EVALUATING THE ROLE OF IN-CHANNEL NATURAL FLOOD MANAGEMENT INTERVENTIONS IN HABITAT IMPROVEMENT AND CREATION FOR LAMPREY SPECIES

A thesis submitted to the University of Gloucestershire in accordance with the requirements of the degree of Masters by Research. In collaboration with the Wildfowl and Wetlands Trust.

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Author's declaration

I declare that the work in this thesis was carried out in accordance with the regulations of the University of Gloucestershire and is original except where indicated by specific reference in the text. No part of the thesis has been submitted as part of any other academic award. The thesis has not been presented to any other education institution in the United Kingdom or overseas.

Any views expressed in the thesis are those of the author and in no way represent those of the University.

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Abstract

Lamprey are known to be valuable ecosystem drivers, transporting, and introducing deep marine sediments and nutrients into freshwater systems. *Lampetra* species in particular have been declining in number over recent decades, which has been the result of anthropogenic interference and habitat fragmentation. *Lampetra fluviatilis* and *Lampetra planeri* have been recognised as vulnerable, demonstrating the need for further research into locating suitable habitats around UK freshwater catchments.

This project aimed to create a concise fieldwork and laboratory methodology for the collection and analysis of environmental water samples. The samples were used to determine the presence-absence of *Lampetra* species during a single spawning season (April 2023) at a positive control site located in the Forest of Dean. Current eDNA techniques were combined with genus-specific qPCR assays to monitor temporal patterns of eDNA concentration across the spawning season in four locations These results were compared with sediment grain size data and physical characteristic measurements to understand influences on *Lampetra* spawning.

The main finding of this study was *Lampetra* species eDNA presence found within water samples taken from the field site during the spawning period investigated. Additionally, it located possible spawning and nursery sediments along a stretch, with physical characteristics known to support both *Lampetra* adults and ammocoetes. The physical characteristics of the sites are similar to those produced by the inclusion of leaky barriers as part of NFM work in rivers and therefore it is assumed that these interventions could create new habitats that are suitable for lamprey spawning. Projects such as this contribute to the growing evidence that eDNA techniques such as qPCR can be used as an alternative to more traditional methodology, often producing rapid and sensitive results without invasive or harmful interaction.

Contents

List of Figures and Tables

Table of Abbreviations

Full	Abbreviation
Bovine Serum Albumin	BSA
Dimethyl Sulfoxide	DMSO
Environmental DNA	eDNA
Master Mix	M.M
Minutes	Min
Natural Flood Management	NFM
No-Template Control	NTC
Polymerase Chain Reaction	PCR
Quantitative Polymerase Chain Reaction	qPCR
Seconds	Sec
Single Nucleotide Polymorphism	SNP
Volts	V

Ethical Considerations

The University of Gloucestershire's Handbook of Research Ethics was considered and understood before either field or lab work was undertaken. Additionally, risk assessments completed for both the lab and fieldwork were completed and signed off before samples were collected or analysed. Permissions were sought before fieldwork commenced and protected areas were not disturbed at any time during sampling. There was no use of other humans or animals during any of the proposed research, and ecological impact was minimised through ensuring environmental biosecurity measures were adhered to at all times whilst out in the field. There was no invasive or direct impact on either *L. planeri* or *L. fluviatilis* so due to the circumstances of the research, there was no requirement to submit an ethics form with the university.

1. Introduction

To fully understand the scope of this research, it was necessary to remark upon the following themes: conservation of vulnerable species, anthropogenic influences within the natural environment, and advances of rapid monitoring techniques. The first chapter provides an overview of the importance of these themes with regard to elusive species, focusing on two in particular where many current monitoring techniques have proved laborious and costly. Streamlining conservation strategies begins with the broader context of the research and ends with the main project aims as shown in the following sections.

Flood excess occurs when the volume of water during a flood event exceeds the threshold of a catchment and generates damage (Chen et al., 2009). The concept of flood-excess volume has a strong influence on mitigation strategies. Before and during such events, it is important to minimise flood damages through a number of mitigating interventions. These flooding events have become increasingly frequent and extreme as a result of multiple factors, both environmental and anthropogenic. Data from the Intergovernmental Panel on Climate Change (IPCC) shows a predicted 7% increase in extreme daily precipitation events by 2030 and has called for a global effort to increase flood mitigation infrastructure (United Nations, 2022). Official data has shown a trend of increasing flooding events globally due to several working factors, with flooding accounting for 44% of all global natural disasters in 2020 (Cooper et al., 2021). Anthropogenic factors that affect the frequency of floods include land use change such as urbanisation, and climate change (Tang, 2020).

1.1 Flood Mitigation

Flood mitigating structures can be divided into soft and hard engineering, utilising different concepts and materials (Mobley et al., 2020). Hard engineering strategies use permanent interventions and artificial materials to reduce the risk and effects of flooding along aquatic systems. For example, hard engineering includes sea walls, gabions, rip rap and groynes as flood defences for coastal management (Kantamaneni et al., 2022). These structures are used to reduce the negative impacts of coastal flooding, erosion, and sediment transportation. Soft engineering approaches to mitigate flood events along coasts involve beach replenishment or nourishment and restoring natural habitats (Morris et al., 2018). Additionally, within the freshwater environment hard structures such as dams, weirs and channels are used to mitigate the social and economic effects of flooding on urban areas (Kuriqi and Hysa, 2021). Nature-based solutions utilise natural processes to promote economic, social, and environmental changes (Lafortezza et al., 2018). Natural Flood Management (NFM) utilises soft, natural materials such as wood to limit flooding impacts through natural processes, specifically infiltration (Grabowski et al., 2019). NFM gained momentum after an early governmental report was published by the Environment Agency (2017), which noted the use of NFM interventions in flood mitigation strategies as results of UK floods in 2015 and 2016.

1.2 Natural Flood Management

NFM is classed as a subset of nature-based solutions used to mitigate the impacts of climate change and natural events (Wilkinson et al., 2019). NFM can be defined as the restoration

and utilisation of natural floodplain or channel characteristics around a catchment (Short et al., 2019). These interventions can be used to support the use of natural processes within freshwater environments and can help to address catchment-scale gaps through long-term monitoring. Natural processes can be used as a tool to reduce flooding through the implementation of measures that emulate natural functions within rivers, floodplains, and catchments. These measures aim to restore or protect ecosystem services and can be implemented in both rural and urban areas. According to Gov.uk (2021) when utilising natural processes, the main benefits to environmental services include improved water quality, flood mitigation, climate regulation and habitat quality. Characteristics can be utilised through a range of interventions that affect riparian hydrology (Venkataramanan et al., 2020), summarised in Figure 1.1. The main aim of NFM projects is to attenuate the flow of water within a catchment and increase infiltration upstream (Nicholson et al., 2020). This encourages water storage upstream to help mitigate the damages caused by flood events on urban areas downstream (Johnson et al., 2022).

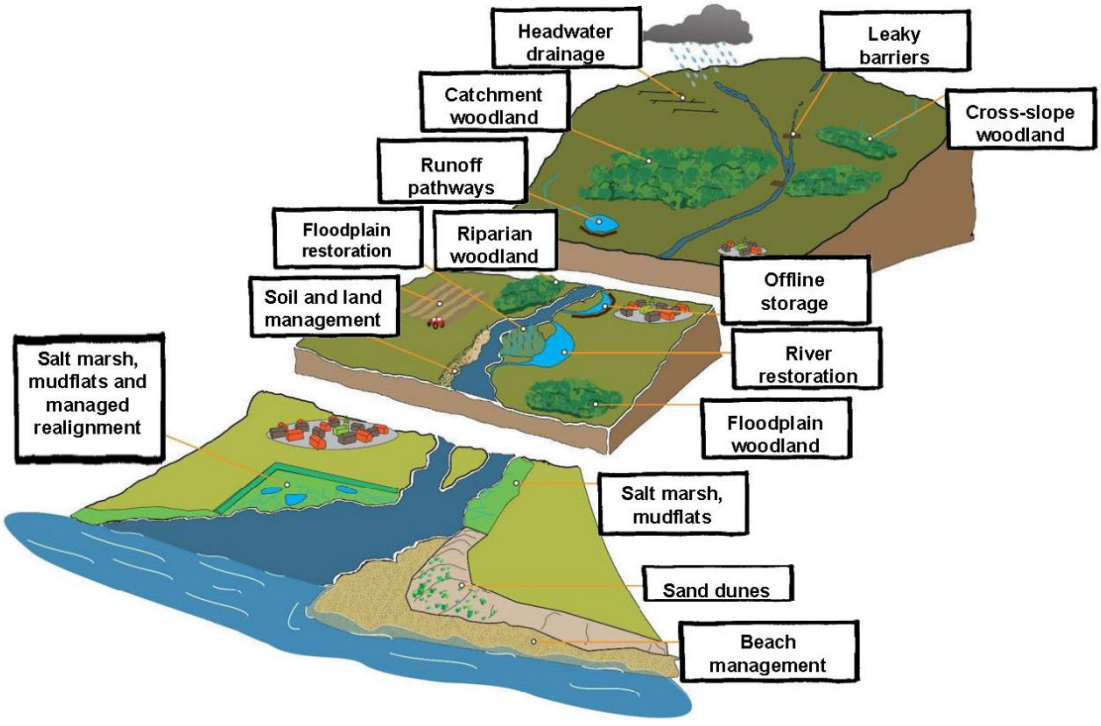


Figure 1.1 Diagram depicting the range of flood management interventions across the upper, middle, and lower course of a drainage basin (Burgess-Gamble et al., 2017).

NFM projects can incorporate woodland management, run-off management as well as floodplain and river management (Gov.uk, 2021). Woodland, field, in-channel, and riparian interventions can have both ecological and social impacts (Mondal and Sahoo, 2022). Woodland based measures span from wet woodlands to tree planting, which divert water from the watercourse and encourage higher infiltration rates during months of increased precipitation (Dittrich et al., 2019). Field interventions comprise mainly of tree planting and

the addition of ponds which accumulate and hold water higher up within the catchment (Collentine and Futter, 2018). The most commonly used in-channel infrastructure are wooden/leaky dams, which decrease water flow downstream and encourage flooding of wild land in autumn or winter months (Gunnell et al., 2019). Many of the NFM structures can be key to habitat creation in freshwater systems (Black et al., 2021).

There are socio-economic advantages of implementing NFM measures within a catchment (Wells et al., 2020). Economic costs of repairing hard infrastructure post flooding are higher than NFM materials and labour costs (Iacob et al., 2014), with the floods in summer 2007 costing £3.2 billion and winter flooding in 2016 costing £1.3 billion in damages to rural and urban areas (Cooper et al., 2021). The comparative cost of implementing soft NFM interventions versus hard engineering structures has been estimated based on the type of intervention, calculating the minimum and maximum cost. For example, Short et al. (2019) estimates that per leaky dam, the cost ranges between a minimum of £77 and a maximum of £400. These estimates are relatively low when compared to hard infrastructure such as sea walls, which can cost upwards of £8000 depending on the length and height (Khazai et al., 2007). NFM projects are a collaborative effort from stakeholders which can encourage partnerships and improve communication within communities (Garvey and Paavola, 2021).

There are multiple ecological benefits that stem from NFM projects. Interventions that accumulate water and sediment such as silt traps, can help improve water quality by reducing the volume of in-stream pollutants through sediment build-up (Liu et al., 2020). For example, agricultural run-off from farms that may contain effluents and pesticides can be prevented from entering the aquatic system (Wingfield et al., 2019). Increasing the attenuation of water and sediments around interventions can aid climate change mitigation (Ellis et al., 2021) as carbon becomes sequestered and buried in freshwater sediments over time, termed ‘Teal Carbon’ (Zinke, 2020). Organic matter also accumulates around NFM structures, promoting an increase in filter feeders that remove decomposing material from the aquatic environment (Lo et al., 2021).

Although such projects have multiple benefits, there are a number of negatives that can result from NFM infrastructure (Kotowski et al., 2004). A negative social impact from introducing NFM to a catchment is conflicts between stakeholders regarding the implementation and maintenance responsibilities of infrastructure (Bark et al., 2021). These disadvantages are similar to negative impacts of hard engineering structures such as sea walls and dams (Rahel and McLaughlin, 2018). Using soft engineering techniques can be less effective when trying to control large flooding events, compared to hard methods like channelisation (Nakamura, 2022). Muhawenimana et al. (2023) proposes that there is a lack of design optimisation or guidance for leaky dams in relation to flood management. Additionally, materials used for soft interventions have a reduced longevity compared to harder materials such as metal and concrete (Dadson et al., 2017). Using natural materials can increase economic costs overtime as soft structures are damaged more easily and require repairs more frequently. Hard structures such as sea walls have a life span of decades and in some cases centuries (Toimil et al., 2020). Green infrastructure such as leaky dams are considered to have a life span of up to 30 years before needing to be replaced (Alves et al., 2020).

1.2.1 Leaky Dams

Leaky dams (Figure 1.2) are constructed by securing large pieces of natural material across a stream or river, stretching from one bank to the other (Bokhove et al., 2018). This in-channel infrastructure continues to allow base flow through, reducing the negative impacts that other barriers may pose to migratory aquatic species. Allowing low water levels through these structures ensures that habitats downstream of such interventions still maintain a flow (Lo et al., 2021). The material used for leaky dams is most often wooden trunks which can be repurposed into these soft structures (Grabowski et al., 2019).

Leaky dams can affect riparian water dynamics by decreasing in-channel water velocity (Hankin et al., 2020), which affects the suspension of sediment particles within the water column (Bazarov et al., 2020). This increases the volume of fluvial sediment deposition, most notably either side of the structure (Huang et al., 2022). Deposits can include finer sediments such as sand, silt, and clay which are particularly important for creating ecological habitats (Follett and Wilson, 2020). One of the main threats to freshwater biodiversity is the degradation of habitats (Colin et al., 2018). Leaky dams have been shown to increase biodiversity in their surrounding environments as a result of this habitat creation (Deane et al., 2021). This increases the importance of research on measures that help to produce or preserve degrading habitats. A disadvantage of leaky dams is that they may obstruct the pathways of larger migratory aquatic species which are unable to pass through these structures (Moser et al., 2021). Unlike hard structures, there are fewer adaptations for leaky dams to accommodate larger migratory species without compromising their effectiveness (Müller et al., 2021).

Figure 1.2 Leaky dam positioned along the Painswick stream in the Cotswolds as part of the Wildfowl and Wetland Trust (WWT) Natural Flood Management project.

1.3 Lamprey Ecology

Lampreys are jawless, cartilaginous agnathans (of the order Petromyzontiform) with a physical morphology similar to eels (Hume et al., 2021). Like eels, they lack scales or paired fins, with only dorsal fins to aid swimming (Borowiec et al., 2021). Two species of European lamprey, the larger River Lamprey (*Lampetra fluviatilis*) (Figure 1.3a) and smaller Brook Lamprey (*Lampetra planeri*) (Figure 1.3b), can be found around Europe (Figure 1.4) including the waterways of the UK (Igoe et al., 2004). Both species are legally protected through the European Habitat Directive Annex II and V, whereby members of the agreement must protect habitats that are known to support lamprey populations (Shephard et al., 2019). Additionally, Annex V prohibits removing the named species and sub-species from the wild (Docker et al., 2015). Habitats that support lamprey populations can also be protected through Special Areas of Conservation (SAC) status, which legally cannot be disturbed (Albright and Lucas, 2021). Lamprey presence in the UK (Figure 1.5) spans from the South of England to the Highlands of Scotland (Hume et al., 2018). There are several known landlocked populations of *Lampetra fluviatilis* (*L. fluviatilis*) in Scottish Lochs which are considered of high ecological importance within the EU (Morris, 1989). Previous research of these two species has primarily focussed on distribution and genetic similarities through phylogeny studies, aiming to decipher whether these are ecotypes of the same species or two separate species of lamprey (De Cahsan et al., 2020).

A.

1 cm

B.

1 cm

Figure 1.3 A) *L. fluviatilis* smolt specimen taken from Kucheryavyy et al. (2022). B) *L. planeri* adult specimen taken from Sperone et al. (2019).

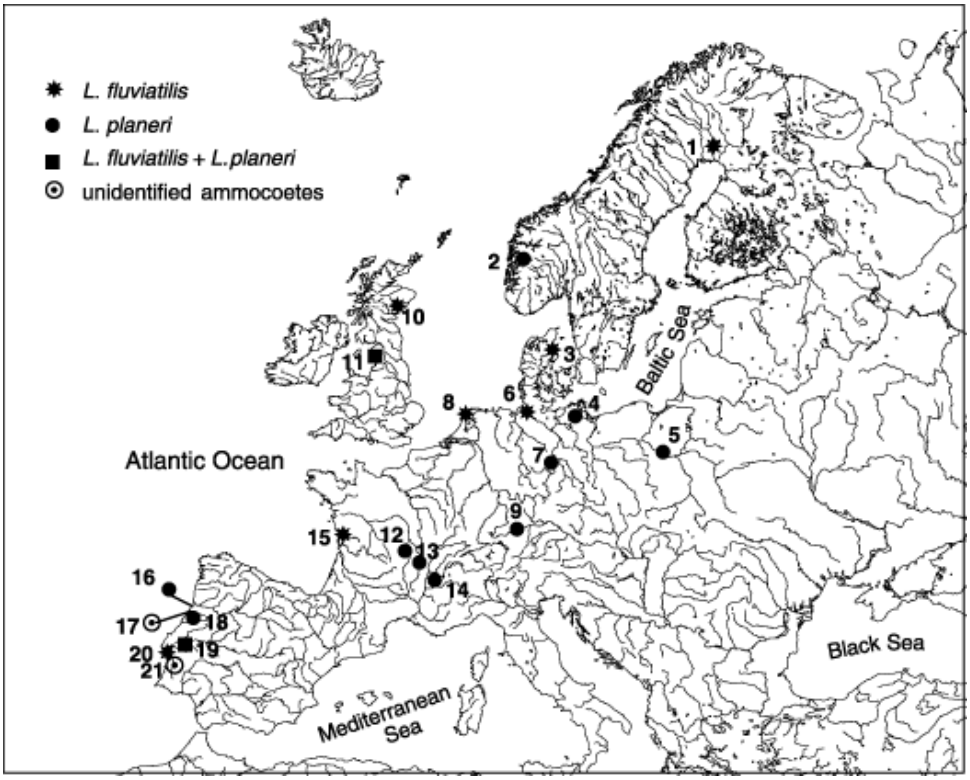


Figure 1.4 Map showing the distribution extent of *Lampetra* species throughout Europe based on the sampling sites of Espanhol et al. (2007).

A. B.

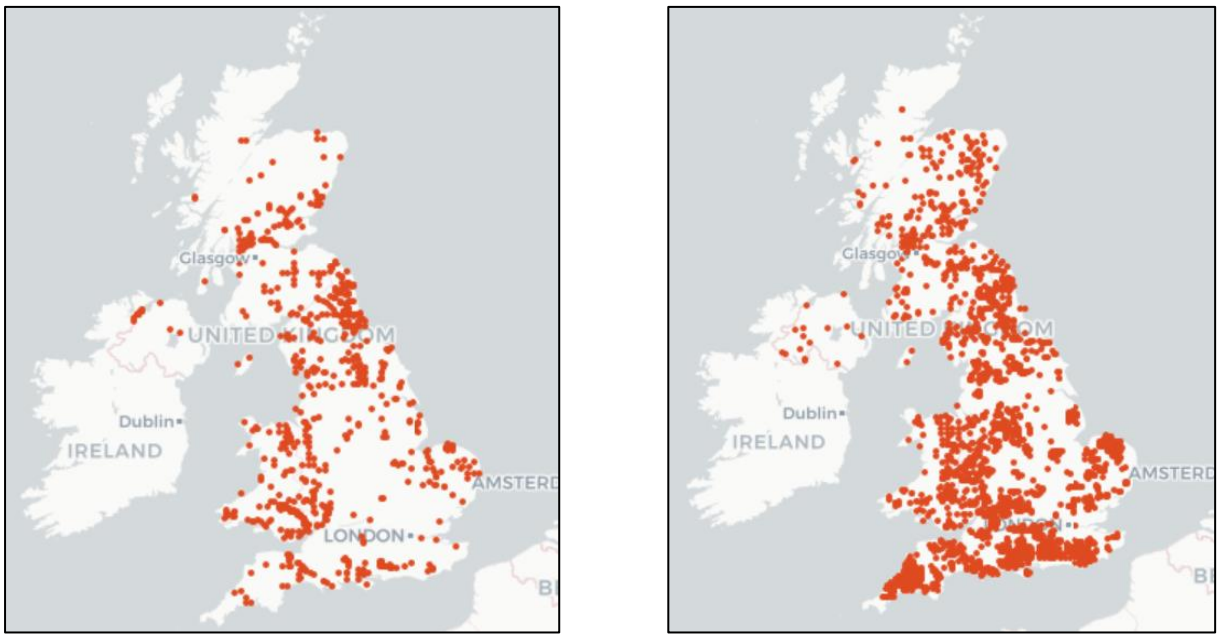


Figure 1.5 A) Map showing the distribution of *L. fluviatilis* throughout the UK based on 1172 confirmed records. B) Map showing the distribution of *L. planeri* throughout the UK based on 4678 confirmed records. These maps were taken from NBN Atlas (2023).

