

Plastics and the Hidden Majority

Conservation Implications for a Threatened UK Freshwater Fish

UNIVERSITY of
STIRLING



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Statement of Originality

I hereby confirm that this PhD thesis is an original piece of work conducted independently by the undersigned and all work contained herein has not been submitted for any other degree.

All research material has been duly acknowledged and cited.

Signature of Candidate:

Flora Anna Rendell-Bhatti

Date: 07/02/2024

General Abstract

Humanity is currently operating outside the planetary boundary for novel entities, with plastic pollution emerging as a significant concern. Global plastic mass, including current use and plastic waste, is estimated at double the mass of all living animal biomass. River sediments can accumulate microplastic contamination, with particularly high contamination in urban hydrometric areas. Higher loadings of microplastic contamination have been observed in sediments compared to the water column and areas with both depositional habitats and low water velocity. Lamprey larvae burrow in depositional freshwater sediments for up to seven years and feed on deposited organic matter. Despite this overlap, the extent of microplastic contamination in lamprey larvae, to the best of my knowledge, has not been documented.

Few would say that lamprey species are charismatic, it's parasitic life stages, unusual image and contrasting management views globally result in these species falling low on the priority list. However, this important ecosystem engineer, protected across Europe is one which this thesis aims to bring front and centre. Considerable knowledge gaps present both research opportunities and challenges. To date management and research efforts have focused on the upstream migration of adult lamprey, due to the threats from anthropogenic barriers. Certainly, an important factor in the conservation of lamprey across Europe, however there is a lack of research into other emerging threats to this primitive species. This thesis has filled in knowledge gaps concerning the exposure of lamprey larvae to microplastic contamination and the ecotoxicological effects associated with such exposure.

The research develops novel methods to optimise the isolation of microplastic particles from complex organic tissue, microplastics identification and automated data analysis. The findings suggest that microplastic contamination is present in wild lamprey populations from Scottish rivers, with different age classes of larvae ingesting distinctive polymer types. To understand whether exposure to microplastics leads to identifiable effects in lamprey, behavioural and developmental end points were considered in experimental systems. These studies suggest microplastic exposure elicits impacts on larvae behaviour and early development. The results obtained from can be used to support further research and to aid development of future studies aimed at conservation management of this ecologically important species.

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Chapter 6

Table 6.1 Wildtype development times by stage, including description of phenotype.

Abbreviations and definitions

μFTIR	Micro-Fourier-transform infrared
ABS	Acrylonitrile butadiene styrene
Ammocoete	larval stage of lamprey
ANOVA	Analysis of variance
APV	Acrylates, Polyurethanes and Varnish
ASA	Acrylonitrile styrene acrylate
ATR	Attenuated total reflection
EDS	Energy dispersive X-ray spectroscopy
EVAc	Ethylene-vinyl acetate
EVOH	Ethylene vinyl-alcohol
FTIR	Fourier transform infrared
GIT	Gastrointestinal tract
HDPE	High-density Polyethylene
HPF	Hours post fertilisation
LDPE	Low-density polyethylene
LOD	Limit of detection
LOQ	Limit of quantification
MilliQ	Ultrapure water, purified using an ion exchange cartridge
MP	Microplastics
MS-222	Tricaine methanesulfonate
PA	Polyamide
PAN	Polyacrylonitrile
PBT	Polybutylene terephthalate
PC	Polycarbonate
PE	Polyethylene
PET	Polyethylene terephthalate
PLA	Polylactic acid
PMMA	Polymethyl methacrylate
POM	Poly oxyethylene
PP	Polypropylene
PPSU	Poly phenylene sulfone
PS	Polystyrene
PSU	Polysulfone
PTFE	Polytetrafluoroethylene
PU	Polyurethane
PVC	Polyvinylchloride
RPi	Raspberry Pi
SAC	Special Areas of Conservation
SCENE	Scottish Centre for Ecology and Natural Environment
SEM	Scanning electron microscopy
Si	Silicone
SOP	Standard operating procedures
SPT	Sodium polytungstate
TWP	Tyre wear particles
WT	Wild type

Chapter One - An introduction to the biology of lamprey (family: *Lampetra*), with particular reference to sensitivity to anthropogenic stressors

1.1 Introduction to Lamprey

Lamprey belong to a vertebrate group that diverged approximately 500 million years ago, sometimes referred to as 'living fossils' as evidenced by their presence in ancient rocks (Morris, 1993). Lampreys share multiple traits with vertebrates, including rudimentary vertebrae; closed circulatory system, neural control of the heart rate and ability to osmoregulate (Nieuwenhuys and Nicholson, 1998). Lampreys fall into the superclass group Agnatha, meaning "no jaws" and are not true fish, but are often referred to as such. Lampreys have a temperate and Arctic distribution with approximately 40 species worldwide, occupying habitats from the Arctic circle southward to Patagonia (Salewski, 2003). The UK is home to three species of lamprey, the sea (*Petromyzon marinus*), river (*Lampetra fluviatilis*) and brook (*Lampetra planeri*) lamprey (Fig.1.1; Renaud, 2011). All lampreys spawn in freshwater streams, with larvae filter feeding by drawing water in to the burrows and feeding on microalgae and suspended fine detritus (Dawson *et al.*, 2015). It is after this stage the parasitic (sea and river lamprey) and non-parasitic (brook) life forms diverge (Fig.1.2).

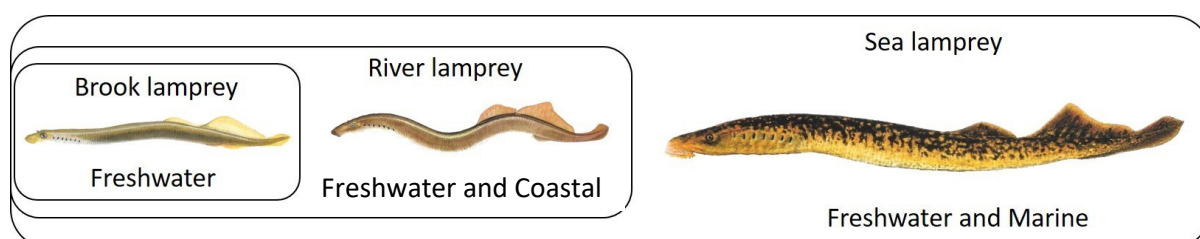


Figure 1.1 The three species of lamprey which are native to the UK, the sea (*Petromyzon marinus*), river (*Lampetra fluviatilis*) and brook (*Lampetra planeri*). Reaching an adult size of ~15-19cm the Brook lamprey is the smallest of the three lamprey species present in the UK and are an entirely freshwater species. River and Brook lamprey are a sympatric paired species, difficult to distinguish apart based on morphological features during larval stages. Image credit: Colin Bean.

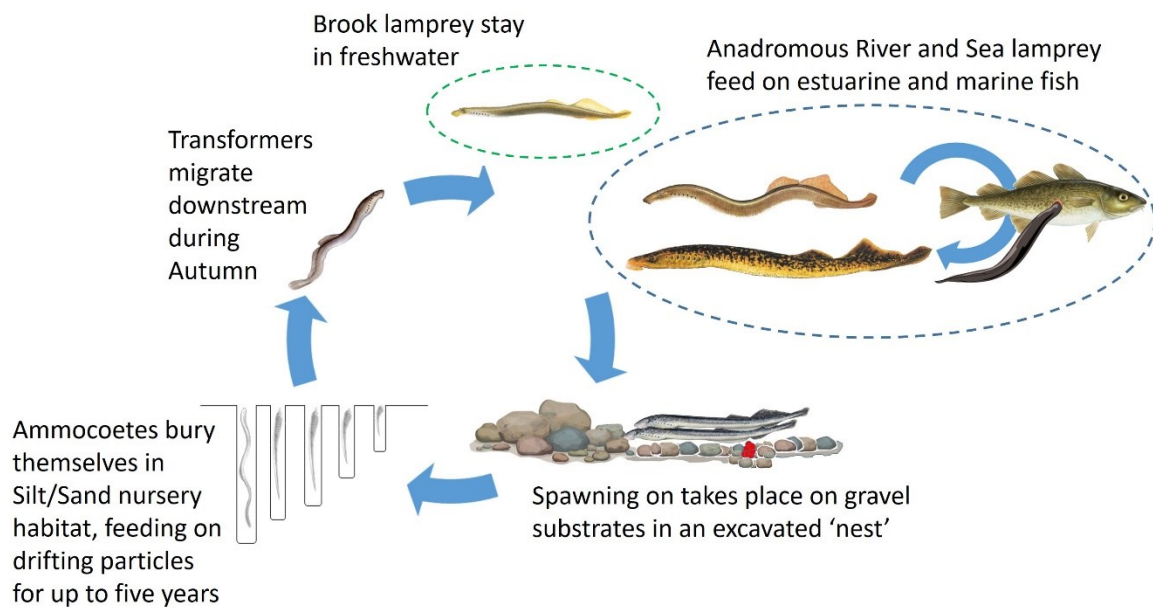


Figure 1.2 River and sea lamprey are anadromous spending 18-24 months in coastal and marine habitats. These are larger fish and adult river lamprey attain a size of 30-50 cm, but sea lamprey can reach a length of 1 m. River lamprey return to freshwaters between October-December, but sea lamprey migrate upstream during April-May. Brook lamprey typically spawn between the months of April-June and this overlaps with the river lamprey spawning period of April-May. Sea lamprey spawn during May-July. Regardless of species, all adult lamprey die after spawning. Whilst spawning typically takes place in pockets (or nests) excavated in gravel substrates, juvenile lamprey (often called ammocoetes) can live for up to five years in silt/sand nursery habitats, where they feed on particulate items such as diatoms, algae, bacteria and detritus. Pre-adult lamprey (known as Transformers) metamorphose and begin their migration to sea from late Autumn onwards. Image credit: Colin Bean.

Sea lamprey (*Petromyzon marinus*) migrate downstream to enter a marine parasitic phase, feeding on the blood and flesh of estuarine and fish and cetaceans (Fig.1.2; Quintella, 2021). Most UK sub-populations of river Lamprey (*Lampetra fluviatilis*) also migrate to sea to feed. However, in some isolated populations, such as Loch Lomond and Lough Neagh, river lamprey complete the parasitic phase in freshwater (Maitland *et al.*, 2015). In contrast, the European brook lamprey (*Lampetra planeri*) remain in freshwater habitats. Brook lamprey are sympatric paired species to river lamprey, being difficult to distinguish based on morphological features during larval stages (Hardisty and Potter, 1971; Renaud, 2011). Both species have a prolonged larval stage; *L. fluviatilis* between three to four years and *L. planeri* at least five years in length (Hardisty, 2006). However, their adult life histories differ; *Lampetra fluviatilis* is an anadromous species metamorphosing into first a transformer and then migrating downstream to forage as an adult on inshore and estuarine teleost species for 12-18 months (Fig.1.2; Hardisty and Potter, 1971; Maitland *et al.*, 1984; Renaud *et al.*, 2009). *Lampetra*

planeri, in contrast, is a non-parasitic species remaining within freshwater where it does not feed after metamorphosis (Fig.1.2; Hardisty, 2006).

1.1.1 Larval stage

The larval stage of all lamprey species, often named ammocoetes, burrow in freshwater sediments for between 3 and 6 years (Fig. 1.2; Quintella *et al.*, 2015). The larvae are nocturnal microphagous filter-feeders, creating burrows within sand banks of temperate streams (Hardisty and Potter, 1971). Larvae of most lamprey species occupy mainly fluvial sedimentary habitats. However, there are species which have an entirely lacustrine lifecycle, such as the Miller Lake lamprey *Entosphenus minimus* (Lorion *et al.*, 2000). After the prolonged larval period, lamprey undergo metamorphosis into adult forms, which as described are either parasitic or never feed again dependent on the species (Fig. 1.2).

