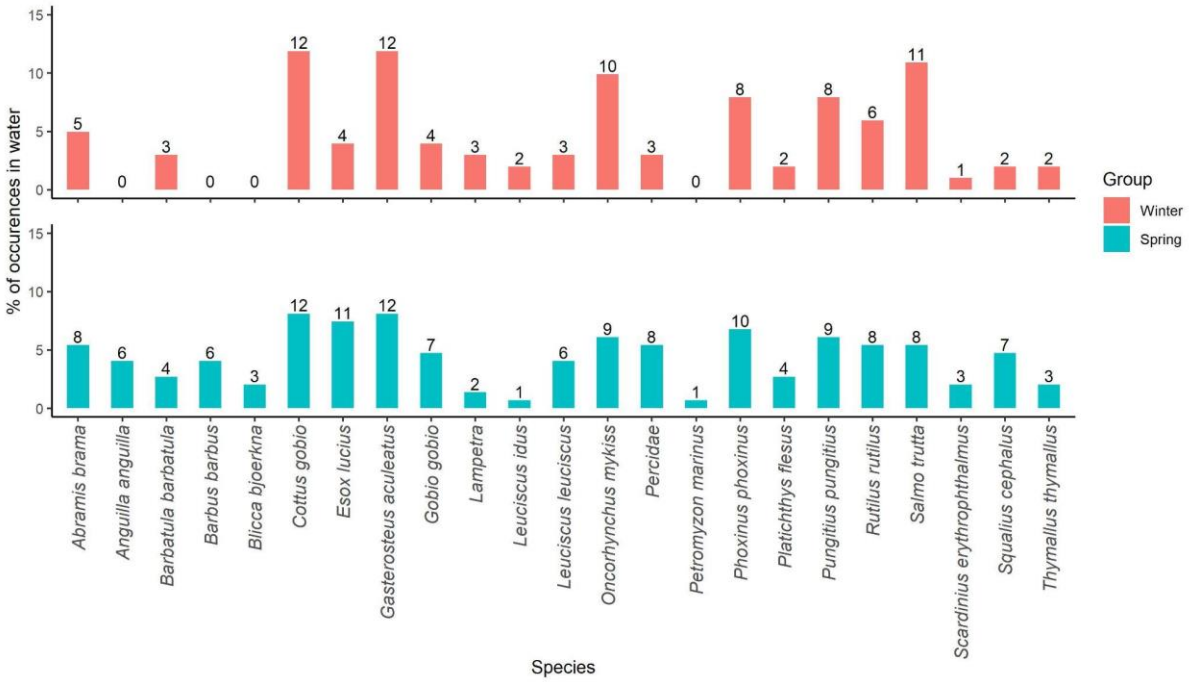


Figure 15: The percentage occurrence of fish taxa from water samples between seasons across the River Hull. Barplots are coloured by location (winter: red, spring: blue), the numbers above the bars indicate the number of positive detections for each species.

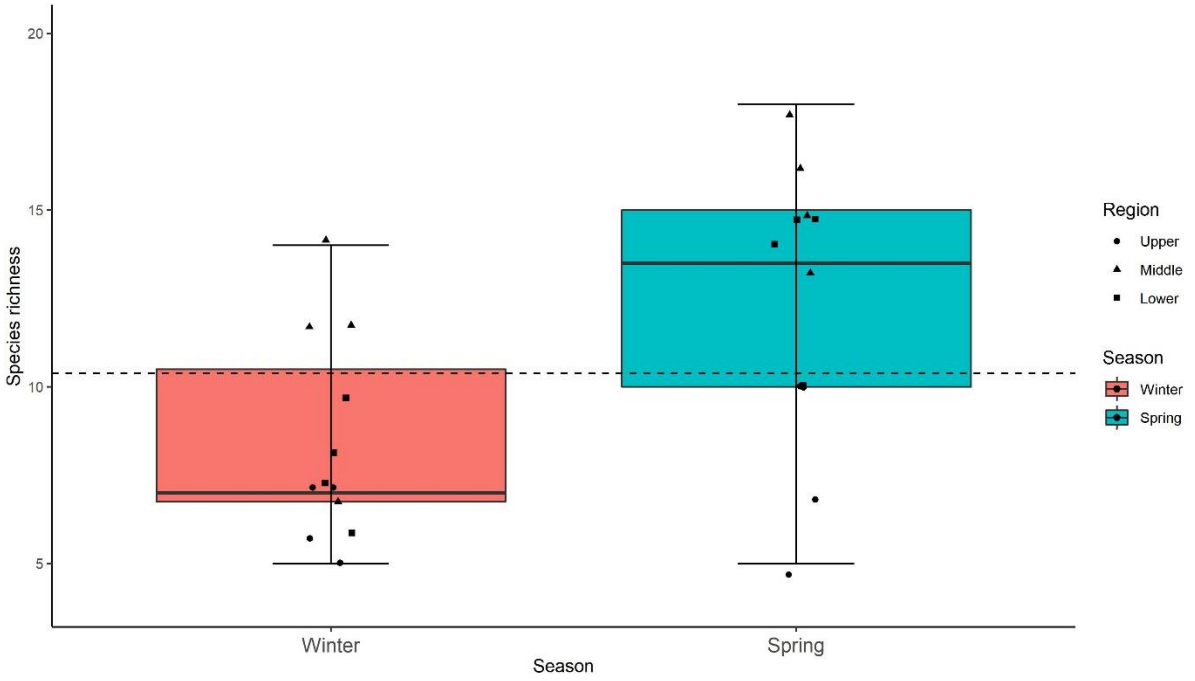


Figure 16: Boxplots of species richness from water samples taken from the River Hull. Boxes are coloured by season (winter: red, spring: blue). The shape of points shows the section they were collected (circles: Upper, triangles: Middle, square: Lower). The mean species richness across all 24 samples (10.375) is represented by the dashed line (paired *t*-test test, t = -5.6731 df = 11, P=0.0001).

3.3 Dietary selection across the River Hull

While 23 fish taxa were detected across the water samples, only 15 fish taxa were detected in the otter diets. Common carp were found in otter diets, however, they were not detected in water samples from the River Hull and were thus omitted from the selectivity calculations. The Ivlev preference index was calculated by section and season for the 14 species found across both otter diet and water samples (Appendix 5, Figure 17).

Across all three sections a consistent strong (>0.35) selective preference was demonstrated towards the European bullhead (Upper: 0.439, Middle: 0.503, Lower: 0.523). In the Lower section, strong preferences were demonstrated towards the

European eel (0.654) and European flounder (0.411). In the Middle section, a strong preference was observed towards stone loach (0.394), Three-spined stickleback (0.407), Perch (0.394) and a moderate preference (>0.2) was observed towards European eels (0.327). Otters in the Upper River Hull demonstrated a preference towards (1.0), Perch (0.439), Eurasian minnow (0.316) and a weak preference was observed towards rainbow trout (0.212). Despite being abundant in the Upper section, the Eurasian otter demonstrated a consistent negative preference (<0) towards brown trout (*S. trutta*), similarly, across all three sections a negative preference was demonstrated towards roach, gudgeon (*Gobio gobio*) and pike.

Across both seasons a consistent selective preference was demonstrated towards the European bullhead (winter:0.191, spring:0.522) (Figure 18). In winter, a strong preference was demonstrated towards the European eel (1.0). Contrastingly, in spring, a strong preference was observed towards stone loach (0.545) and European eels (0.423), a moderate preference (>0.2) was observed towards Perch (0.298) and three-spined stickleback (0.298). Across both seasons a negative preference was demonstrated towards roach, gudgeon, pike and rainbow trout.

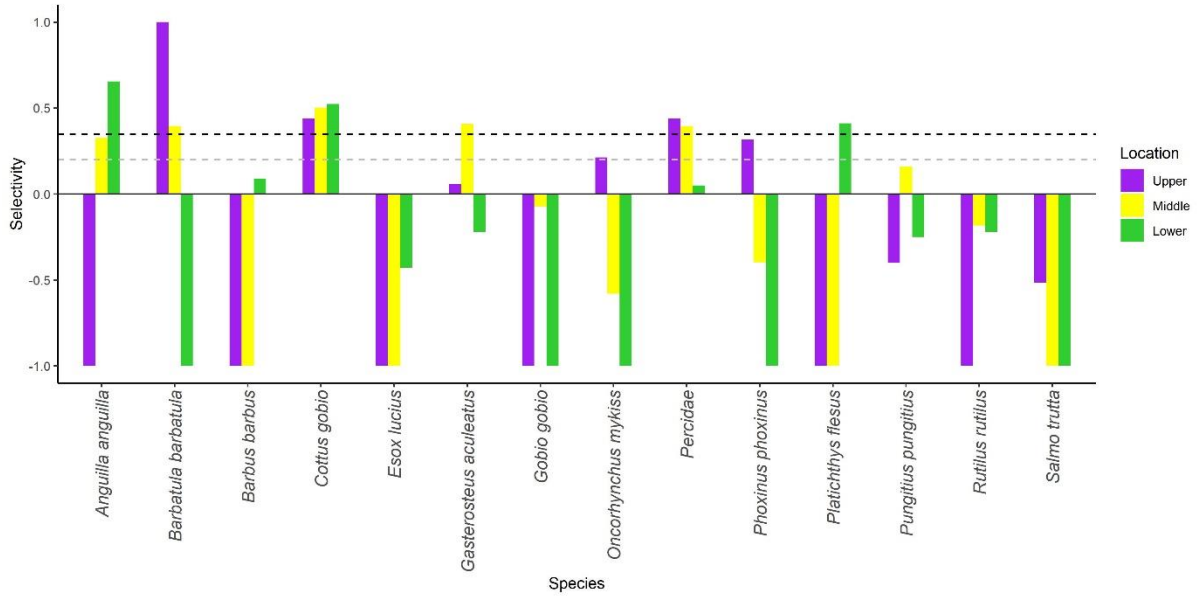


Figure 17: Ivlev's Electivity index values for each species across the three sections of the River Hull. Bars are coloured by location (Upper: purple, Middle: yellow, Lower: green). The grey dashed line indicates the value (0.2) at which we accepted a moderate dietary preference, the black dashed line indicates the value (0.35) at which we accepted a strong dietary preference.

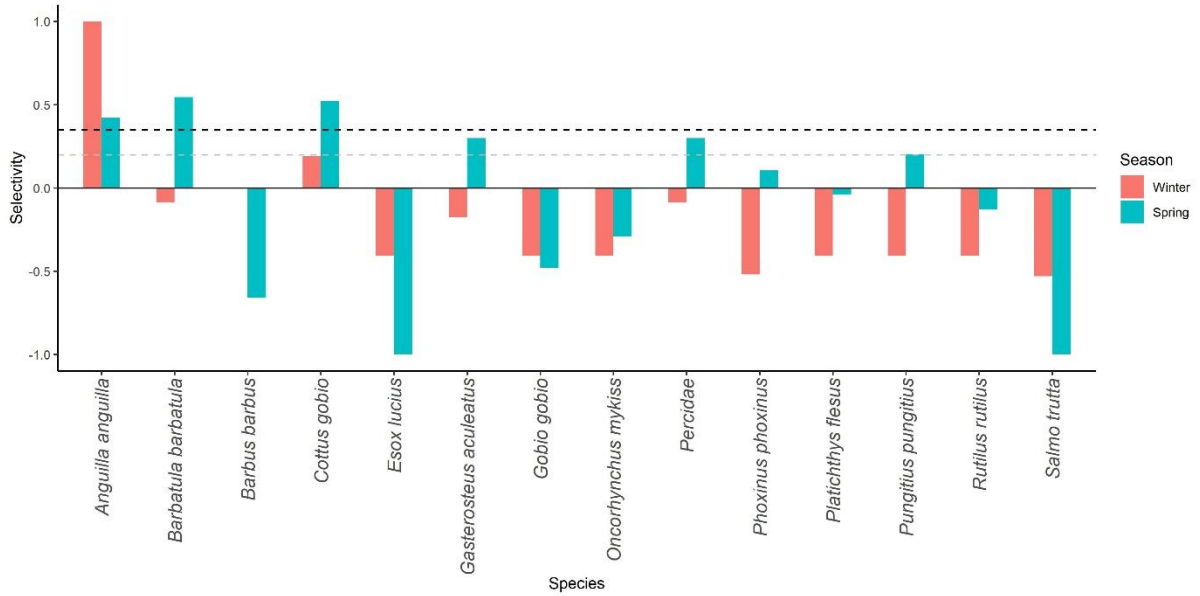


Figure 18: Ivlev's Electivity index values for each species across winter and spring. Bars are coloured by season (winter: red, spring: blue). The grey dashed line indicates the value (0.2) at which we accepted a moderate dietary preference, the black dashed line indicates the value (0.35) at which we accepted a strong dietary preference.