1.3.1 *Lampetra fluviatilis*

Lampetra fluviatilis (Linnaeus, 1758) can only be found within western Europe due to a rapid decline in populations elsewhere in the EU (Kujawa et al., 2019). They are larger than *L. planeri*, with adults growing to approximately 30-45 cm in length (Figure 1.3a) and appearing grey-blue in colour (Kucheryavyy et al., 2022). *Lampetra fluviatilis* have diadromous life cycles (Figure 1.6), sharing marine habitats with species such as Sea lamprey (*Petromyzon marinus*), and freshwater habitats with *L. planeri* (Tsimbalov et al., 2015). *Lampetra fluviatilis* adopts an anadromous life stage for spawning as individuals reach reproductive maturity, migrating inland up freshwater rivers and streams from estuarine habitats (Zvezdin et al., 2022). Migrations begin from late autumn and continue until the end of spring, coinciding with the start of the spawning season (Tummers et al., 2016). Adults migrate during hours of darkness to avoid predation and maximise chances of reaching spawning sites (Mateus et al., 2021). Lamprey migration is a source of material and nutrient transport between the marine and freshwater environments (Nislow and Kynard, 2009). Ammocoetes spend between 3-5 years in burrows before maturing into macrophthlamia (Goodwin et al., 2008). Macrophthlamia migrate out to oceanic feeding grounds, where individuals display parasitic-hematophagous behaviours (Quintella et al., 2021). When fully developed, *L. fluviatilis* adults form a row of teeth around their mouthpart which they use to latch onto fish and rasp at the tissue to yield blood (Adams et al., 2008). This is a distinguishing feature of *L. fluviatilis* adults and can enable species identification when compared with *L. planeri* (Polyakova et al., 2019).

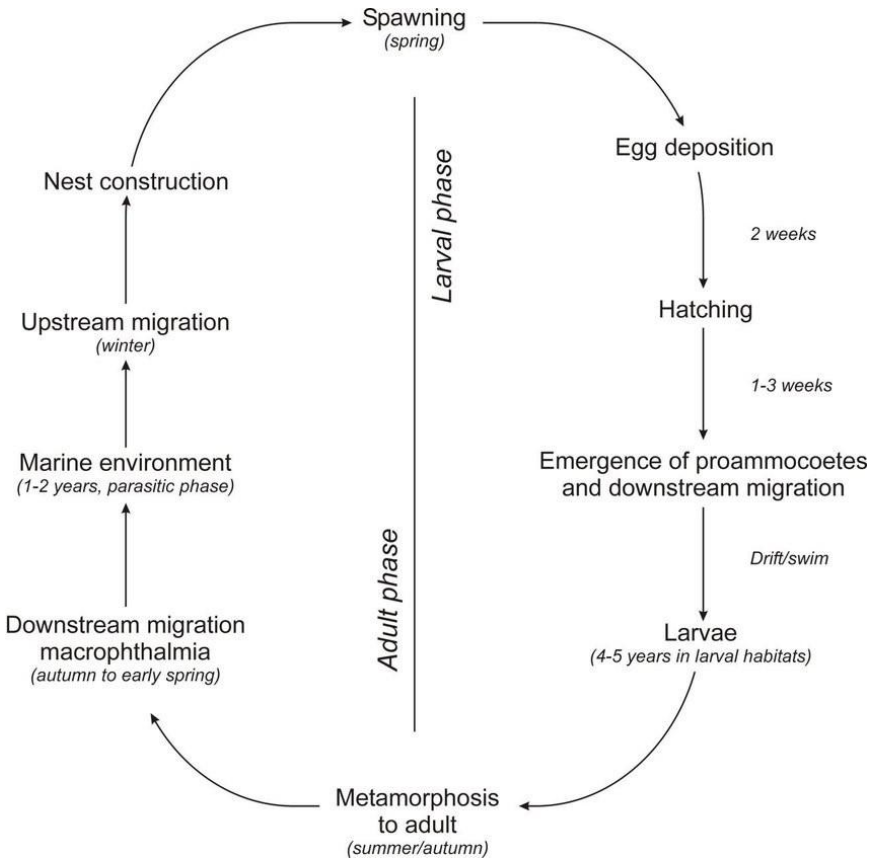


Figure 1.6 Diagram detailing the life stages of *Lampetra fluviatilis* taken from I.C.E.S (2015).

1.3.2 *Lampetra planeri*

Lampetra planeri (Bloch, 1784) is a smaller lamprey species, with adults growing between 11-20 cm in length (Byrne et al., 2000). When fully matured into adults (Figure 1.3b), they appear a grey-brown colour with white undersides (Sperone et al., 2019). *Lampetra planeri* have suffered a decrease in number throughout the UK but are more easily detected than other species due to the accessibility of their habitats (Hume, 2017). As this species of lamprey do not migrate large distances through waterways, they can be found in locations where other migratory species are not present (Schreiber and Engelhorn, 1998). An example would be that *L. planeri* are often present in habitats further upstream of watercourses that have a migratory barrier. *Lampetra fluviatilis* may struggle to pass these migratory barriers, creating a delay in spawning and result in a wider distribution of *L. planeri* in freshwater systems (Russon and Kemp, 2011). *Lampetra planeri* ammocoetes spend an average of 7 years buried in nursery sediments (Figure 1.7) where, similarly to *L. fluviatilis*, they filter feed on organic matter (Dawson et al., 2015). Both species feed on plant and animal detritus, diatoms, and algae within sediments at the ammocoetes stage (Loshakova and Knizhin, 2015) Unlike parasitic *L. fluviatilis,* mature *L. planeri* adults do not have a working mouthpart and do not feed, instead relying on stored resources for reproduction (Malmqvist, 1980). ~~*L.*~~ *Lampetra planeri* are known to have a lower fecundity than *L. fluviatilis*, which is a contributing factor to their decline (Silva et al., 2015).

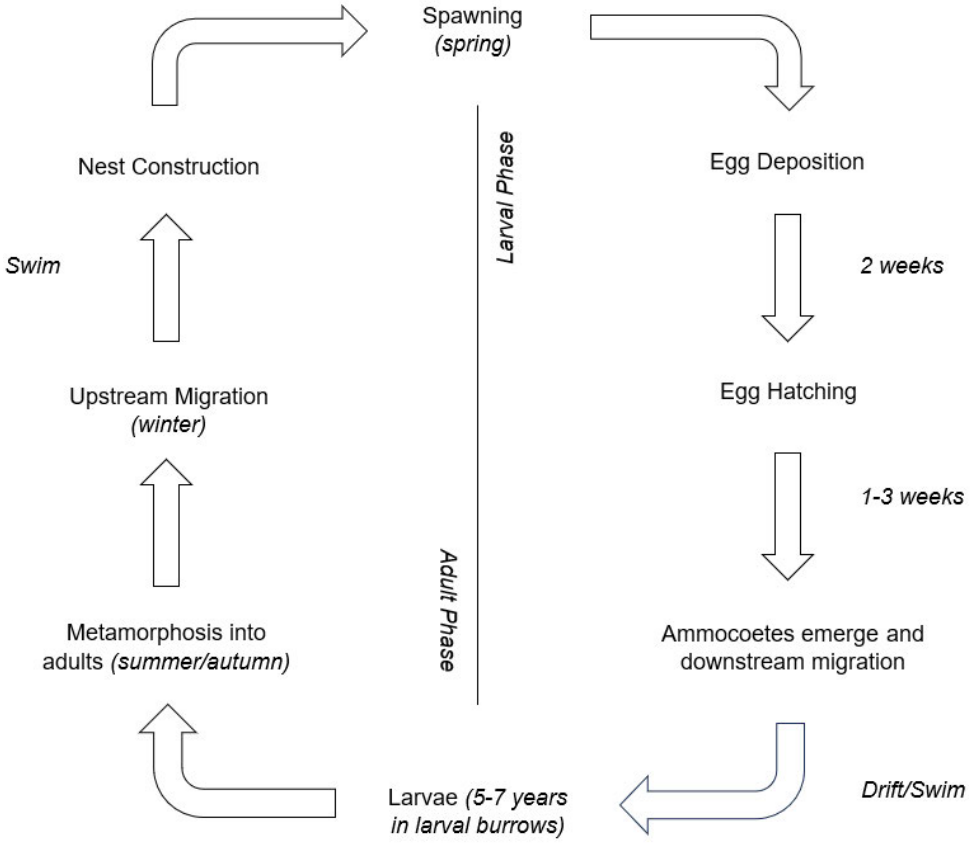


Figure 1.7 Diagram showing the life stages of *Lampetra planeri*.

1.3.3 Lamprey Spawning

Freshwater sites are chosen for spawning due to many factors such as water flow velocity, which aids fertilisation (Clemens et al., 2016). Previous studies have shown that lamprey choose their spawning sites based on the stream or river gradient and the sediment particle size (Aronsuu and Tertsunen, 2015). Suitable and favourable spawning habitats consist of larger particles of sediment such as pebbles and gravel (Oliveira et al., 2022). Lampreys have a low fidelity to natal spawning sites and in some cases do not return to the same catchment to spawn (Davies et al., 2022). Once suitable spawning substrate has been located, male lampreys arrange larger rocks to form a circular nest or redd (Zvezdin et al., 2017). These redds appear as bare patches of small gravel surrounded by a ring of larger pebbles and rocks (Dhamelincourt et al., 2022). The female individuals will settle in the redd, and a number of males will attach by latching on with a mouthpart (Cochran et al., 2008). Males wrap themselves around a female in order to position themselves for the release of gametes (Johnson et al., 2015). Once intertwined, females can release up to 25,000 eggs in one dispersal using the current to transport them into gaps between sediment (Docker et al., 2019). All 41 known species of lamprey are lithophilic, producing gametes which are released during a single spawning event (Daupagne et al., 2022).

Lampetra fluviatilis and *Lampetra planeri* share the same spawning season, reproducing between April and July (Lasne et al., 2010). Spawning occurs in darkness to avoid predation of the eggs, starting at 17:00 and finishing by 03:00 the next morning (Russon and Kemp, 2011). Lamprey eggs are coated in an adhesive substance which enables the eggs to accumulate sand and gravel sediments (Yorke and McMillan, 1979). Once enough substrate has adhered to the eggs, they become weighted and sink to reduce passive displacement (Staponkus and Kesminas, 2014). Additionally, this keeps eggs hidden from predators, which increases the number of hatchlings that survive to the larval stage (Kemp et al., 2011). Once the lampreys have successfully fertilised and dispersed the eggs, the adults will die due to the resource strain (Kujawa et al., 2019). Fertilised eggs hatch after roughly 3 weeks and release ammocoetes into the river current (Silva et al., 2015). Ammocoete is the term given to a lamprey larva upon hatching where there are no distinguishable physical characteristics. It precedes the juvenile stage where differentiating characteristics develop (Dawson et al., 2015). Through a combination of active swimming and passive drift, the larvae migrate to suitable nursery habitats where they create burrows in the silt (Maitland, 1980). Each species of lamprey has a burrow-dwelling stage, highlighting the importance of these sediments for larval recruitment (Quintella et al., 2007). After the ammocoete stage, the larvae develop into juveniles termed macrophthlamia (Moser et al., 2015). Once ready to migrate, macrophthlamia mature into their adult morphs over 3 to 4 months and stop feeding (Dziewulska and Domagała, 2009).

Lampetra planeri are more difficult to observe due to their extended period buried in nursery sediments, however they have a wider distribution than *L. fluviatilis* (Golovanov et al., 2019). Where lamprey populations have been identified in the Southern Hemisphere, there is little to no conservation effort, with many lamprey species lacking enough abundance data to accurately assess their conservation status (Lucas et al., 2021). Methods to capture lampreys have previously been grouped into two separate categories: passive or active sampling. Passive methods are non-invasive and include eDNA techniques or sampling when individuals are within the water column. Active methods can be more destructive and include substrate excavation or encouraging individuals out of burrows (Clemens et al.,

2022). *Lampetra* species are most commonly captured through more traditional methods such as netting, trawling, walk-over surveys, and electrofishing in key habitats. Monitoring and surveillance of both lamprey species is usually conducted through electrofishing (Harris et al., 2023). These techniques can provide strong observational datasets, showing species population trends and identifying suitable spawning or nursery substrates (Jones et al., 2020). There are disadvantages when using these more traditional methods, for example trawling methods are highly invasive within the lamprey habitats. The distribution of lamprey is determined by the physical characteristics of the freshwater environment that they live in, and in particular the grain size of the channel (Moser et al., 2019). Habitat connectivity and fragmentation has been highlighted as a key factor for the decline of lamprey throughout the UK (Lothian et al., 2020). Invasive methods can negatively affect habitat quality for aquatic species, adding to freshwater habitat degradation throughout Europe (Lecaudey et al., 2019).

1.4 Environmental DNA

Environmental DNA (eDNA) are collectively the biological traces left after an organism has moved through an environment (Thomas et al., 2020). These traces may be in the form of gametes (excretory products such as cells or faeces), and can be sampled from air, water, or terrestrial ecosystems (Rees et al., 2014). Conservation uses of eDNA include the ecological monitoring of endangered or vulnerable species, species identification, and determining the prevalence of invasive species (Thomsen et al., 2012). Within both terrestrial and aquatic habitats, eDNA has been adopted for ecological biomonitoring (Moser et al., 2007). Globally, these methods help contribute to the production of socioeconomically or commercially important harvest species (Yates et al., 2019). Similarly, eDNA can determine ecological interactions that are key to environmental services such as pollination (Banerjee et al., 2022). Methods utilise mitochondrial, ribosomal, or nucleic genetic material for species identification which is extracted from eDNA capture techniques (Blank et al., 2008). Mitochondrial DNA is found within the mitochondria of animal cells; ribosomal DNA and nucleic DNA are found within the nucleus of animal cells. Mitochondrial DNA is the most common DNA captured in environmental samples. The most common method of obtaining eDNA from aquatic environments is capturing genetic material in filter paper pores (Lekang et al., 2015).

Genetic material can be extracted from a wide range of matter including sediments and is suitable for palaeontology studies (Evans and Weber, 2020). eDNA can be extracted from sediments collected in cores to understand past species or community distribution in chronological order (Wesselmann et al., 2022). Wider applications of eDNA methods can be seen within the public health sector, where wastewater analysis provided community-scale tracking of covid RNA prevalence during the 2020-2021 pandemic (Palmer et al., 2021). Research such as this has solidified eDNA as a rapid and highly sensitive diagnostical tool which can be utilised across a wide range of sectors. Currently the predominant application of eDNA is to determine the presence or absence of target species in circumstances where it is not possible to collect observational data (Xia et al., 2021). Very little genetic material is needed to enable species identification or presence-absence testing (Bylemans et al., 2019). This is beneficial when the target species is endangered, protected, or rare and obtaining a physical tissue sample may not be possible (Piggott, 2016). As sampling techniques are

non-invasive, collecting eDNA has little to no effect on organisms and their environments (Baltazar-Soares et al., 2022).

There are many benefits to using eDNA techniques for species identification and calculating biodiversity (Larson et al., 2020). eDNA methods can produce results quicker than conventional observational methods such as electrofishing capture and release using nets or trawling (Pereira et al., 2021). Traditional methods can have increased time, labour and resource costs when compared to eDNA techniques which can provide rapid results within 24 hours of sample collection (Jerde, 2021). When using the more traditional methods such as trawling or dredging, costs increase due to the need of trawling equipment. Similarly, when collecting samples via research vessels, a trawling crew is required for extended periods of time, which can also increase labour costs (Virdin et al., 2022). Unlike other biomonitoring methods, eDNA capture requires fewer and much cheaper resources whilst still maintaining reliable, rapid results (Wang et al., 2021). The average length of time needed to collect trawling samples varies depending on the research area, the storage capacity of the vessel and weather conditions (Docker and Hume, 2019). Total time dedicated to walkover and electrofishing surveys can range from hours to multiple days depending on the size of the site and the number of individuals involved (Chambers et al., 2021). Additionally, traditional techniques like trawling or dredging can be highly destructive to the organisms and their environment, often resulting in fatalities. eDNA methods are both non-invasive and non-destructive which is key when working with protected, vulnerable species (Zou et al., 2020).

Within a project, eDNA techniques can be combined with more traditional capture methods such as trawling, where water samples are taken at different depths. Afzali et al. (2021) determined that when combining eDNA metabarcoding and trawling at depths of up to 250 m, eDNA proved more effective at quantifying species richness. This study found that 53% of total detected species overlapped between the eDNA and trawling method - with metabarcoding detecting a higher number of species that are known to be resilient to trawling gear. Afzali et al. (2021) similarly determined that eDNA metabarcoding can be used to compliment trawling surveys and provide reliable data for vertical fish distribution in water columns and relative quantitative estimates of fish biodiversity. Similar research conducted by Przybyla-Kelly et al. (2023) showed that eDNA techniques such as ddPCR and qPCR had a higher sensitivity compared to trawl catch data at the same depths ranging from 8 to 83 m deep. An advantage of combining conservation methods is that eDNA techniques can be used across all sediment types, whereas trawling gear is restricted to softer substrates therefore neglecting largely rock substrate habitats (You et al., 2021).

Despite an increase in eDNA methods since its first use, there are persistent inconsistencies and a lack of standardisation for many species (De Cahsan et al., 2020). Well documented limitations when using eDNA for conservation include the inability to determine species abundance or biomass (Lawson-Handley et al., 2019). Currently there few reliable eDNA techniques for measuring species total abundance, which limits the use of such methods to primarily presence-absence testing (Beng and Corlett, 2020). These techniques cannot determine the life stage or age of the target species, unlike observational methods which can also provide gender identification (Huisman, 2017). Challenges of working with eDNA are often beyond the control of the researcher due to biotic and abiotic factors (Barnes and Turner, 2016). Due to the turbulence of the natural aquatic environment, there are several abiotic factors which can influence the quality of eDNA collection (Pilliod et al., 2014). Much

of the literature states that the quality of samples is greatly affected by disturbances of the benthic substrate, as this can cause a decrease in the total volume of water filtered (Capo et al., 2020). eDNA methods are able confirm the presence of a target species at the sample location, but this may be from eDNA transported by currents from upstream habitats (Carraro et al., 2020). Water turbulence may also cause issues within larger catchments.

Fish DNA within the aquatic environment is known to decay overtime and can be compared across several decay models (Tsuji et al., 2019; Shogren et al., 2018; Andruszkiewicz et al., 2021). eDNA has been found to go through an initial rapid decay which is then followed by a much slower decay period (Rourke et al., 2022). Decay rates differ across individual fish species and environmental conditions, with aquatic DNA half-life ranging from 1 hour up to 10 days after the initial rapid decay (Collins et al., 2018). Many studies utilising eDNA methods suggest DNA degrades at a faster rate in marine environments when compared to freshwater (Lamb et al., 2022). Abiotic influences such as solar radiation, dissolved oxygen, and pH similarly induce decay in eDNA. It is understood that the presence of humic acid produced by decomposing vegetation can degrade the quality of eDNA in aquatic environments (Hallam et al., 2021). Despite several abiotic factors affecting eDNA degradation, research has shown that temperature has the greatest impact and can accelerate these rates (Barnes et al., 2014). The literature shows that the range of influences on eDNA sample quality includes biological conditions and the characteristics of the DNA itself (Holman et al., 2022). Research conducted by Friebertshauser et al. (2019) determined that DNA decay is positively correlated with biotic factors such as the presence of filter feeding organisms within aquatic habitats.

eDNA can help ecologists to better understand a species past and present habitat preferences, as well as distribution, through genetic signatures (Thomsen and Willerslev, 2015). Techniques have advanced in recent decades to allow whole communities to be detected, including trophic interactions through stomach contents analysis both in aquatic environments and terrestrial environments (Garlapati et al., 2019). eDNA can determine how species are interacting within an ecosystem and decipher complex food web interactions by analysing an organism’s diet from stomach contents (Weber et al., 2023). Alternatively, the same techniques can be used to determine mutualistic relationships between species in environments (Johnson et al., 2023). DNA techniques can be optimised to have high sensitivity for the target species and have the potential to be as accurate as observational identifications without the need to observe individuals when sampling (Stoeckle et al., 2017). Similarly, for conservation projects where the target species may be protected, eDNA methods can be used without the need to acquire handling licenses or special permissions in order to conduct the research (Veilleux et al., 2021).

1.4.1 eDNA for Lamprey Identification

Reoccurring identification issues have arisen with regards to *L. planeri* and *L. fluviatilis* observational data as adults have very few distinguishable characteristics apart from body size or mouthpart (Mateus et al., 2013). During the ammocoete stage, the larvae of all lamprey species show no distinguishing morphological characteristics (Moser et al., 2007). Although there are differences in life cycle stages and adult morphologies, many studies have considered *L. planeri* and *L. fluviatilis* as paired species (Rougemont et al., 2017). The genetic differentiation of the two lampreys is low in sympatric environments where they

share the same habitats, and these two species are considered partially reproductively isolated ecotypes (Docker and Potter, 2019). As the species share similarities in their DNA and are found in many of the same environments, the two European *Lampetra* species are considered by some to be the same species with different phenotypes (Decanter et al., 2023). Similarly, like eel species during the spawning season, an increase in genetic material is released into the environment through gametes and skin cells where mating individuals intertwine (Takeuchi et al., 2019). Additionally, these particular species expire after mating, causing the carcasses to breakdown within the aquatic system and releasing more DNA. It is most common to conduct lamprey surveys and sampling during the spawning season to better the chances of observations or capturing eDNA (De Souza et al., 2016). Additionally, another issue which can hinder species identification is hybridisation between *L. planeri* and *L. fluviatilis* due to their genetic likeness (Docker, 2009).