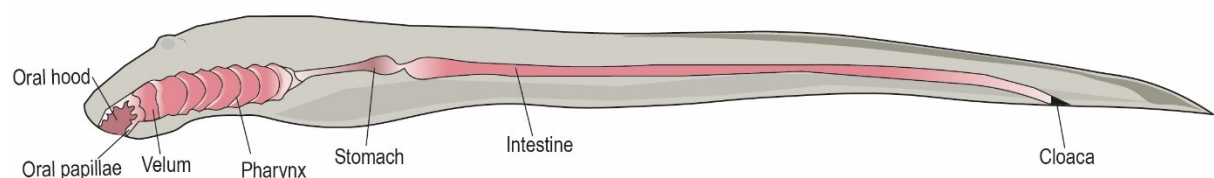


Figure 1.3 Lamprey larva with key anatomical features labelled.

The anatomy of larval lamprey is adapted to its extended life history stage buried in dark sediments, for example the lack of developed eyes (Fig.1.3). Instead, larvae have rudimentary eyes, buried beneath the skin. These basic optical structures, buried beneath a translucent patch of skin, aid in negative phototactic behaviour (Ullen *et al.*, 1997; Richardson *et al.*, 2010). Lamprey larvae lack many distinctive features, and unlike adult forms of lamprey, which have a characteristic oral disc for parasitic feeding, larvae instead have a blunt anterior end (Kelly and Kind, 2001). The mouth structure consists of an overhanging upper-lip and partially enclosed by a lower lip, forming the oral hood (Fig.1.3). The oral hood, an important feeding structure, consists of branching cirri (Whitaker, 1999), with seven branchiopores (Richardson *et al.*, 2010). These branchiopores are located on either side of the head and are used for both respiration and the expulsion of water. Preceding the mouth, the velum, a pair of muscular paddles act to create respiratory currents by rhythmically pulsing forwards and backwards (Mallat, 1981). Larvae gill structures lead directly into the ciliated tracts of the pharynx, at which point food particles are strained off (Richardson *et al.*, 2010). Food particles

are subsequently captured and passed to the end of the pharynx, into the oesophagus and intestine (Hardisty and Potter, 1971). The intestine runs through the body of the larva, where it opens at the posterior end as the cloacal opening.

1.1.2 Lamprey larva bioturbation

Charles Darwin was one of the first to document the ability of faunal organisms to modify their physical environment through particle reworking, using earthworms as a study organism (Darwin, 1892). Almost, 100 years later, this phenomenon was studied in aquatic ecosystems, and deemed as one of the most important processes that modify the structure and composition of aquatic sediments (Cullen, 1973; Grant, 1983; Rhoads, 1967). Since then, the ecosystems services provided by sediment fauna, through particle reworking and burrow ventilation, have been studied and documented (Mermillod-Blondin *et al.*, 2003; Shirakawa *et al.*, 2013; Boeker *et al.*, 2016). Such behaviour is termed bioturbation (Kristensen *et al.*, 2012) and represents a process which can alter the physical and chemical nature of the streambed, particularly streams with low hydrologic energy (Moore, 2006; Rice *et al.*, 2016; Shrivastava, Stewardson and Arora, 2021).

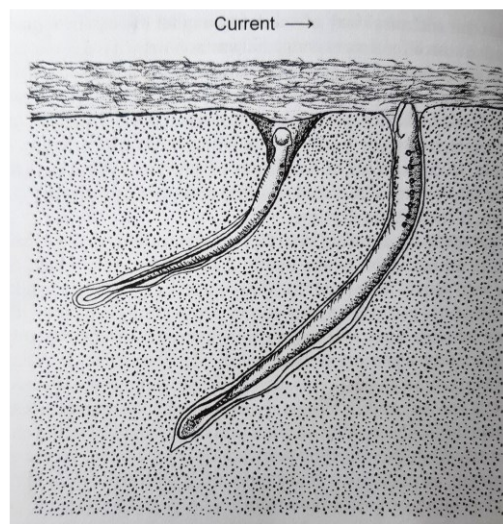


Figure 1.4 Burrowed lamprey larvae, reproduced from Hardisty (2006), based on drawings by Applegate. (1980).

Burrowing behaviour in areas of slow flow and fine sediment enables larvae to avoid predation, whilst feeding in the water column or on surrounding sediment (Applegate, 1950; Hardisty, 2006). Larvae are documented to typically burrow in the surface layers of substrate, with larger larvae (90-160mm) burrowing approximately 75-150mm below the surface

(Hume, 2013). Larvae bioturbation activities increases habitat variability, microbial community composition, oxygen availability and generate suitable habitats for other species (Shirakawa *et al.*, 2013; Boeker *et al.*, 2016). This increase in habitat complexity has been shown to benefit other fishes and stream invertebrates (Sousa *et al.*, 2012). Considered downward conveyers, larvae are vertically orientated head-up feeders, actively ingesting particles at the surface and egesting faeces in deeper sediment strata (Fig.1.4). Burrow construction and maintenance results in particle movement through displacement, ingestion, and defecation. Maintaining sufficiently high oxygen tension is suggested to be a possible function for the depth of burrows, as shown in captive lamprey larvae (Galloway *et al.*, 1987).

1.1.3 Habitat

Microenvironmental and macroenvironmental factors have a significant effect on the presence of lamprey larvae. Larvae habitat preferences have been studied in detail both observationally and experimentally, suggesting that one of the most important microenvironmental variables to determine habitat suitability is substrate composition (Aronsoo and Virkkala, 2013). It is vital that larvae can construct borrows and maintain vital water flux within their habitat. Softer substrates are preferred over clay and gravel substrates, an ideal habitat encompasses fine soft sediments, with organic matter and low water velocity (Hardisty, 2006; Smith, Welsh and Turk, 2011). Current research has not yet identified sediment preference changes in larvae with increasing age and size. Where suitable conditions exist, larvae can make up a significant proportion of the streambed biomass (Beamish and Youson, 1987). In addition to areas of high densities, larvae are very mobile animals, and can recolonise areas through drift (White and Harvey, 2003; Bracken and Lucas, 2013). It is important to note this ability of larvae to travel downstream, highlights that conclusions drawn about the locality in which they are found must be made on a broader habitat scale.

1.1.4 Conservation management

All lamprey species in the UK are protected under one or more national or international legislative provisions (Table 1.1). Perhaps the most powerful of these is the European Union (EU) Habitats Directive (HD) on the Conservation of Natural Habitats and of Wild Fauna and Flora. This Directive aims to promote the maintenance of biodiversity, taking account of

economic, social, cultural, and regional requirements (Council of the European Communities, 1992). As a result of decreasing population sizes, five lamprey species are listed in Annex II of the HD. The Annex II listing refers to species for which conservation requires the establishment of Special Areas of Conservation (SACs), often termed Natura 2000 sites, as part of the EU Natura 2000 network of protected areas (Shephard *et al.*, 2018). Three of these species (*Petromyzon marinus*, *Lampetra fluviatilis* and *Lampetra planeri*) occur in Scotland, and a series of SACs have been established to aid in protecting the status of these, unexploited, populations. However, a recent review of the IUCN Red List status of all three of these lamprey species describes all as being ‘Least Concern’ within the latest Great Britain (GB) Red List (Table 1.1; Nunn *et al.*, 2023).

Table 1.1. Detailing national and European legislative provisions for lamprey species, *Lampetra fluviatilis*, *Lampetra planeri* and *Petromyzon marinus*.

Legislation				Relevant schedules and appendices as listed in Table above		
UK Biodiversity Action Plan (BAP)				UK BAP priority fish species list		
EU Habitats Directive (HD)				Annex II - designation as qualifying feature within SACs for the species listed. Annex V – exploitation may be subject to management.		
The Conservation (Natural Habitats etc.) Regulations 1994 (Cons Regs)				Schedule 2 – European Protected Species in GB Schedule 3 – animals that may not be taken in certain ways		
Bern Convention (Convention on the Conservation of European Wildlife and Natural Habitats)				Appendix III – regulation of the exploitation of species listed.		
Red Lists (IUCN Global, and National)				CR – Critically endangered, VU – Vulnerable, LC - Least Concern, EX – Extinct.		
Scientific name	UK BAP	HD Annex	Cons Regs Schedule	Bern Conv Appendix	IUCN (Global)	GB Red List
<i>Lampetra fluviatilis</i> (L.)	Y	II, V	3	III	LC	LC
<i>Lampetra planeri</i> (Bloch)	-	II	-	III	LC	LC
<i>Petromyzon marinus</i> L.	Y	II	-	III	LC	LC

Brook lamprey are still widespread throughout Scotland and the rest of the UK, however river and sea lamprey have a more restricted distribution (Hume, 2017; Shephard *et al.*, 2018). Populations of *P. marinus* are considered to be small, indicated by the monitoring of SACs,

which typically fail to locate suitable larval habitat containing abundant numbers of sea lamprey (Hume, 2017). Also, important to note is the occurrence of ‘freshwater resident’ river lamprey. These populations of river lamprey, of which there are two known UK populations (Loch Lomond in Scotland and Lough Neagh in Northern Ireland), grow to maturity in freshwater rather than going to sea (Hume *et al.*, 2013). This is evidence to suggest that some populations may be of particular conservation importance (Hume *et al.*, 2013; Docker and Potter, 2019).

It is important to note that there are difficulties in our ability to morphologically differentiate between brook and river lamprey larvae (Lucas *et al.*, 2021), which could have implications for underestimating river lamprey population abundances. The same could be said with regards to sea lamprey populations because, whilst we can differentiate between the larvae of *Lampetra* and *Petromyzon*, we may not be sampling these life stages effectively. This may be a result of *Petromyzon* larvae residing in areas where protocols are not established to sample for them effectively, such as in deeper water habitats (Moser *et al.*, 2007; Mueller *et al.*, 2012).

The stark contrast between the declining populations of lamprey in Europe and North America, with the invasive status of *Petromyzon* in the Laurentian Great Lakes in North America, leads management in opposing directions (Lucas *et al.*, 2021). However, restoration programmes of lamprey populations in Europe can utilise information gained by the control management programs of North America (Hansen *et al.*, 2016). Additionally, knowledge surrounding the ecology of lamprey stems predominantly from efforts to control *Petromyzon marinus* populations from the North American Great Lakes (Lampman *et al.*, 2020). In Europe, however, the declining populations of all species of lamprey leads to heightened concern (Davis *et al.*, 2021).

1.1.5 Lamprey larvae feeding and diet

The means by which Lamprey larvae feed is foremostly by passively filtering particles from the seston (Moore and Mallatt, 1980, Malmqvist and Brönmark, 1982; Goodwin *et al.*, 2008; Richardson, Admiraal and Wright, 2009; Docker, 2015). However, experiments suggest larvae can also feed on particles within the substrate pore water (Docker, 2015). Lamprey larvae burrowing activities enables the head to lay at an angle of about 60-70° to the surface of the

sediment (Fig.1.3; Mallat, 1982), and allows for outgoing water to pass through the gill openings directly into the surrounding sediments (Hardisty, 2006).

Larvae are considered detritivores, feeding on organic matter breakdown, including fungi and microorganisms that exist within the biofilm complex (Moore and Beamish 1973; Sutton and Bowen 1994; Mundahl *et al.* 2005). The digestive tract contents of lamprey larvae consist of mainly (>90% of weight) of sand, detritus, algae and invertebrates. Diatoms (0.03-1.50%), other plankton (0.03%), and periphyton unicellular algae (0.05%) and coenobia, as well as plankton rotifers (0.1-0.81%) were also documented (Polyakova *et al.*, 2019). With regards to size, larvae ingest particles within the range of 5-340µm, as found within the guts of both small and large wild larvae (Moore and Mallatt, 1980). Particle selection is primarily thought to be based on size of the oral cilia, with small particles becoming trapped in mucus in networks within the pharynx, which is constantly swallowed (Mallatt, 1981; Hardisty, 2006). Larger particles are suggested to be ejected out of the gills and into the surrounding sediment (Limm and Power, 2011). As the gut passage time of larvae varies from 54 to 70h (temperature dependant), the analysis of gut contents of larvae only preserves a snapshot of the dietary composition (Moore and Beamish, 1973; Evans, Bellamy and Bauer, 2019). Therefore, authors have employed techniques such as stable isotope analysis to reflect assimilated sources and diet composition (Jolley *et al.* 2015; Evans and Lampman, 2019; Evans, Bellamy and Bauer, 2019; Evans and Weber 2020).