3.4 Dietary overlap between the Eurasian otter and American mink

Following predator assignment 191 otter spraints and 31 mink scats remained. 2 otter spraints (JM94 and MC19) and four mink scats (HA08, JM77, MC50 and RH03) did not contain any reads following predator removal, these were subsequently removed from the dataset. The final dataset consisted of 189 otter spraints and 27 mink scats which following taxonomic refinement and removal of domestic species contained 35 vertebrate prey taxa (30 assigned to species level), consisting of 3 amphibian taxa, 7 bird taxa, 19 fish taxa and 6 mammal taxa.

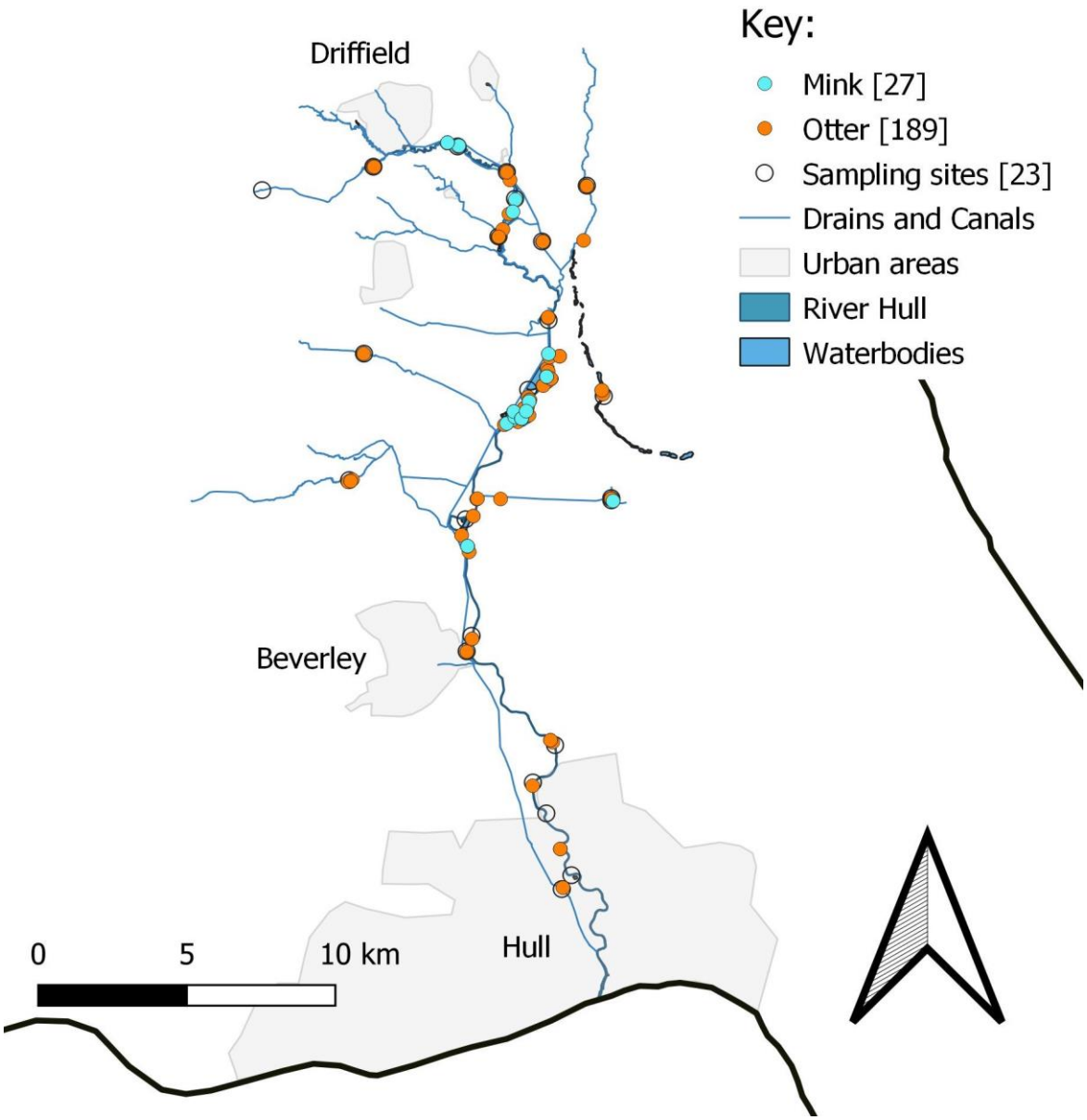


Figure 19: Sampling locations of 216 confirmed otter and mink faecal samples collected across the River Hull between 2015 - 2021. Points are coloured based on the predator (Mink: blue (n=29), Otter: orange (n=189)), spraint collection sites are marked by clear circles (n=23).

Otter vertebrate diet primarily consisted of fishes and amphibians accounting for 88.93% and 6.90% of prey occurrences respectively, contrastingly the mink diet predominantly contained mammals (37.5%) and birds (35%) (Figure 20). The two predators shared 14 prey taxa: common frog (*Rana temporaria*), waterfowl (family: *Anatidae*), Eurasian coot (*Fulica atra*), common moorhen (*Gallinula chloropus*), common starling (*Sturnus vulgaris*), common bream (*Abramis brama*), European

bullhead, three-spined stickleback, rainbow trout, perch, common roach, water vole, European rabbit and brown rat (*Rattus norvegicus*) (Figure 21).

17 prey taxa were detected in mink scats compared with 32 prey taxa found in otter spraints. Prey taxa unique to mink were European hare (*Lepus europaeus*), *Microtus* spp., and water shrew (*Neomys fodiens*). Water vole and common moorhen were the most common prey species in mink diets accounting for 17.5% and 15% of prey occurrences respectively. European bullhead and three-spined stickleback were the most common prey species in otter diets comprising 19.58% and 17.01% of prey occurrences respectively (Figure 21).

Predator influenced alpha diversity of faecal samples (Mann-Whitney U Test, W=671, N=216, p-value=<0.001)., and the number of prey species per sample was significantly higher in otter spraints (Mean= 3.286) than mink scats (Mean= 1.481) (Figure 22). Predator had a weak significant effect on total beta diversity of prey communities (PERMANOVA, R2=0.05208, DF=1, P=<0.001). Multivariate dispersions were also different between predators, where mink scats had significantly higher dispersion than otter spraints (ANOVA, F=13.65, DF=1, P=<0.001).

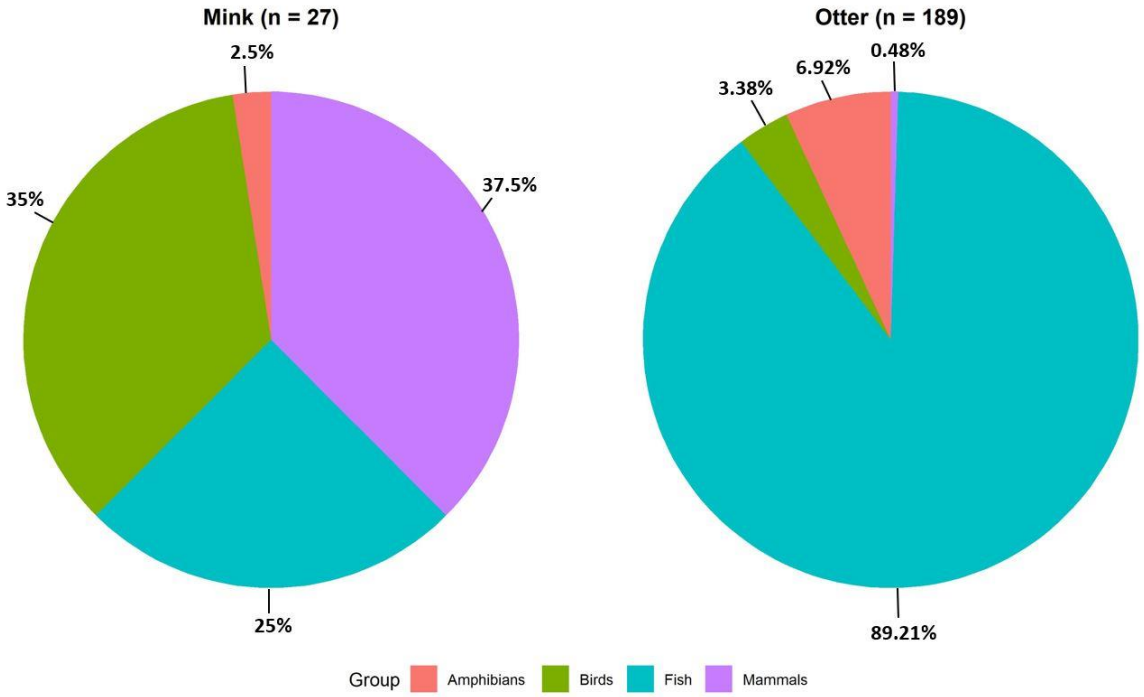


Figure 20: Diet by vertebrate group for the Eurasian otter and American mink showing the percentage of dietary occurrences assigned to each prey group. Segments are coloured dependent on their vertebrate group.

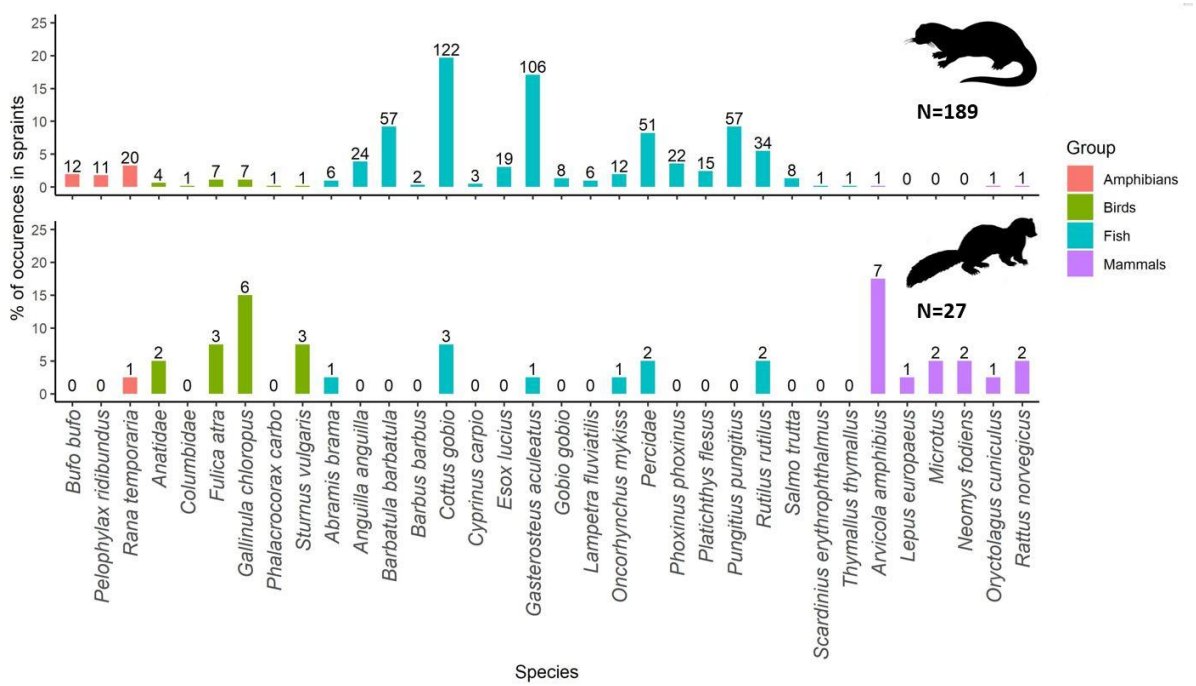


Figure 21: Bar plots showing the percentage occurrence of prey taxa from otter spraints and mink scats across the River Hull. Species detected are coloured dependent on their vertebrate group and the numbers above the bars indicate the number of occurrences for each species. This data is a combination of the current study and data from Harper *et al*., (2020b) (PERMANOVA, R2=0.05208, DF=1, P=<0.001).