DNA analysis has been an important tool in understanding the genetic similarities of the two lamprey target species, especially with regards to the question of shared phylogeny (Pereira et al., 2021). Online open-source databanks, such as the National Centre for Biotechnology Information (NCBI), provide a large resource of well-defined genomes including *L. fluviatilis* and *L. planeri*. This allows the identification of species from eDNA through comparative DNA sequencing (Rausch et al., 2019). A study by Souissi et al. (2022), located a Single Nucleotide Polymorphism (SNP) that can be used to genetically identify the separate species when both are present in the same location. Prior to the genetic analysis conducted by Souissi et al. (2022), a report was published regarding proof of concept for using eDNA sampling in order to test the presence-absence of *Lampetra* species. Within their study, Zancolli et al. (2018) listed 6 pairs of probes and primers which could be used to determine the presence-absence of specifically *Lampetra* species, even when other lamprey species may be present. One disadvantage of this study however was the lack of data relating to the efficiency of these primers and probes, or any data to show they had been used with true environmental samples. Further research is needed to confidently identify the presence-absence of both *Lampetra* species, particularly in locations where both species may be present and gene flow may be occurring (Bracken et al., 2015). There is a need to standardise and optimise a working method for the Zancolli et al. (2018) primers and probes, and to field test the presence-absence of both *Lampetra* species. eDNA as a conservation tool has a high potential for bridging such knowledge gaps, either complementing more traditional methods or as a stand-alone technique. Published applications such as Zancolli et al. (2018) have focused on outlining prospective field methods and recommendations for capturing lamprey eDNA, however there few studies that have conducted these methods in a field environment. Additionally, many of these applications have used laboratory trials to proof test methods but have yet to be conducted in the field (Souissi et al., 2022).

1.5 Polymerase Chain Reaction

Since its first use, Polymerase Chain Reaction (PCR) techniques have helped to revolutionise both genetic and biological research (Garibyan and Avashia, 2013). The uses of PCR range from the biomedical industry to environmental and commercial food standards monitoring (Zhu et al., 2020). Although there are some applications which require space-domain systems, most current methods used are time-domain based protocols (Nekrutenko et al., 2000). Developing from initial research, the introduction of new DNA polymerases with

increased stability and efficiency, as well as the development of highly sensitive quantitative methods such as digital PCR (dPCR) and quantitative PCR (qPCR), have increased the utility of PCR further still (Hunter et al., 2017). PCR is a reaction which enables the exponential and specific synthesis of a region of DNA (Figure 1.8) using two specifically designed DNA fragments, which are complimentary to a target DNA molecule within the sample (Van Pelt-Verkuil et al., 2008). These small, specific DNA fragments are either termed oligonucleotides or primers, and are required by the DNA polymerase to initiate synthesis of each strand of the DNA molecule (Valentini and Pompa, 2016).

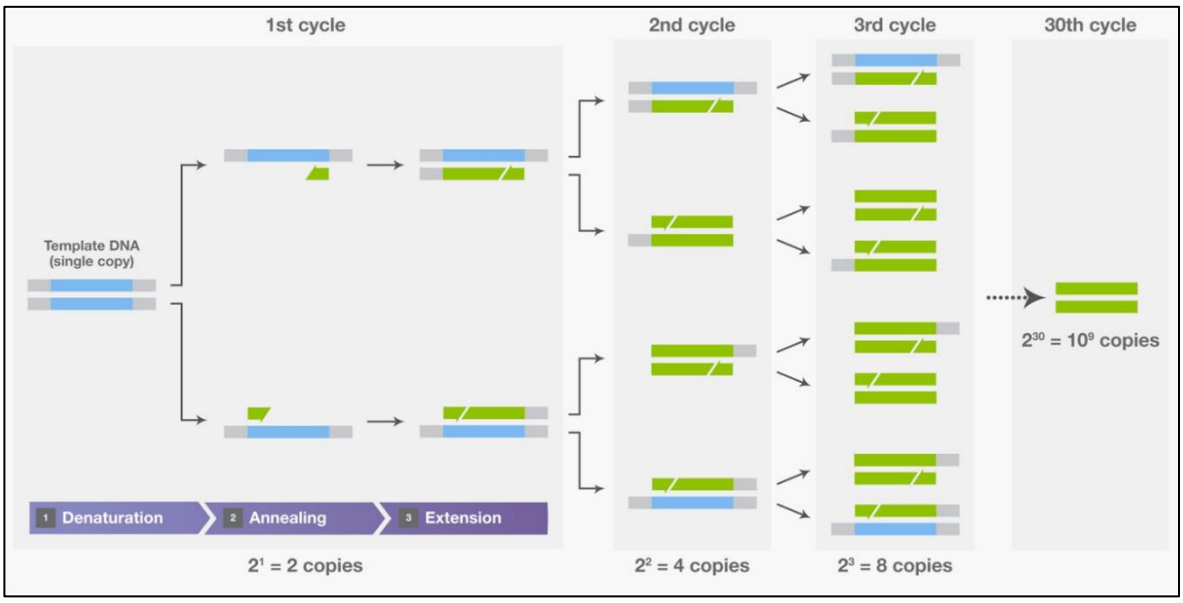


Figure 1.8 Graphic depicting the cyclical process of PCR, used to amplify target amplicons (Thermo Fisher Scientific, 2023).

Most PCR assays generally have a high specificity which is determined through the hybridisation of complementary base pairing (Sachse, 2003). Correct hybridisation occurs when the specific primer sequence is complementary to the sequences present on the target DNA that is being amplified (Wittwer et al., 2001). The addition of a reporting dye such as SYBR Green or other fluorescent probes, allows for amplicon quantification (Zhang et al., 2015). PCR protocols are often coupled with gel electrophoresis which allows the size (in base pairs) of any reaction products present within the sample to be determined, helping to differentiate between amplicon or show the presence-absence of a target amplicon (Koskinen et al., 2009). The main principle of PCR is that by using an initial small volume of target DNA, this can be amplified into much larger volumes that are required for subsequent protocols (Valentini and Pompa, 2016).

Although PCR methods have evolved in accuracy and specificity over previous decades, there are persistent issues that can arise when carrying out assays. One example of such issues is the over-estimation of low DNA concentrations through bias sources (Hajia, 2018). These sources of bias include false positives, non-linear relationships between expected and measured DNA concentrations and non-specific amplification or contamination (Smith and

Osborn, 2009). False positives can be defined as background signals or artefacts which are produced by PCR instrumentation rather than the target DNA being amplified. Without the use of a positive control sample, false positives can go undetected and affect the final results. PCR inhibitors can skew the results of these processes, for example any substance which will disturb the fluorophore function impairs the analysis (Schrader et al., 2012). When working with eDNA, inhibitors from the natural environment such as humic acid can disrupt PCR methods and result in misleading data (Hallam et al., 2021). PCR is a process used exclusively for amplifying small volumes of DNA, making it the one of the most useful tools for analysing eDNA samples with regards to presence-absence testing (Rishan et al., 2023). eDNA samples produce very small yields when extracted due to several reasons, such as capture time and degradation in the natural environment. For these reasons, PCR is necessary to increase the volume of and concentration of eDNA for further analysis.

1.6 Project Aims and Objectives

There are inconsistencies in lamprey taxonomy which stem from a lack of in-field methods that are able to distinguish between species and ongoing discussions between scientists regarding lampreys' evolutionary history and nomenclature system. Lamprey phylogeny is complicated by disparities in the scientific language used between studies, creating confusion as to where lamprey sit within taxa (Docker et al., 2015). Continuing uncertainty remains when trying to distinguish between paired species using the same habitats, with a limited number of new methods attempting to resolve this issue (Alfino and Roberts, 2019). Additionally, there is limited research associated with *L. planeri* which may have resulted from a decline in populations since the 1950s and difficulties obtaining accurate voucher specimens. This study is rooted in ecological conservation and strives to improve upon current methodologies to identify suitable lamprey habitat using eDNA methods. This study aims to evaluate whether in-channel NFM interventions contribute to habitat creation for lamprey species and develops eDNA methods as a rapid assessment tool for monitoring and surveillance of lamprey at suitable sites.

The objectives of this study are:

1. Evaluate current eDNA methods available to differentiate between *L. planeri* and *L. fluviatilis* and create a workflow for the data collection, preparation, and analysis of samples to allow application of eDNA.
2. Characterise the grain size and flow characteristics of river sites to identify potential lamprey habitat.
3. Collect eDNA samples over one spawning season to determine the presence-absence of *L. fluviatilis* and *L. planeri* at a positive control site.
4. In a field setting, evaluate the effectiveness of eDNA presence/absence methods at positive control site and correlate this with the physical characteristics of the site to determine if leaky barriers do create new habitats suitable for lamprey.

2. Methodologies

2.1 Site Selection

To develop in-field and laboratory eDNA methodologies to confirm the presence-absence of *Lampetra* species, a positive control site with past confirmation of lamprey presence was required. The fieldwork site was selected based on observational data from Harrison and Pinder (2020), which confirmed the presence of both *L. fluviatilis* and *L. planeri* at locations around Soudley Ponds, Cinderford (51°47’56’’N, 2°29’30’’W). Harrison and Pinder’s findings confirmed the presence of lamprey individuals at two sites out of twenty-five electro-fished around the catchment. In this study, a single adult *L. planeri* and two larval *L. fluviatilis* / *L. planeri* were recorded. There were no leaky barriers present at this site, however the conditions of the site were similar to those of NFM catchments.

A walkover of the site was conducted ahead of the fieldwork season (01.04.23), to determine site suitability for the proposed sampling methodology, and the sampling points were identified during this site walkover. Permissions were sought ahead of the fieldwork Forestry England. All water samples for eDNA analysis were collected from 03.04.2023 – 28.04.2023, at four sampling points along the stream connecting two of the Soudley Ponds (Figure 2.1). These points were chosen due to accessibility from the stream bank and were evenly spaced geographically along the reach. Samples were collected at sunrise three times per week (Monday, Wednesday, and Friday) during the sampling period. The samples were filtered on-site within 1 hour of collection and within 2 hours of collection on the University of Gloucestershire (UoG) Francis Close Hall Campus, Cheltenham, when on-site filtering was not possible; these steps are outlined in the sections below.

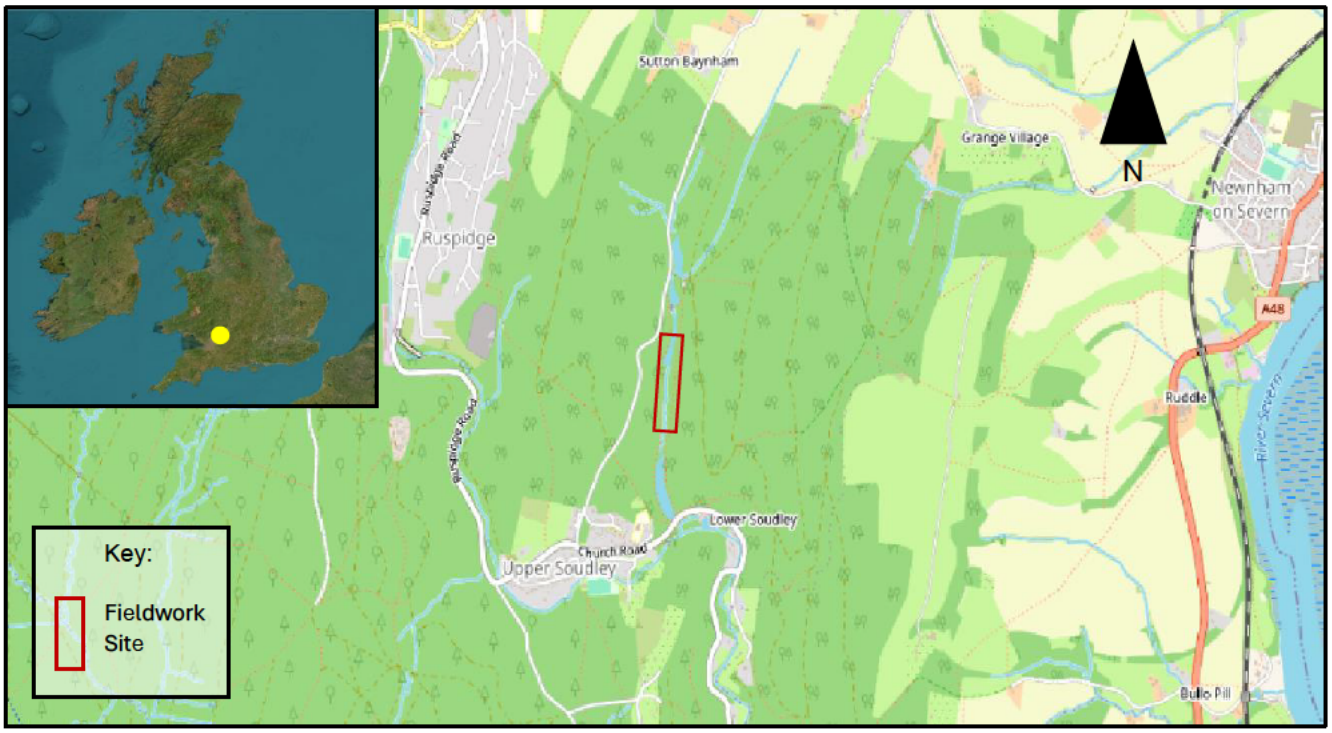


Figure 2.1 Fieldwork site located at the stream between the Soudley Ponds, Cinderford, within the Forest of Dean. The inlaid map shows the relative location of the sampling site within the United Kingdom.

2.2 Physical Characteristics

In addition to the water samples, one sediment sample was taken, and the physical characteristics of the river channel were measured at each sampling point on the final day of the fieldwork season. Sediment samples were taken using a trowel and gloves from the bank of the stream at each point. Approximately one full trowel of sediment was taken from the stream bed and placed into individual plastic bags labelled with the collection date and the sampling point code (Table 2.1). The four sediment samples were transported back to the laboratory for drying. The physical characteristics measured included water temperature, pH, dissolved oxygen (DO) and water flow velocity. The first three variables were measured at the fieldwork site using meters (the Hanna HI991300 Multimeter and HI9146 Dissolved Oxygen Meter) that were calibrated on-site and then placed into the stream channel to take readings. Water flow velocity was measured with a Valeport 801 Electromagnetic Flow Meter. This was held in the water for 30 sec at 40% of the channel depth and the average flow measurement recorded. All equipment was cleaned in the UoG laboratory before use at the field site.

Table 2.1 List of each sampling point with the given code for the paired water samples taken and their geographic location. Each filter was labelled with the code and date of collection.

Name	Sample Code	Location
Sampling Point 1	S1a/S1b	51°47’52’’N, 2°29’31’’W
Sampling Point 2	S2a/S2b	51°47’51’’N, 2°29’31’’W
Sampling Point 3	S3a/S3b	51°47’47’’N, 2°29’32’’W
Sampling Point 4	S4a/S4b	51°47’45’’N, 2°29’32’’W

2.2.1 Sediment Analysis

At the UoG laboratory, the contents of each sediment sample bag were oven-dried in a metal tray at 105 ℃ for 12 hours and weighed the following day using a fine balance to determine total dry weight of each sample (Figure 2.2a). Each dried sample was tipped one at a time into the top sieve of a sieve cascade which was secured onto a shaking plate. The sieve cascade was made of six steel mesh sieves, beginning with the largest mesh size (diameter 16 mm) at the top and the smallest size at the bottom (diameter 1 mm) of the cascade (Figure 2.2b). The samples were shaken at an amplitude of 5 for a total of 5 min. After shaking the sieves were separated, and the contents of each weighed on the fine balance. These data were used to determine the percentage fraction of each grain size category through dividing the dry contents of each sieve by the dry weight of the whole sample and multiplying by 100.

A. B.

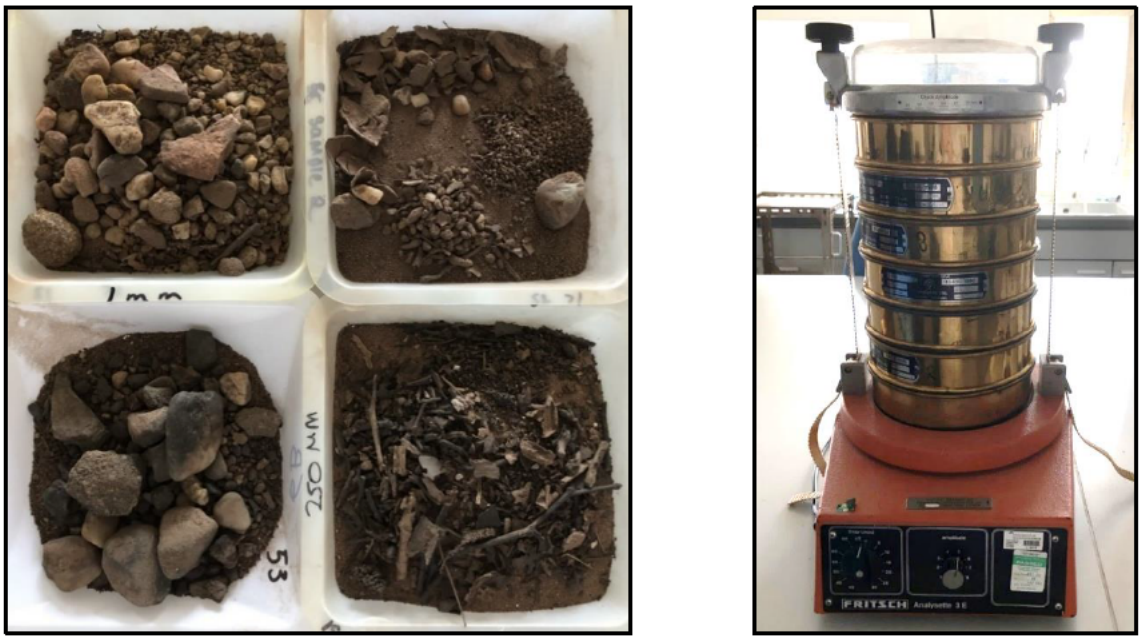


Figure 2.2 A) Each sediment sample after 12 hours drying at 105 ℃. Top left- sampling point 1, top right- sampling point 2, bottom left- sampling point 3 and bottom right- sampling point 4. B) The sieve cascade and shaking plate. The top sieve comprises of a 16-8 mm diameter steel mesh and the bottom sieve comprises of a 1 mm diameter steel mesh.

To decrease the volume of sediment <1 mm for each sample point for the laser spectroscopy process, the sediment was passed through a riffle box. Sediment entered the top of the riffle box where the grains were randomly split between two collection trays. This process was repeated until 10 mL of sediment <1 mm was transferred into a glass beaker. A loose slurry was created for each sample by combining a small volume of sediment with 10% Sodium Hexametaphosphate solution in the 10 mL glass beaker using a metal spatula. Sediment finer than 1 mm was processed using the Granulometer HydroMU 2000 (Malvern) which determines the ratio of sand, silt, and clay in each sample through laser spectroscopy (Figure 2.3). This instrument connected to a desktop computer where the Mastersizer 2000 software recorded the data of each sample.

Figure 2.3 Malvern Laser Granulometer HydroMU 2000 used to determine the ratio of sand, silt, and clay in the four sediment samples.

The system was emptied and flushed with 800 mL of clean tap water three times before a sample was tested. A beaker containing 800 mL of tap water was placed under the stirring mechanism and the sediment slurry was added gradually using the metal spatula until the Mastersizer 2000 software indicated enough slurry had been added. Each sample underwent 60 sec of ultrasonic mixing to disperse the grains before the ratio measurements were taken. Once a sample had been analysed the Granulometer was drained and rinsed another three times before the next sample slurry was added to a fresh beaker containing 800 mL of tap water. This process was repeated for all sediment samples and all data outputs were exported into a Microsoft Office Excel file.

2.3 eDNA Methodologies

This section outlines the optimisation of a working qPCR laboratory methodology for environmental water samples using the ATPase6 primers and probe taken from Zancolli et al. (2018). The primers and probe had not previously optimised, demonstrating the need for a section outlining the qPCR methodology development. Additionally, the results of this section support the findings of the project and are therefore mentioned within the discussion chapter.

2.3.1 Obtaining Samples

Tissue samples of *L. fluviatilis* and *L. planeri* were obtained from Natural Resources Wales, and the Monarch Genomic DNA Extraction and Purification kit sourced from New England

BioLabs (NEB) was used to extract the DNA. The samples had been preserved and transported in 70% ethanol solution. Prior to travelling to the field site, all in-field equipment was initially soaked in a 10% bleach solution for a minimum of 10 min in a laboratory environment to thoroughly degrade all surface and trace DNA. To create the dilute bleach solution, 100 mL of bleach was poured into a 1 L measuring cylinder. 900 mL of cold tap water was added to the cylinder to produce a 10% mixture. This protocol was also repeated after each sampling event at the laboratory located on the Francis Close Hall campus, Cheltenham. To remove trace DNA from in-field equipment and prevent degradation of target eDNA, the items were removed from the solution and thoroughly rinsed under cold tap water before returning to the fieldwork site. A fresh pair of gloves was worn every time a new sample was collected and cleaned, DNA-free equipment was kept separate from the used items to avoid cross-contamination.