The significance and extent of selection for detritus or allochthonous material (i.e. terrestrial) to larvae diet has been an ongoing topic of debate in the literature (Hardisty and Potter, 1971; Kelly and King, 2001; Hardisty, 2006; Dawson *et al.*, 2015; Evans and Lapman; 2019; Hayden *et al.*,2019). Allochthonous material is of lower nutritional value than autochthonous (in-stream) material, such as algae, however detritus is consistently found at large volumes in the intestine of larvae (Hardisty, 2006). This observation is consistent with isotopic studies, which highlight the importance of substrate and deposited organic matter in the larvae diet (Limm and Power, 2011; Evans and Bauer, 2015; 2016). These data suggest that lampreys may acquire food particles within the sediment pore water, functioning as a deposit feeder (Docker, 2015). Both Hayden *et al.* (2019) and Evans and Lapman (2019) highlight that our knowledge surrounding the diet and particle selection by larvae is not complete and needs

further study, however larvae occupy a unique position within river food webs and specialise in a niche foraging on terrestrially derived detritus (Hayden *et al.*, 2019).

1.1.6 Ecological role

Lamprey larvae perform important ecological roles at all stages of their life cycle (Docker, 2015). On a trophic level, larvae can represent a significant proportion of the biomass in some streams (Beamish and Youson, 1987). Therefore, a multitude of animals within this trophic food web utilise larvae as a food source. Predators of lamprey include birds (Cochran, Leisten and Sneen, 1992; Sjöberg, 2011), fish (Sjöberg, 2011) and mammals (Close, Fitzpatrick and Li, 2002). In addition to provisioning biomass and supporting upper trophic levels, larvae contribute importantly to nutrient cycling. Larvae facilitate the conversion of nutrients derived from detritus and algae into stored biomass (Docker, 2015).

Hypoxic sediments are common throughout aquatic environments (Kristensen *et al.*, 2012), therefore buried organisms must develop strategies to overcome anoxic conditions. Lamprey larvae carry out burrow ventilation to flush oxygen into their burrows, thereby increasing oxygen availability within the sediments. This behaviour, termed bioturbation, also contributes to why larvae form a key component within riverine ecosystems, acting as ecosystem engineers; larvae alter their habitat by influencing sediment structure and function (Shirakawa *et al.*, 2013). Faunal bioturbation is all transport processes carried out by animals that directly or indirectly affect sediment matrices, including, particle reworking and burrow ventilation (Kristensen *et al.*, 2012). Bioturbation activities include burrowing and feeding actions, that organisms complete which results in changes in physical and chemical properties of the sediment (Mermillod-Blondin *et al.*, 2011; Kristensen *et al.*, 2012; Bartoli *et al.*, 2020). Recognised as a crucial component in models of sediment dynamics (Paarlberg *et al.*, 2005), the removal of keystone bioturbators could result in changes in habitat structure and subsequent impacts on local biodiversity and ecosystem functioning (Coleman and Williams, 2002). As a species which aids in nutrient cycling within stream beds and water catchments, lamprey larvae represent a hidden majority which are fundamental to freshwater ecosystems. Declines in the population abundance of some lamprey species have been observed over the last hundred years, with significant losses seen in the anadromous species (Maitland and Campbell, 1992; Kelly and King, 2001; Maitland, 2003). However, these declines could be

attributed to lack of reliable protocols and efficient methods to survey these populations (Nunn et al., 2023).

1.2 Ecological impacts - a focus on microplastic contamination in riverine habitats

Despite some species of lamprey being ecologically and culturally important, many lamprey species face population declines because of a multitude of anthropogenic stressors (Docker *et al.*, 2015; Lucas *et al.*, 2020). Lamprey species face a range of anthropogenic stressors, with the most significant threats being barriers to migration, pollution and habitat degradation (Renaud, 1997; Close *et al.*, 2002), leading to decline of lamprey populations throughout Europe (Hume *et al.*, 2021). Increasing temperatures, due to climate change, are also impacting physiological processing in lampreys, as documented in the survival rate of Pacific lamprey embryos (Meeuwing *et al.*, 2005). Being impacted across a multitude of stressors, lamprey could represent a sentinel species within freshwater habitats to monitor contaminants of concern and wider ecosystem impacts of cumulative anthropogenic stressors.

As of 2020, the total material output of human endeavour surpasses the sum of all living biomass on earth (Elhacham *et al.*, 2020). As a result of such profound human impact on the earth, it has been proposed that the current geological epoch be renamed the Anthropocene (Crutzen, 2006; Steffen *et al.*, 2007; Zalasiewicz, 2012; Lewis and Maslin, 2015; Waters *et al.*, 2016). Global plastic mass, both in current use and plastic waste, is estimated at double the mass of all living animal biomass (Elhacham *et al.*, 2020). Plastic pollution is one of the key candidates as evidence for the onset of the Anthropocene, along with increases in carbon dioxide and methane (Steffen, 2011). Microplastics are a major threat to biosphere integrity and planetary health, alongside with climate and biodiversity impacts (Elhacham *et al.*, 2020). As a novel and complex stimulus, plastic contamination the potential to threaten already damaged ecosystems and biological processes, and ultimately ecosystem services we rely on as a global community.

1.2.1 The challenges of microplastic research

Microplastics are currently defined using ambiguous terminology, resulting in confusion and miscommunication within the scientific community and more broadly across sectors. Rochman *et al.* (2019) and Hartmann *et al.* (2019) suggest the need for the establishment of

a universal criteria for identifying microplastics. Hartmann *et al.* (2019) proposes clear and well-defined categories: (i) nanoplastics: 1 to <1000 nm; (ii) microplastics: 1 to <1000 µm; (iii) mesoplastics: 1 to <10 mm; (iv) macroplastics: 1 cm and larger. The following chapters will use this classification to aid a common language across the field.

Microplastic research is fraught with challenges; to gain robust and reliable data it is a resource and time intensive process (Hersen *et al.*, 2017; Meng *et al.*, 2020; Zhu and Wang, 2020). This stems from the complications of microplastics existing ubiquitously (Lim, 2021), to the challenges of isolating micro-sized particles from complex organic matrices (O'Connor *et al.*, 2020). To produce data on which valid conclusions can be drawn, it is essential to follow a series of procedural methods which abide by strict criteria, this includes both positive and negative controls, contamination controls and reliable analysis techniques (Hermsen *et al.*, 2018; Collard *et al.*, 2019). However, often studies do not adopt these procedural methods, leading to lack of relevance for their findings (Hermsen *et al.*, 2018).

There are multiple challenges associated with extraction and detection of microplastics from complex matrices, particularly for biota (Kukkola *et al.*, 2021). This is due to a lack of standardised procedures for many species, and as each species is composed of varying amounts of proteins, lipids and chitin it is difficult to use procedures which are applied all organisms (von Friesen *et al.*, 2019). There are currently no standard operating procedures for the digestion of lamprey to isolate microplastic particles. Although methods from the digestion of fish and bivalves can form a starting point, it is essential to determine protocols which consider lamprey specifically. This is because physiological differences between biota can present challenges for efficient digestion to isolate synthetic particles. For example, as opposed to a mineralised skeleton multiple structures within lamprey larvae are formed of cartilage. This cartilage can be resistant to digestion protocols and hinder microplastic detection on the final filter (Dehaut *et al.*, 2016).

It is also important to note that challenges arise as microplastics are recognised not just as a particle or single contaminant, but as a diverse suite of contaminants which can exist as both macroplastic debris, but also in particulate form (Wagner *et al.*, 2018; Rochman *et al.*, 2019; Bank *et al.*, 2022). This is of particular importance to bear in mind when assessing the impacts of different polymer types, origins, pollutant history and subsequent ecotoxicological impacts on biota. Considering plastic debris as a diverse suite of contaminants reflects its complex mix

of synthetic and environmental history. Most additives such as plasticisers, flame retardants and antimicrobials are not covalently bound to the polymer matrix, therefore they can disassociate from the polymer backbone and leach into the surrounding environment (Hermabessiere *et al.*, 2017; Paluselli *et al.*, 2019; Schmidt *et al.*, 2019). Such additives can have negative impacts on biota, ranging from developmental toxicity (Oliviero *et al.*, 2019; Capolupo *et al.*, 2020; Gardon *et al.*, 2020) to mortality (Li *et al.*, 2015). Conclusive evidence for the transfer of hazardous compounds from the polymer to organisms via environmental media is lacking; however, studies have identified compromised physiological functions as a result of plastic ingestion (Teuten *et al.*, 2009; Wright *et al.*, 2013; Syberg *et al.*, 2015). Furthermore, plastic debris is lipid rich and provides an ideal colonisation surface for bacteria, presenting microplastic particles as a potential food source to biota (Ye and Andrady, 1991; Zettler *et al.*, 2013; Harrison *et al.*, 2014; McCormick *et al.*, 2014; Nauendorf *et al.*, 2016). These factors among other logistical and resource challenges prevent the of monitoring such a complex pollutant and a full emergence of microplastics as a regulated contaminant.

1.2.2 Microplastics in Freshwater Ecosystems

The global health of freshwater ecosystems, and the species inhabiting them has been declining more rapidly compared to terrestrial and marine biomes (Sala, 2000; Jenkins, 2003). These declines in both species and overall health are a result of multiple anthropogenic stressors, including climate change, pollution, exploitation, invasive species, and habitat degradation (Ormerod *et al.*, 2010). Research highlights the complexity of the issue, with multiple and often conflicting demands on water and land-use and management, alongside key knowledge gaps in freshwater pollution (Harrison *et al.*, 2010). Management of important multifunctional ecosystems, including lentic and lotic freshwaters, which provide key ecosystem services is underpinned by evidence-based research (Harrison *et al.*, 2010). The freshwater environment is reliant on both biotic interactions between trophic positions and between abiotic macro and microenvironmental compartments. Aquatic biota can significantly alter stream bed properties, increasing oxygen availability and redox potentials within the stream bed (Boeker *et al.*, 2016). Such natural alteration of the physical environment by organisms is a crucial interaction in most ecosystems (Shirakawa *et al.*, 2012). Fluvial systems accumulate some of the highest levels of microplastic contamination (Hurley *et al.*, 2018; Rochman, 2018; Rowley *et al.* 2020), with particularly high contamination

associated with urban areas (Woodward *et al.*, 2021). Rivers represent both a major microplastic pathway to oceans (Lebreton *et al.*, 2017; Meijer *et al.*, 2021), and act as long term microplastic sinks (Drummond *et al.*, 2020; Margenat *et al.*, 2021).