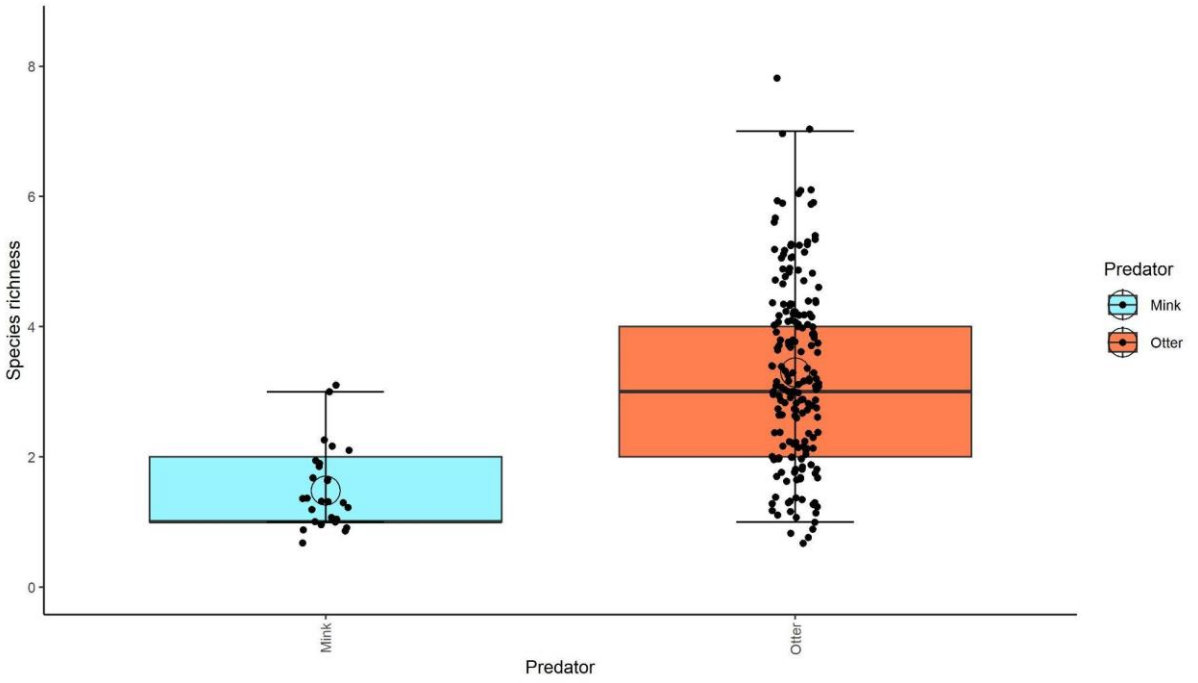


Figure 22: The number of vertebrate prey species detectedfrom otter (n=189) and mink (n=27) faecal samples collected at the River Hull. Boxes are coloured by predators (mink: blue, otter: orange). The clear circles show the mean number of species per faecal sample (mink= 1.481, otter= 3.296) (Mann-Whitney U Test, W=671, N=216, p-value=<0.001).

3.5 Replicability of our extraction method

Following threshold application, all 30 samples remained, and the predator had successfully been assigned as otter. Samples were initially visualised via NMDS (Figure 23), for 6 of the 10 spraints all 3 replicates were consistent for species detected. Four spraints had one replicate which differed: stone loach was detected in 1 replicate on 3 (BG4, TL9, SW2) separate occasions and three-spined stickleback was detected in a single replicate once (PB5) (Figure 24). Despite these differences, community composition did not vary significantly between replicates (PERMANOVA, R2=0.00564, DF=2, P=0.491, Figure 23) and multivariate dispersions were homogeneous (ANOVA, F=0.6941, DF=9, P=0.706) suggesting our extraction method was broadly replicable.

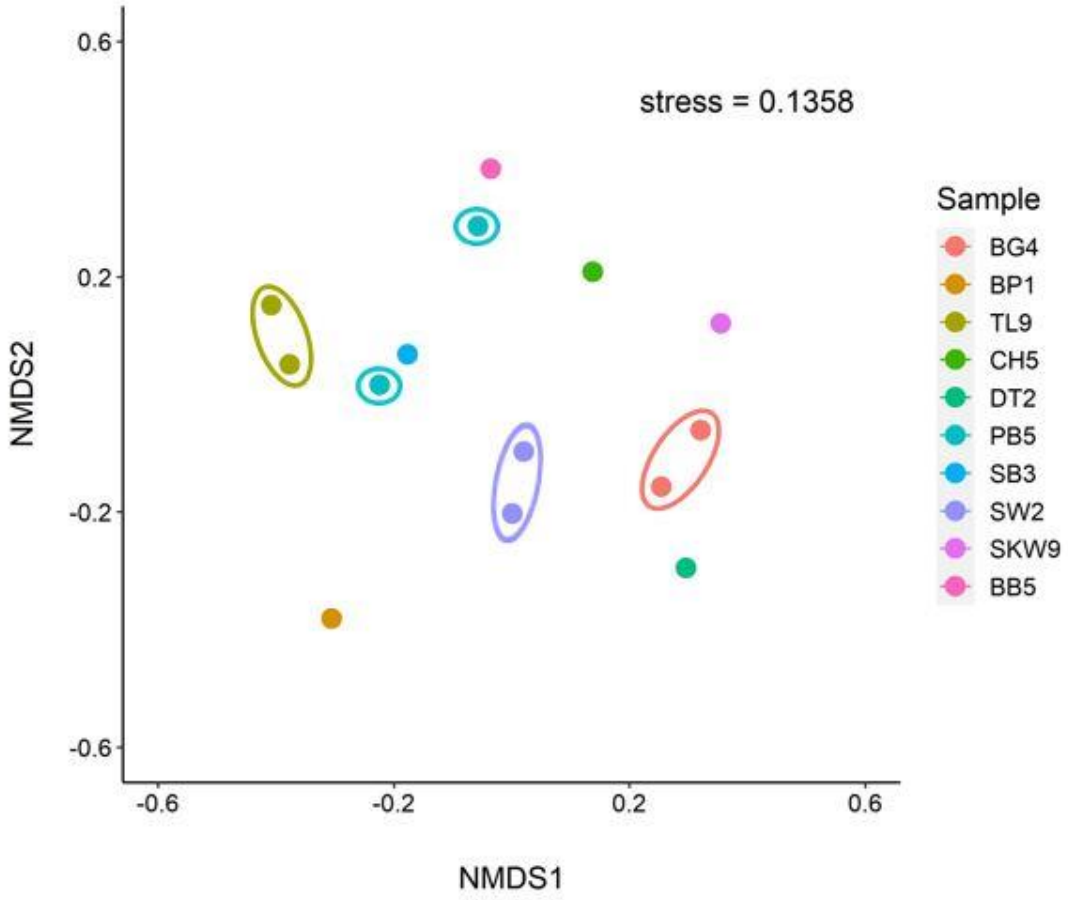


Figure 23: Non-metric Multidimensional Scaling (NMDS) plots of prey communities from 10 otter spraints. Points and ellipses are coloured by sample. In 6/10 samples all 3 replicates were identical, therefore they cluster directly on top of each other (PERMANOVA, R2=0.00564, DF=2, P=0.491).

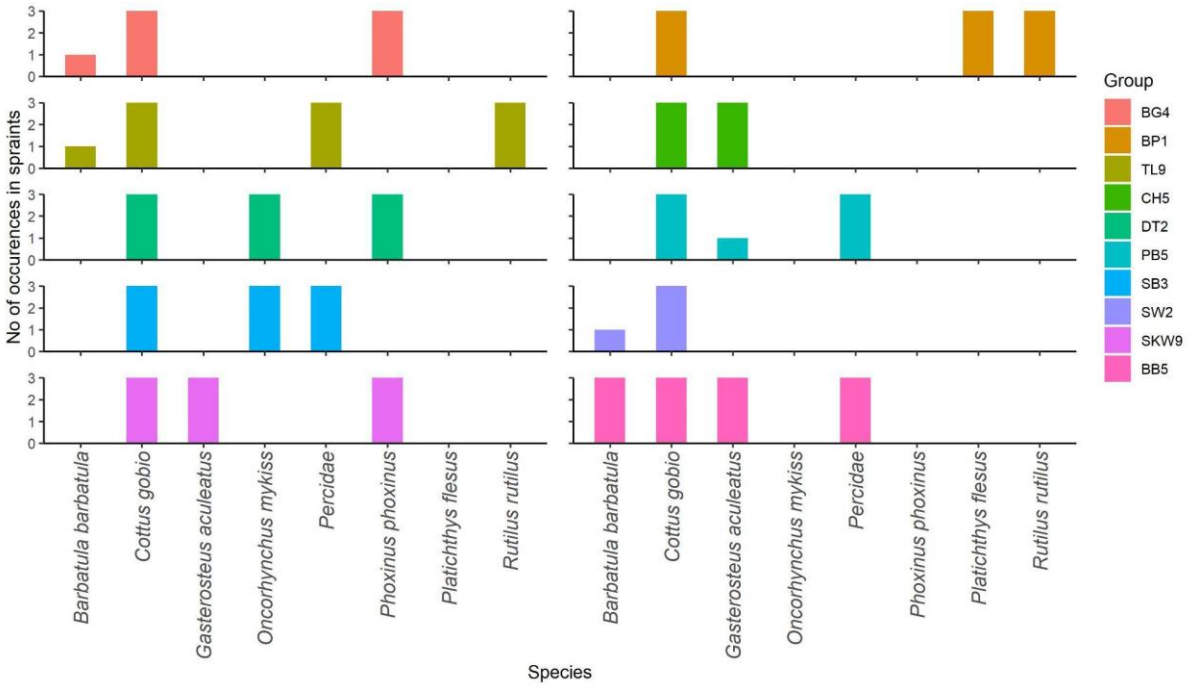


Figure 24: The number of occurrences for each prey species across the 3 extraction replicates. If the same prey species were detected in all 3 extraction replicates species bars would be at 3 occurrences.

4.Discussion:

This research has provided further understanding into the feeding behaviours of the Eurasian otter across the River Hull catchment. Section specific dietary preferences were found across the upper, middle and lower River Hull with a consistent selective preference across all three sections towards the European bullhead. The calculation of selectivity coefficients has shown how eDNA can be incorporated in modern dietary studies to provide a molecular approach to understanding dietary preference. While the overall otter diet did not vary significantly between seasons, Eurasian otters fed on significantly more different prey items in spring (Mean= 3.125) than winter (Mean= 2.482). The replicated DNA extraction experiment demonstrated that faecal metabarcoding of spraint samples is broadly consistent suggesting that a single extraction replicate is sufficient for detecting most prey items. Finally, comparisons between the Eurasian otter and American mink have provided further evidence of

niche differentiation between the two species, thus allowing for coexistence with minimal trophic competition.

4.1 General patterns in otter diet

Across the River Hull, otters fed predominantly on fish, the most common prey species were bullhead, three-spined stickleback, perch and ninespine stickleback.This is consistent with previous dietary analyses where small, slow moving species have been shown to be the dominant fish species in otter diets (Brzeziński, Romanowski and Kopczyński, 2006; Kruuk, 2006; Almeida, Copp and Masson, 2012; Britton *et al.*, 2017; Krpo-Ćetković, Subotić and Skorić, 2019; Drake, 2020; Harper *et al*., 2020b; Moorhouse-Gann *et al.*, 2020). Lanszki et al., (2001) found that otters living beside eutrophic fish ponds had a preference (*Ei*= 0.79) for fish between 500g - 1000g and irrespective of the species otters negatively selected for fish heavier than 1000g. They also found a negative preference towards species living in open water (*Ei*= 0.64). Remonti et al., (2010) discusses that while larger species provide more energy, they also require a longer handling time, meanwhile smaller species provide less energy but are easier to catch. In addition, Harper *et al*., (2020b) discuss the vulnerability of bullhead to predation suggesting that the camouflage amongst boulders is an ineffective defence against otters. The results of our eDNA water samples demonstrated that these fish are abundant along the River Hull, and previous research has shown that otters typically exploit the most abundant prey species throughout their habitats (Copp and Roche, 2003; Kruuk, 2006; Dettori *et al.*, 2021). Therefore, the preference towards small slow-moving species such as bullhead and three-spined

sticklebacks observed in our study may be explained by their ineffective escape mechanism, shorter handling time and high abundance throughout the River Hull.