Two water samples were collected at each sampling point. At three of the sampling points, two 1 L water samples were taken from the surface of the stream. At the fourth sampling point two 1 L water samples were taken at a depth approximately 10 cm from the channel bed to allow a comparison of eDNA presence at a range of depths. A clean pair of gloves was worn for every individual water sample to prevent cross contamination between samples. 50 mL of water was drawn into a 50 mL syringe, and a 5 µm syringe filter (Sartorius) was secured to the top of the syringe before expelling the water. The syringe filter was removed, and this process was repeated until the water was unable to pass through the filter. To assist with maximising the volume of water pushed through the filter, the syringe was placed into a caulking gun and compressed. After noting the volume of water sample filtered the syringe filter was removed and 50 mL of air was drawn into the syringe. This air was then pushed through the filter in order to expel any remaining water trapped within the filter unit. The 50 mL syringe and empty 1 L collection bottle were placed into a bag to keep them separate from DNA-free equipment.

Using a sterile 2 mL syringe, 1 mL of Longmire's preservative buffer solution was pushed all the way through the filter and two sterile locking end caps (luer) were secured onto either side of the filter. Each filter was labelled using the sampling code (Table 2.1) and date before being kept in a clean, separate bag. These steps were repeated for the other 7 water samples using a DNA-free 1 L bottle, fresh gloves, sterile filter, sterile end caps, 50 mL syringe, and 2 mL syringe. A 1 L bottle of filtered water taken to the site each sampling day was also filtered using the method outlined above as the daily filtration control to show no cross contamination occurred during the filtering process. All equipment was transported to and from the site on each sampling day. The filters and equipment were transported back to the laboratory where they were stored in a cold room and kept at 4℃ until the DNA was extracted within three weeks of sampling.

2.3.2 DNA Extractions

Using a pipette, the eDNA was removed from within the filters by pushing air through the bottom of the filter and expelling the Longmire's buffer out through the top of the filter. Fresh buffer was pushed through the bottom of the filters to wash the eDNA from the filter membrane. The contents of each filter were collected in a 1.5 mL Eppendorf tube which had been labelled with the corresponding collection date and sampling point code. The same Monarch Genomic DNA Extraction and Purification kit (NEB) was used to extract the eDNA

from the buffer solutions. The solutions underwent the Mammalian Whole Blood extraction and purification protocol (NEB), resulting in a final volume of 100 µl of each sample.

Skin samples underwent DNA extraction using the Animal Tissue extraction protocol (NEB), where the samples were incubated in a water bath for 1 hour at 56 ℃ at step three. At 5 min intervals the tubes were removed from the water bath and vortexed for 30 sec to aid with tissue breakup. These extractions similarly resulted in a final volume of 100 µl. To quantify the extracted tissue sample DNA, 2 µl was pipetted from each sample onto a Nanodrop (Fisher) that was cleaned between each sample using standard cleaning procedure. Additionally, four of the DNA extracted environmental water samples were selected at random and quantified. The final volume of extracted and purified eDNA was divided, pipetting 25 µl of the samples into another 1.5 mL Eppendorf so the original tube contained the remaining 75 µl. This step was taken with all of the extracted samples to prevent the whole sample degrading from repeated freeze-thaw action. All tubes were labelled with the sampling point code and the date of collection. The Eppendorfs containing 25 µl were kept in the freezer at -20 ℃ for the entirety of the research project.

2.3.3 PCR and qPCR Testing

Gradient polymerase chain reaction (PCR) was conducted to determine the optimum annealing temperature of primers and probes (Table 2.2). For a 100 pmol/µl solution, primers were rehydrated using DNA-free H_2O (dH_2O). A 100 mM stock concentration of each primer was prepared and diluted to a working concentration of 10 µM. 399 µl dH_2O was pipetted into the forward primer tube and 385 µl into the reverse primer using a Fisherbrand Lite pipette. 5 µl of each reconstituted primer with a concentration of 100 µM was pipetted into 45 µl of dH_2O to create a working stock. 1 µl of 18.6 µg/ml *Lampetra* template DNA was pipetted into five reaction tubes (Table 2.3). 1 µl of dH_2O was pipetted into a reaction tube for the No Template Control (NTC).

Table 2.2 Sequences for the probe, forward and reverse primers tested using a gradient PCR. The probes correspond to a *Lampetra* species, and the bold alleles highlight the position of a Single Nucleotide Polymorphism. Marker length is 83 base pairs.

Primer Sequence 5'-3'	Marker		Probe Sequence 5'-3'	Reference
Forward CACACCTGCAGGGATGATGT Reverse CGTAGCACGAGACGATTGTG	diagLpf	*L. fluviatilis* *L. planeri*	GCCGC**C**GCCTAATTACTGGA GCCGC**A**GCCTAATTACTGGA	Souissi et al. 2022

Table 2.3 PCR reaction components, recommended volumes, and final volumes (a maximum of 8 reactions). Final volume of each reaction was 50 µl using primers from Souissi et al. (2022).

Reagent	Protocol Volume / µl	Final Volume / µl
2x PCRBIO HS Taq Mix	25	200
Forward Primer (10 M)	2	16
Reverse Primer (10 M)	2	16
Template DNA	1	(1 per reaction tube)
PCR Grade dH_2O	20	160
Total Volume	50	392

The PCR temperatures were set from 60-65 ℃ using the gradient calculator on a Prime PCR Thermal Cycler (Techne). Before the cycling protocol began, the lid preheated to a temperature of 105 ℃ for two min. The temperature decreased to 95 ℃ for a total of one cycle to activate the Hot-Start (HS) Taq Mix. After one cycle, the temperature remained at 95 ℃ for 15 sec before decreasing to the gradient temperatures for 1 min. The temperature increased to 72 ℃ for a 10 sec final stage and these stages repeated for 40 consecutive cycles. For the final cycle, the temperature was kept at 72 ℃ for 10 min before decreasing to a holding temperature of 10 ℃. The products were held at 10 ℃ overnight in the Prime Thermal Cycler.

The PCR protocol outlined above was repeated using two different primer pairs (Table 2.4). Changes to the previous gradient PCR method included a wider temperature range of 55-65 ℃ and a M.M for a total of 12 reactions (Table 2.5) was produced for both primer pairs. A M.M for this PCR used the same reagents as previously listed (PCRBiosystems). Ten tubes contained 1 µl of 18.6 µg/ml *Lampetra* DNA and one reaction tube per pair contained 1 µl of dH_2O as the NTC. The two primer pairs were tested simultaneously, and all 22 reaction tubes were also held at 10 ℃ overnight to match the previous PCR experiment.

Table 2.4 Sequences for the probe, forward and reverse primers tested using gradient PCR.

Gene	Length (bp)	Primer Sequence 5'-3'	Probe Sequence 5'-3'	Reference
CO1	84	Forward GCCTTCCCACGTATAAACAACA Reverse TGCTTCAACTCCTGCGGAA	ACTTCCACCCTCACTCCTTC	Zancolli et al. 2018
ATPase6	90	Forward GCCTTAGCCCACTTATTACCAG Reverse AGGTCGGATGAAAAGGCTAAT	CACCCCAATTGCACTCATCC	Zancolli et al. 2018

Table 2.5 PCR reaction components, recommended protocol volumes and the final volume of each reagent. The final volume was 50 µl per reaction for both sets of Zancolli et al. (2018) primers.

Reagent	Protocol Volume / µl	Final Volume / µl
2x PCRBIO HS Taq Mix	25	300
Forward Primer (10 M)	2	24
Reverse Primer (10 M)	2	24
Template DNA	1	(1 per reaction tube)
PCR Grade dH_2O	20	240
Total Volume	50	588

qPCR was conducted for the probes (Table 2.2), labelled 'diagLpf' in the form of a molecular beacon (ThermoFisher Scientific). Included in the molecular beacon for *L. planeri* was the Single Nucleotide Polymorphism (SNP) at position 47. Similarly, for *L. fluviatilis*, the molecular beacon highlighted the same SNP at position 47. A M.M was produced using the reagents listed (PCRBiosystems), and the same primer stock solutions were used for this M.M (Table 2.6). A 1:5 serial dilution was created by pipetting 10 µl of *Lampetra* DNA with a measured concentration of 18.6 µg/ml into 40 µl of dH_2O in a PCR reaction tube. To continue the series, 10 µl of the first dilution was pipetted into a second reaction tube containing 40 µl of dH_2O. This process was repeated to produce a set of 11 serial dilutions and 1 µl of dH_2O was added to a twelfth reaction tube as the NTC. 20 µl of the dilution series was pipetted from the PCR tubes into wells on the top row of a 96 well qPCR plate and transferred into the machine (Azure Biosystems). The qPCR protocol consisted of 1 cycle at 95 ℃ for a total time of 2 min, followed by 40 cycles of 95 ℃ for 5 sec and was lowered to a temperature of 62 ℃ for 30 sec. The plate was held at 4 ℃ overnight in the machine.

Table 2.6 PCR reaction components, recommended protocol volumes and the final volume of each reagent (maximum of 14 reactions) on a 96 well qPCR plate.

Reagent	Protocol Volume / µl	Final Volume / µl
qPCRBIO Probe Blue Mix no ROX	10	140
Forward Primer (10 µM)	0.8	11.2
Reverse Primer (10 µM)	0.8	11.2
Probe (10 µM)	0.4	5.6
Template DNA	1	(1 per reaction tube)
PCR Grade dH_2O	7	98
Total Volume	20	266

Similarly, qPCR was also conducted for CO1 and ATPase6 probes (Table 2.7). The probes were rehydrated using dH_2O and ten working stock solutions were created in 1 mL Eppendorf tubes. A M.M was produced for a maximum of 9 reactions per probe using the listed reagents (PCRBiosystems), with 6 serial dilutions and 1 µl of dH_2O as the NTC. The dilution series was produced in PCR tubes using the same method outlined previously and then pipetted into the top three rows of the qPCR plate to show a comparison between each series. The same protocol was used for this qPCR run as previously listed, however the temperature used for the 40 cycles was 60 ℃ and again this plate was stored in the machine overnight at 4 ℃ (Azure Biosystems).

Table 2.7 PCR reaction components, the recommended volume and final reaction volumes per probe taken from Zancolli et al. (2018). The final volume in each well was 20 µl.

Reagent	Protocol Volume / µl	Final Volume / µl
qPCRBIO Probe Blue Mix no ROX	10	90
Forward Primer (10 µM)	0.8	7.2
Reverse Primer (10 µM)	0.8	7.2
Probe (10 µM)	0.4	3.6
Template DNA	1	(1 per reaction tube)
PCR Grade dH_2O	7	63
Total Volume	20	171

An initial pilot qPCR assay was conducted to assess the specificity of the ATPase6 primers and probes taken from Zancolli et al. (2018) against genomic DNA. Genomic DNA from *L. planeri* and *L. fluviatilis* tissue was standardised to create 20 ug/ml separate stocks of both species. To produce the stock for *L. planeri*, 8 µl of DNA was pipetted into a PCR tube containing 12 µl dH_2O. Similarly, to produce the stock for *L. fluviatilis* 18 µl of DNA was pipetted into a PCR tube containing 2 µl of dH_2O. Using the standardised stocks, three 1:5 dilution series were produced: one series for *L. planeri*, one series for *L. fluviatilis* and a combined series for both species. The combined series was produced by pipetting 10 µl of 10 µg/ml of *L. fluviatilis* and *L. planeri* genomic DNA into a PCR tube. Each series consisted of 6 dilutions from 40 µg/ml to 0.0128 µg/ml concentration and were run in duplicate on the 96 well assay plate (Table 2.8). To make the dilutions for each series, 10 µl of the stock was pipetted into a PCR tube containing 40 µl of dH_2O as outlined previously. Additionally, a No-Template Control (NTC) was run alongside each replicate dilution series to ensure no cross contamination occurred during the preparation or amplification steps. The four environmental samples which had been previously quantified using a Nanodrop were also run on the same plate, being tested in triplicate with additional NTCs (Figure 2.4).

Three M.Ms were produced for the *L. planeri*, *L. fluviatilis* and combined series, and a M.M was created for the environmental samples. The M.Ms for the dilution series were made for a maximum of 16 reactions including the NTCs, and the M.M for the environmental samples were made for a maximum of 9 reactions in separate 1.5 mL Eppendorf tubes. 2 µl of each dilution series and environmental sample was pipetted into the corresponding well. The four random environmental samples had been sampled on the following dates: 03.04.23, 21.04.23, 26.04.23 and 28.04.23 (Figure 2.4). These four samples were run alongside the dilution series to determine if lamprey DNA was present and cross reference the samples against know concentration curves. When each well had a total of 20 µl, a clear plastic film was stuck on to the plate and pressed down to ensure all wells were sealed tightly. The plate was spun in a centrifuge for 1 min at 1000 rpm before being loaded into the qPCR machine (Azure Biosystems) to ensure all reagents were gathered at the bottom of each well. The protocol for this pilot has been outlined previously. All reagents were kept on ice whilst the M.Ms were prepared to prevent DNA degradation, and were returned to the -20 ℃ freezer once finished.

Table 2.8 Reagent list, recommended volumes, and the final volumes for *L. planeri*, *L. fluviatilis* and combined dilution series. Final volumes for the environmental samples are also outlined.

Reagent	Protocol Volume / µl	Dilution Series Final Volume / µl	Water Sample Final Volume / µl
qPCRBIO Probe Blue Mix separate ROX	10	160	90
Forward Primer (10 µM)	0.8	12.8	7.2
Reverse Primer (10 µM)	0.8	12.8	7.2
Probe (10 µM)	0.4	6.4	3.6
Template DNA	1	(2 per well)	(2 per well)
PCR Grade dH_2O	Top up to total vol	96	54
Total Volume	20	320	180

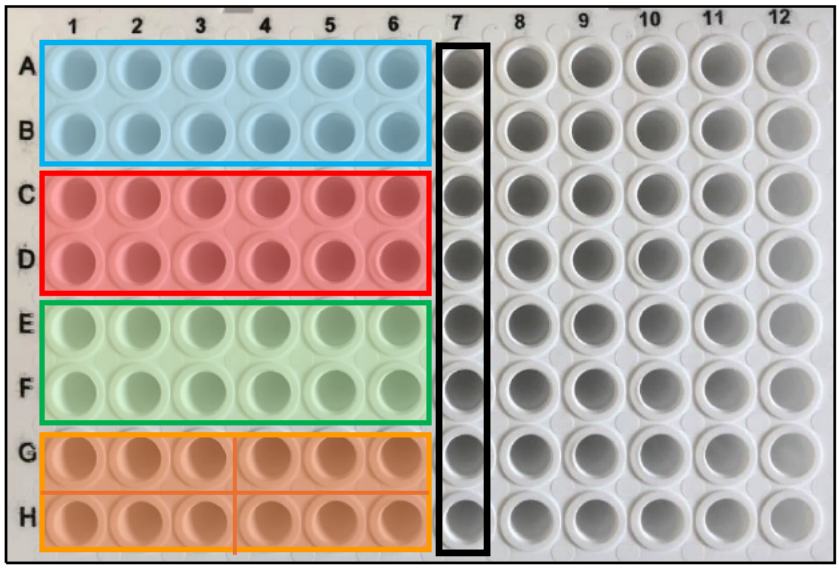


Figure 2.4 96 well assay plate layout for the pilot qPCR run. Blue) *L. planeri* dilution series replicates, (Red) *L. fluviatilis* series replicates, (Green) Combined DNA series replicates. The orange wells show the layout of the four environmental samples. Wells outlined in black were NTCs.

To optimise the method and create stronger quantification curves, the volume of DNA within the dilution series was doubled from 2 to 4 µl per well (Table 2.9). This modified the dilution series concentration range to 80 µg/ml 0.00512 µg/ml. Additionally, each dilution series was extended by one dilution (Figure 2.5) to allow better comparisons with environmental samples of lower DNA concentrations. Additionally, the volume of environmental sample was also increased to 2 to 7 µl per well (Table 2.9), increasing the sample concentrations to 238.49, 76.72, 129.71 and 130.06 µg/ml. This step was changed to reduce the cycle number of the environmental samples for better comparison against the standardised curves. The number of replicates was decreased by one (Figure 2.5) to preserve the volume of remaining sample. For all dilution series replicates the volume of M.M pipetted into each well was 16 µl and the volume of M.M pipetted into the environmental sample wells was 13 µl. The steps that followed remained the same as previously outlined.

Table 2.9 Reagent list, recommended volumes, and the final volumes for the serial dilutions (maximum of 18 reactions) and environmental samples (maximum of 12 reactions).

	Serial dilutions		Environmental samples	
Reagent	Protocol Volume / µl	Final Volume / µl	Protocol Volume / µl	Final Volume / µl
qPCRBIO Probe Blue Mix separate ROX	10	180	10	120
Forward Primer (10 µM)	0.8	14.4	0.8	9.6
Reverse Primer (10 µM)	0.8	14.4	0.8	9.6
Probe (10 µM)	0.4	7.2	0.4	4.8
Template DNA	1	(4 per well)	1	(7 per well)
PCR Grade dH_2O	Top up to total vol	72	Top up to total vol	12
Total Volume	20	360	20	240

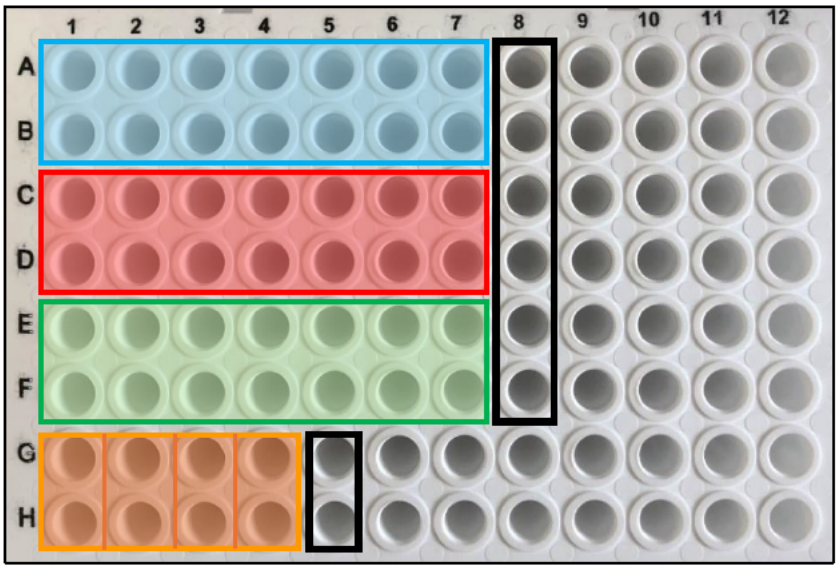


Figure 2.5 96 well qPCR assay plate. Blue) *L. planeri* dilution series replicates, (Red) *L. fluviatilis* series replicates, (Green) Combined DNA series replicates. The orange wells show the four environmental samples tested. Wells outlined in black were NTCs.

For the next qPCR run the number of environmental samples was increased to a full plate of 25, allowing for NTCs and replicates. A M.M was created in a 1.5 mL Eppendorf tube for combined DNA dilution series replicates (Table 2.10). The volume of DNA pipetted into each well of the series was decreased from 4 µl to 2 µl. Additionally, a M.M was produced in a 2 mL Eppendorf tube for the environmental sample replicates (Table 2.10). Each sample was tested for presence-absence using three replicates for an average and compared against the known concentrations of the dilution series. The volume of sample was decreased from 7 µl to 2 µl. Another development for this qPCR assay was the addition of Bovine Serum Albumin (BSA) and Dimethyl Sulfoxide (DMSO). A working stock of BSA was created by weighing out 0.006 g on a fine balance and adding that to 10 mL of dH_2O. This solution was then mixed on a vortex for 30 sec to combine the two components. 18 µl of M.M was pipetted into each well and 2 µl of sample was pipetted in after to ensure each well had a total volume of 20 µl. The protocol followed previous steps.

Table 2.10 Reagent list, recommended volumes, and the final volumes for the dilution series and for 25 environmental samples (maximum of 80 reactions).