Microplastics are one of the least studied groups of contaminants in river systems and research focused on microplastics in freshwater is still in its infancy (Eerkes-Medrano, Thompson and Aldridge, 2015; Blair *et al.*, 2017; Blettler *et al.*, 2018). However, current evidence from freshwater systems suggests microplastic presence and impacts are equally as far reaching as what has been observed in marine systems (Faure *et al.*, 2012; Eriksen *et al.*, 2013; Imhof *et al.*, 2013; Free *et al.*, 2014; Eerkes-Medrano *et al.*, 2015; Nava *et al.*, 2023). The presence of microplastic in freshwater systems, in some instances, exceeds estimates for the marine environment for some areas, at ~517,000 particles m⁻² (Hurley *et al.*, 2018). Sources of microplastics to rivers include effluent from wastewater treatment plants (Habib, Locke and Cannone, 1996; Magnusson and Noren, 2014; Talvitie *et al.*, 2015; Mahon *et al.*, 2016; Murphy *et al.*, 2016; Ziajahromi *et al.*, 2017; Woodward *et al.*, 2021), inappropriate waste management (Free *et al.*, 2014), run-off from agricultural soils (Nizzetto *et al.*, 2016), urban environments (Baldwin *et al.*, 2016; Ballent *et al.*, 2016; Horton *et al.*, 2017) and debris from roads in the form of tyre wear particles (TWP) (Kole *et al.*, 2017; Wagner *et al.*, 2018; Knight *et al.*, 2020). Lentic systems are also heavily impacted by microplastic contamination, particularly those close to densely populated and urbanised areas with elevated deposition areas, long water-retention times (Horton *et al.*, 2017; Nava *et al.*, 2020; Talbot and Chang, 2022; Nava *et al.*, 2023). Microplastics, therefore, contribute a novel stressor to freshwater habitats and the organisms that live within them, which already face a range of anthropogenic pressures.

It is important to consider that microplastic research is in a phase of rapid advancement and method development is at the heart of much of the current research. To take tyre wear particles (TWP) as an example; research suggests that this type of microplastic is suspected to be abundant, yet is currently widely unreported, most likely due to methodological limitations for quantifying black microplastic particles (Knight *et al.*, 2020). Instead, estimations are most often based on quantification using chemical markers (e.g. Zn, or organic compound markers) for the particle's presence. It has recently been estimated that 18% of micronized tyre rubber is discharged from land to freshwater (Unice *et al.*, 2019a,

2019b), however little research has been conducted to understand the potential impacts of such inputs on freshwater biota (Halle *et al.*, 2019). New studies have identified a preservative used in tyres as a major contaminant within tyre and road wear particles in freshwater ecosystems, causing consistent mortality in Coho salmon (*Oncorhynchus kisutch*). This pioneering research narrowed down 6PPD-quinone from thousands of unidentified chemicals leaching from tyres (McIntyre *et al.*, 2021; Tian *et al.*, 2021). Ubiquitous in roadway environments, 6PPD-quinone can leach readily from TWP leading to transport into receiving waters, thereby posing high potential for adverse effects in local aquatic environments (Hu *et al.*, 2023). Subsequent research has since both confirmed and found no evidence of the toxicological effects of tyre rubber-derived 6PPD-quinone on aquatic species, suggesting it may be a species-specific response (Hiki *et al.*, 2021; Varshney *et al.*, 2022; Maji *et al.*, 2023).

1.2.3 Deposition of microplastics within freshwater sediments

Riverine systems are highly heterogeneous in their biophysical components and this complexity underpins their long-term vitality (Fig. 1.5, Stanford, 1998). The hyporheic zone is defined as the area below the streambed interface that is equally influenced by surface and groundwater flow dynamics (Fig. 1.5, Boano *et al.* 2014). This zone is an ecologically sensitive compartment of fluvial ecosystems, acting as an important habitat for many organisms at the base of the riverine food web (Boulton *et al.*, 1998).

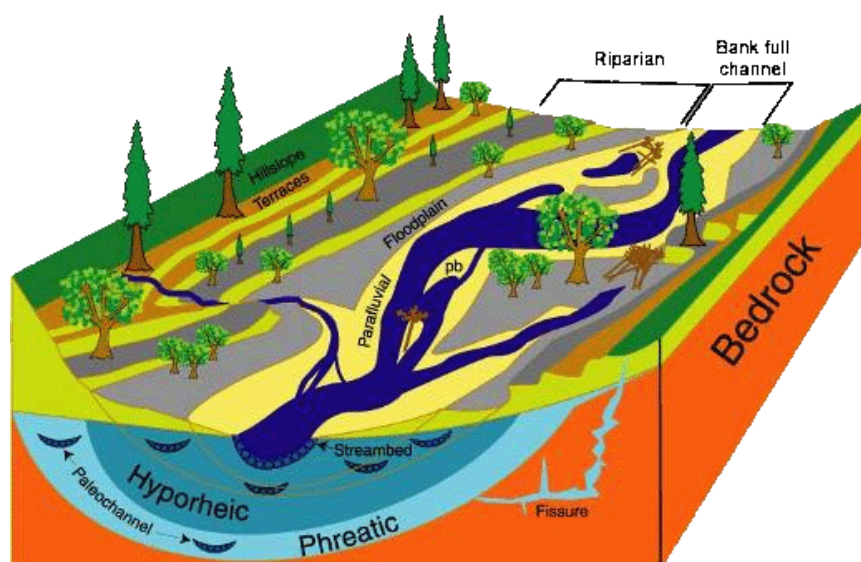


Figure 1.5 Microplastics can accumulate in the hyporheic zone of riverine systems. The hyporheic zone is a subsurface area beneath and alongside a river or stream, where surface water interacts with groundwater. It's a dynamic and ecologically important zone where water flow, sediment transport, and biogeochemical processes occur. Image credit: Stanford, 1998

Organisms which reside within this zone, and undergo sediment reworking and burrow ventilation (i.e., bioturbation) significantly alter the hyporheic flux, residence times and penetration depth of solutes, as shown in laboratory experiments (Shrivastava, Stewardson and Arora, 2021). The larval stage of lamprey is one of these important organisms which reside within the sediments of the hyporheic zone and contribute to ecosystems services within the stream bed.

However, the hyporheic zone is also identified as an important accumulation zone and temporal sink for microplastics (Frei *et al.*, 2019). Factors ranging from the hydrodynamics of river systems to input sources will determine both deposition of sediments and microplastic concentrations within each zone (Vianello *et al.*, 2013; Zhang *et al.*, 2017; Rodrigues *et al.*, 2018; Tibbetts *et al.*, 2018; Corcoran *et al.*, 2019). For example, when a river loses energy the lower water flow velocity may result in sedimentation of plastic debris (Vianello *et al.*, 2013; Ballent *et al.*, 2016; Zhang *et al.*, 2017). Drawing on knowledge from a diversity of fields, understanding and techniques from sediment research can aid in overcoming challenges in the field of microplastic research (Waldschläger *et al.*, 2022). Microplastic particle distribution within a catchment drainage network may also be determined by characteristics of the polymer itself, for example density, as well as other external factors, such a river flow, turbidity, water depth, substrate type and river topography (Castañeda *et al.*, 2014; Eerkes-

Medrano *et al.*, 2015; Klein *et al.*, 2015; Simon-Sánchez *et al.*, 2019). Density modification processes of synthetic particles, such as aggregation, photo-degradation, biofouling, interactions with biota, will affect the characteristics of the polymer and therefore the fate of microplastics within fluvial systems (Frei *et al.*, 2019).

During flood events, there is clear evidence of partitioning of microplastic load, with fibres flushing downstream (Woodward *et al.*, 2021). It is also important to note that evidence of hydraulic sorting and dilution produces reduced microplastic concentrations in the overbank, when compared to the river channel (Woodward *et al.*, 2021). The dynamic nature of rivers may result in local temporary sinks being rapidly altered due to changes in hydrodynamics, such as floods (high flow rates), channel currents (variations in flow rates, changes in depth) and stagnant water (depositional environments) (Klein *et al.*, 2015; Nizzetto *et al.*, 2016).

The interplay between dynamic biotic interactions within the sediment of the hyporheic zone, and microplastic deposition is not yet fully understood. However, as a biologically important zone, which is also highlighted as an accumulation zone for microplastics, there could be potential for biota to be exposed to contamination at higher occurrence rate, when compared to the open channel. Understanding the accumulation of microplastic debris and associated contaminants in depositional hotspots within riverine systems is key to aid management of habitats and associated biota within river compartments. A wider evidence base on the levels and characteristics of microplastic deposition in sediments is developing, but there are a limited number of studies investigating the extent of microplastic contamination in freshwater sediments (e.g. Wagner *et al.*, 2014; Fischer *et al.*, 2016; Bessling *et al.*, 2017).

1.2.4 Microplastic exposure in freshwater fish

Freshwater fish represent a greatly diversified group (Dennis, 1989), which facilitate vital ecosystem functions (Jackson *et al.*, 2001; Dugan *et al.*, 2006; Brummett *et al.*, 2013) and are highly economically valued (Dugan *et al.*, 2006; Arismendi and Nahuelhual, 2007; Gupta *et al.*, 2015). Freshwater fish have also been identified as a key receptor and bioindicator of microplastic pollution, representing a strong model taxon for developing knowledge on how microplastics affect ecology and behaviour of animals (Andreou *et al.*, 2021). They represent one of the most studied groups of taxa with regards to microplastic ingestion in the freshwater environment (O'Connor *et al.*, 2019). However, despite negative impacts being

well-documented in marine species and high estimates of microplastic pollution in the freshwater environment, research on the interactions between microplastics and freshwater fish is still in its infancy (Wagner *et al.*, 2014; Blettler *et al.*, 2018; Andreou *et al.*, 2021). Therefore, global understanding of microplastic ingestion rates by freshwater fish is hindered by small sample size, inconsistencies between methodologies, alongside limitations in taxa studied and research location (Collard *et al.*, 2019). It is also important to note that no specific method has been defined as the standardised method to be used in fish gut contents analyses (Collard *et al.*, 2019).

Plastic contamination has been identified in multiple freshwater fish species (Possatto *et al.*, 2011; Andrade *et al.*, 2019; Hurt, O'Reilly and Perry, 2020; Kusmieriek and Popiolk, 2020; Aytan *et al.*, 2023); however, concentrations vary between taxa, life stages, trophic guild and feeding strategy (Ferreira *et al.*, 2016; Horton *et al.*, 2018; McGoran *et al.* 2018; McNeish *et al.*, 2018; Costa *et al.*, 2023). Microplastic uptake patterns are diverse across aquatic biota, with microplastics shown to be consumed size-selectively by some aquatic organisms such as mussels, and conversely no distinct patterns in fish (Kankiliç *et al.*, 2023). Microplastic ingestion by freshwater fish is expected to occur during active feeding and gill ventilation (Abbasi *et al.*, 2018; Hurt *et al.*, 2020; Park *et al.* 2020). Accumulation of microplastics via the gills has been observed in experimental studies (Mak *et al.*, 2019; Roch *et al.*, 2020). Once ingested or taken up via the gills, microplastics can present a stressor to freshwater fish in three broad ways: (1) physical blocking of the GIT or injury to tissues (2) leaching of polymer additives (3) desorption of environmental contaminants bound to the particle. Each one of these stressors present themselves in non-mutually exclusive ways or together as a dynamic stressor. Therefore, the implications of microplastic exposure on freshwater fish is highly complex and heterogeneous. The impact of synthetic contamination depends on a huge range of factors, from concentration, size, polymer type, and the ecology of the species (Andreou *et al.*, 2021).

One key finding across studies has been the correlation between the number of ingested microplastics and exposure gradient within the environment or distance from pollution source (Ferreira *et al.*, 2016; Horton *et al.*, 2018). It is important to note that some studies classify particles as microplastics based on visible characteristics and no analytical techniques are employed to confirm the polymer type (Kusmieriek and Popiolk, 2020). Therefore, studies

such as these must be interpreted with caution, as the results may not reflect the true number of actual microplastics. Furthermore, often when using visual techniques particles <0.5mm are omitted from such studies due to the limitations associated with observational methods used to identify the suspected microplastics.