While a consistent preference was demonstrated towards bullhead, the highest selective preference across the River Hull was seen on European eels in the lower section.This is concordant with literature where several studies across the UK (Miranda *et al.*, 2008; Almeida, Copp and Masson, 2012; Kruuk, 2014; Groves and Smith, 2021) and Europe (Beja, 1996; Taastrøm and Jacobsen, 1999; Smiroldo, Balestrieri, *et al.*, 2019) cite the European eel as a preferred prey species. It has been suggested that the preference towards slow moving species such as bullhead is a result of a reduction in the availability of eels forcing otters to diversify their diets towards alternative prey sources (Almeida, Copp and Masson, 2012; Kruuk, 2014; Drake, 2020; Moorhouse-Gann *et al.*, 2020).

Amphibians accounted for a small component of the otter diet in our study. Predation was highest on marsh frogs (*Pelophylax ridibundus*) accounting for 75% of amphibian occurrences. Despite their lower calorific value (Nelson and Kruuk, 1997), amphibians have been shown to be an important secondary prey resource for otters (Jędrzejewska *et al.*, 2001; Clavero, Prenda and Delibes, 2005; Pagacz and Witczuk, 2010; Smiroldo, Villa, *et al.*, 2019).

Predation on birds was primarily made up of waterfowl where Eurasian coots were the most common bird eaten. Studies from Shapwick Heath Nature reserve found up to

61% of spraints contained feathers and the primary bird species eaten was coots occurring in 15/87 (17.24% of spraints) (De la Hey, 2008), which demonstrates the feeding plasticity of otters.

Mammals were rare in our study, the only prey species was brown rat as a single occurrence. This is concordant with previous morphological (Taastrøm and Jacobsen, 1999; Sittenthaler, Koskoff and Pinter, 2019; Dettori *et al.*, 2021) and molecular studies (Hong *et al.*, 2019; Harper *et al*., 2020b; Jang-Liaw, 2021) in which mammals have contributed little to no prey occurrences in otter diets.

4.2 Spatial variation in otter diets

There were significant differences in the dietary composition across the three geographic sections which largely reflected the gradual change of fish communities along the river as shown by our eDNA water samples. This is consistent with previous research across the UK and Europe which has shown that otter diets are primarily dictated by the available fish community (Kruuk and Moorhouse, 1990; Taastrøm and Jacobsen, 1999; Copp and Roche, 2003; Harper *et al*., 2020b; Dettori *et al.*, 2021).

The increased predation on marine and catadromous species such as the European eel and European flounder in the lower sections of the River Hull is consistent with a study carried out on coastal otters in Cornwall which highlighted both eels and flatfish to be important species in estuarine habitats (Groves and Smith, 2021). This study demonstrated that the relative proportion of marine species found in otter diets decreases with distance from coastline which is similar to the trends found in our study

along the River Hull where flounder were only fed on in the lower River Hull. This is also supported by our eDNA metabarcoding of water samples, where flounder decrease from 8 detections in the lower section to 3 in the middle which highlights the increased flounder abundance in the lower sections.

Similarly, our findings that eels are a preferred species in the lower reaches is concordant with a PhD study at Cardiff University which demonstrated that the importance of eels in otter diets declined with distance from the coast (Drake, 2020). This study suggested that after the tidal limit eel abundances decline and therefore, otters switch towards bullhead as they are a more abundant prey (Ibbotson *et al.*, 2002; Drake, 2020). Our results show a similar pattern where towards the tidal reaches in the middle River Hull, otter diet diversifies from 8 to 11 species. Again, this is supported by our eDNA metabarcoding of water samples, where eel detections are highest in the lower River Hull demonstrating the increased availability of eels in the lower sections.

European bullhead, three-spined stickleback, perch and stone loach were the most common prey species in the middle River Hull. This section contains two large nature reserves in Tophill Low and Pulfin bog which provide an array of different habitats from large lakes and reservoirs, to small ponds joined by slow flowing streams. The eDNA water sample data collected has shown that three-spined sticklebacks are abundant in this section, thus it is unsurprising that the otters are positively selecting for them. A similar increase in predation on three-spined sticklebacks and bullhead was described by Moorhouse-Gann *et al.*, (2020) during a study of otter stomach contents across

England and Wales. However, they suggested that this predation was the result of a decline in abundance of the preferred species, European eel, highlighting concerns over the nutritional disparities between eels and these smaller species. A similar observation was described in Scottish lochs, where a reduction in eel occurrences in spraints reflects a decrease in eel numbers in the surrounding tributaries (Kruuk, 2014).

Perch were a significant component of otter diets in the middle section of the River Hull. This section contains several large reservoirs in Tophill Low and Pulfin Bog and our eDNA water sample data has shown they are common throughout this section. Similarly, a molecular study of otter diets across the Agri river system in Italy found that perch accounted for 44.72% of dietary occurrences in pool 4 which was a large lake (Buglione *et al.*, 2020b). This highlights the importance of perch as a prey resource in lentic habitats.

Predation on common carp was also recorded in the middle region of the River Hull, despite no detections in the eDNA water samples. This carp has potentially come from stocked private fishponds as there is a large amount of recreational angling in this middle region. Previous research in both the UK (Almeida *et al*., 2010) and Europe (Wisniowska, 2006), have shown carp to form a small component (<10% of spraints) of otter diets in pond habitats, with both papers highlighting concerns surrounding conflict with anglers.

In the upper River Hull, otters were seen to feed primarily on European bullhead, Eurasian minnows, three spined stickleback and rainbow trout. This section is an important habitat for both brown trout and Grayling. Despite this, neither species are selectively predated upon, grayling are not eaten at all, meanwhile brown trout are negatively selected for. Our findings are consistent with a study carried out on a brown trout stream in Serbia, where despite the river being dominated by brown trout, otters fed primarily on bullhead, accounting for 71% of dietary occurrences across the whole river (Krpo-Ćetković, Subotić and Skorić, 2019). They suggest that the higher energy expenditure required to catch fast moving salmonids is unprofitable when compared with the abundance of easily catchable prey (Erlinge, 1968a; Jacobsen, 2005; Alderton *et al.*, 2015; Krpo-Ćetković, Subotić and Skorić, 2019; Harper *et al*., 2020b).

Minnows were the second most common prey species in the upper River Hull. This is concordant with studies in Poland where brook minnows were an important prey resource in upland streams, particularly in Autumn and Winter (Brzeziński, Romanowski and Kopczyński, 2006).

In the upper River Hull rainbow trout were detected in 7 spraints and a weak selective preference was observed. By contrast, rainbow trout was only found in one spraint from the middle section and zero from the lower River Hull. The River Hull was traditionally stocked yearly with large rainbow trout by the West Beck Preservation Society, however, this was stopped in 2007 and the likelihood of a wild population remaining is rare (Pedley, 2016). Therefore, the predation on rainbow trout in the upper

sections is most likely a result of predation on escapees or individuals from inside Wansford trout farm. Stocked individuals are typically larger and poorly adapted to wild habitats, thus making them more vulnerable to predation by otters. A study in two Danish rivers demonstrated that following the introduction of stocked fish to a trout stream, otter predation on trout intensified highlighting a shift from wild to stocked individuals, however, when stocked fish were added to a river dominated by cyprinids the otter diets remained unchanged (Jacobsen, 2005). Similarly, research in Finland has suggested that stocked trout streams and fish farms may constitute important winter feeding grounds for otters, particularly in times of food shortages (Ludwig *et al.*, 2002). These studies suggest that the otter predation on rainbow trout in our study may just be a result of an abundant prey resource from the fish farms.

4.3 Seasonal variation

Overall otter diets did not change between winter and spring with bullhead and three-spined stickleback remaining the dominant prey items across both seasons. This is concordant with research in Poland where bullheads were seen to be the most common prey species throughout the year (Brzeziński, Romanowski and Kopczyński, 2006).

We demonstrated otters feeding on a significantly higher number of prey items per spraint in spring than winter. This is consistent with a study in Norfolk ponds where the proportion of river associated species in otter diets increased from winter to spring, they attributed this to an increase in the detectability of prey species due to fish becoming more active prior to spawning (Nunn *et al.*, 2010; Almeida *et al.*, 2013).

Additionally, otter home ranges are known to increase in size from winter to spring as individuals re-establish their territories, with spring travels being known to encompass both the summer and winter ranges (Erlinge, 1967). Therefore, this increase in prey occurrences per spraint from winter to spring might be a result of the larger home ranges providing new habitats for foraging (Almeida, Copp and Masson, 2012; Krawczyk *et al.*, 2016).

Predation on European eels increased from winter to spring. This is coherent with several studies in which predation on eels peaked in spring (Parry *et al.*, 2011; Almeida *et al.*, 2013), and is most likely a result of the influx of thousands of elvers (juvenile eels ranging from 81-120mm (Piper, Wright and Kemp, 2012) migrating into freshwater rivers providing an abundant, easily catchable prey resource for the otters to exploit (White and Knights, 1997; Piper, Wright and Kemp, 2012). This is supported by our eDNA metabarcoding of water samples, where eels detections increased from winter to spring demonstrating the increased eel activity in the catchment during spring.

Similarly, predation on perch increased from winter to spring. Perch typically spawns in spring (Jones, 1982) therefore, the increased predation in spring may be a result of perch becoming more active and thus, vulnerable to predation by the otter. This is once again supported by our eDNA metabarcoding of water samples, where perch detections increased from winter to spring highlighting the increased activity of perch in the catchment during spring.

Predation on brown trout was rare and was only observed in winter. Brown trout typically spawn between October and December in the UK (Armstrong *et al.*, 2003), at which point they are more vulnerable to predation by otters. This is consistent with the results of our eDNA water sampling, where brown trout detections were higher in winter than spring, highlighting their increased activity. Similarly, research in Austria found that significantly more salmonids were eaten in Stream O during winter which coincided with the brown trout spawning season (Sittenthaler, Koskoff and Pinter, 2019). The increase in predation on brown trout in winter from our study may be a result of the spawning season making individuals more vulnerable to predation.

In winter otters can be seen to feed on more non fish prey items than in spring. Otters have been documented to diversify their diet from fish towards birds (De la Hey, 2008) and amphibians (Weber, 1990; Lanszki and Molnar, 2003; Clavero, Prenda and Delibes, 2005; Remonti *et al.*, 2008; Pagacz and Witczuk, 2010; Almeida *et al.*, 2013; Smiroldo, Villa, *et al.*, 2019) in times of low food availability and this may be reflected in the current study. A study in Northern Ireland comparing otter diets from 1980 to 2003 has shown that otters have diversified their diet to feed on significantly more non fish prey (Preston, Portig and Montgomery, 2007). Earlier studies had shown that otter signs (runs, holts, spraints and tracks) across Northern Ireland had declined (Preston *et al.*, 2006). They hypothesised that the reasons behind this was a shortage of food which has subsequently caused individuals to feed on less optimal prey (Preston, Portig and Montgomery, 2007).

Predation on amphibians in our study was only observed in winter. This consisted of marsh frogs and common frogs. It is well documented that otters will prey on amphibians during times of low fish abundance, and amphibian spawning seasons, which are primarily in spring (Weber, 1990; Lanszki and Molnar, 2003; Clavero, Prenda and Delibes, 2005; Remonti *et al.*, 2008; Pagacz and Witczuk, 2010; Almeida *et al.*, 2013; Smiroldo, Villa, *et al.*, 2019). Unfortunately, we were unable to detect this shift in our study, however, it is worth noting that 4 out of 7 samples collected in May during the small sampling campaign contained marsh frogs. Therefore, it is possible we sampled too early and may have missed the seasonal shift towards amphibians.

In summary, the increase in prey occurrences per spraint from winter to spring is most likely due to a combination of: spawning seasons providing increased prey availability (Jones, 1982; Almeida *et al.*, 2013), changes in activity levels of prey species as the temperatures rise making prey more vulnerable (Nunn *et al.*, 2010) and finally, the expansion of otter home ranges providing a wider range of habitats for foraging (Erlinge, 1967; Almeida, Copp and Masson, 2012; Krawczyk *et al.*, 2016).