	Dilution series		25 environmental samples	
Reagent	Protocol Volume / µl	Final Volume / µl	Protocol Volume / µl	Final Volume / µl
qPCRBIO Probe Blue Mix separate ROX	10	250	10	800
Forward Primer (10 µM)	0.8	20	0.8	64
Reverse Primer (10 µM)	0.8	20	0.8	64
Probe (10 µM)	0.4	10	0.4	32
Template DNA	1	(2 per well)	1	(2 per well)
BSA	NA	0.5	NA	1.5
DMSO	NA	6.25	NA	18.75
PCR Grade dH_2O	Top up to total vol	143.25	Top up to total vol	569.75
Total Volume	20	500	20	1,600

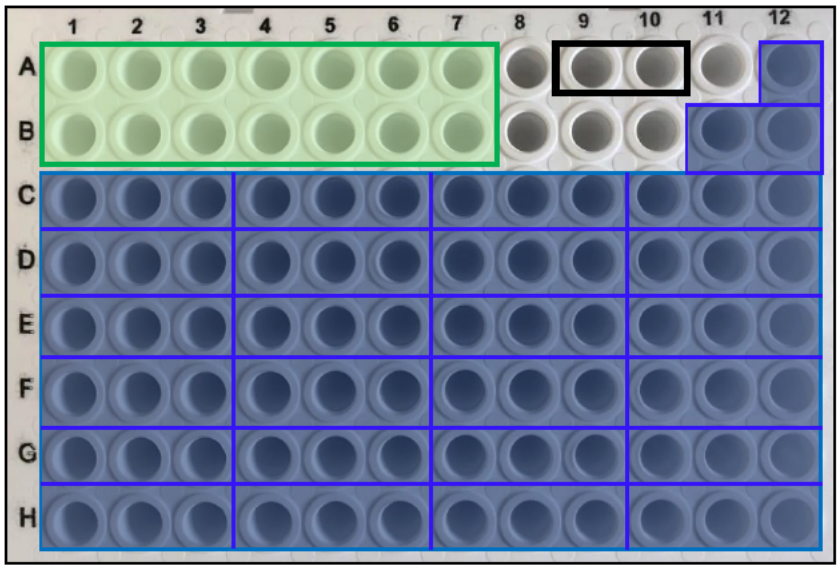


Figure 2.6 Plate layout for 25 environmental samples with three replicates (blue). Combined *Lampetra* DNA serial dilution replicates (green). NTCs are outlined in black, well A9 was an NTC for the dilution series and well A10 was an NTC for the environmental samples.

Due to poor amplification curves in the previous qPCR run, five random negative environmental samples were chosen from the previous assay plate of 25 to undergo testing to check whether PCR inhibitors were present in the samples (Figure 2.7). These five samples were collected: 07.04.23, 10.04.23, 12.04.23, 17.04.23 and 26.04.23. The samples were spiked with 1.6 µg/ml of combined *Lampetra* DNA. 2 µl of the 8 ug dilution series sample was pipetted into 8 µl of a chosen environmental sample to make the final concentration of DNA 1.6 µg/ml. This step was repeated for the other four environmental samples. Two combined DNA dilution series were run alongside the five samples with the addition of a lower dilution, increasing the range of concentration 40 µg/ml to 0.000512 µg/ml to allow for improved comparisons between the standards and environmental samples. Each spiked sample was tested in triplicate and the dilution series were tested in duplicate. The reagent volumes were the same for both the dilution series and spiked environmental sample M.Ms which were pipetted into 1.5 mL Eppendorf tubes (Table 2.11). The protocol temperatures and timings were kept identical to the previous runs; however, the number of cycles was increased from 40 to 45 in order to extend the amplification curves produced by the environmental samples.

Table 2.11 Reagent list, recommended volumes, and the final volumes for the combined DNA dilution series replicates and spiked samples (maximum of 20 reactions for the standards and samples).

Reagent	Protocol Volume / µl	Final Volume / µl
qPCRBIO Probe Blue Mix separate ROX	10	200
Forward Primer (10 µM)	0.8	16
Reverse Primer (10 µM)	0.8	16
Probe (10 µM)	0.4	8
Template DNA	1	(2 per well)
BSA	NA	0.4
DMSO	NA	6
PCR Grade dH_2O	Top up to total vol	113.6
Total Volume	20	400

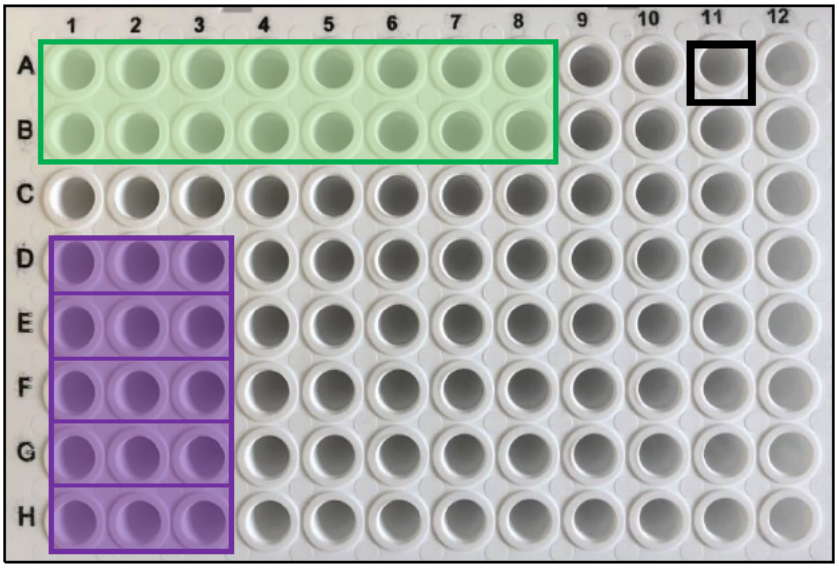


Figure 2.7 Plate layout of five spiked negative environmental samples tested in triplicate (purple), and a duplicate dilution series of combined *Lampetra* DNA (green). The well outlined in black was the NTC.

2.3.4 Final qPCR Protocol

The final iteration of the qPCR method was used to test the remaining environmental samples. A duplicate dilution series with a total 8 standards was pipetted at the top of each subsequent plate in rows A and B. All plates with a total of 25 samples had the same layout as the initial plate (Figure 2.6). Additionally, the only change to the M.M volumes of reagents was an increase from 2 µl to 4 µl of sample and standard in each reaction well to ensure a higher chance of detecting DNA present, all other volumes were kept the same (Table 2.10). The M.Ms for the dilution series was produced in 1.5 mL Eppendorf tubes and the M.Ms for the samples were created in 2 mL Eppendorf tubes. For the samples that resulted in neither a clear positive or negative, for example a higher quantification cycle (Cq) value or an early fluorescence plateau, a second set of replicates were tested on the final assay plate. In total, two pilot qPCR assays were used to optimise the primers and probe, one assay plate was used to test for inhibition and the remaining six plates tested environmental samples.

2.3.5 Agarose Gel

PCR amplicons were visualised on a 2% agarose gel was produced to determine optimum annealing temperature. 100 mL of a 1 x Tris Acetate EDTA (TAE) running buffer solution was made by diluting 10 mL of 10 x TAE buffer into 90 mL of distilled H_2O in a measuring cylinder. A fresh solution of 1 x TAE buffer was produced on the day of each gel electrophoresis. The TAE solution was decanted into a 200 mL flask and 2 g of dry research-grade agarose powder (ThermoFisher Scientific) was added into the flask. The flask was heated at 900 W for 15 sec periods in a microwave, removed and shaken until all agarose powder dissolved and there were no air bubbles remaining. The solution was left to cool until all steaming ceased before 10 µl of Sybr Safe red gel stain (ThermoFisher Scientific) was pipetted into the flask and gently mixed. 10 µl was the recommended volume of gel stain.

2.3.6 Gel Electrophoresis

Two rubber seals were fitted to each open end of the dock to ensure that the gel solution remained inside. An 8 well gel comb was placed at one end of the dock to test the six reaction tubes for Souissi et al. (2022) and the gel solution was poured in. Air bubbles in the solution were removed gently using a sterile pipette tip. Whilst the gel was setting, 600 mL of 1 x TAE buffer was prepared using the previous method in a 1 L flask. Once set, the gel comb and rubber seals were removed before the gel dock was placed into the gel tank. The TAE buffer solution was added to the gel tank up to the maximum fill indicator line. To confirm the expected PCR amplificon had been amplified, the amplicons were compared with a 50-150 base pair (bp) ladder. 10 µl of purple loading dye was pipetted and mixed into each of the reaction tubes by drawing the product into the pipette tip and expelling it several times. 20 µl of each PCR product was pipetted into a well until all reaction tube products had been loaded onto the gel and the tank lid was secured.

20 µl of NTC was pipetted into the final well as a negative control. The gel was run at 110 V for 30 min and placed in a gel scanner (Azure Biosystems). After primer optimisation, the probes taken from Souissi et al. (2022) underwent qPCR and these products were run through gel electrophoresis. This gel was produced as outlined previously; however, the larger gel dock was used with a 16 well gel comb to test 12 qPCR reactions. The same 50-150 bp ladder and loading dye volumes were used as listed above. The loading dye was pipetted into the reaction wells of the qPCR 96-well assay plate. 40 µl of each qPCR product was pipetted into each well and was similarly run for 30 min at 110 V before being scanned on the gel scanner.

Methods as previously described were repeated for all primer pairs but with the following modifications for the Zancolli et al. (2018) primers. A larger gel dock was used for these primers to allow electrophoresis of each pair to occur simultaneously. Additionally, two 16 well gel combs were placed into the large gel dock- one located at the top of the dock, the other situated in the middle of the dock to form two equally spaced rows of wells. To confirm the expected PCR amplificon had been amplified, the amplicons were compared with a 100-1000 bp ladder. This gel was run for 45 min at 110 V to allow the bands to move through at least 50 % of the gel length. The gel was removed from the tank and transferred to the gel scanner.

2.3.7 Sequencing

Positive qPCR products representative of environmental water samples were retained; these were cleaned using a Qiaquick PCR purification kit (Qiagen, N.V.) quantified using a Qubit 4.0 (Thermo Fisher Scientific) and sent to Source Bioscience UK for Sanger sequencing. The amplicons were derived from samples collected during April sampling. The sequence data were visualized, checked for miscalls and aligned using MEGA version 10.0.4 (Kumar et al., 2018). Sequences were then compared to *Lampetra sp.* sequence on the National Centre for Biotechnology database using the basic local alignment search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the presence of the target species.

3. Results

3.1 Physical Characteristics Analysis

The flow of the stream at the field site varied between the sampling points. The velocity at sampling point 1 was faster (0.201 m/s) compared to sampling point 2 which had a slower velocity of 0.054 m/s (Table 3.1). Sampling point 4 had the slowest velocity of all the points with a rate of 0.02 m/s (Table 3.1). Water temperature across the sampling points differed, with sampling point 4 producing the lowest measurement (9.4 ℃). This was compared to sampling point 1 which was recorded as the warmest point (9.8 ℃). Across the sampling points, table 3.1 showed the pH was most alkaline at point 4 (8.04). The pH remained the same at points 2 and 3 (8.01), however the pH became more neutral at sampling point 1 (7.95). Water at sampling point 4 had the highest DO content (12.06 ppm) of all sampling points (Table 3.1). Additionally, sampling points 1 and 3 shared similar DO contents (9.72 and 9.83 ppm respectively), with little difference between the two points. Sampling point 2 had the second highest DO content when compared to the rest of the points, with a concentration of 10.07 ppm (Table 3.1).

Table 3.1 Physical characteristic averages taken on 28.04.2023 at each of the sampling points at Soudley Ponds, Cinderford.

Sampling Point	Temperature (℃)	pH	DO (ppm)	Flow (m/s)
1	9.8	8.04	9.72	0.201
2	9.7	8.01	10.07	0.054
3	9.6	8.01	9.83	0.122
4	9.4	7.95	12.06	0.020

3.2 Grain Size Analysis

Figure 3.1 showed that the sampling point with the highest percentage of larger grain sediments was point 1; the results from this showed that 51% of the sample consisted of grains 16-8 mm in diameter, compared to sampling points 3 and 4 which showed 19% and 29% respectively (Figure 3.1). The sampling point with the lowest percentage of larger grains was point 2. At sampling point 2, the percentage of grains 16-8 mm in diameter present at was 0% (Figure 3.1). Sampling point 2 also had the lowest percentage of grains 8-4 mm in diameter (6%) but showed a high percentage of mid-sized grains 4-2 mm in diameter (23%). These results were compared to sampling points 1 and 4, which both had higher percentages of medium grains (15% and 20% respectively). Sediment of that size is often described as granules (USDA, 2023). To contrast, sampling point 2 had the highest percentage of small grains, with 33% of grains 2-1 mm in diameter and 38% of grains less than 1 mm (Figure 3.1). Sampling point 1 had the smallest percentages of small grains; for example, 3% of grains less than 1 mm in diameter (Figure 3.1). When comparing the contents of each sediment sample, figure 3.1 showed that the sampling point with the most

even percentage distribution was point 4. Point 4 had the most similar percentages for all grain sizes tested in comparison to sampling point 2 which had was composed mainly of smaller grains.

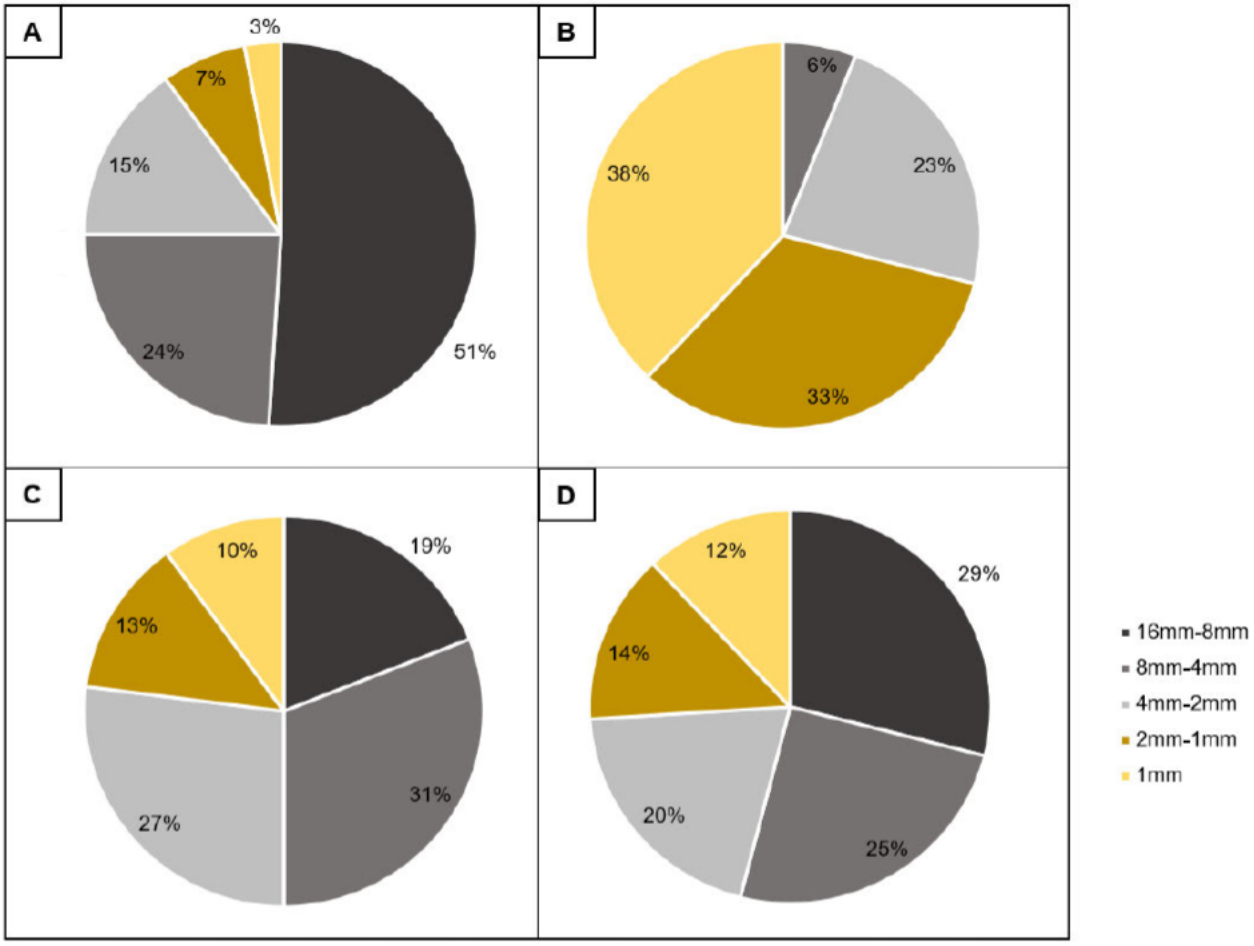


Figure 3.1 Bedload sediment distribution for sample 1 (A), sample 2 (B), sample 3 (C) and sample 4 (D). Distribution was calculated using a sieve cascade. Colours indicate sediment types, with the coarsest as dark grey and the finest as pale yellow.

Similarly to the sieve cascade results, figure 3.2 showed that sampling point 1 had the highest percentage of large particles. For example, point 1 had the highest percentage of particles 0.5 microns in diameter (17%). Particles of these sizes are known as coarse sand (USDA, 2023). When comparing the sampling points, point 2 showed the highest percentages of grains 2-1 mm and less than 1 mm in diameter (Figure 3.2). Over two thirds (71%) of grains in the sediment sample taken from point 2 were 2 mm in diameter or less (Figure 3.2). Particle analysis also showed that point 2 had the highest volume of fine sand particles of all sampling points with 44% (Figure 3.2). It can be seen from figure 3.2 that point 3 had a higher percentage of larger particles such as coarse and medium sand (9.5% and 30% respectively) when compared to sampling point 4 (2% and 22%). According to

figure 3.2, sampling points 3 and 4 were the only points with particles 0.0001 microns in diameter (0.5% and 0.51% respectively).

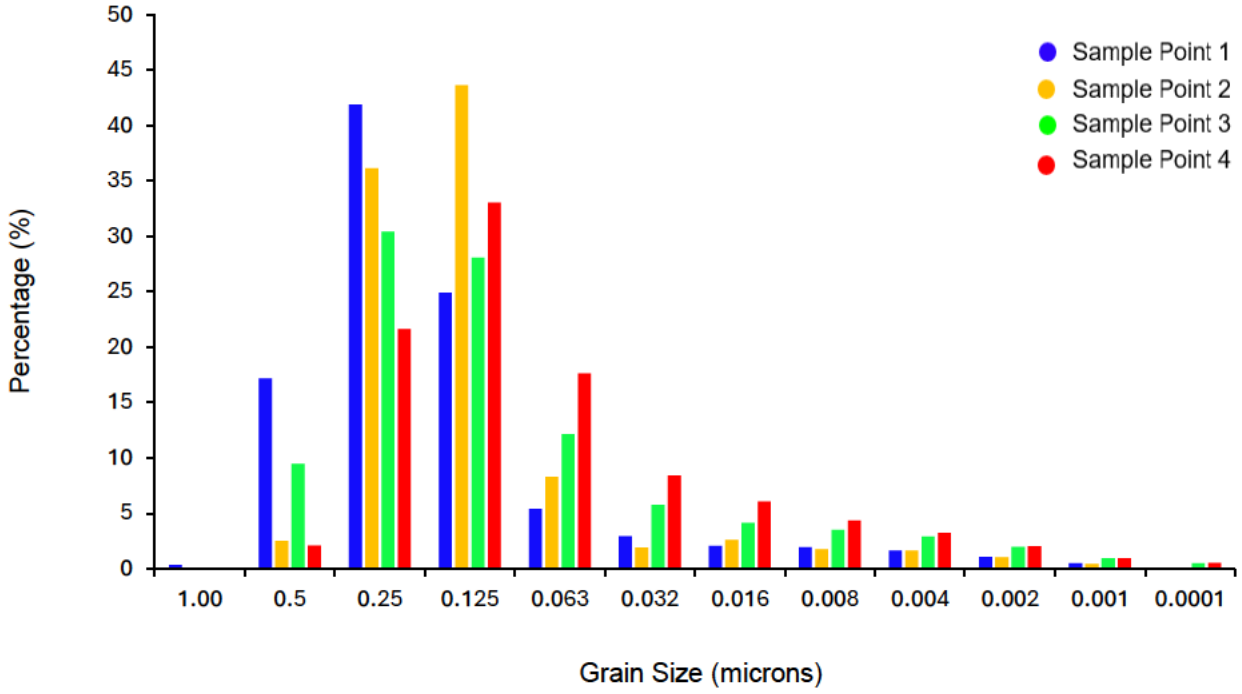


Figure 3.2 Percentage of Sand:Silt:Clay grains for each sampling point measured using a Granulometer 2000 (Malvern Panalytical).

When categorising the bedload sediment based on particle sizes, point 1 was described as a sandy substrate (Figure 3.3). The spectroscopy analysis of sampling point 1 reflected the data shown in figure 3.1, further promoting point 1 as a suitable habitat for spawning individuals based on the abundance of larger particles. This result was key to determining the category of bedload sediment as sampling point 2 was also described as sand (Figure 3.3). As the same description was used to describe sampling points 1 and 2, it was determined that the two samples showed the most similar silt:clay particle ratio of all samples shown by the proximity of the data points (Figure 3.3). The difference in the percentages across the range of particle sizes can be observed in figure 3.3, where there is a distinct separation between points 3 and 4 based on the ratios of sand:silt:clay. Sampling point 3 was classed as loamy sand bedload, whereas point 4 was located on the border between loamy sand and sandy loam. Sampling point 4 had the highest ratios of silt and clay particles compared with the other samples (Figure 3.3). Additionally, sampling point 4 had higher percentages for the smaller particles measuring between 0.125-0.0001 microns in diameter.