Experimental exposure of freshwater fish to microplastics allows a comprehensive and controlled view of the physiological and genetic effects that microplastic exposure elicits. However, experimental studies are highly variable with regards to exposure type and length, therefore making broad conclusions from these data is difficult. Microplastic ingestion has been observed experimentally to cause a reduction in immunity, and diversity and composition of gut microbiota in juvenile freshwater guppies (Huang *et al.*, 2020). Oceanic fish larvae that have been exposed to microplastics have exhibited significant toxic effects, including death, decrease in growth, behavioural changes and alternations to the DNA (Pannetier *et al.*, 2020). Ingestion and subsequent excretion are dependent on fish size, and re-consumption of microplastic beads is evident (Ory *et al.*, 2018; Hoang and Felix-Kim, 2020). In contrast some laboratory studies do not find evidence that microplastics cause acute harm to fish after dietary exposure, with effective elimination of microplastics from the body (Jovanovi *et al.*, 2018; Ory *et al.*, 2018).

There is evidence to suggest fish reject microplastics, except when they are in combination with food items (Kim *et al.*, 2019; de Sa *et al.*, 2015; Xiong *et al.*, 2019). Therefore, as lamprey larvae feed in a non-selective manner, exposure of microplastic contamination could present during feeding on organic detritus, diatoms and algae or burrowing within the sediment (Hardisty, 1971; Moore and Mallatt, 1980). Furth more, older, weathered microplastics with a developed ecocorona, masked within the detritus, could further increase ingestion probability. Food particles are likely to be inextricably linked to microplastic contamination, if it is present. The feeding strategy of larvae coupled with the matrices in which they passively forage could predispose them to a higher incidence rate of microplastics. For example, marine fish species with the most efficient filtration apparatus, were found to ingest the highest number of anthropogenic particles (Collard *et al.*, 2017).

These studies highlight ingestion and occurrence of microplastics in freshwater fish, alongside the physiological and toxicological impacts elicited from microplastic ingestion. Additionally, these studies highlight that microplastic input sources and individual fish characteristics (size,

feeding strategies) are associated with ingestion rates and occurrence in the gut. Furthermore, studies highlight that microplastics can reside in other organs of the fish (e.g. gills) and dissection of the gestational intestinal tract from a sample may hinder a complete picture of microplastic particle occurrence. However, with freshwater fish having a wide range of life histories and feeding strategies dissimilar to larvae, the following section focuses on other freshwater and terrestrial biota in which comparisons can be drawn to gain further understanding of potential larvae vulnerability.

1.2.5 Implications of microplastic contamination for lamprey larvae

Due to the protracted larvae stage of the lamprey life cycle in freshwater, anthropogenic contaminants in fluvial sediments are a potential concern, particularly given their benthic filter feeding behaviour. (Soimasuo *et al.*, 2004). Larvae may be sensitive to a multitude of anthropogenic stressors, including metal contamination (Myllynen *et al.*, 1997), persistent organic pollutants (POP; Tapie *et al.*, 2011) and pesticides (Andersen *et al.*, 2010). However, the concentrations of these contaminants in lamprey wild are below lethal levels (Madenjian *et al.*, 2020), suggesting that a series of sub-lethal effects may be acting on lamprey life history traits, such as growth, behaviour, reproduction and feeding. The following section draws on evidence largely from animals with similar feeding and life history strategies to lamprey larvae, which allows discussion of potential larvae vulnerability to microplastics.

Suspension and filter feeders are suggested to be particularly vulnerable to microplastic pollution as they commonly feed on suspended or depositional particulate matter (Galloway *et al.*, 2017; Su *et al.*, 2018). The filtration system of filtering fish defines the size limit of microplastic particles that are retained and subsequently swallowed. Research from the marine environment suggests that as the size of plastic particles decrease, the diversity of organisms that can ingest the particles increases (Auta *et al.*, 2017). For filter feeding organisms the size of microplastic particles that organisms can ingest will depend on the size of particles that they naturally ingest (Wagner and Lambert, 2018). Particle selection in lamprey larvae is thought to be based on size of the oral cillae, with small particles becoming trapped in mucus in the pharynx (Hardisty, 2006). Therefore, there is a positive relationship between the body size of larvae and the size of particles which can be ingested. Algae collected from larvae guts range from 260-400 μm , whilst organic particles and sand grains in the gut can be as small as 30 μm (Hardisty, 2006). Adverse effects of microplastic ingestion by

non-selective filter feeders includes blockages, reduced dietary intake and internal injuries. Additionally, it is important to consider variation in gut retention time between species and plastic size, which is an area which is not well understood (Collard *et al.*, 2019). Once incorporated into the food web, plastic particles may be retained and transported up the food web as a consequence of predation (Nelms *et al.*, 2018; Welden *et al.*, 2018).

Lamprey larvae burrow and feed in sediments in a similar fashion to oligochaete freshwater sludge worms (*Tubifex tubifex*), marine polychaetes (lugworms *Arenicola marina* and ragworms *Nereis diversicolor*), terrestrial earthworms and nematodes. Both oligochaetes and polychaetes have been shown to accumulate microplastics and exhibit subsequent ecotoxicological effects associated with exposure (Van Cauwenberghe *et al.*, 2015; Hurley *et al.*, 2017; Revel *et al.*, 2018; Muller-Karanassos *et al.*, 2019; Mueller *et al.*, 2020). Terrestrial earthworms ingest microplastics with effects on reproduction (Lahive *et al.*, 2019), energy reserves (Wright *et al.*, 2013), oxidative stress (Rodriguez-Seijo *et al.*, 2018), tissue damage and immune response (Rodriguez-Seijo *et al.*, 2017), egestion rates and long-term retention (Lahive *et al.*, 2022). Further experimental research also highlights the possibility of microplastics to move additives and pollutants to lugworms (*A. marina*), thereby reducing fitness and physiological functions (Browne *et al.*, 2013).

It is important to consider the complex interactions that biota can have with microplastics, such as the ability for animals to alter the behaviour of synthetic particles within substrates. Micro- and nanoplastics are both incorporated into the burrows of earthworms (Huerta Lwanga *et al.*, 2017) and transported in both the horizontal and vertical soil horizon (Maass, Daphi, Lehmann, & Rillig, 2017; Rillig, Ziersch, & Hempel, 2017; Heinze *et al.*, 2021). This could have implications for freshwater ecosystems, as the way in which water-saturated sediments of river catchments are reworked is remarkably like terrestrial soils (Meysman, Middelburg and Heip, 2006). Furthermore, retention of microplastics in sediments during high flow conditions has been evidenced through the binding of sediments with silk strands from the caddis fly (Frei *et al.*, 2019). As an ecosystem engineer and bioturbator, lamprey larvae, could influence the fate of microplastics within sediments.

Referring back to the important consideration of microplastic contamination as a diverse suite of contaminants (Rochman *et al.*, 2019), the heterogeneity of plastic shape, polymer type and size results in variations in interactions with biota. For example, the freshwater shrimp

Hyaella azteca has been shown to take a significantly greater time to excrete microplastic fibres, when compared to microplastic particles or natural food items. The gut clearance time for fibres was additionally shown to increase in a dose-dependent manner (Au *et al.*, 2015). Fibres are found as the most common type of microplastic ingested by coastal fish species, with black being the most common colour (Koraltan, Mavruk and Güven, 2022). Furthermore, tyre wear particles have a longer gut-retention time when compared to microplastics (Redondo-Hasselerharm *et al.*, 2018a). Therefore, results for one type of microplastic cannot easily be extrapolated to all types of microplastic contamination.

Based on the discussed literature, the vulnerability of lamprey larvae to microplastic contamination as a sub-lethal stressor could be high, due to a range of factors. Firstly, higher loadings of microplastic contamination have been observed in sediments compared to the water column (Bondelind *et al.*, 2020; Boucher *et al.*, 2019; Li, Geng *et al.*, 2019). Therefore, larvae foraging and bioturbating in the benthos of riverine habitats, may encounter higher concentrations of microplastic contamination than pelagic species. Secondly, considering the ingestion and toxicological impacts in other biota with similar feeding strategies and life histories, it suggests that larvae lamprey larvae could be vulnerable to microplastic contamination. However, further research is needed to elucidate whether this is the case.

For ecosystems already affected by anthropogenic contaminants and stressors, the potential for microplastic contamination to contribute to altering ecosystem functioning is high (Hooper *et al.*, 2012; Cornejo *et al.*, 2019). European lamprey populations are highly vulnerable to sediment contamination and anthropogenic stressors (Maitland *et al.*, 2014), with mitigation challenged by a combination of land-use management, anthropogenic inputs, hydrological processes and population pressures, which are also strongly linked to plastic contamination hotspots and behaviour (Baldwin *et al.*, 2016; Nizzetto *et al.*, 2016; Hitchcock and Mitrovic, 2019). However, despite this, no research to date has investigated plastic contamination or subsequent ecotoxicological impact on larval or adult lamprey. As a sentinel freshwater fish, which provides numerous ecosystems services, it is expected that further declines of threatened larvae populations could result in implications for provisioning and functioning of freshwater ecosystems. Such services which could be impacted include, nutrient cycling and assimilation and oxygen regulation (Webster, 1975; Shirakawa *et al.*, 2013; Boeker *et al.*, 2016).

1.2.6 Lamprey larvae susceptibility and vulnerability to microplastic contamination

With regards to lamprey larvae susceptibility and vulnerability to microplastic ingestion, it is important to firstly consider how microplastics could be interacting with the feeding mechanisms of lamprey. Secondly it is important to discuss how this could contribute to main effect mechanisms for toxicity of microplastics. In freshwater habitats biofilms are important sites for carbon and nutrient transformation, forming the base of food webs and contributing to local and global and ecosystem functioning (Battin *et al.*, 2016; Harrison *et al.*, 2018). Whilst these biofilms form the basis of lamprey larvae diet, lipid-rich biofilms form a colonisation surface for bacteria on lipophilic microplastic pollution (Kettner *et al.*, 2019). The sludge worm (*T. tubifex*) exhibits preferential ingestion of particles associated with increased organic material (Rodriguez *et al.*, 2001). Synthetic particles with a biofilm (ecocorona) are also evidenced to be more readily ingested (Murano *et al.*, 2021), translocated (Ramsperger *et al.*, 2020) and elicit increased toxicity within biota (Murano *et al.*, 202).

The means by which lamprey larvae detect high value patches on which to feed is not yet fully understood, however it is likely that olfactory stimuli (i.e., volatile organic compounds) induce behavioural response in larvae, due to lamprey evolution in a world of dissolved chemicals (Barreiro-Iglesia *et al.*, 2010). Chemoreception is functionally important for the entire life cycle of lamprey, from spawning (Bjerselius *et al.*, 2000; Meckley *et al.*, 2012; Daghfous *et al.*, 2012) to feeding (Barreiro-Iglesias *et al.*, 2010). Dimethyl sulfide (DMS) is evidenced to be an important foraging cue for marine fish (DeBose *et al.*, 2008; DeBose *et al.*, 2010), seabirds (Nevitt *et al.* 1995; Savoca *et al.*, 2016; Dell’Ariccia *et al.*, 2014) and other aquatic animals (Cunningham *et al.*, 2008; Endres and Lohmann, 2012). As DMS signals the degradation of lignin by microorganisms (Lomans *et al.*, 2002), the presence of DMS could therefore act as a chemosensory cue for lamprey larvae to forage and navigate in the freshwater environment. The production of DMS is also linked to the degradation of microplastic particles and biota have been shown to alter their feeding behaviour because of DMS being present (Savoca *et al.*, 2016). This could have implications for the ingestion of microplastics for lamprey larvae, however there is currently no research investigating the influence of DMS on lamprey larvae and microplastic ingestion.