4.4 Resource partitioning between the Eurasian otter and American mink

The comparisons between the Eurasian otter and invasive American mink diets in this study have highlighted the niche differentiation between the two mustelids. Otter diets were primarily focused on fish and amphibians accounting for 89.21% and 6.92% of

prey occurrences respectively, meanwhile, mink diets were focussed towards mammals and birds accounting for 37.5% and 35% of prey occurrences respectively.

Interestingly, the proportion of fish prey in mink diets was considerably higher in our study accounting for 25% of prey occurrences compared with only 4.5% of occurrences in their study (Harper *et al*., 2020b). Our results are consistent with research across Europe where fish were seen to be an important prey resource in American mink diets accounting for 27% (SE 3.9) of prey in samples collected from streams and rivers (Jędrzejewska *et al.*, 2001). Predation on fish in our study consisted of the most abundant prey species in bullhead, perch and roach. Studies into otter and mink diets in Sweden have demonstrated both species feeding primarily on fish, accounting for 90.8% and 60.2% of prey occurrences respectively (Erlinge, 1969). This study suggested that both species tend to exploit the most abundant prey species, however, in summer when fish availability is lower, mink shift their diets to exploit waterfowl, meanwhile otters continue to prey on fish particularly slow-moving species such as burbot (*Lota lota*).

The differences in prey proportions in mink diets between the current study and Harper *et al.,* (2020b) can most likely be explained by differences in the sampling locations. 8/12 of the new mink samples were collected from the main river channel and its surrounding tributaries, meanwhile in the previous study 13/15 samples from the River Hull were collected at Tophill Low. Tophill Low is an important nature reserve managed by Yorkshire Water with high densities of small mammals and waterfowl, therefore it is unsurprising that the mink exploits this abundant prey. Additionally, there are

established resident otter populations at Tophill Low, this is typically 3 individuals consisting of one dog otter and two females either side of the reserve. Bonesi et al., (2004) demonstrated the shift in mink diets to be more pronounced in habitats where otter densities were higher. Therefore, the high densities of otters at Tophill Low may explain why the niche differentiation between the two species is more pronounced in the previous study (Harper *et al*., 2020b).Mink predation on birds in our study largely consisted of waterfowl with common moorhen as the most common prey item. This is concordant with studies from a reserve in Poland where mink predated heavily upon waterfowl, particularly coots killing 278 nesting individuals during spring (Bartoszewicz and Zalewski, 2003).

The most common prey species in mink diets was water vole with 7 occurrences (25.93% of mink scats). Water voles are a conservation priority species in the UK Biodiversity Action Plan and are currently listed as endangered in England and Wales and near threatened in Scotland (Mammal Society, 2023). The detrimental impacts of mink on water vole are well known and they were attributed to their near extinction, which subsequently prompted extensive culls to eradicate the mink (Macdonald, Sidorovich and Anisomova, 2002; Macdonald and Harrington, 2003). Barreto et al., (1998) found that mink was the most important factor in determining water vole distribution across the Thames catchment. More recently, research in Poland showed that the probability of water vole occurrence was significantly lower at sites where mink were present (Brzeziński *et al.*, 2018). Therefore, the predation observed in this study is a serious concern and highlights the need for continued culls to prevent further

damage to water vole populations (Fraser *et al.*, 2018; Harrington *et al.*, 2020; Martin and Lea, 2020).

Faecal metabarcoding has provided further evidence towards the coexistence of these two species through niche differentiation (Jędrzejewska *et al.*, 2001; Bonesi, Chanin and Macdonald, 2004; Harper *et al*., 2020b). These results highlight concerns surrounding the impacts of mink predation on conservation priority species such as the water vole (Barreto *et al.*, 1998; Macdonald, Sidorovich and Anisomova, 2002; Macdonald and Harrington, 2003; Brzeziński *et al.*, 2018) and provide further evidence towards the need for coordinated approaches (Lambin, Horrill and Raynor, 2019) and new technologies such as electronically triggered smart traps (Martin and Lea, 2020) to improve mink culling across the UK. This will prevent further damage to native species (Macdonald and Harrington, 2003; Fraser *et al.*, 2018).

4.5 Replicability of the metabarcoding approach

The extraction replicate experiment found that for 7/9 fish species the method was consistent, however, difficulties arose with smaller species such as stone loach and three spined sticklebacks where species were missed in some replicates. The current Mu-DNA faecal extraction protocol relies on taking a 0.25g subset of each faecal sample, this can lead to challenges with taking a representative sample as a single otter spraint can weigh over 1g. Gosselin *et al.,* (2017) described similar challenges. They found that faecal sampling location influenced prey detection in coyote (*Canis latrans*) scats. They concluded that multiple sampling locations may be necessary to determine predator diets with a combination of the homogenised sample and side

locations yielding comparable dietary coverage to all locations. Similarly, previous research carried out on seal diets have homogenised scats manually in ethanol to prior to extraction (Bowles *et al*., 2011, Thomas *et al*., 2016; 2022). Therefore, it may be worthwhile to homogenise the otter spraints prior to taking the 0.25g subset for extraction to maximise prey detection.

The DNA extraction method used may also impact the detectability of prey species (Harper *et al.,* 2020b). Oehm *et al*., (2011) found that the cetyltrimethyl ammonium bromide (CTAB) protocol they created had much higher success rates for detecting prey DNA in than the commercial kits when investigating mealworms in avian scats. However, this was only applicable to small, homogenised samples, prey detection from larger pieces of faeces was broadly comparable between commercial and homemade extraction kits. Therefore, it may be worthwhile trialling both different extraction protocols and extracting from different parts of the otter spraint to maximise prey detectability.

In summary, for most prey species our results seem to be broadly consistent between extraction replicates. This is consistent with several other faecal metabarcoding studies which suggest that the focus should be on biological over technical replicates (Alberdi *et al.*, 2019; Mata *et al.*, 2019; Ando *et al.*, 2020). Therefore, with a sufficient sampling size a single extraction will be suitable to understand overall patterns in otter diet.

4.6 Limitations of the current study

While the read counts from eDNA water samples have been shown to correlate with relative abundances in lentic environments (Li *et al.*, 2019; Di Muri and Bean, 2020), it is more challenging in lotic environments. In the current study, the presence of Wansford trout farm in the upper reaches and the downstream transportation of eDNA in flowing environments (Deiner and Altermatt, 2014; Roussel *et al.*, 2015) means that rainbow trout are assigned an artificially high read count which does not reflect their true abundance in the environment. Therefore, presence/absence was chosen as a more conservative approach than read counts. However, this may result in the most common species being underrepresented, which will subsequently influence the selectivity calculations.

As a result of the large size of otter home ranges (up to 40km of river for a male) (Kruuk, 2006; Harper *et al*., 2020b), and the habitual nature of dog otters patrolling their territories, where a spraint is found might not always correlate with where the prey was eaten. In addition, the lack of eDNA water sample data to inform the prey communities from surrounding tributaries means that certain species such as stone loach (*B.barbatula*), which was found in spraints from the upper section but not water samples may be given an artificially high selectivity value. Therefore, future studies should expand the eDNA water sampling to incorporate tributaries to investigate the entire prey community available. This could be carried out in conjunction with individual genotyping and PCR sex typing to investigate how each otter feeds within their home range.

One of the main challenges in dietary studies is secondary predation, smaller species such as minnows are widely predated by larger fish. Carss *et al*., (1996) highlights the challenges of secondary predation when working with otter spraints, where minnows can be seen inside the bodies of ingested rainbow trout. Therefore, we cannot rule out the chances of secondary predation causing an over representation of minnows in otter diets for our study.

During our current study we were unable to investigate the invertebrate component of the otter diet, however, previous morphological analyses from undergraduate dissertations at the University of Hull have demonstrated invertebrates being eaten (Grey, 2017; Treddell, 2018; Weldon, 2019). Several studies across the UK (Grant and Harrington, 2015; Britton *et al.*, 2017) and Europe (Beja, 1996; Jędrzejewska *et al.*, 2001; Martínez-Abraín *et al.*, 2020) have demonstrated crayfish to be important prey resources particularly in spring-summer months. Therefore, further study should look to sequence otter spraints with invertebrate primers to understand the invertebrate component of otter diets within the River Hull catchment.

5.Conclusions

In conclusion, this research has provided further understanding into the feeding behaviours of the Eurasian otter across the River Hull catchment. We have demonstrated section specific dietary preferences across the upper, middle and lower River Hull with a consistent selective preference across all three sections towards the

European bullhead. Through the calculation of selectivity coefficients, we have shown how eDNA water samples can be incorporated in modern dietary highlighting how molecular methods can enhance dietary studies.

There is still a need to understand the role of which individual plasticity, social hierarchy and sex-based differences in ecology influence the diet. However, preliminary studies with microsatellite genotyping have demonstrated the challenges of working with otter spraints where only very fresh samples will amplify successfully (Appendix 11) (Dallas *et al.*, 2000). One potential methodology may be to remove all samples the night before collection, therefore any samples which are collected are <24 hours old and have a better chance of amplifying (Buglione *et al.*, 2020a). In addition, some studies instead chose swabbing of faecal samples to collect mucus, thus minimising prey DNA (Lampa *et al.*, 2015).