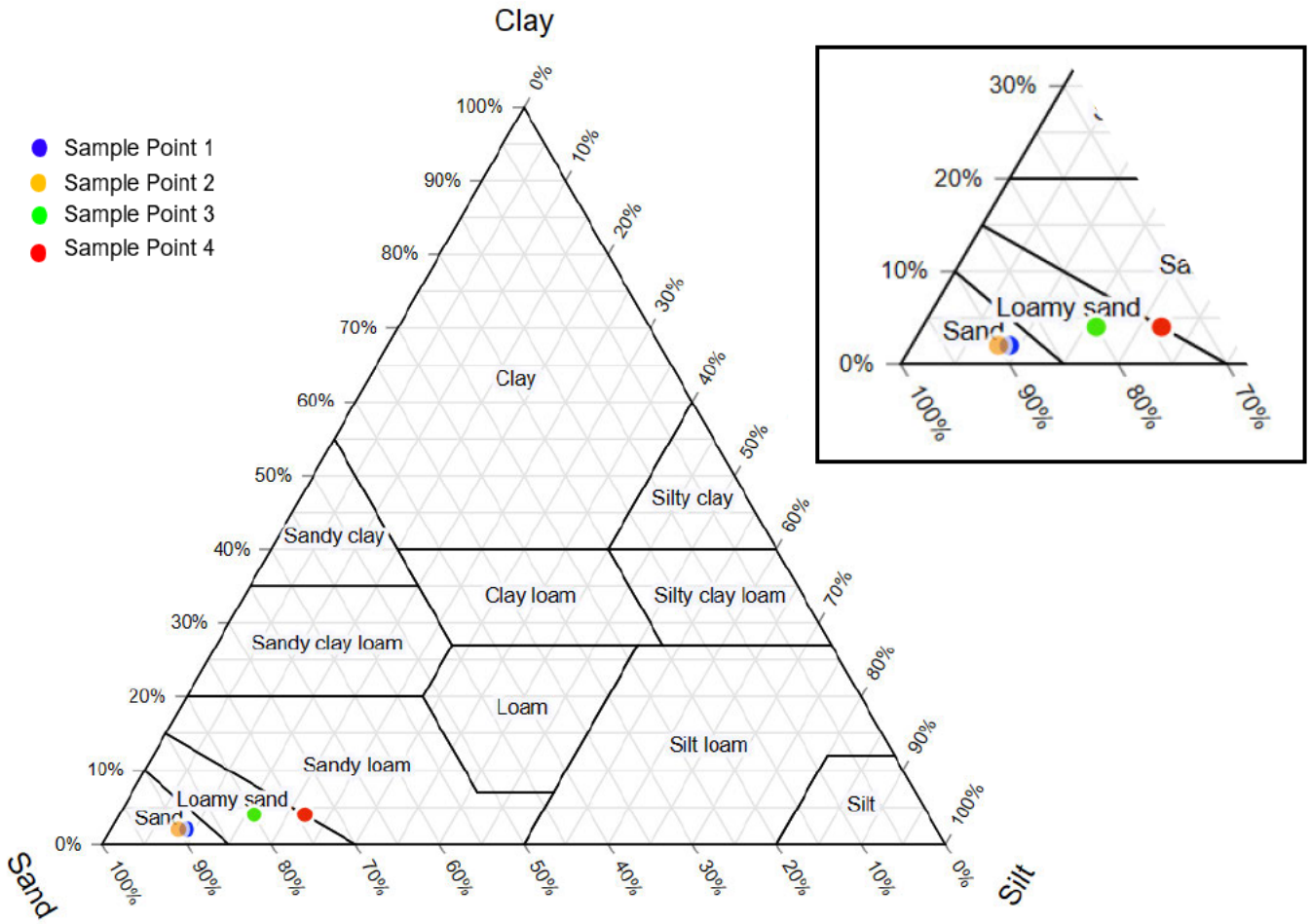
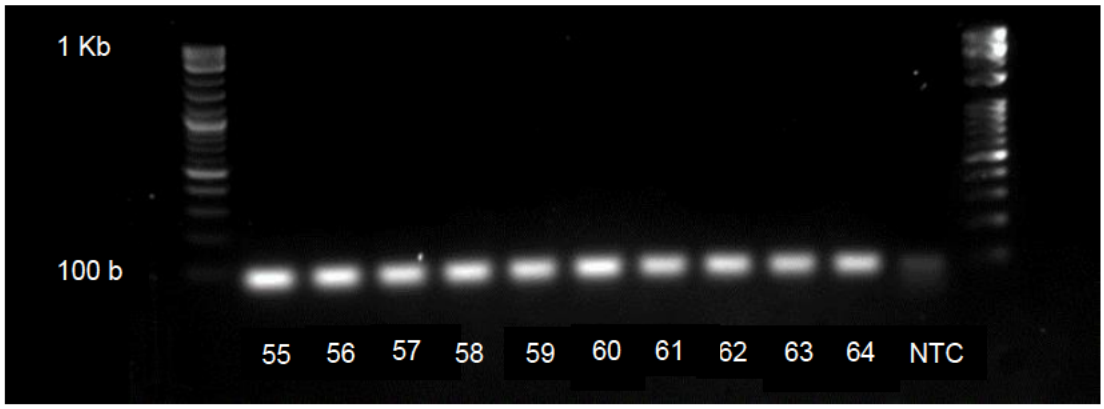


Figure 3.3 Bedload sediment classification of four samples from Soudley Ponds. Percentages were determined using a Granulometer 2000 (Malvern Panalytical). Sediment categories are based on the United States Department of Agriculture (USDA) classification system.

3.3 Gel Electrophoresis Analysis

CO1 primers taken from Zancolli et al. (2018) showed positive amplification of the *Lampetra* target amplicon, with bright banding visible across the entire temperature range of 55-65 ℃ (Figure 3.4a). The bands were shown to correspond to the 84 base pair (bp) fragment length on the 100-1000 base ladder. Bands at temperatures 55, 56 and 60 ℃ had the strongest fluorescence of all the temperatures tested using the CO1 primer pair (Figure 3.4a). ATPase6 primers also showed positive amplification of the target amplicon, with little visible difference between the bands (Figure 3.4b). All bands were located at the expected fragment position, in line with the 90 bp length on the same 100-1000 base DNA ladder. The No-Template Control (NTC) for the ATPase6 primers showed less primer-dimer fluorescence than the CO1 primer pair (Figure 3.4b).

A.



B.

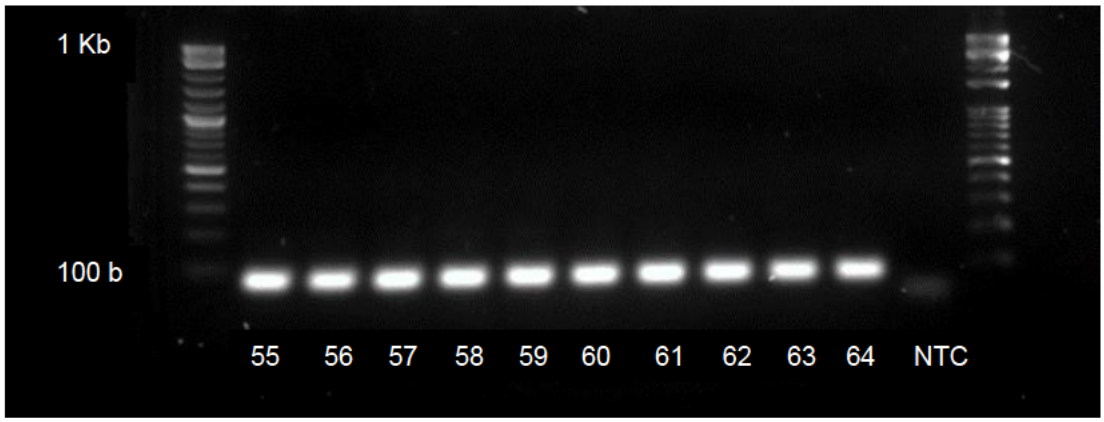


Figure 3.4 Gel of gradient PCR used to test optimum annealing temperatures with a temperature range of 55 – 65 ℃. A) CO1 primers and (B) ATPase6 primers. Both sets of primer were taken from Zancolli et al. (2018).

Each environmental sample tested on a gel showed positive amplification for the target amplicon using the ATPase6 primers (Figure 3.5). All bands aligned with 90 bp on the 100-1000 base ladder, which was the expected length of the amplified fragments. There appeared to be variation in the strength of the bands, with sample 4b (well 2) having the weakest fluorescence and sample 4a (well 5) having the strongest fluorescence (Figure 3.5). The NTC did show possible non-specific binding as fluorescence can be seen on the gel at well 6, with an initial band followed by a second in line with the environmental samples.

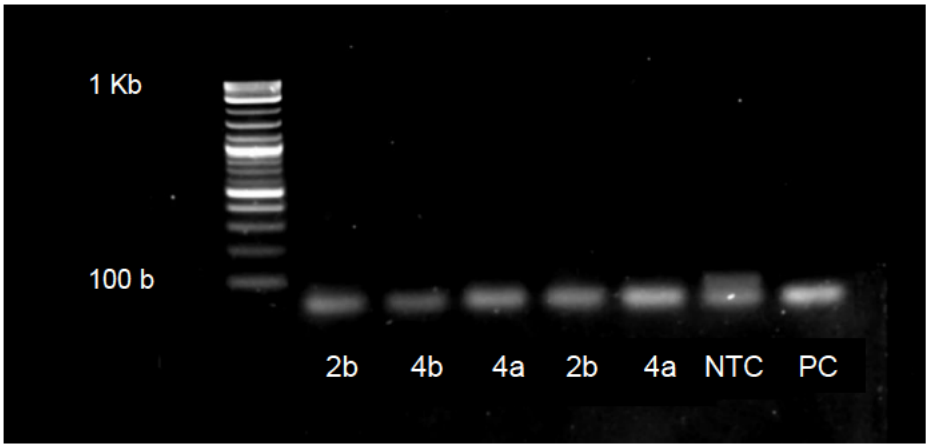


Figure 3.5 Gel of environmental water sample positive qPCR products. Well 1) collected 12.04.2023, (Well 2) collected 28.04.2023, (Well 3) collected 21.04.2023, (Well 4) collected 26.04.2023, (Well 5) collected 21.04.2023, (Well 6) No Template Control and (Well 7) Positive Control.

3.4 qPCR Presence-Absence

The results from the qPCR inhibitor test determined that there were no in PCR inhibitors present within the extracted and purified DNA (Figure 3.6). Spiked samples showed positive detections for all replicates. The quantification of cycles (Cq) values matched the third serial dilution at a concentration of 1.6 µg/ml (Figure 3.6). Figure 3.7a showed a successful qPCR assay from a plate of 25 environmental samples alongside a 1:5 dilution series. The replicate amplification curves for dilutions 1-6 showed matching Cq values, indicating the concentration of *Lampetra* DNA was standardised across those replicates (Figure 3.7a). The lower concentration dilutions (0.00512 and 0.001024 µg/ml) showed a larger difference in Cq value between the replicates. Environmental sample curves started from cycle 35 and were compared to the eighth dilution in the series (Figure 3.7a). A standard curve from the same qPCR assay showed the quantity of *Lampetra* DNA in relation to the Cq value of each pair of serial dilutions (Figure 3.7b). This showed a higher quantity of DNA resulted in a lower Cq value. For example, the first dilution pair had a concentration of 0.001024 µg/ml and a Cq value of 36, whereas the final dilution pair had a concentration of 40 µg/ml and a Cq value of 16 (Figure 3.7b).

There was no *Lampetra* eDNA detected at the sampling points between 03.04.23 and 17.04.23 (Figure 3.8a). On 19.04.23, points 1 and 2 showed 83% positive detection for *Lampetra* eDNA, and points 3 and 4 showed 100% positive detection (Figure 3.8b). Total number of positive replicates for points 1 and 2 was five, and the total number of positive replicates for points 3 and 4 was six (Figure 3.8b). On 21.04.23, sampling points 1 and 3 showed 67% positive detection of *Lampetra* eDNA with four positive replicates out of six (Figure 3.8c). Sampling point 2 remained unchanged from the previous sampling day with an 83% positive detection for eDNA and the results of point 4 also remained unchanged, showing a 100% detection of *Lampetra* presence with six positive replicates (Figure 3.8c). Figure 3.8d shows that on 24.04.23, the number of positive replicates for sampling point 1

increased from four to six, with 100% positive detection. The number of positive replicates at sampling points 2, 3, and 4 all decreased from the previous day, with point 3 showing the lowest positive detections of 34% (Figure 3.8d). At the end of the fieldwork season, the presence of *Lampetra* eDNA fell to 17% for all four sampling points across the site and only one replicate at each point showed positive detection (Figures 3.8e and 3.9).

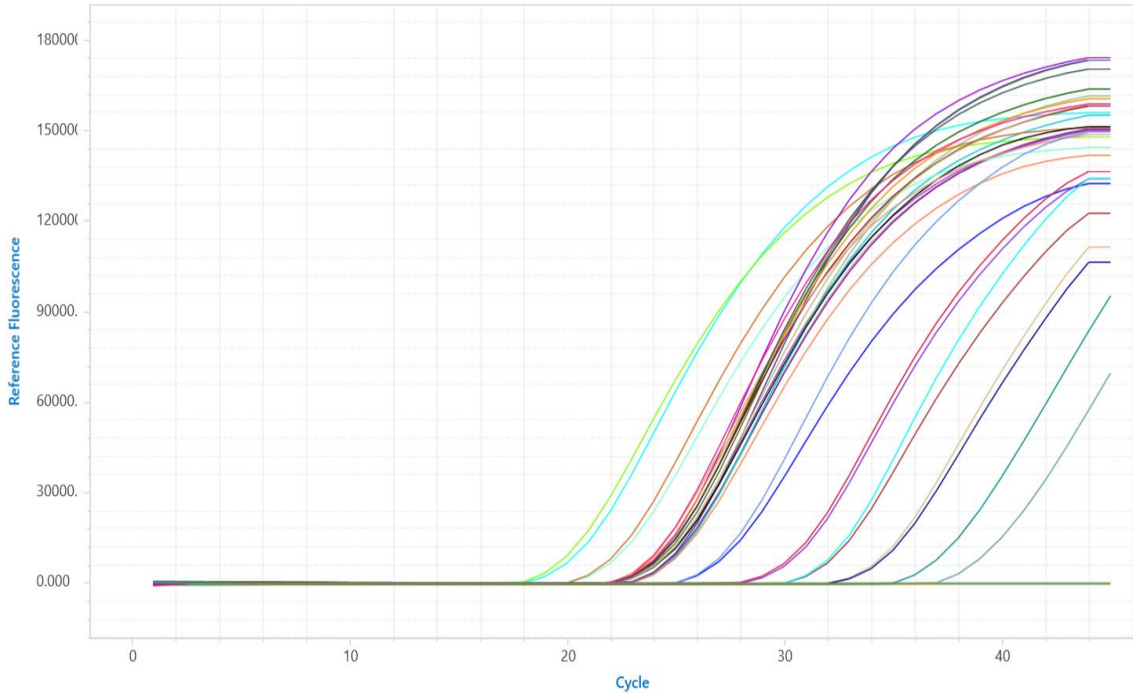
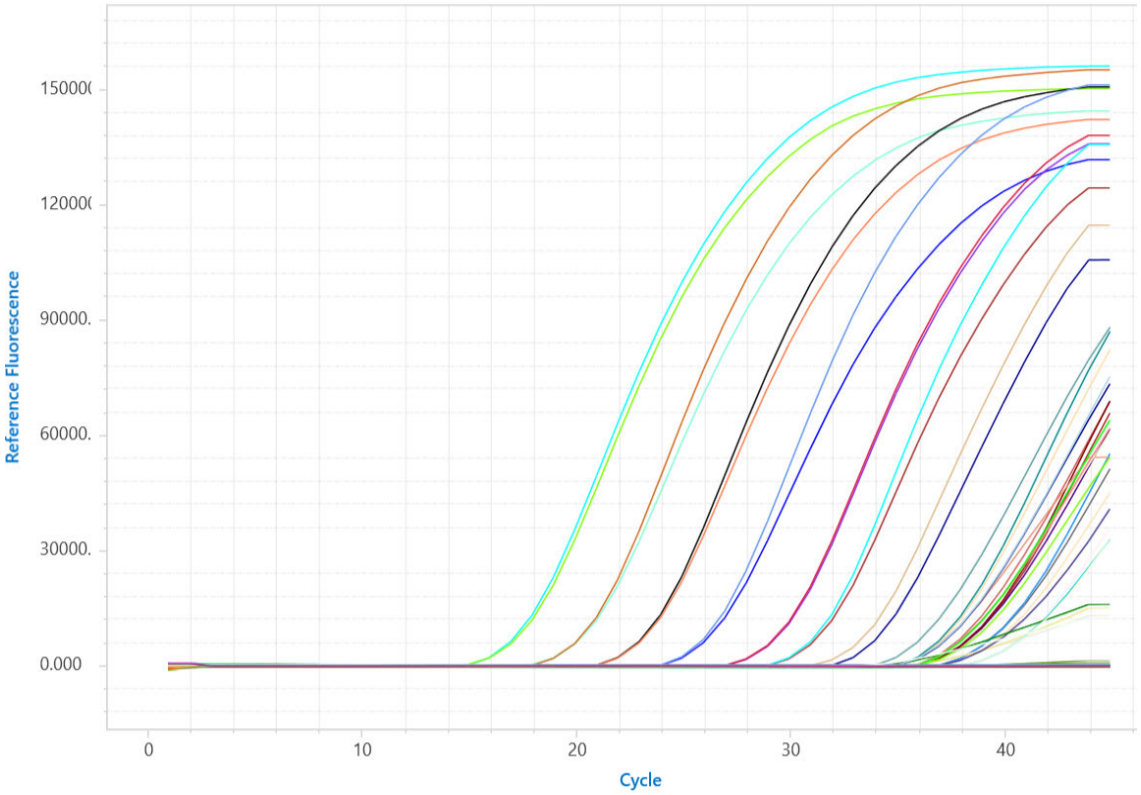


Figure 3.6 qPCR assay results from a PCR inhibitors check using a real-time qPCR machine (Azure Biosystems).

A.



B.

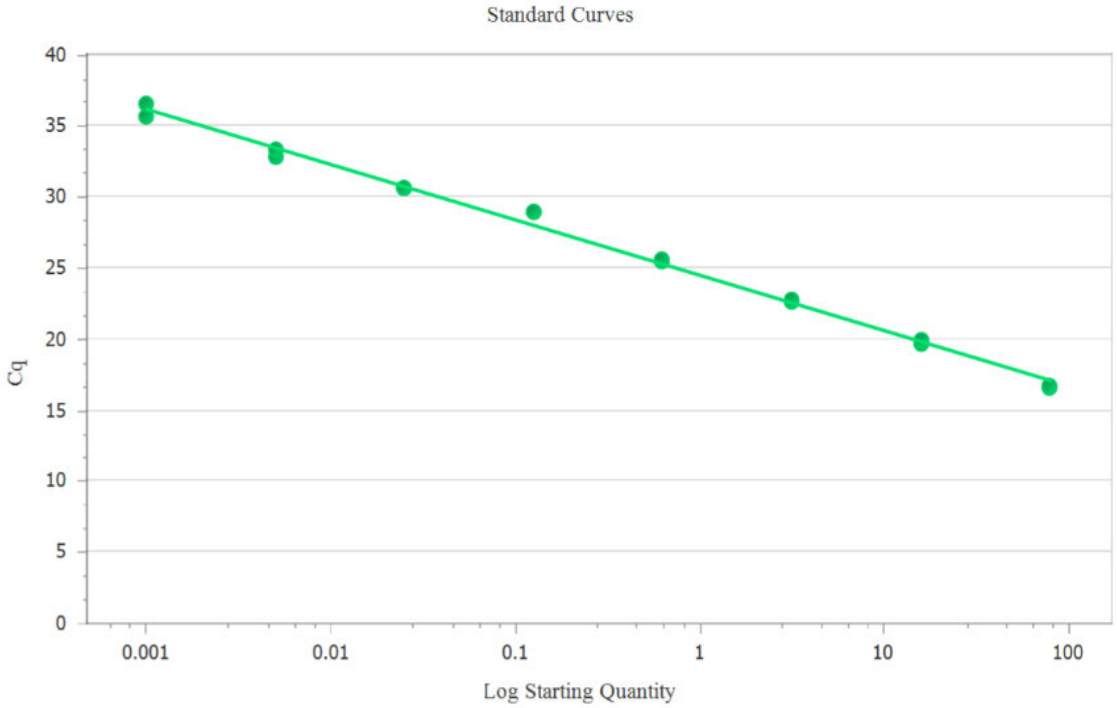


Figure 3.7 A) qPCR assay results showing the amplification of target amplicons in 25 environmental water samples compared with a 1:5 dilution series of genomic *L. planeri* and *L. fluviatilis* DNA and (B) Dilution series standard curve from the same qPCR analysis. All analysis was conducted on real-time qPCR machine (Azure Biosystems).