Decreased nutritional value of food (i.e., food dilution) due to microplastic ingestion, is a commonly proposed effect mechanism for toxicity of microplastics (Koelmans *et al.*, 2020;

Rauchschwalbe *et al.*, 2021). Lamprey larvae are continuously exposed to natural organic particles, therefore damage from exposure to the particles themselves may be negligible for digestive function and processes. However, microplastics may pose an ecotoxicological risk through their inherent particulate properties (size, shape), leaching of polymer additives (Paluselli *et al.*, 2019) or increase microbial load (Amaral-Zettler *et al.*, 2020), compared to natural particles, within biota tissue. Microplastics also interact with contaminants such as polycyclic aromatic hydrocarbon differently to natural sediment particles, having a high sorption capacity (Wang and Wang, 2018). The hazard from desorption of contaminants from microplastics is expected to be small, however due to the quantity of natural sediment compared to microplastics in aquatic environments (Koelmans *et al.*, 2016; Koelmans *et al.*, 2013). However, if large quantities of microplastics (e.g., wastewater effluent) enter pristine systems, it could result in concern of receiving regions due to high sorption capacities (Teuten *et al.*, 2009). Additionally, microplastic aggregates can be formed from algae, organic matter, sediment, or dissolved substances, which lamprey larvae may mistake for algae rich food substances, thereby increasing exposure during feeding (Wagner and Lambert, 2018). Such microplastic aggregates can foster significantly different microbial communities compared to natural particles, thereby presenting risk to individuals (Zettler *et al.*, 2013; McCormick *et al.*, 2016; Ogonowski *et al.*, 2018; Miao *et al.*, 2019; Mughini-Gras *et al.*, 2021).

If larvae select particles based on the microbial load or DMS signals and the particle is simply acting as a vector, synthetic particles could be suspected to make up a higher composition of an larvae diet. This is suggested based on the evidence that microplastic particles act as vectors for complex microbial communities, which significantly differ from particles in the water column and suspended organic matter (Zettler *et al.*, 2013; McCormick *et al.*, 2014; Kettner *et al.*, 2019; Amaral-Zettler *et al.*, 2020).

1.3 Objectives of the thesis

The objective of this thesis is to fill in key knowledge gaps on microplastic contamination in both larval lamprey and the habitats in which they inhabit. To reduce uncertainties around microplastics as a contaminant of concern and to inform conservation management it is first essential to overcome barriers in the collection of microplastic research. Once standard operating procedures for isolating microplastics from lamprey larvae have been established, environmental data can be gathered reliably. This data can then be used to inform

laboratory behavioural and toxicological studies using environmentally realistic concentrations of microplastics. Below I will outline the specific aims of each chapter, clearly defining the expectations for the work.

Chapter 2: Prior to providing robust and reliable estimates of microplastic ingestion in lamprey larvae, it is necessary to determine and optimise methods to isolate microplastics from complex tissue samples. As this has not been developed for lamprey larvae before, chapter two begins with this objective. The expectations for this work are to utilise a small sample of lamprey larvae to provide key recommendations and standard operation procedures to enable the analysis of a broader suite of samples from across Scotland in chapter three. The aim here is to also strongly consider key experimental designs to overcome the challenges of microplastic research, such as contamination and recovery controls, including negative and positive controls to ascertain reliable estimates of microplastic particles ingested by lamprey larvae.

Chapter 3: The aim of chapter three is to take the learnings of chapter two forward, with the objective of providing a robust understanding of the levels of microplastic ingestion across habitats in which lamprey larvae are protected (Special Areas of Conservation, SACs) and urban areas in which they are not protected. The expectations are to analyse paired samples of lamprey and sediment samples to determine if microplastic particles ingested are correlated with those in the surrounding sediment. This will provide an understanding of whether the general environment in which lamprey reside has implications for microplastic contamination. It will also explore whether microplastics could be a contributing stressor to the population status of lamprey larvae, aiding future conservation management of UK lamprey species.

Chapter 4: The aim of chapter four is to utilise knowledge gained from chapters two and three, digging deeper into our understanding of microplastic ingestion by larval lamprey. The objective is to understand whether size/age of lamprey larvae influences the composition and count of microplastics in the gut. This piece of research considers a conservation management perspective, determining risk to each cohort of a population to enable directed advice for site conditioning reports within SACs. The expectation of this work is to analyse the variation of microplastic particle count, size and polymer type in lamprey larvae of different aged samples from the River Teith, Scotland. This study will provide further insights into the influence of

age on microplastic ingestion of fish and will contribute baseline data to assess the ecological risk of microplastics in Special Areas of Conservation in Scotland.

The aims of chapters five and six are to draw on data collected in the field studies to consider the behavioural and toxicological implications of microplastic ingestion for lamprey larvae.

Chapter 5: This chapter aims to gain an understanding of whether exposure to microplastic contamination has behavioural implications with regards to microplastic avoidance. Laboratory studies will be adopted to monitor lamprey larvae in response to microplastic exposure with behavioural endpoints. As little research has been conducted on assessing lamprey larvae to contamination, it is expected that this chapter will include method development to identify ecologically relevant end points.

Chapter 6: The objective of chapter six is to provide an understanding about one of the most sensitive and important stage of a lamprey's life cycle. The expectations for this chapter are to identify whether microplastics elicit developmental toxicity in lamprey embryos. The specific aims are to determine and compare the toxicity of leachates and particles from tyre wear microplastics to the development of key embryonic tissue structures of lamprey larvae. This research will adopt immunohistochemistry protocols and monitor development from fertilisation to allow us to determine any effects tyre wear particles elicit.

Together these chapters will complement each other to contribute to our understanding of whether microplastic contamination in lamprey larvae firstly is present, and secondly whether such contamination elicits a toxicological response of which we should be concerned from a conservation perspective. Therefore, finally **Chapter 7** will discuss this, interpreting and explaining the importance and relevance of the results in a wider context.

Chapter Two - Developing Methods for the Isolation of Microplastics from Larval Lamprey and Paired Freshwater Sediment

2.1 Abstract

Despite widespread documentation of microplastic ingestion by freshwater biota, the lack of standardised methods for extracting and quantifying microplastics from biological samples hampers accurate assessment of microplastic burdens in wildlife. Without consistent methodologies and reporting of recovery efficiencies, temporal and geographical comparisons of microplastic hazards remain challenging. In this study, we developed methodological recommendations to establish an efficient protocol for extracting and quantifying microplastics from the gastrointestinal tract of lamprey larvae (*Lampetra*). Our pilot study aimed to identify suitable methods for purification and to assess the presence of microplastic particles in riverine sediment and larval *Lampetra*, marking the first documentation of microplastics in larval lamprey. Our findings reveal detectable numbers of microplastic particles in *Lampetra* larvae and associated sediments, although the variation in abundance, size distribution, and polymer composition between biota and sediment samples was relatively small. Moving forward, methodological considerations to streamline sample processing and reduce microplastic losses and contamination were identified, based on previous oxidative methods identified in the literature. Due to limited sampling, conclusions cannot be drawn about individual sites but should be treated as group or nonspecific to location. By providing methodological recommendations and highlighting key considerations, this research contributes to the ongoing efforts to improve the accuracy and reliability of microplastic assessments in aquatic ecosystems.

2.2 Introduction

In the natural environment, plastic pollution is persistent to degradation due to the complete lack of or low activity of enzymes that have the potential to degrade them (Yoshida *et al.*, 2016; Alshehrei, 2017). Therefore, synthetic plastics are instead broken down mechanically or by photo and thermal-oxidative degradation, into microplastics (<5mm; Arthur *et al.* 2009) and further into nanoplastics (<0.1 μm). It is important to note that the definition of microplastics and how it is defined is constantly progressing within the field, based on the relatively new research area with frequent in-flow of scientific discoveries altering the way in which we see such a complex pollutant (Lusher *et al.*, 2017). Rochman *et al.* (2019), highlights

the need to rethink microplastics as a single pollutant and instead consider it as a diverse contaminant suite. Rochman *et al.* (2019) and Hartmann *et al.* (2019) suggested the need for the establishment of a universal criteria for identifying microplastics, with current ambiguous terminology resulting in confusion and miscommunication. Therefore, the work within this thesis will use the following categories defined by Hartmann *et al.* (2019): (i) nanoplastics: 1 to <1000 nm; (ii) microplastics: 1 to <1000 µm; (iii) mesoplastics: 1 to <10 mm; (iv) macroplastics: 1 cm and larger.

Microplastics have been identified globally in a range of environments (Rochman, 2018) and across multiple species (Sequeira, 2020), however no research to date has documented whether lamprey larvae ingest microplastics. As stated previously, there is an absence of standard operating procedures for quantifying microplastics in freshwater biota, with no methods for lamprey available. Microplastic research is a complex field that presents several challenges and difficulties, which can impact the accuracy, reliability, and comprehensiveness of studies (Ivleva, 2021). The field is still relatively new and rapidly evolving, and as a result, researchers are continually refining methods and expanding our understanding of microplastics, leading to updates in best practices and recommendations (Hermesen *et al.*, 2018). Preliminary research has been focused on documenting levels of microplastics within ecosystems and organisms. Such descriptive studies are crucial in our understanding of the extent of microplastic contamination of ecosystems. However, developing consistent methodologies that generate reliable data on the ingestion of microplastics by biota are essential for environmental monitoring of biota and to support the risk assessment of microplastic contamination in the environment. Although numerous studies investigate microplastic uptake in fish (Sequeira *et al.*, 2020), no studies have documented the occurrence of microplastic ingestion by lamprey larvae. To establish ecologically relevant data for a protected species, it is essential to develop best practice methods which allow reliable estimates of microplastic ingestion by lamprey larvae.

Compared to larger macroplastics, the quantification of microplastics in environmental samples is challenging, due to the issue of separating the small microplastic particles from organic content (Cashman *et al.*, 2020). Isolating microplastic particles from environmental matrices in a robust manner requires high sampling effort (i.e. temporal and resources) (Hersen *et al.*, 2017), therefore standard operating procedures must be established which

consider a representative sample. Isolating microplastics from lamprey or any biological sample involves careful extraction and separation techniques to avoid both losses and contamination, thereby ensuring reliable results. Establishing best practice methods for isolating microplastic from fish samples are essential in our understanding and provide background data for species management and conservation. Despite intensive research to resolve methodological issues in the last decade, there are still many challenges that exist in reliably estimating microplastic particles in biota. Challenges include identifying the correct sample preparation procedure for the tissue (Löder *et al.*, 2017), whilst controlling for contamination using blank recoveries and quantifying recovery of microplastics through positive controls (Prata *et al.*, 2021). Many studies to date do not represent a high standard of quality assurance, particularly with regards to positive and negative controls (Koelmans *et al.*, 2019). Standard operating procedures (SOP) are currently not defined for all biota, and studies adopt a wide range of methods to firstly prepare the sample and isolate microplastic particles, and secondly to analyse the results (Costa *et al.*, 2018; Hermesen *et al.*, 2018; Renner *et al.*, 2018; Silva *et al.*, 2018). This lack of standardisation across methods prevents comparability of results (Hamm *et al.*, 2018) and it is important to focus efforts on identifying preferred or standardised methods for species.

Prior to the identification of microplastics within samples, it is recommended that biogenic matter is removed to prevent interference with analysis and the process is dependent on the size and tissue composition of the species of interest (Kershaw, Turra & Galgani, 2019). The effectiveness of the chemical treatment of samples to isolate microplastics varies, affecting the microplastic particles by degrading the physical properties (Fig. 2.1; Hamm *et al.*, 2018). Several methodologies exist to reduce organic matter in environmental samples, from enzymatic purification (Loder *et al.*, 2017) to physical and chemical separation techniques (Mani *et al.*, 2019; Lusher *et al.*, 2020). The most common agents are oxidizers (e.g., H₂O₂ and NaClO; Jabeen *et al.*, 2017, Pazos *et al.*, 2017, Collard *et al.*, 2018, Fig. 2.1), hydroxides (e.g., KOH; Dehaut *et al.*, 2016, Zhang *et al.*, 2017, Fig. 2.1), enzymes as digesting agents (Loder *et al.*, 2017, von Friesen *et al.*, 2019). Both enzymes and Fenton's agent could be a solution to achieving the efficient, rapid, and inexpensive digestion of the organic matter in the gastrointestinal tract of freshwater fish. However, it is important to consider the effectiveness

of these treatments and how this can negatively affect the synthetic microplastics present (Fig. 2.1).