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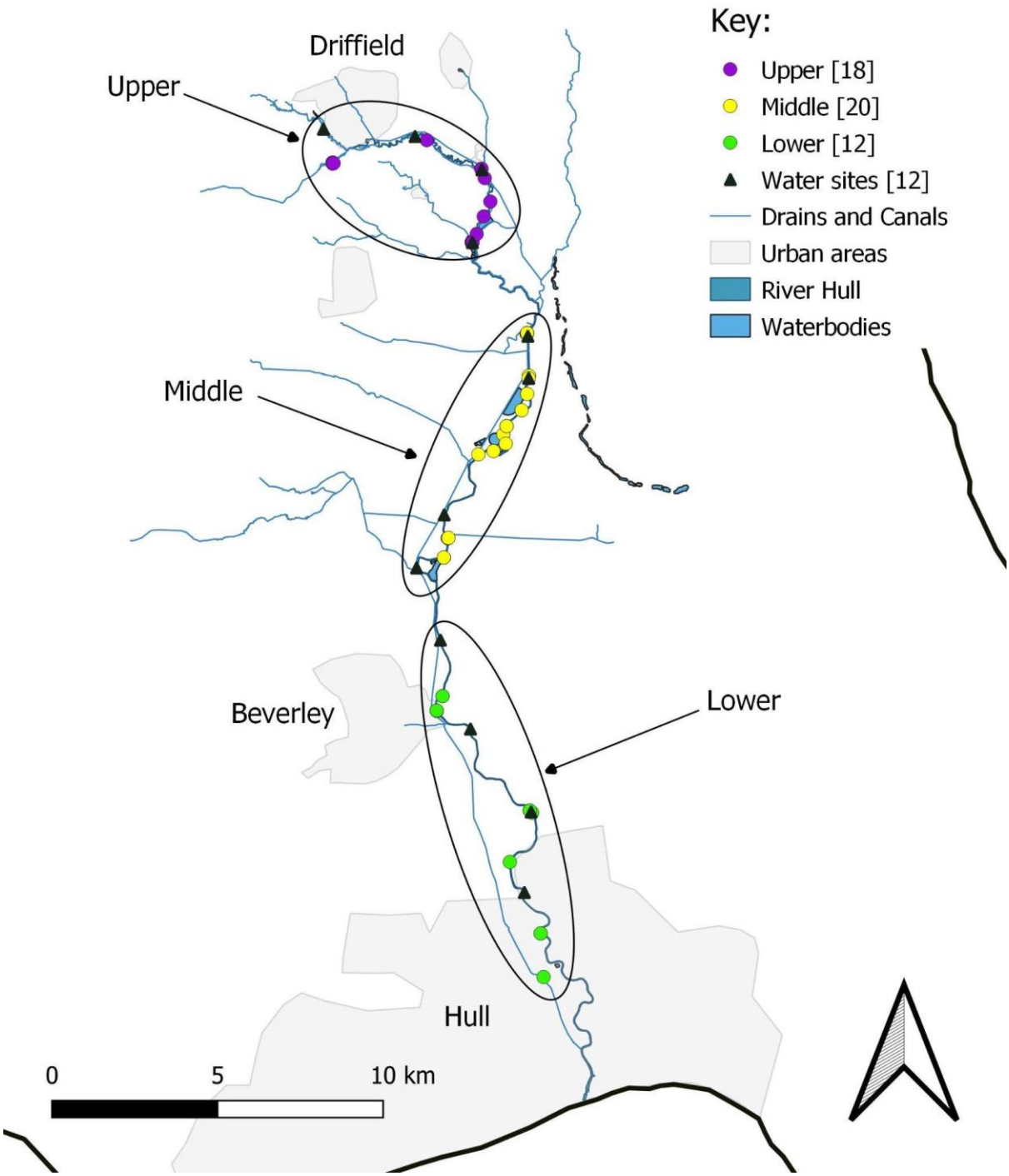
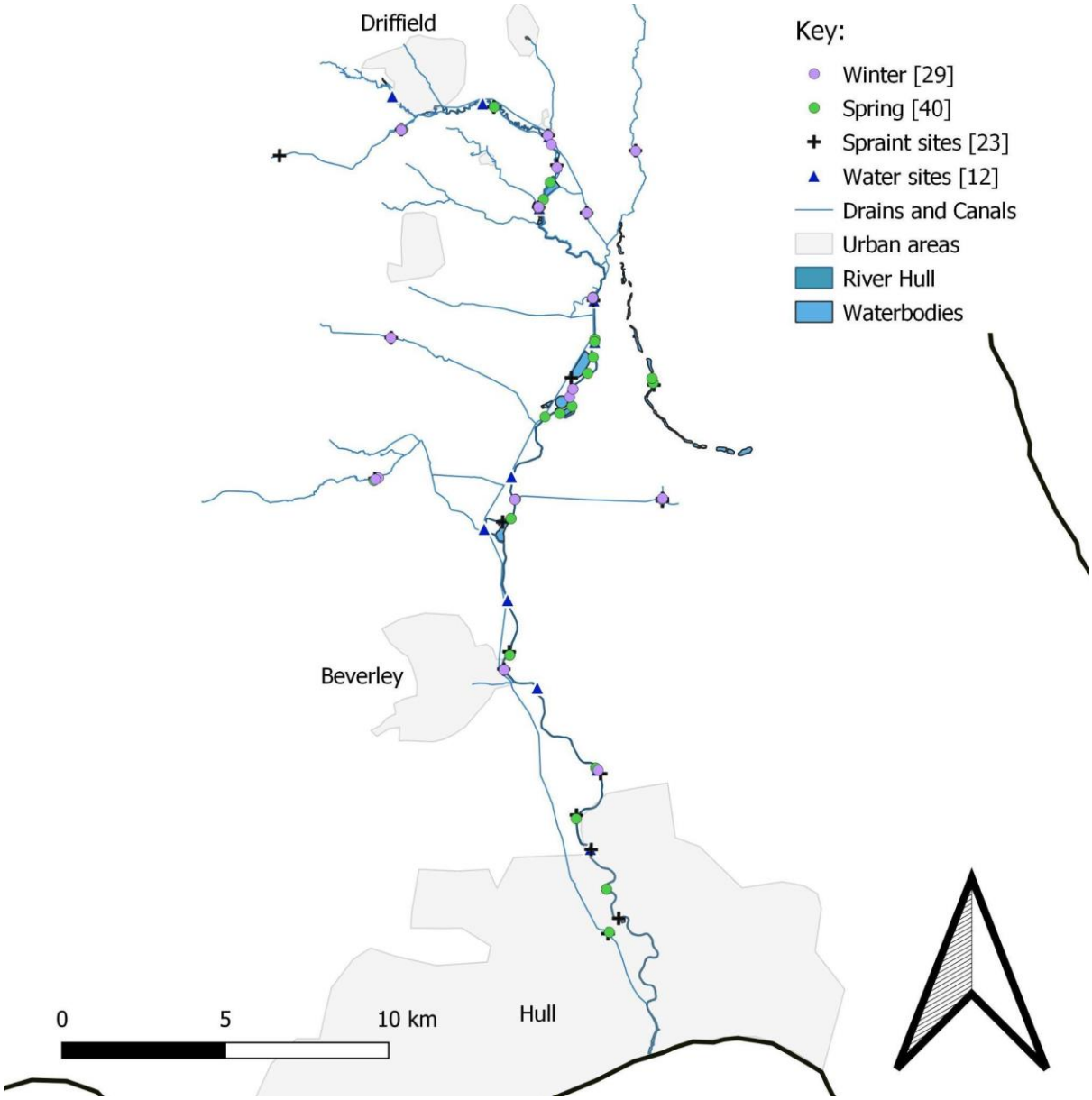
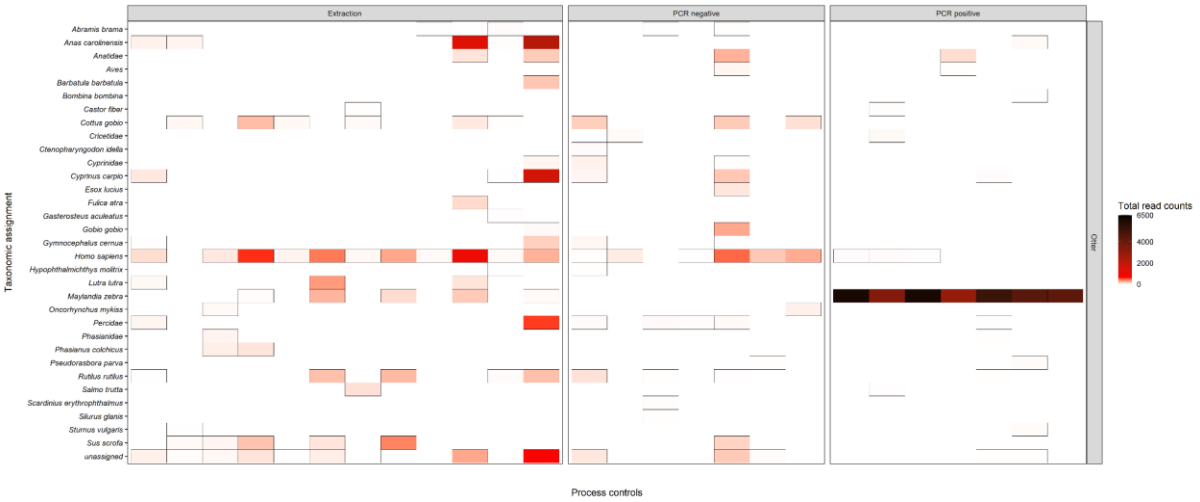
7. APPENDIX



Appendix 1: A map depicting the 50 confirmed otter spraints collected across the three sections of the River Hull, points are coloured based on their section (Lower: Green (n=12), Middle: Yellow (n=20), Upper: Purple (n=18)). eDNA water sampling points are marked by black triangles, ellipses demonstrate the three sections the river was partitioned into for the spatial analysis.

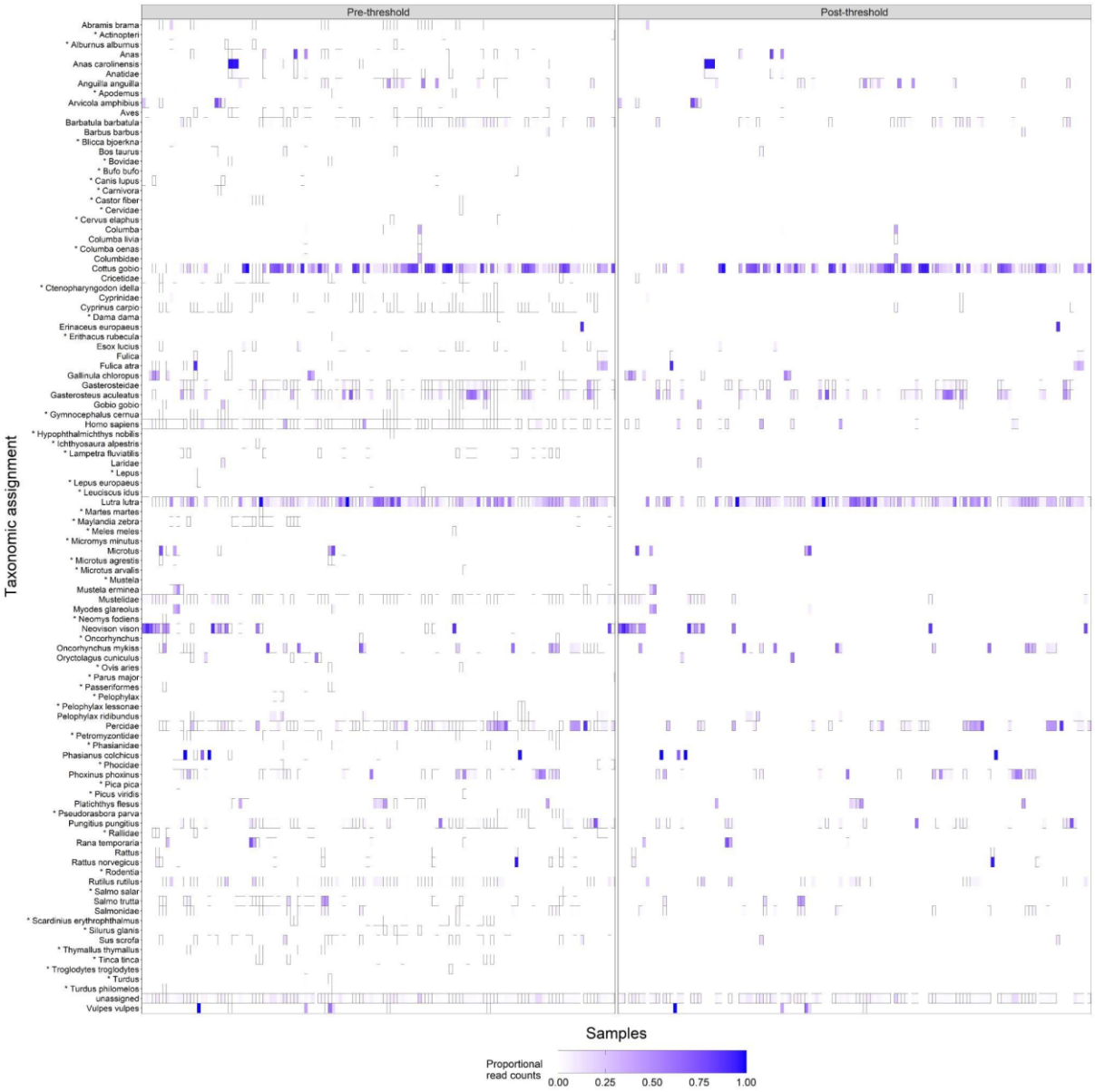


Appendix 2: A map depicting the 69 confirmed otter spraints collected across the three sections of the River Hull, points are coloured based on the season (winter: purple (n=29), spring: green (n=40)). eDNA water sampling points are marked by blue triangles, spraint collection sites are marked by black crosses.



Appendix 3: A proportional contamination heatmap, highlighting contamination across the extraction blanks and PCR controls.

This heatmap map demonstrates the contamination in the process controls across the different stages of the lab workflow. Read counts are shown by a colour gradient where the darkest colours represent the highest read counts. Colour gradients are scaled based off the read counts of *Maylandia zebra* in the positive control.



Appendix 4: A proportional contamination heatmap, highlighting the impact of our 1% threshold value, species which were removed following threshold application are marked by an *.

This map demonstrates the proportional contamination in samples prior to the application of our 1% threshold. Proportional read counts are shown by a colour gradient where the darkest colours represent the highest read counts. Species which were removed following the threshold application can be seen by the *. 52 taxa were removed as a result of the 1% threshold application.