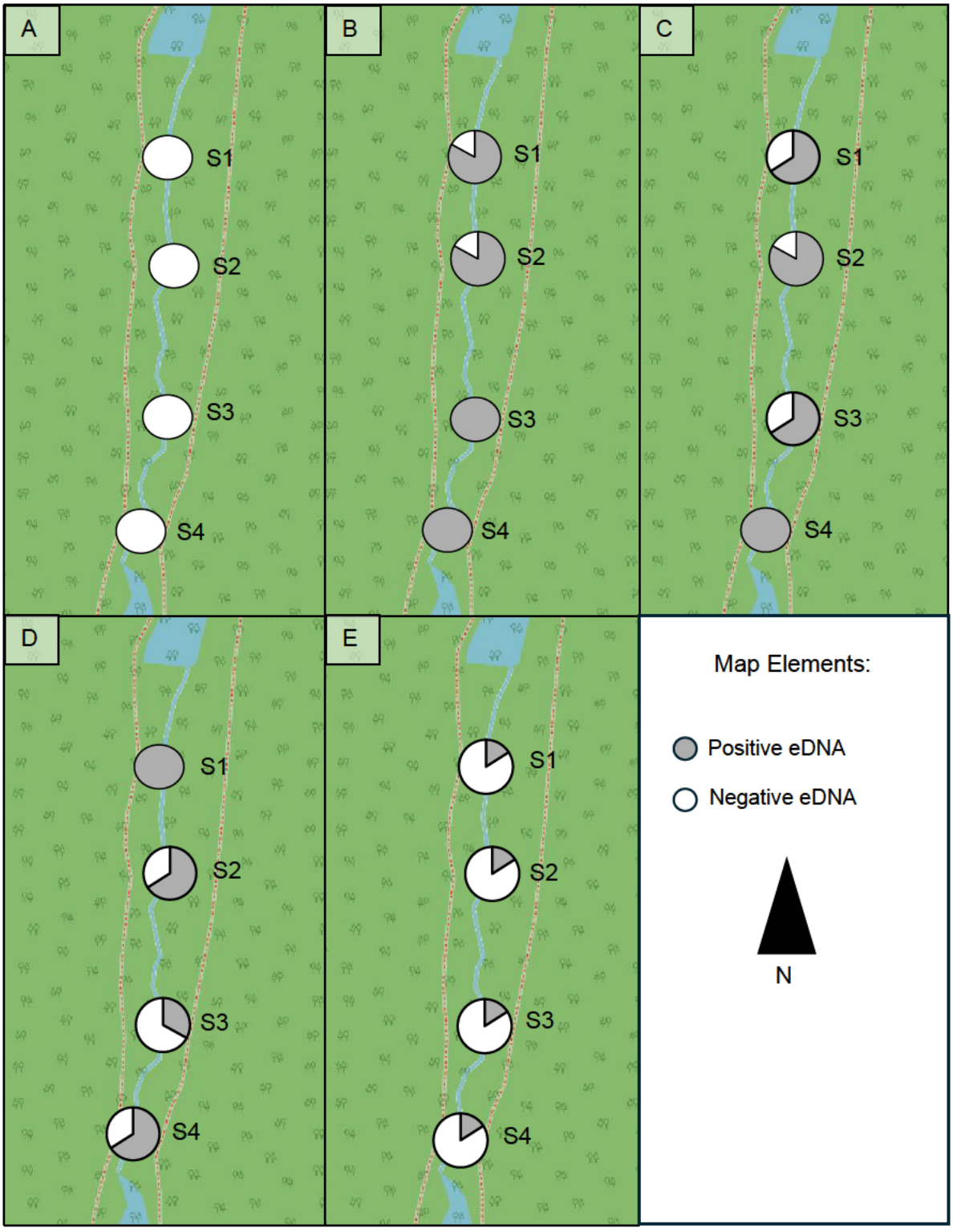


Figure 3.8 A) Map highlighting the presence of *Lampetra* eDNA at each sampling point for water samples collected from the 03.04.2023 to the 17.04.2023, (B) Presence on the 19.04.2023, (C) Presence on the 21.04.2023, (D) Presence on the 24.04.2023 and (E) Presence on the 26.04.2023 and 28.04.2023 (the same for both days).

No positive amplification detections occurred between 03.04.23 and 17.04.23 (Figure 3.9), which implied an absence of *Lampetra* eDNA at the field site. Positive detections were only recorded on the final five sampling days of the fieldwork season. Results showed a spike in positive amplification on 19.04.23, with a median of 5 positive reactions for the sampling points on that date. The 19.04.23 samples had the highest number of positive detections of all sampling days across the fieldwork season and showed presence of *Lampetra* eDNA throughout the sampling site (Figure 3.9). Additionally, qPCR results from the 21.04.23 showed a higher number of positive wells than the 03.04.23 to the 17.04.23. There was an overall decrease in the median of positive wells (4) compared to the 19.04.23. The number of positive detections decreased again on the 24.04.23, with a median of 3.5. For the final two sampling days on the 26.04.23 and 28.04.23, the median number of positive wells was 1. Each sampling point on these days resulted in one positive well out of a maximum of six, showing a large decrease in detections compared with previous sampling days (Figure 3.9). DNA sequencing showed that a 41bp sequence was a 100% match to *L. planeri*, *L. fluviatilis* and *Lampetra* species.

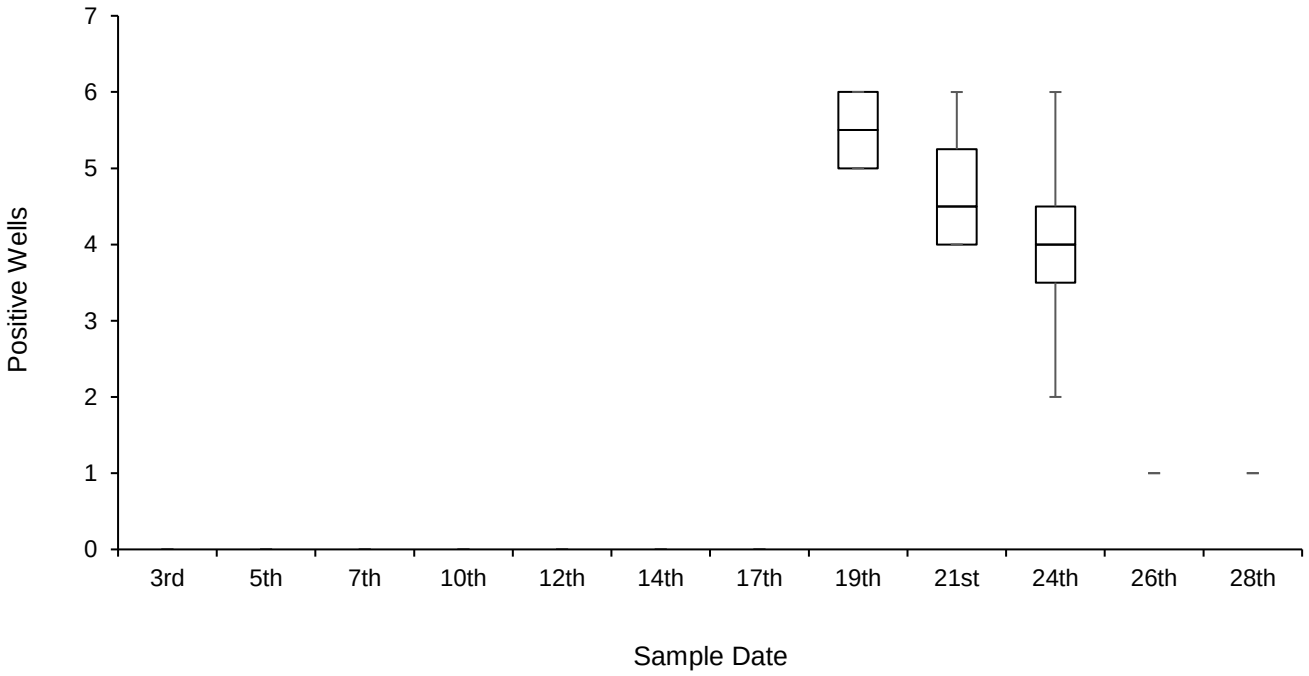


Figure 3.9 Box and Whisker plot showing the number of positive wells per sampling day. The top line represents the upper quartile, the mid-line represents the median, the bottom line shows the lower quartile, and the bars show the range.

Precipitation rates during April can be variable, often resulting in changes to the physical characteristics of a field site. During the fieldwork season, the highest volume of water sample filtered was 1 L and the lowest volume of sample filtered was 850 mL (Figure 3.10). This can be seen in figure 3.10, as there were no results for volumes 0-700 mL. There was a single positive amplification detection for the lowest volume of environmental water sample filtered (850 mL), compared to 16 positive detections for 900 mL of filtered water sample.

The highest number of 48 positive detections occurred when the entirety of the 1 L environmental water sample was filtered (Figure 3.10).

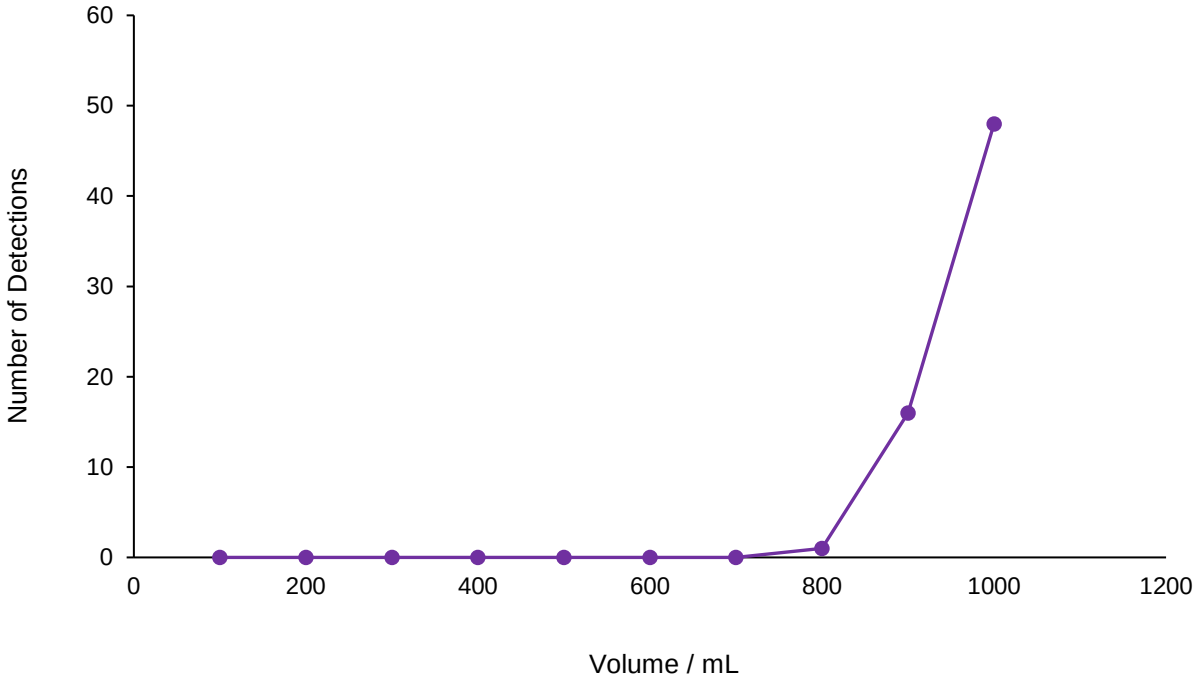


Figure 3.10 Line graph showing the number of positive amplification detections compared with the volume of environmental water sample filtered.

4. Discussion

4.1 Grain Size and Physical Characteristics

Previous studies have described the physical characteristics and bedload sediment composition required for *Lampetra* spawning in European rivers (Clemens et al., 2016). Each sediment fraction within the range of sizes plays an important role in the spawning process (Aronsuu and Tertsunen, 2015). For example, larger grains such as pebbles or gravel must be present in order for male *Lampetra* to build redds (Table 4.1). These redds are used to attract female *Lampetra* and encourage spawning (Zvezdin et al., 2017). Section 3.2 showed that the sampling point with the highest percentage of larger grain sediments was point 1; these grains can be described as medium gravel according to the USDA classification system. Medium gravel is a larger sediment size compared to silt or clay particles; however, research has shown that sediment of varying sizes is needed for optimum *Lampetra* spawning (Oliveira et al., 2022). From the findings shown section 3.2, it was determined that sampling point 1 would be well suited to support the sediment requirements of *Lampetra* spawning events. It has been found previously that both *L. fluviatilis* and *L. planeri* share many bedload sediment requirements for spawning and this can often result in the two species sharing many of the same spawning habitats (Decanter et al., 2023). Although no individuals were sighted during the fieldwork season, based on the findings of section 3.2, it was determined that suitable spawning habitats were present at the field site.

Table 4.1 Optimum physical characteristics and particle size for each stage of *Lampetra sp.* life cycle.

Physical Characteristics	Particle / Grain Size	Life Stage Suitability
Increased Flow Rate Decreased Stream Depth	8 - 16 mm	Nest Construction
Increased Flow Rate Decreased Stream Depth	0.0001 microns - 16 mm	Spawning
Increased Flow Rate Decreased Stream Depth	0.125 microns - 2 mm	Egg Deposition
Increased Flow Rate Decreased Stream Depth	0.125 microns - 2 mm	Hatching
Increased Flow Rate Decreased Stream Depth	0.0001 - 0.5 microns	Downstream Migration
Decreased Flow Rate Increased Stream Depth	0.0001 microns - 1 mm	Ammocoete Burrows
Decreased Flow Rate Increased Stream Depth	0.0001 microns - 1 mm	Metamorphosis
Decreased Flow Rate Increased Stream Depth	4 - 16 mm	Upstream Migration

When considering the results of section 3.2, it was important to consider the range of grain sizes present at each sampling point. Data from points 1, 3, and 4 showed a more varied range of sediment sizes when compared to sampling point 2. In particular the percentage of medium and fine gravel, which are the preferred grain sizes for spawning, were higher at these points in comparison. This could provide adult *Lampetra* with a larger number of potential spawning sites and reduce resource competition or hybridisation (Docker, 2009). To contrast, sampling point 2 showed a complete absence of medium gravel and had the smallest percentage of fine gravel grains 8-4 mm in diameter. This lack of relatively larger sediments may have made this location less desirable to upstream migrating *Lampetra* (Table 4.1). It is possible however, that grains 4-2 mm in diameter at sampling point 2 may have provided enough anchoring weight for smaller *L. planeri* individuals during mating and upstream migration. This could make point 2 a suitable spawning habitat for small *L. planeri* individuals and again reduce possible resource competition or hybridisation (Akat et al., 2022). Migrating individuals may have continued further upstream in search of more suitable spawning sites. Lampreys are known to make several passes through a site during the migration season in order to locate the most optimum spawning substrate. Additionally, *Lampetra* are known to lack spawning site fidelity and therefore may not return inland via the same route as previously attempted (Davies et al., 2022).

Overall, sampling point 1 was described as sandy (USDA, 2023), which implied that this location would be beneficial for spawning *Lampetra* based on the larger particles present. Similarly, the percentage of 1 mm grains found at point 1 were much lower than any other sampling point. When comparing the percentages of 1 mm grains to the particle percentages, point 1 had the highest percentage of larger fine particles than points 2, 3, or 4. The fine faction of the sediment (less than 1 mm that was measured using laser spectroscopy) from this research also showed variation across the sampling points. Although male *Lampetra* require larger sediments for constructing redds, smaller sediments also play a key role in the spawning process. In order to overcome egg drift and possible predation, eggs are released with an adhesive coating that enables small grains to attach to the surface of each egg and weigh them down (Yorke and McMillan, 1979). Once the eggs become coated, they sink to the bottom of the water column and become safely lodged between larger substrate or vegetation (Staponkus and Kesminas, 2014). The high percentage of fine particles present at point 2 could potentially increase the number of successful egg hatchings. This may also increase the number of *Lampetra* individuals surviving to adulthood, which would be advantageous for vulnerable species such as these. Previous research detailed the need for both large and fine sediments at *Lampetra* spawning sites (Table 4.1). The results of section 3.2 indicated that sampling points 1, 3, and 4 met these criteria.

According to section 3.2, sampling point 2 had the largest percentage of small grains of all the points. The high percentage of smaller grains could make point 2 more desirable to female *Lampetra* individuals. Females prefer spawning sites with a number of small grains such as sand present as this can aid in egg protection (Fissette et al., 2021). When considering the habitat requirements of *Lampetra* species, it is important to acknowledge that these differ between the adults and the offspring. Particles of less than 1 mm are required by lampreys in the early ammocoete and juvenile life stages (Almeida et al., 2002). From the findings, it was determined that sampling point 2 had the highest percentage of smaller grains; therefore, providing the most suitable nursery habitat at the field site. Previous studies have found that sites which are able to support both spawning events and provide nursery habitats such as the Forest of Dean, are ecologically important for the two *Lampetra* species (Quintella et al., 2007). Both *Lampetra* species have been recognised as vulnerable within European legislation, with anthropogenic influences being the main threat to these species (Shephard et al., 2019). Locating field sites with both spawning and nursery habitat present is key in supporting *Lampetra* populations, which have suffered a decline in numbers as a result of habitat fragmentation or loss (Lecaudey et al., 2019).

Understanding how physical characteristics of a stream impact the bedload sediment size fractions is important. These characteristics can be classed as optimum or undesirable for *Lampetra* spawning. Section 3.1 showed variations in physical characteristics across the sampling points, specifically when focusing on temperature and velocity. A stream's velocity can have a large impact on sediment erosion, transportation, and deposition throughout a freshwater catchment (White, 1990). Findings from section 3.1 showed a higher velocity at sampling points 1 and 3. Water in streams with a higher flow rate have an increased carrying capacity, which helps with the lamprey fertilisation and larval drift processes (Pratt et al., 2021). Increased mixing of the gametes occurs naturally when the flow rate is faster, compared with the slower rates measured at sampling points 2 and 4 (Kholodnyy et al., 2020). Additionally, the faster the flow rate, the less metabolic cost is required by ammocoetes to locate suitable nursery habitat as they can drift on the currents (Maitland,

1980). Sites with high flow velocities are favourable for *Lampetra* spawning as the smaller, lighter grains remain suspended and transported downstream (Naidu, 2020). This leaves the larger grains at the fast flow habitats which are a fundamental requirement for lamprey redd construction (Oliveira et al., 2022). Sampling point 1 had the fastest flow rate of all points and this correlated with the high percentage of larger grains present.

In addition, a decrease in water flow rate enables smaller grains suspended within the water column to descend and accumulate over time on the stream bed (Gerolin et al., 2020). The reduction in flow observed at sampling points 2 and 4 could account for the higher percentages of smaller grains found at these points. The velocity of the stream at sampling point 4 was the slowest stream flow at the site by 0.034 m/s. When determining the most suitable habitat for lamprey spawning, section 3.1 suggested that point 4 would be desirable in terms of bedload composition. Comparing results from sections 3.1 and 3.2, it could be determined that the decrease in flow rate at sampling point 4 enabled the accumulation of fine particles necessary for *Lampetra* nursery habitat. Additionally, the decrease could also promote an accumulation of larger grains as the potential carrying capacity was reduced. Sampling point 4 was located at a sharp meander of the stream which had resulted in the formation of a stream pool. Stream pools are known to exhibit an above average depth compared to the rest of the stream, and a below average flow rate (Paudel et al., 2021). The carrying capacity of the stream may have been too low for any sediment transportation downstream of point 4, resulting in the most even distribution of grain size percentages out of all sampling points. The slower velocity may have also accounted for the increase in silt and clay particles found at point 4.

Habitats with higher percentages of silt and clay are the most suitable nursery environments for *Lampetra* ammocoetes and juveniles post spawning (Negro et al., 2021). Unlike the heavier grains seen at the start of section 3.2, the lighter particles observed do not undergo compaction. A higher number of fine particles are required for compaction to occur, whereas larger grains such as gravel or pebbles compact more quickly. This is due to the weight of the surface grains pushing down on the sediment layers beneath (Dasgupta et al., 2020). Lighter particles are able to remain free and uncompacted which allows *Lampetra* ammocoetes and juveniles to burrow easily into the softer habitats found within the stream bed. Juveniles of both *Lampetra* species require burrows for filter feeding and protection from predators (Quintella et al., 2007). Juveniles remain in these nursery burrows until they complete the final metamorphosis to become adults, highlighting the need for silt and clay particles (Moser et al., 2007). Nursery habitats are equally as important to spawning sites as they provide a source of nutrition and protection for the *Lampetra* juveniles, which are required to undergo larval drift in order to search for these types of habitats (Maitland, 1980). Other studies have found that the optimum spawning habitats would be in close proximity to nursery habitats. This would support both mating individuals and their offspring (Bracken et al., 2019).

When discussing the effects of physical characteristics on lamprey spawning, it is necessary to consider the influence of temperature. Lampreys have well described patterns of behaviour during the final ten-month period of their life cycle. During these last ten months they undergo a migration and spawning event before dying (Jubb et al., 2023). Water temperature is a stimulus for individuals to begin migrating inland in search of spawning and nursery sites (Boulêtreau et al., 2020). For example, a decrease in water temperature after the summer months signifies the start of the migration season from October to February.

Additionally, the reverse is true for the spawning season when temperatures increase after the winter months from April to July (Baer et al., 2018). According to section 3.1, the average temperature at the field site on the final sampling day (9.6 ℃) could reflect the increase in water temperature from the winter months. This would be a key trigger for male *Lampetra* to begin constructing redds for mating (Heath et al., 2021). Sampling point 4 had the lowest average water temperature compared to the other points, which may be a result of increased water depth. Previous studies have outlined a negative trend between water depth and water temperature, where water temperature decreases as the depth increases (Morales-Marín et al., 2019).