Due to the ubiquitous presence of microplastics and the potential for toxicity, particularly for smaller particles, it is important that the analysis of microplastics is accurate and efficient to critically assess and describe microplastic contamination (Ivleva, 2021). Analysis here is referred to as the identification and quantification of synthetic particles, namely microplastics. Following sample preparation and purification, microplastics are commonly analysed through visual identification (Löder and Gerdt, 2015), the hot needle method (Devriese *et al.*, 2015) or Nile red staining (Cole, 2016). However, most of these methods risk human bias, resulting in an over or underestimation of microplastics. Alternatively, one of the most powerful methods of chemically analysing microplastic samples is through Fourier-transform infrared (FTIR) spectroscopy (Löder *et al.*, 2015). This study focuses specifically on

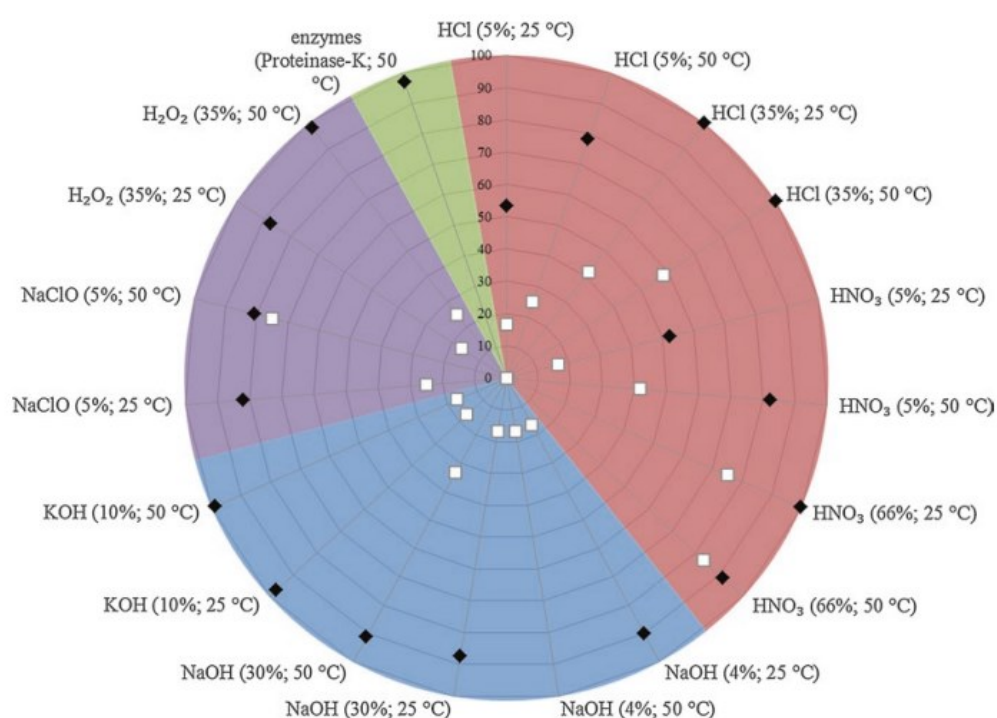


Figure 2.1 Reproduced from Hamm *et al.* (2018) showing the effectiveness of different digestions treatments (black symbols, in %) and maximum percentage of microplastics negatively affected by the treatments (white symbols, based on 12 polymers). Different coloured sectors highlight the different treatments = red: acid, blue = alkaline, violet = oxidative, green = enzymatic.

this spectroscopic method which adopts focal plane array (FPA) based micro-Fourier-transform infrared (μ FTIR) imaging which is proven to be a highly efficient method for microplastic analysis of sample filters. This technique enables chemical imaging of particles of

a whole sample filter, whilst simultaneously recoding thousands of spectra, results in a time efficient and powerful tool (Primpke *et al.*, 2017).

For a species which has not yet been studied, method development of sample preparation is critical to understand technicalities, such as which tissues to target, the mass of biomass suitable for analysis, dissection method and how to separate this biogenic material from the synthetic microplastic particles (Löder *et al.*, 2017). Therefore, this chapter gathers preliminary data to build on existing literature and methods to identify a suitable methodologies and standard operating procedures for the isolation of microplastic particles from lamprey larvae. This groundwork will lay a foundation for subsequent research identifying microplastic uptake in lamprey larvae. The aims of this chapter are to undertake the following:

- 1) Consider both contamination control and blank sample procedures to obtain robust results.
- 2) Develop sample preparation methods to successfully and efficiently isolate microplastics from lamprey larvae samples for analysis with micro-Fourier infrared spectroscopy (μ FTIR).
- 3) Identify the polymer type and size of microplastics that are ingested by lamprey larvae and whether they correlate with that of the surrounding sediment.
- 4) Identify and address any issues which arise from processing samples of sediment and lamprey.

2.3 Methodological approaches, results and discussion

2.3.1 Contamination control and blank sample procedures

Contamination potential of samples with microplastics in clothing or indoor air is widely acknowledged in the literature, leading to the reporting of inaccurate results (Wesch *et al.*, 2017; Hermesen *et al.*, 2018; Koelmans *et al.*, 2019; Scopetani *et al.*, 2020). Unfortunately, contamination control measures are often lacking in microplastic research with many studies including only a few essential measures (Prata *et al.*, 2021). As quality control in microplastic research is vital in reporting accurate results, a contamination control checklist was firstly devised and adhered to, based on recommendations from the literature, to reduce contamination potential and subsequently error rates (See Appendix A).

Procedural blanks are essential to measure background contamination levels as the samples are being processed (Hidalgo-Ruz *et al.*, 2012; Hermesen *et al.*, 2018). To rigorously report and account for the contamination in the final data, a series of steps were undertaken. Firstly, blank corrections from the average of the procedural blanks for each polymer, were run on the raw data. Secondly, the limit of detection (LOD) and limit of quantification (LOQ) were calculated (See Appendix A). This rigorous method ensures that false positive values are not overestimated and should be considered strongly when reporting microplastic concentrations, as recommended by Horton *et al.* (2021) and the Association of Official Agricultural Chemists (AOAC International). Specifically, for this thesis and associated publications, the LOD was defined as the mean of the blanks sample plus 3.3 x the standard deviation of the blank. The LOQ was expressed as the mean of the blank samples plus 10 x the standard deviation of the blank. A set of three blanks per workflow were run for the sediment and biota samples to simulate the sample processing.

The data presented in this chapter were processed inside a fume hood and an atmospheric control was placed out during all steps. To assess the extent of atmospheric contamination from the fume hood environment, an open glass petri dish was left out during all steps. Once sample preparation was complete for all workflows, the contents of the petri dish was rinsed with 50% ethanol three times and collected for contamination control analysis using μ FTIR.

2.3.2 Developing methods to isolate microplastics from lamprey larvae samples for analysis with micro-Fourier infrared spectroscopy.

To identify appropriate methodologies to assess the microplastic contamination of lamprey larvae, a total of 44 *Lampetra* were sampled from the River Carron and River Devon on 28th and 29th January 2020. At each site paired sediment samples were collected in the lamprey survey patch area. A total of four sample sites were selected, two sites on the River Carron main stem (cd83, NS 80856 83083 and dc19, NS 83576 81619) and two sites on the Devon main stem (td84, NS 90980 96484 and cd26, NS 85273 94126). Collected larvae ranged between 57 – 176 mm in length, with a median length of 111mm. At each site four sediment samples were collected at random intervals in the lamprey survey patch area. The sediment surface was sampled in all cases to approximately 10 cm depth using a stainless-steel scoop, combined to fill a 700ml glass Kilner jar, ensuring that minimal excess water was retained. Sediments were stored in darkness at 4°C until further preparation (Horton *et al.*, 2017). See

Appendix A ‘Sediment sample processing’ and Figure 2.3 for methods used for the isolation of microplastics from collected sediments.

Lampetra larvae were collected via electrofishing using smooth direct current (DC) at a 180-Hz frequency. Larvae were collected in a 1L glass beaker in river water and immediately euthanised by an overdose of anaesthetic (Tricaine methane sulphonate, MS222, 300mg/L, Matthews and Varga, 2012). Once euthanised, *Lampetra* were measured on wooden measuring boards. Larvae were wrapped in aluminium foil and snap-frozen in a dry-shipper for transport back to the laboratory. Larvae were stored at -80°C freezer until further analyses. The biota blanks consisted of deionised water (Milli-Q, 0.2µm), which went through the same processes described for the biota samples.

Table 2.1 Number of pooled larvae per sample with corresponding sample code, watercourse, and mean length of larvae (mm) for each site.

Sample Code	Watercourse	Pooled Larvae	Median Length (mm)
cd26	Devon	8	151.13
dc19	Carron	11	124.19
dc83	Carron	11	81.73
td84	Devon	14	93.21

2.3.2.1 Lamprey larvae dissection

To target and quantify ingested microplastics in biota, many studies firstly isolate the gastrointestinal tract (GIT) through dissection of the animal (Lusher *et al.*, 2017). As our primary research question is to understand microplastic ingestion by lamprey larvae, localising investigation to the gastrointestinal tract was relevant here. This removes the ability to detect microplastics which have been translocated from the GIT into adjacent tissues, however this was not the focus of this study and is more relevant for nanoplastics (McIlwraith *et al.*, 2021; Clark *et al.*, 2022). The cartilaginous structures within lamprey larvae are also resistant to digestion protocols and hinder microplastic detection during analysis (Dehaut *et al.*, 2016). Therefore, it is essential to describe an appropriate method to dissect the digestive tract from the larvae.

Larvae were initially defrosted and dissected as shown in figure 2.2, to remove the cartilage backbone, head and gill structure from the larvae. The faeces were carefully removed using a clean metal tool (Fig.2.2b) and added to the glass petri dish. The caudal fin was removed, and animal cut dorsally down the centre line from the eye spot with a pair of sharp metal scissors (Fig.2.2c). The material from the mouthpiece and gills were removed with a sharp metal implement along with the GIT intact (Fig.2.2d). The method described here ensures that the GIT remains fully intact, whilst removing the main cartilaginous structure which is difficult to digest from the sample. All steps were completed in a clean environment, under a fume hood and implements washed with Milli-Q three times between each larva. Lamprey larvae GIT from each site were pooled together to compose one sample per site and the final tissue was either processed directly or stored in aluminium foil at -80°C until processing.

2.3.2.2 Biogenic matter removal



Figure 2.2 Dissection of the gastrointestinal tract (GIT) of lamprey larva prior to sample preparation to isolate microplastic particles. a) intact defrosted larval lamprey, b) faeces removal, c) removal of caudal fin and animal cut dorsally down the centre line from eye spot, d) GIT removed intact, including tissue from the gills and mouth, e) final combined GIT tissue after dissection. Work conducted under contamination control procedures.

Following a literature search of appropriate methods to digest aquatic organisms (Appendix A Table 1), the universal enzymatic purification protocol (Loder *et al.*, 2017) presented the most efficient means of digesting the biogenic material in lamprey larvae. In contrast to chemical digestion, enzymatic digestion of biota samples has been suggested as a method which is both efficient at removing biogenic material and prevents losses, damage, and surface change of microplastics within samples (Fig 2.1; Loder *et al.*, 2017; Lusher *et al.*, 2020). As no methods exist for the digestion of lamprey larvae to isolate microplastics, it was decided that due to the complexity of lamprey tissues (chitin, protein, lipids) and gut contents,

enzymatic digestion presented the most efficient way to remove the biogenic matter. This was also determined based on the needs of the analysis technique, which requires samples to have very low levels of biogenic matter to analyse the final filter accurately. If natural particles are retained, a filter cake builds up on the final filter which results in increased error and uncertainties of the data (Rasmussen *et al.*, 2021).