Species	Preference	Location
Anguilla anguilla	-1	Upper
Anguilla anguilla	0.327	Middle
Anguilla anguilla	0.654	Lower
Barbatula barbatula	1	Upper
Barbatula barbatula	0.394	Middle
Barbatula barbatula	-1	Lower
Barbus barbus	-1	Upper
Barbus barbus	-1	Middle
Barbus barbus	0.09	Lower
Cottus gobio	0.439	Upper
Cottus gobio	0.503	Middle
Cottus gobio	0.523	Lower
Esox lucius	-1	Upper
Esox lucius	-1	Middle
Esox lucius	-0.43	Lower
Gasterosteus aculeatus	0.058	Upper
Gasterosteus aculeatus	0.407	Middle
Gasterosteus aculeatus	-0.221	Lower
Gobio gobio	-1	Upper
Gobio gobio	-0.074	Middle
Gobio gobio	-1	Lower
Oncorhynchus mykiss	0.212	Upper
Oncorhynchus mykiss	-0.58	Middle
Oncorhynchus mykiss	-1	Lower
Percidae	0.439	Upper
Percidae	0.394	Middle
Percidae	0.05	Lower
Phoxinus phoxinus	0.316	Upper
Phoxinus phoxinus	-0.397	Middle
Phoxinus phoxinus	-1	Lower
Platichthys flesus	-1	Upper
Platichthys flesus	-1	Middle
Platichthys flesus	0.411	Lower
Pungitius pungitius	-0.401	Upper
Pungitius pungitius	0.16	Middle
Pungitius pungitius	-0.251	Lower
Rutilus rutilus	-1	Upper
Rutilus rutilus	-0.183	Middle
Rutilus rutilus	-0.221	Lower
Salmo trutta	-0.514	Upper
Salmo trutta	-1	Middle
Salmo trutta	-1	Lower

Appendix 5: The selectivity values for each prey species across the 3 sections of the River Hull

7.1 Molecular sexing of otter spraints

DNA obtained from faecal samples can additionally be used to genotype individuals and observe genetic variation within a population (Kohn and Wayne 1997). For example, microsatellites can be used for DNA fingerprinting of individuals and Y chromosome-specific markers such as the SRY gene to distinguish between sexes. The Eurasian otter is the perfect candidate for such non-invasive genetic sampling. Otters are habitual, patrolling the same routes throughout their territories, depositing spraints at sites of interest which means regular spraint collection is possible (Erlinge, 1967, 1968b; Mason and Macdonald, 1986; Kruuk, 2006). Scent profiles of otter spraints have been shown to indicate the age, sex, reproductive status and individual identity which confirmed longstanding suspicions that sprainting plays a role in communication (Kean, Müller and Chadwick, 2011; Kean, Chadwick and Müller, 2015; Sittenthaler *et al.*, 2020).

Dallas and Piertney (1998), created the first Eurasian otter microsatellite protocol which used 13 microsatellite primers to identify individuals. In a subsequent study, Dallas et al., (2000) designed primer pairs to amplify the male specific SRY gene. The primer pairs were successful for 60% of freshly collected spraints, however, the success rates dropped to 25% for spraints collected after 18 hours. Subsequent research has suggested that otter spraints should be collected in the early morning of cold months and focus on the anal jellies or mucus part from faeces when possible to maximise the chances of successful microsatellite amplification (Hájková *et al.*, 2009). This information when used in conjunction with the dietary analysis potentially provides the opportunity to analyse individual and sex specific diet preferences.

Aim: Do otter diets vary between sexes or individuals?7.1.1

Methods:

As the Mu-DNA otter spraint extraction protocol (Sellers *et al*., 2018) is primarily suited to dietary analysis, spraint samples underwent two new extraction methods to maximise otter DNA. Initially faecal samples were swabbed to collect the mucous component of the otter spraint. Samples then followed the Mu-DNA tissue protocol (Sellers *et al*., 2018), with one method following a spin column-based extraction where DNA is bound to a silica membrane, and a second method where magnetic beads were used to bind the DNA. These two methods were then compared to see which method worked best.

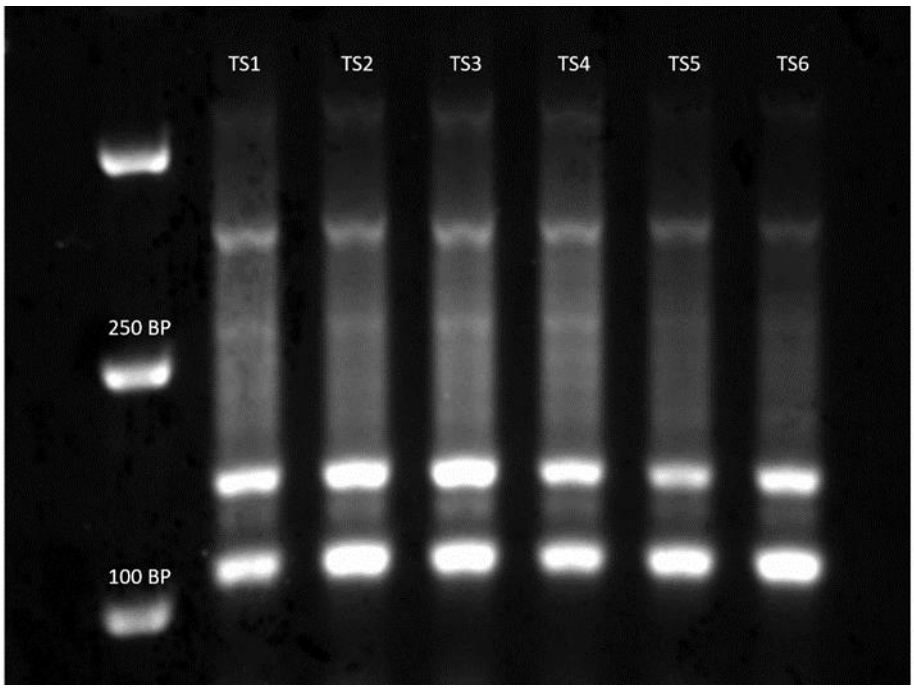
To investigate sex-based differences in the diets of Eurasian otters, a molecular sexing approach using PCR amplification of sex-chromosome specific regions was carried out using the following primer pairs designed at the Cardiff University Otter Project: LL-ZFX720F (GAGGGACTGAGGTTGGTTACC) - LL-ZFX872R (ACGTTATTAGGCAAGCATTCCTA) which amplify a 153 base pair fragment of the X-specific ZFX gene and LL-ZFY1081F (CAGGACCCTGGAATCATGAC) - LL-ZFY1183R (CACCCATAACACTCCCAATCTA) to amplify a 101 base pair fragment of the Y-specific ZFY gene (F Hailer, personal communication, November 2020). Each individual PCR reaction contained 7.5 µL of Q5 High-fidelity 2 x master mix (New England Biolabs, UK), 0.5 µL of each forward and reverse primer (2µl total), 3.5 µl molecular grade water and finally 2 µL of DNA. A positive control quantified at 0.05 ng/µl from a tissue sample of a known male otter and a negative control of molecular

grade water were used throughout. A touchdown PCR was performed on an Applied Biosystems® Veriti Thermal Cycler (Life Technologies, CA, USA) with the following conditions: 95°C for 3 min, 10 cycles of 94°C for 30 s, 65°C-55°C for 20 s and 72°C for 45 s, with -1°C per cycle, followed by 30 cycles of 94°C for 30 s, 55°C for 25 s and 72°C for 45 s. Final elongation step was at 72°C for 10 min.

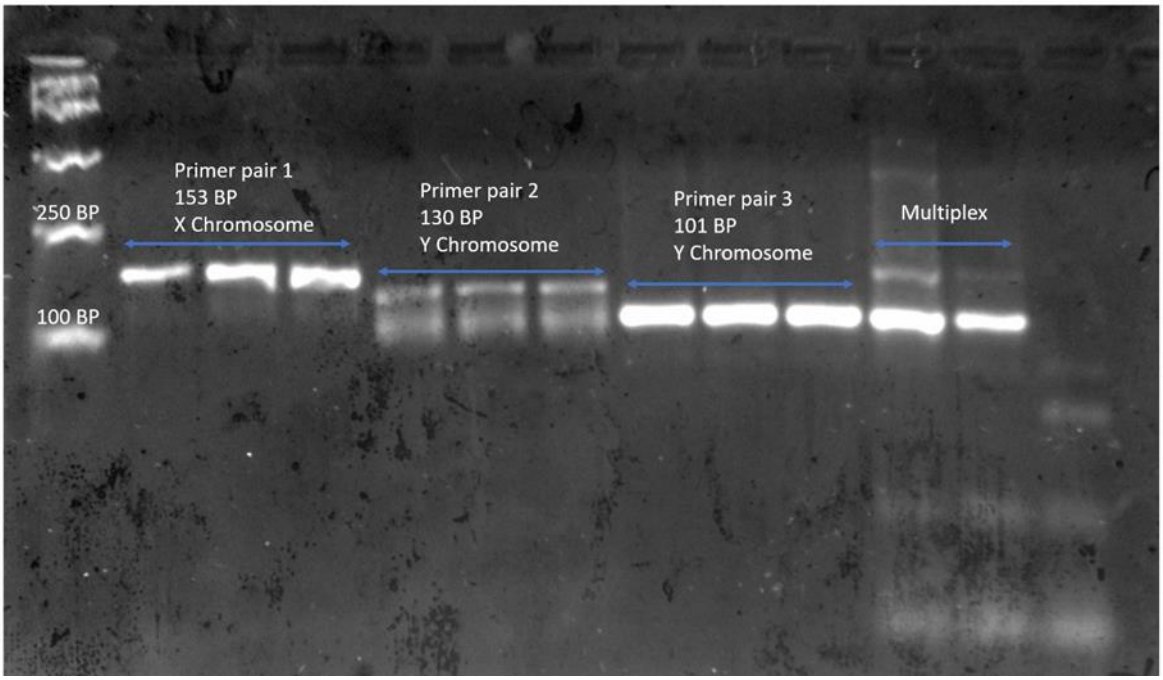
2 µl of each reaction was mixed with 1 µl of loading dye (R0621, Mass Ruler DNA loading dye (6 x), Fisher Scientific UK Ltd., Loughborough, UK) before samples were visualised via gel electrophoresis on a 2% agarose gel (1.6 g Bioline® Agarose in 80 mL 1 x sodium borate) (Brody and Kern, 2004) using a sodium borate buffer (1 x) with gel red (1000 x) (Cambridge Bioscience, Cambridge, UK) as a stain and 2 µl of HyperLadder™ 50 bp (BIO-33040, HyperLadder™ 50 bp (500 Lanes), Meridian Bioscience, Scientific Laboratory Supplies Ltd, Nottingham, UK) for the ladder. The gel conditions used were 150 V for 50 min and gels were imaged using Image Lab Software (Bio-Rad Laboratories Ltd, Watford, UK).

Appendix 6: An annotated gel showing a comparison between spin column and bead-based extraction methods for swabs from otter spraints. Samples WA2 and TL9 beads show male otters (two bands), meanwhile BV3, SKW6 and SKW8 demonstrate a single X band at ~150 bp indicating a female otter. The fact that TL9 spin column only has a single band (Female), meanwhile the same sample following bead extractions contains two bands (Male) thus, demonstrating some of the challenges when working with highly degraded DNA.

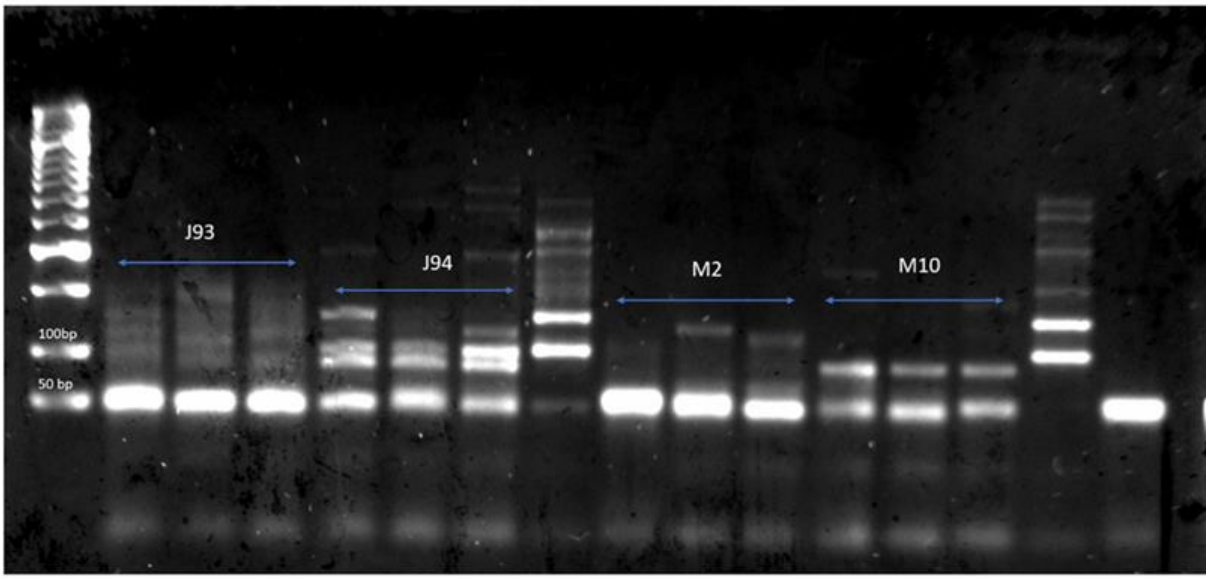
Samples were deemed as males if two bands were present at ~150 bp and ~100 bp representing amplification in both the X and Y chromosome and a female if only a single band was present at ~150 bp representing just the X chromosome (Figure 3). Despite some initial successes with swab samples, consistent results could not be attained due to non-specific amplification. Therefore, the dietary analysis could not be carried out on an individual or sex specific basis (question 3). Following initial success with tissue samples, it seemed that primer pair 2 for the Y chromosome was difficult to see and lead to more of a smear therefore, this primer pair was later omitted to avoid confusion. Samples extracted by mu-DNA struggled with non-specific amplification, therefore a new swab-based approach was trialled (Appendix 9). This method was initially successful and fresh samples BP1 and BP2 were successfully assigned to a male otter (Appendix 9). However, consistent results could not be obtained therefore, this section was removed.