This trend was somewhat reflected at sampling point 4 where there was a difference in temperature of 0.2 ℃. Similarly, studies have determined that the warmest water is found at the top of the water column and the coolest water is at the bottom (Jimenez et al., 2008). Sampling points 1, 2, and 3 had a higher average water temperature which may have been a result of those points being shallower than point 4. Another possible reason for the difference in average water temperature is the understanding that temperatures are more evenly distributed in shallow water (Cyr, 2020). This is due to increased mixing between the layers in the water column. In deeper water bodies such as lakes or ponds, a thermocline is created which divides the warmer water at the surface from the colder water below (Lofton et al., 2022). Water velocity can similarly influence water temperature by reducing stratification between the temperature layers. As can be seen in section 3.1, points 1 and 3 were shallow with higher velocities than sampling point 4. This potentially caused the difference in water temperatures due increased mixing. Point 4 was much deeper with a decreased velocity resulting in less mixing between the temperatures and creating a slightly colder habitat.

Overall, it could be determined from section 3.2 that three of the four sampling points (1, 3, and 4) had grain sizes required for *Lampetra* spawning. Physical characteristics are similarly known to influence both the temporal and spatial distribution of *Lampetra* spawning (Negro et al., 2023). These characteristics are known to play a large role in the spawning of migratory species, specifically the water flow and depth of a stream (Liu et al., 2021). The results of this section revealed that the sediment and physical characteristics of sampling point 2 were not as desirable for *Lampetra* spawning as the other points. This sampling point could be better suited as a nursery habitat which is equally as important for *Lampetra* species. The loss of fine sediments is one of the main threats to these lamprey species; therefore, the identification of these sediments is advantageous to the restoration of *Lampetra* populations (Lecaudey et al., 2019).

4.2 Gradient Polymerase Chain Reaction Analysis

Although the No-Template Controls (NTCs) for both the CO1 and ATPase6 primers were negative, potential primer-dimer binding occurred during the PCR process. This was represented by faint banding in the final well of both gels shown in section 3.3. These wells should have been completely free from *Lampetra* DNA as a result of using DNA-free H_2O. Non-specific binding often occurs when the primers bind to any genomic DNA present within the reaction (Jansson and Hedman, 2019). The very dull bands were non-specific as they did not align on the gel with the *Lampetra* samples. Similarly, the two NTC bands were not positioned with the target amplicon bands on the DNA ladders. The genomic DNA may have contaminated the reactions via a range of sources. For example, the pipette used when

producing the M.Ms or when loading the PCR products onto the gel (Minich et al., 2019). When conducting the literature review, there was no evidence of real-life application or optimisation of the CO1 or APTase6 primers taken from Zancolli et al. (2018). This lack of optimisation may have resulted in reduced sensitivity of the primers. This could have enabled them to bind to genomic DNA present that was not associated with the *Lampetra* target amplicon (Garafutdinov et al., 2020).

Although Zancolli et al. (2018) designed both forward and reverse primer sequences, a lack of testing meant there were no recommendations for primer optimisation. A large temperature range was chosen to test optimum annealing temperatures for both primer pairs (Section 3.3). This range was based on similar temperatures used in previous PCR research (Valentini and Pompa, 2016). Following the results of the gradient PCR, the ATPase6 primer pair were chosen to test the environmental samples for *Lampetra* eDNA presence-absence. This pair of primers showed less primer-dimer and non-specific binding when compared to the CO1 primer pair (Section 3.3). Additionally, the ATPase6 pair showed better thermal flexibility which was advantageous when conducting qPCR assays. Much of the literature recommended a 10 ℃ difference in annealing temperature between the primers and probe during qPCR testing (Hunter et al., 2017).

4.3 Methodology Optimisation

A review of the most current laboratory methodologies for determining presence-absence using qPCR was conducted ahead of the fieldwork season. Recommendations from studies such as Zancolli et al. (2018) and Weldon et al. (2020) provided a starting methodology for assay protocol and reagent volumes for environmental samples. Initial pilots were conducted to determine the efficiency of the ATPase6 probe binding to *Lampetra* eDNA using known concentrations and serial dilutions (Section 2.3.3). The results of the pilot assay concluded that the probe taken from Zancolli et al. (2018) was able to bind effectively to the *Lampetra* DNA present in the dilution series. The probe similarly bound to *Lampetra* eDNA present in the four quantified environmental samples. Samples from 03.04.23 showed no amplification, however amplification occurred for samples collected on 21.04.23, 26.04.23 and 28.04.23. Results of the four environmental samples reflected the pattern of eDNA presence for the remaining samples, as no positive amplification occurred from water filtered before the 19.04.23.

Additionally, the results of the pilot showed that the combined *L. fluviatilis* and *L. planeri* dilution series had the most similar quantification cycle (Cq) values for each replicate. This was in comparison to the separate *L. fluviatilis* and *L. planeri* dilution series. A Cq value is the fractional number of cycles required for the fluorescence of each well to increase above threshold level, which can therefore be measured by the machine (Ruiz-Villalba et al., 2021). Readings below the threshold are classed as background or baseline fluorescence and may result from non-specific binding within samples (Campion and Loughran, 2021). When determining the most effective qPCR assay reagent volumes for detecting the presence of the *Lampetra* species, the recommended volumes and cycle programme were used for the pilot plate (Section 2.3.3). A second pilot was used with minor changes to the volume of DNA in each reaction of the dilution series to optimise the Cq range of the reactions (Section 2.3.3). Through increasing the volume of sample in the reactions, the concentration of DNA was also increased. This resulted in the Cq value for each serial dilution being decreased by

2 cycles. This small change increased the amplification efficiency, as evidenced by the exponential fluorescence detected sooner by the machine within the qPCR protocol (Ruijter et al., 2021).

Previous qPCR research has shown that Cq values can be linked to the number of target amplicon copies within the reaction wells. In addition, the higher the copy number, the lower the number of cycles required for enough product to surpass the threshold (Pancza et al., 2021). Cq values are a representation of the sensitivity of the probe in relation to the quantification cycle threshold. If the Cq values are lower, the amplicon has been detected earlier into the assay protocol (Khoury and Tran, 2020). Further optimisation occurred for the first full assay of 25 environmental samples tested, each with three replicates (Section 2.3.3). Three replicates were chosen to ensure there was a deciding factor if the replicate results were divided into one negative and one positive. BSA was added to the M.M as a measure to supress the effect of any PCR inhibitors within the environmental samples and enhance the specificity of secondary structure region amplification (Wong et al., 2020). DMSO was added to improve the strand separation of C-G rich regions within the DNA present (Carvalho et al., 2021). The addition of both reagents was used to improve the efficiency of the qPCR process. Despite reducing the volume of sample per reaction, this was still a higher volume than the recommended (Section 2.3.3).

On the first full assay plate, none of the environmental samples showed amplification despite the addition of BSA and DMSO. Negative amplification within environmental samples can be caused by a multitude of factors, such as target amplicon concentration being below the limit of detection or the complete absence of target amplicon (Thomas et al., 2020). The presence of PCR inhibitors can similarly affect the results of qPCR assays and are usually captured on filter membranes alongside eDNA (Hallam et al., 2021). Inhibition occurs during PCR when compounds such as tannins and humic acid cause a reduction in the activity of DNA polymerases. Similarly, these compounds can bind to nucleic acids or change the composition of preservative buffer solutions (Borchardt et al., 2021). In addition, inhibitor compounds can specifically affect qPCR assays by quenching the fluorescence of the dyes. These dyes are functional components that bind to the target DNA during the annealing stage of the protocol (Lance and Guan, 2020). During the DNA extraction process, the presence of inhibiting compounds should have been removed at the purification stage by remaining on the column membrane. Five of the negative samples were chosen randomly to be spiked with known concentrations of extracted *Lampetra* DNA in order to test for inhibition (Section 2.3.3).

Section 3.3 showed that there was no inhibition of amplification in the environmental samples and therefore those samples resulted in a true absence of *Lampetra* eDNA. After conducting further environmental sample assays, it was determined that the results from the first full plate of samples matched the presence-absence pattern of the rest of the samples. When testing for inhibition, the protocol was changed by increasing the total number of cycles (Section 2.3.3). The increase to 45 cycles was added to provide clearer amplification curves for environmental samples with lower concentrations of eDNA which had high Cq values. Previous assays had shown exponential amplification starting during the 40^{th} cycle for environmental samples. It was determined that these would be better observed with added protocol length through a higher number of cycles. To further optimise the assays, another dilution was included with the serial dilution replicates (Section 2.3.3). This lower dilution provided amplification curves with more similar Cq values to that of the

environmental samples. This addition provided a known concentration comparison for the environmental samples and could aid in determining eDNA quantities in each reaction.

To confirm the results of section 2.3.3, the qPCR products were extracted from the inhibition assay plate and loaded onto a gel (Section 3.3). This gel showed that the amplified product bands were aligned with the corresponding band of the DNA ladder. It was determined that the probe had amplified the target *Lampetra* amplicon as the eDNA fragments matched the expected length of 90 bp. NTCs were included on each assay plate as a form of quality control. This showed that amplification within environmental samples was from *Lampetra* eDNA, rather than contamination between the positive or negative samples. Quality control was key during the optimisation of the qPCR protocol and several measures were taken into consideration during the development stages. For example, fresh pipette tips were used when pipetting reagents or changing between samples to prevent cross contamination across reactions. An optimisation period of up to three months was suggested by Zancolli et al. (2018), however the methodology of this project was optimised over two months. The results show that the laboratory methodology developed during this study proved effective in determining the presence-absence of *Lampetra* eDNA at the sampling site of Soudley Ponds, Cinderford, across a spawning season during April 2023.

Fluorescence detected by the qPCR machine occurred between cycle number 35 and 40 for the environmental samples which reflect those found in other qPCR studies (Thomas et al., 2020). These cycle numbers were expected as research has shown that eDNA concentrations are lower in environmental water samples compared to other environmental sources (Thalinger et al., 2021). This often results in higher Cq levels as the amplification curves start much later within the qPCR protocol (Section 3.4). The standard curve showed that the dilution series replicates amplified and fluoresced within a minimum of one cycle of each other. According to section 3.4, the dilution series replicates of known concentrations fit the general trend line.

4.4 *Lampetra* Presence-Absence

An important finding from the qPCR assays conducted through May and June of 2023 showed the presence of *Lampetra* species within the Soudley Ponds, Cinderford catchment during the fieldwork season from the 03.04.23 to 28.04.23. Positive qPCR reactions indicated presence of *Lampetra* eDNA at each of the sampling points along the chosen stretch of the stream (Section 3.4). Based on the findings of Lasne et al. (2010), it was expected that a spawning event would occur in April around the River Wye and Severn catchment. Using this knowledge, fieldwork was planned for April 2023 to maximise the opportunity of capturing eDNA if *Lampetra* species were spawning in the catchment. Previous studies were used to guide sampling strategy, such as the sampling frequency and time of sample collection. According to Russon and Kemp (2011), lampreys are known to spawn from 17:00 pm until 03:00 am and therefore sample collection took place at sunrise every sampling day. eDNA begins to degrade within six hours of a release event such as a spawning (Saito and Doi, 2021). Based on this understanding the collection was set at sunrise to limit degradation and capture the highest eDNA yield possible. No *Lampetra* individuals were observed during the fieldwork period, which likely reflects the overnight spawning patterns described in literature.

Additionally, there was no observed evidence of spawning, such as redds, during the fieldwork season. This would suggest that *Lampetra* present at the field site were in low numbers which reflects the findings of Harrison and Pinder (2020). According to Moser et al. (2021), *Lampetra* adults would be in-situ at suitable spawning habitats by April, well in advance of spawning, season to improve their chances of successful mating. Additionally, male individuals start constructing redds ahead of the spawning week and it would be expected that if *Lampetra* were present at the fieldwork site, redds would have been visible from the beginning of April (Dhamelincourt et al., 2022). As only a single stretch of the catchment was sampled, it was not possible to determine the exact spawning location or locations. eDNA such as gametes released during spawning are transported downstream by the water flow away from the release event. This can make locating the spawning event difficult when using eDNA compared to other methods such as physical observations or catches (Jones et al., 2020).

The results of section 3.4 showed a sharp increase in eDNA detections on the morning of 19.04.23. All sampling points showed positive detection of *Lampetra* eDNA from water samples filtered on the 19.04.23 (Section 3.4). Using these results, it was confirmed that *Lampetra* species eDNA was present at each of the sampling points on the morning of the 19.04.23. It was estimated that the *Lampetra* species spawning event happened between the evening of 18.04.23 and the morning of 19.04.23. This time frame could be determined using the temporal limitations of eDNA within the aquatic environment, which can affect the quality of the fragments (Saito and Doi, 2021). The spawning event likely occurred upstream of sampling point 1 and this was deciphered from the presence of *Lampetra* eDNA at all four points along the site. In the case of the spawning event happening between sampling points, there would have been an absence of eDNA upstream of the event and presence downstream, causing a split in the detection results. This project was able to provide evidence on *Lampetra* species, however due to the low specificity of the ATPase6 primers it was not possible to determine if either or both *L. fluviatilis* and *L. planeri* species were present within the river catchment.

Subsequent water samples taken on the 21.04.23 similarly highlighted the presence of *Lampetra* eDNA at each of the sampling points (Section 3.4). The number of positive replicates however fluctuated when compared to the previous sampling day. This decrease in the number of positive replicates for points 1 and 3 may have resulted from the higher water velocity at these points (Section 3.1). eDNA fragments may have been transported more efficiently downstream from points 1 and 3, when compared to the lower velocities at points 2 and 4 (Section 3.1). Smaller eDNA fragments (often in the form of cells) such as gametes are usually suspended within the water column of aquatic environments and can be easily transported down a water course (Yu et al., 2019). The rate at which eDNA is transported through an environment is dependent on the rate of water flow, resulting in differences of eDNA presence between sites along the same stream (Carraro et al., 2022). 100% detection at point 4 may have resulted from the geological characteristics of that sampling point. The meander at point 4 could have caused the decrease in velocity (Section 3.1) and enabled an accumulation of eDNA around this sampling area (Livanov, 2023). In addition, the decreased water temperature at point 4 (Section 3.1) may have helped to maintain the eDNA fragments and delay the impacts of thermal degradation (Qian et al., 2022). Similarly, sampling point 2 had a slower velocity, which may explain the higher number of positive replicates for point 2 compared with points 1 and 3 (Section 3.4). Literature states that a spawning event for *Lampetra* species may span across multiple days

depending on the number of individuals present, which may explain the high eDNA presence two days after the estimated spawning date (Daupagne et al., 2022).

Adult *Lampetra* morphs do not have working mouthparts and are unable to feed after the final metamorphosis to adult form. As a result of this morphological change, the metabolic cost of migration and spawning is too great to overcome (Kujawa et al., 2019). After spawning, adult *Lampetra* individuals die which creates another potential source of eDNA when cells, blood and gut contents are released during predation events and decomposition (De Souza et al., 2016). This second source of eDNA may account for the results obtained from sampling point 1 on the 24.04.23 where all six replicates were positive for *Lampetra* eDNA (Section 3.4). Daupagne et al. (2022) determined that usually only a single spawning event occurs within the *Lampetra* spawning season. The increase in eDNA presence occurred after a two-day break with no sampling and it would be expected that fragments from the spawning event would have degraded. It was determined that the spike in eDNA presence was likely the result of settled or buried eDNA being disturbed upstream of sampling point 1. When reviewing the literature, a disturbance of eDNA upstream was more likely than a second spawning event.

Results from section 3.4 add further plausibility that the spawning event occurred upstream of sampling point 1 during the third week of April 2023. This was seen in the results as on the 24.04.23 the number of positive detections increased at the most upstream sampling point of the field site. At the end of the fieldwork season, the number of *Lampetra* eDNA detections fell at all four sampling points across the site (Section 3.4). Only one replicate at each point showed positive detection on the 26.04.23 and 28.04.23, which was nine days post estimated spawning event. This decrease in detections and presence would be expected due to the degradation of eDNA fragments after a release event such as spawning. Additionally, it was expected that any *Lampetra* eDNA remaining intact would have been transported downstream of the sampling points before the final two days of sampling, although fragments may have become buried in upstream sediments. The negative control samples were filtered on-site along with the environmental samples and showed no amplification during the qPCR assays. This further validates that amplification in the environmental sample assays was the true presence of *Lampetra* eDNA, rather than contamination. Disinfecting the equipment between sampling days also ensured that positive amplifications were true detections, rather than detecting remaining eDNA from prior sampling. These steps were included to maintain quality control during the fieldwork season.

5. Conclusions and Recommendations

5.1 Conclusions

Over the course of this research project, a working fieldwork and laboratory methodology for detecting the presence-absence of *Lampetra* species was developed. Sampling strategies were based on recommendations from previous research focussing on similar aquatic species, methodology reviews and proof of concept literature. The main finding of this research was the presence of *Lampetra* eDNA in water samples collected at the Soudley Ponds sampling site during a spawning season in April 2023. From the findings, it was estimated that a spawning event did occur between 18.04.2023 and 19.04.2023 as a large spike in eDNA detection could be seen within the environmental sample assays, compared with no detections before this sampling date. Although no *Lampetra* individuals were observed over the fieldwork season, eDNA techniques such as qPCR showed true amplification of the target amplicon which was cross referenced with gel electrophoresis imagery. This piece of research has helped to expand the limited literature available regarding *Lampetra* spawning in the Forest of Dean catchment, building upon previous projects that used more traditional methods. The eDNA results suggested that Soudley Ponds could be a site of interest for further research during the next *Lampetra* spawning season.

When analysing the sediment taken from the sampling site, it was determined that each sampling point had grain or particle sizes required for either or both *Lampetra* spawning and nursery habitats. Locating waterways that could potentially support lamprey spawning events, and subsequently support offspring, is vital for the continued protection of these species. Sites identified as having a possible *Lampetra* species presence are protected under European legislation, demonstrating the vulnerability of these species and the need for further research. One of the main threats to *Lampetra* species is the loss of sediments through anthropogenic interference resulting in habitat fragmentation via the addition of impassable in-channel structures. Physical characteristics play a role in creating and maintaining aquatic habitats and Soudley Ponds was no exception. The physical characteristics tested at each sampling point showed favourable spawning and nursery characteristics for *Lampetra* species. Most notably, the sampling points had both fast flows which support spawning and slow flows which support the deposition of nursery sediments. These physical characteristics are created when NFM interventions are implemented in waterways, such as leaky barriers, and therefore it can be assumed that the new habitat created by these NFM structures will be suitable for lamprey spawning.

The methodology development within this study was much more complex than expected. It was determined that establishing a working methodology using a positive control site was more beneficial than conducting analysis at an NFM site without confirmed lamprey presence. Knowledge gaps were identified during the literature review, some of which have been answered over the course of this project. The optimisation of the primers and probe taken from Zancolli et al. (2018) has provided an understanding of their use within an in-field application. This research has contributed to the evidence that eDNA can be used as a sensitive technique to identify rare or vulnerable species. Additionally, the project has also highlighted the advantages of eDNA and qPCR as rapid tools for locating possible *Lampetra* species spawning events and verifying results from previous projects with traditional

methods. When considering species conservation in relation to anthropogenic interventions in nature, it is beneficial to understand the key habitat characteristics. Increasingly this is occurring through the use of non-invasive monitoring techniques such as eDNA to inform conservation management plans for rare and vulnerable species.

5.2 Recommendations

To improve the streamlining of similar research projects in the future it is recommended that the filters undergo DNA extraction within one week of collection. This will ensure that any degradation effects are limited, and qPCR analysis can begin much sooner, giving more time for assay optimisation and repeats. Additionally, although Longmire's buffer is a preservative for upwards of six months, degradation can still occur within the filters which could affect the yield of DNA extracted.

It is recommended that the serial dilution be extended further to provide a better understanding of the detection and quantification limit of the primers and probe taken from Zancolli et al. (2018). By extending the dilutions, an increased number of amplification curves could be used as a comparison for the quantity of *Lampetra* eDNA within environmental water samples. Additionally, the serial dilutions could also be made to a 1:10 concentration rather than a 1:5 in order to further determine the limit of detection of eDNA from environmental samples. This dilution factor would create lower concentrations of control DNA and provide a visual reference when comparing water sample results to the serial dilutions. Extending the serial dilution could also be beneficial for estimating the limit of quantification and copy number of the eDNA from environmental samples. In addition, it is recommended that all fieldwork sampling equipment should be disinfected for a minimum of 20 mins to ensure all remaining eDNA traces are removed.

Furthermore, to validate that the target amplicon has been amplified it is recommended that the positive amplification results from the qPCR assays undergo gel electrophoresis. This would provide a visual comparison between the expected length of the eDNA fragments against the DNA ladder. One of the main recommendations from this project is that future research should focus on the primers and probe taken from Souissi et al. (2022). Further optimisation of these primers and probe is required to determine whether *Lampetra* species can be distinguished based on positive eDNA amplification using environmental water samples. To better understand how the physical characteristics may affect *Lampetra* spawning, it is recommended that these measurements be taken more frequently throughout the fieldwork season. A larger volume of characteristic data would enable more in-depth analysis and provide insight into the day-to-day influences on spawning events such as weather.

Finally, this research could be expanded to include eDNA testing of NFM sites that have had leaky barriers installed in the river channels to test whether these do indeed create a viable habitat for lamprey. Future studies such as this may be applied on a larger scale, taking into consideration the advantages of modelling analysis.

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