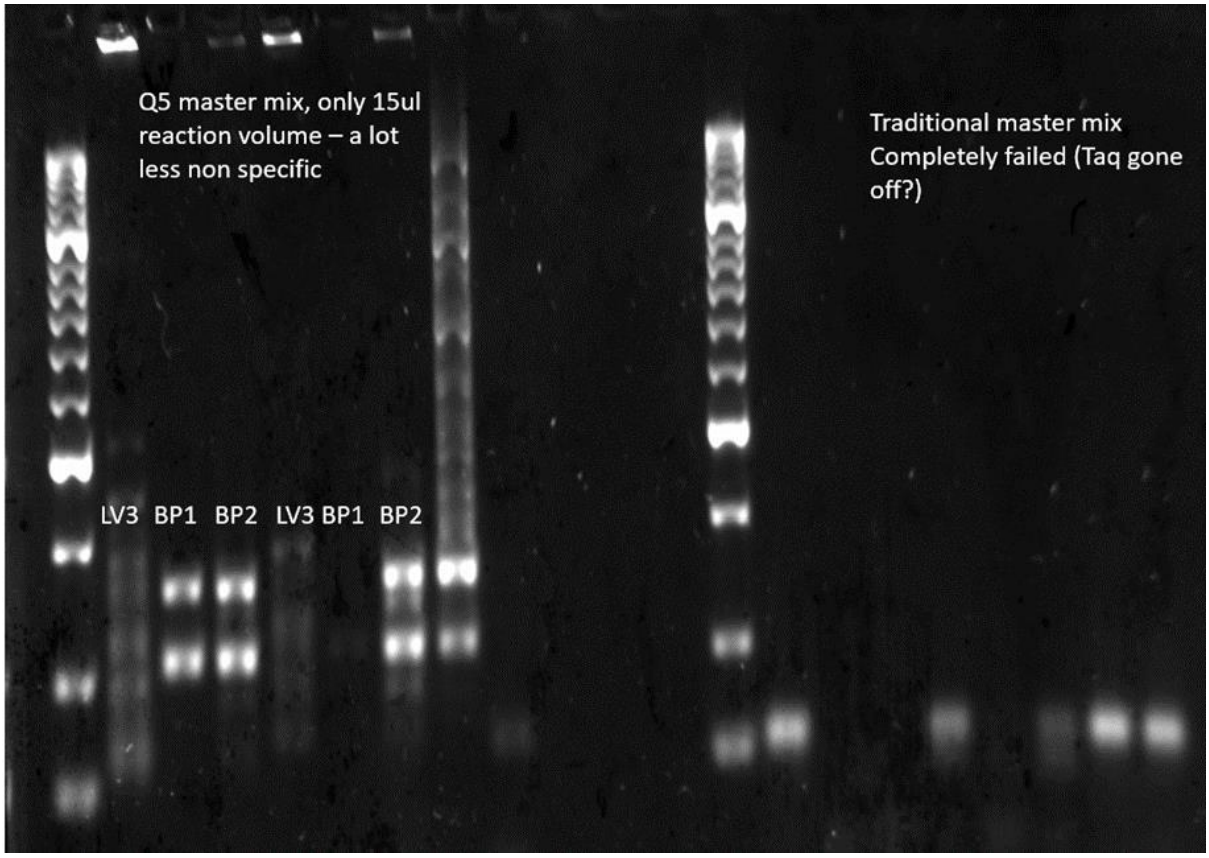
Appendix 7: Tissue samples taken from a male otter run on a 1.5% agarose gel for 30 minutes at 200V as we typically run gels



Appendix 8: Each primer pair run individually on tissue samples from a male otter on a 1.5% agarose gel for 30 minutes at 200V as we typically run gels



Appendix 9: The gel conditions were later optimised at 150V for 50 minutes. However, we had challenges with non-specific amplification.



Appendix 10: A new swab-based extraction method and a change of reaction mix which contained Q5 master mix, this highlighted some success with freshly collected samples where both BP1 and BP2 could successfully be assigned as a male otter.

7.2 Microsatellite genotyping of otter spraints

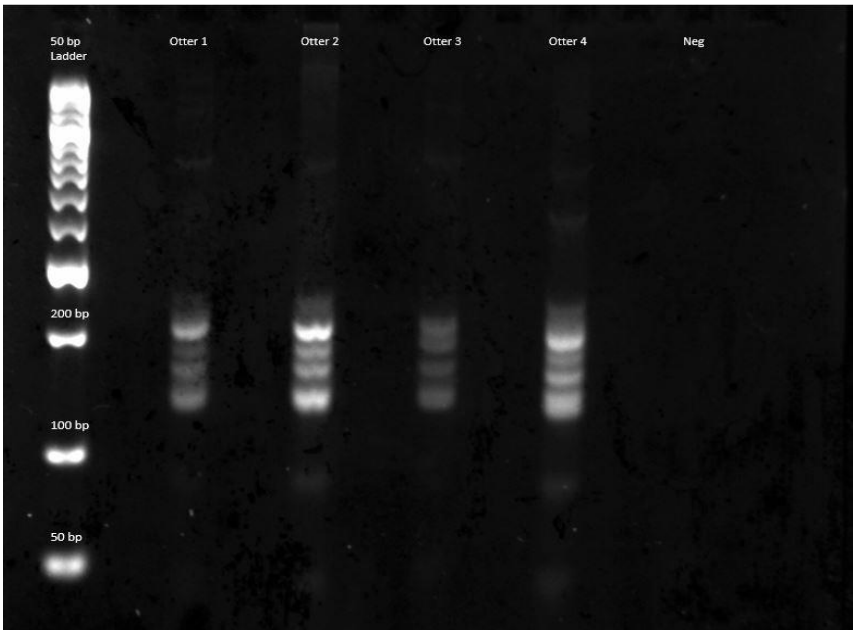
To investigate individual based differences in the diets of Eurasian otters, an initial multiplex PCR was carried out using the following published primer pairs, LUT-435, LUT-453, LUT-717, 04OT05 and 04OT22 (Dallas and Piertney 1998; Huang *et al*., 2005), following a protocol used at the Cardiff University Otter Project (F Hailer, personal communication, November 2020): Each individual PCR reaction contained 5µL of PCR multiplex kit (QIAGEN, Manchester, UK), 0.2µL of each forward and reverse primer (2µl total), 1µl molecular grade water and finally 2µL of DNA. A positive control quantified at 0.05 ng/µl from a tissue sample of a known male otter and a negative control of molecular grade water were used throughout. A PCR was

performed on an Applied Biosystems® Veriti Thermal Cycler (Life Technologies, CA, USA) with the following conditions: 95°C for 15 min, followed by 29 cycles of 94°C for 30s, 58°C for 90s and 72°C for 60s. Final elongation step was at 60°C for 30 minutes.

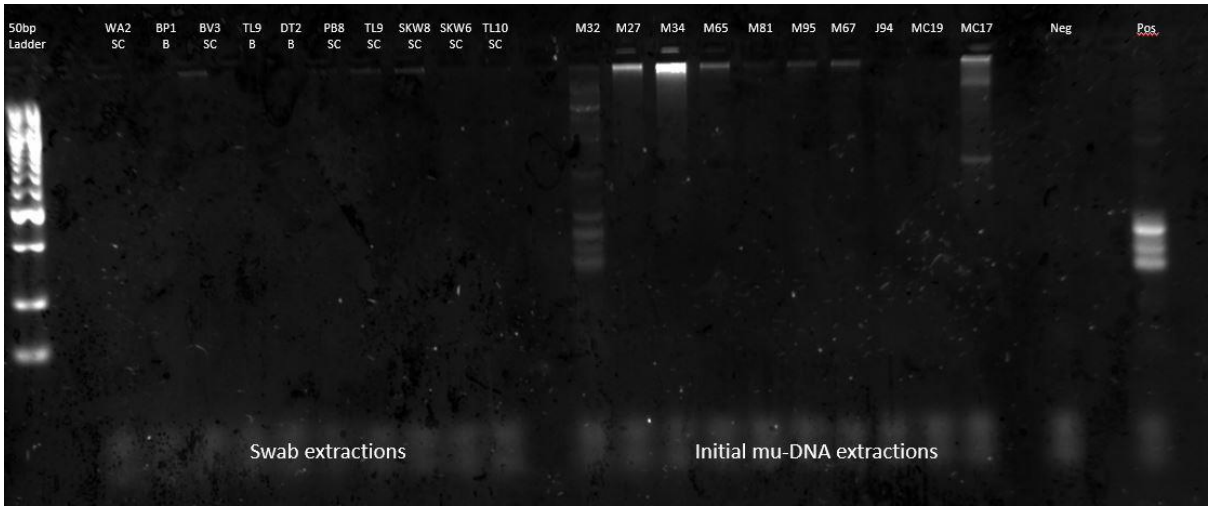
2µl of each reaction was mixed with 1µl of loading dye (R0621, Mass Ruler DNA loading dye (6X), Fisher Scientific UK Ltd., Loughborough, UK) before samples were visualised via gel electrophoresis on a 2% agarose gel (1.6 g Bioline® Agarose in 80 mL 1x sodium borate) (Brody and Kern, 2004) using a sodium borate buffer (1X) with gel red (1000X) (Cambridge Bioscience, Cambridge, UK) as a stain and 2ul of HyperLadder™ 50bp (BIO-33040, HyperLadder™ 50bp (500 Lanes), Meridian Bioscience, Scientific Laboratory Supplies Ltd, Nottingham, UK) for the ladder. The gel conditions used were 200V for 20 minutes and gels were imaged using Image Lab Software (Bio-Rad Laboratories Ltd, Watford, UK).

Despite success with the tissue samples (Appendix 10), only 1 faecal sample (M32) successfully amplified (Appendix 11). Following this, the 4 tissue samples and 1 faecal sample were then processed for 3 separate multiplex PCR reactions consisting of 4 replicates per sample (12 x replicates per sample total). The subsequent two multiplexes consisted of published primer pairs: LUT-604, LUT-733, LUT-615, LUT-902 and LUT-782 for multiplex 2 and LUT-818, LUT-701, LUT-833, LUT-715 and LUT-832 for multiplex 3 (Dallas and Piertney 1998; Huang *et al*., 2005; F Hailer, personal communication, November 2020). The samples were then sent off for fragment analysis at the MRC PPU DNA Sequencing and Services at the University of Dundee. This would allow comparisons to understand how the River Hull otter population sits in relation to the rest of the UK and confirm whether or not they are genetically distinct.

However, following the fragment analysis, the microsatellites have not yet been scored.



Appendix 11: An initial gel of microsatellite multiplex 1 from the tissue samples of 4 otters, highlighting successful microsatellite amplification for the 5 loci.



Appendix 12: A comparison between swab based and mu-DNA extractions for 5 microsatellite loci, only M32 yielded amplification in the positive section. Thus, highlighting the challenges of identifying individual genotypes from degraded faecal samples.