The initial incubation of larvae aimed to macerate biological cells and increase contact surface for the following enzymatic treatments (Fig.2.3). A solution of 5 % (w/w) Sodium Dodecyl Sulfate (SDS), with a volume of 120 ml per incubation vessel was applied. The samples were incubated at 50°C for 24 hours in a hot water bath. Following maceration via SDS, the sample was filtered using a 10 µm stainless steel mesh. The degradation of organic matter and biological material from the *Lampetra* larva samples was then undertaken following the universal enzymatic purification protocol (UEPP), as suggested by Löder *et al.*, (2017) for planktonic samples. Firstly, protease was used to catalyse the decomposition of protein within the samples. 20 ml protease (Protease from *Bacillus* Species, P3111 Sigma Aldrich) was applied to 100 ml Tris HCL 1 M buffer at a pH of 9 at 50°C for 24 h. The remaining solution was subsequently filtered through a 10µm stainless steel mesh. Lipase was applied, as UEPP suggests, after the protease step, to account for lipids released during the digestion (Löder *et al.*, 2017). 5 ml lipase was added to 100 ml Tris HCL 1 M buffer at a pH 9 at 40°C for 24 h. As previous steps, the solution was filtered through a 10µm stainless steel mesh prior to the next step. Following treatment with lipase, the samples were incubated with 5ml chitinase in 100 ml NaOAc 1 M buffer at a pH 5 at 37°C for 72 hours.

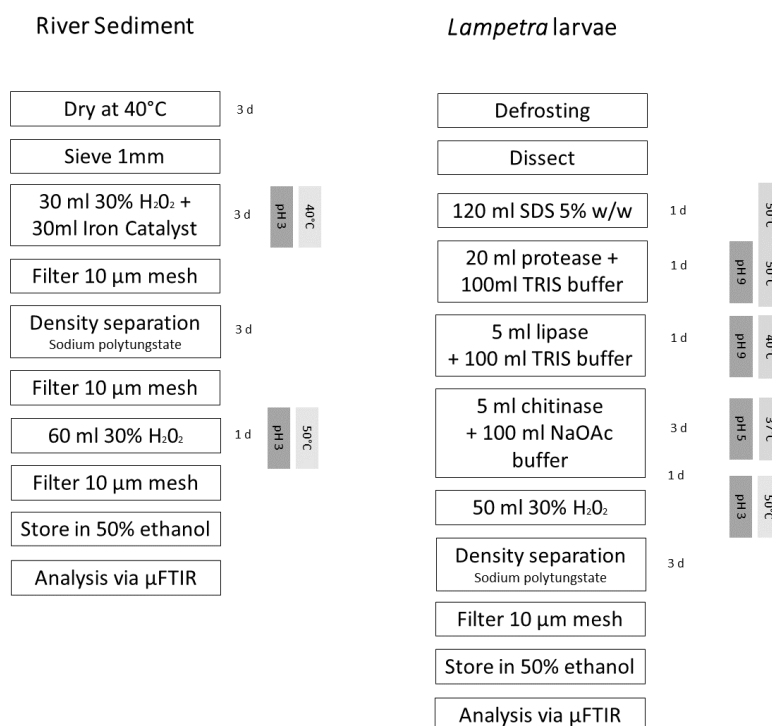


Figure 2.3 Flowchart of the methods used to prepare and purify river sediment and *Lampetra* samples, for quantification of microplastic particles using µFTIR. Methods for lamprey larvae adapted from universal enzymatic purification protocol (Loder *et al.*, 2017). For specifics on sediment sample processing see Appendix A.

To remove organic matter from the stomach contents, 50 ml H₂O₂ was added to each sample and incubated at 50°C for 24 h. The solution was filtered through a 10 µm stainless steel and the remaining material on the filter underwent density separation via pre-filtered (0.2µm) sodium polytungstate (SPT) solution at a density of 1.8 g/cm³. After the last filtration step the filter cake was flushed in a 50 ml beaker using pre-filtered SPT solution. The samples were then transferred to 250 ml or 1000 ml separatory funnels, with 125 ml SPT, where they remained for 3 days. Every 24 hours the settled portion were carefully discarded, and the remaining liquid was swayed to support the separation processes and to pick up any material stuck to the inside of the separatory funnel. The separation funnels were carefully emptied until just a few mL of SPT, containing the light fraction with the microplastics, were left. This light fraction was filtered through a 10 µm stainless steel mesh and the remaining material on the filter was washed off into a 50 ml beaker with 50% HPLC grade pre-filtered (0.2 µm) ethanol. To ensure all material was removed from the filter, a natural bristle brush was used

to brush off any material into the beaker. The contents of the beaker were transferred to a 10ml glass vial for sample storage and analysis.

2.3.2.3 Deposition of samples for analysis via μ FTIR

Samples were analysed in March 2020 at the Centre for Ecology and Hydrology (CEH) in Wallingford with guidance from the CEH ecotoxicology team. All deposition steps were completed under laminar flow (Biosafety Cabinet), with all glass wear and stainless-steel equipment rinsed 5 times under DI water and 3 times with Milli-Q to avoid contamination. Each sample (<1 ml) vial was weighed and recorded on the day of sampling for FTIR. The mass of the vial and lid was subtracted from this to calculate the volume of the sample in 50% ethanol. To deposit samples, a small filter unit was assembled, consisting of a small Buchner flask, a filter holder, and a stainless steel 'top hat' with a 10 ml diameter. The top hat was held in place with bulldog clips and stainless steel. The sample was shaken to homogenise and immediately pipetted (glass) and deposited on the silver filter (Sterlitech, silver membrane filters, 3.0 μ m, 25 mm). Care was taken to ensure that particles did not appear as a mat, with an insufficient spread between particles for FTIR to distinguish between them. Where possible, the whole sample was deposited on the silver filters, but for samples in which substantial material was present after processing, a subsample was deposited. For those samples in which a subsample was deposited, the volume deposited was calculated to allow the total number of particles per gram solids to be back calculated. The silver filters with deposited sample were then transferred to a 12 mm glass petri dish, covered with aluminium foil, and labelled prior to transfer to the μ FTIR. In the case of the lamprey samples acquired from the River Carron (dc19 and dc83), two sets of the samples were deposited (i.e. dc19_2 and dc83_2). The second set of samples underwent an additional Fenton's reaction prior to analysis and less of the sample was deposited (Appendix A Figure 2). This was because of the high number of organic particles covering the silver filters in the first deposition of these samples (Appendix A Table 2). As discussed above, a high organic particle number deposited onto the silver filter can cause making of synthetic particles and hinder a reliable estimation of microplastics in the sample (Rasmussen *et al.*, 2021).

2.3.2.4 Spectral analysis of microplastics

Samples were analysed for plastic and natural particle number, composition, size (25-500 μm) and mass using micro-Fourier Transform infrared (μFTIR , PerkinElmer, Spotlight 400 FT-IR Microscope) spectroscopy. The silver filter, onto which the samples were deposited, reflects infrared (IR) light enabling the light to pass through the particles and be reflected through the particles to the FTIR detector. Groups of spectra are collated via raster mapping to produce a spectral map, which is compared with a database (AAU MPHunter) to identify polymer types. The wavenumber range used was 700–4000 cm^{-1} . The results of four scans were combined and interpreted as the final result. The resulting IR maps were analysed using Simple software, combining the interface of MPHunter with automated analysis pipeline for microplastic analysis, enabling human bias to be excluded from particle identification (Primpke *et al.*, 2019).

2.3.3 Identifying the polymer type and size of microplastics that are ingested by lamprey larvae and whether they correlate with that of the surrounding sediment

Microplastic particles were documented in all lamprey larvae and matched sediment samples (Fig.2.4, Fig.2.5). Two polymer types accounted for all synthetic particles found in sediment and biota samples, which included acrylates, polyurethanes and varnish (APV) and polypropylene (PP). There was no significant correlation between *Lampetra* and sediment microplastic concentrations (ANOVA, $p=0.05465$). There were no significant differences in number of plastic particles found between sites for sediment (ANOVA, $p=0.4181$) or *Lampetra* (ANOVA, $p=0.6514$) samples. No statistically significant difference was detected between microplastic count (items) between repeat one and two of the *Lampetra* samples (ANOVA, $P=0.1569$).

Microplastic abundance was reported by the major dimension of the particle, which was classed into size ranges that are commonly used in other studies (Leslie *et al.*, 2017; Vaughan *et al.*, 2017; Peng *et al.*, 2018; Zhang and Liu, 2018; Liu *et al.*, 2019). Four unevenly distributed fractions were used: 25-50 μm , 50-100 μm , 100-250 μm , 250-500 μm (Fig.2.2). A total of 44 particles were identified as microplastics within sediment samples, among which the highest concentration of particles fell within the 100-250 μm fraction (Fig.2.6A, 59%, 26 items). Within *Lampetra* larvae a total of 39 plastic particles were identified as plastic polymers,

similarly to the sediment size fractions, the highest concentration of particles fell within the 100-250 μm size fraction (Fig.2.6B, 62%, 24 particles).

The results obtained from the sediment matrix revealed all samples contained plastic particles above the limit of detection (LOD), including 89% of all polymer types above LOD (Fig. 2.4; Appendix A Table 2). However, only 25% of all polymers detected within the sediment samples were quantifiable, with blank-corrected values falling above the limit of quantitation (LOQ). Microplastic particles were detectable (LOD) in biota samples at three of the four field sites (Fig.2.5). Within *Lampetra* samples 29% of polymers fell above the LOD and 14% of polymers above the LOQ (Fig.2.5, Appendix A Table 2). A higher number of polypropylene particles were detected in the second analysis of the samples from the River Carron, with 122.5 polypropylene particles in dc83_2 (Fig.2.5, Appendix A Table 2).

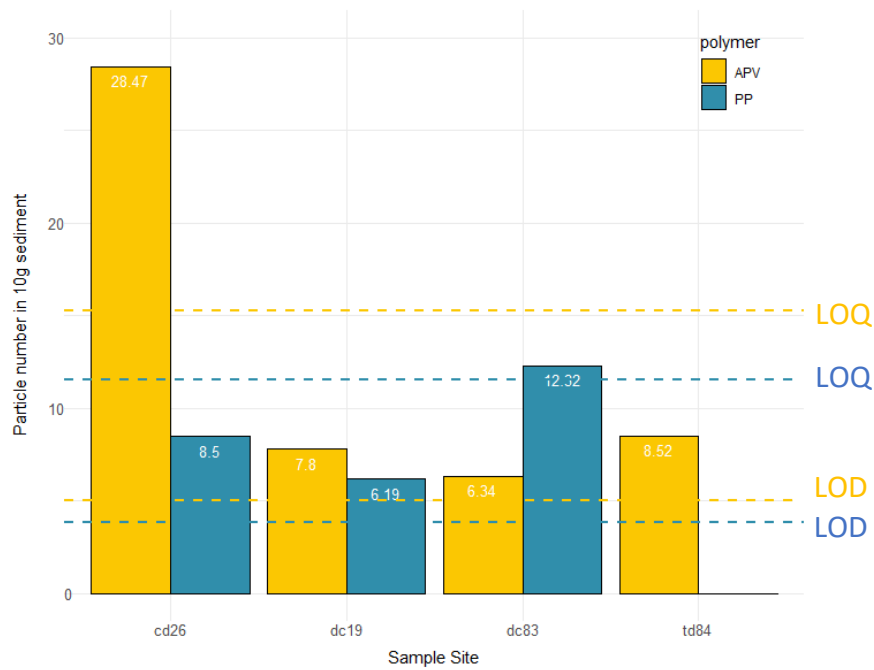


Figure 2.4 Blank corrected particle number of microplastics (APV and PP) detected in 10g of sediment samples collected from four sites. Dashed lines showing limit of detection (LOD, yellow) and limit of quantification (LOQ, blue).

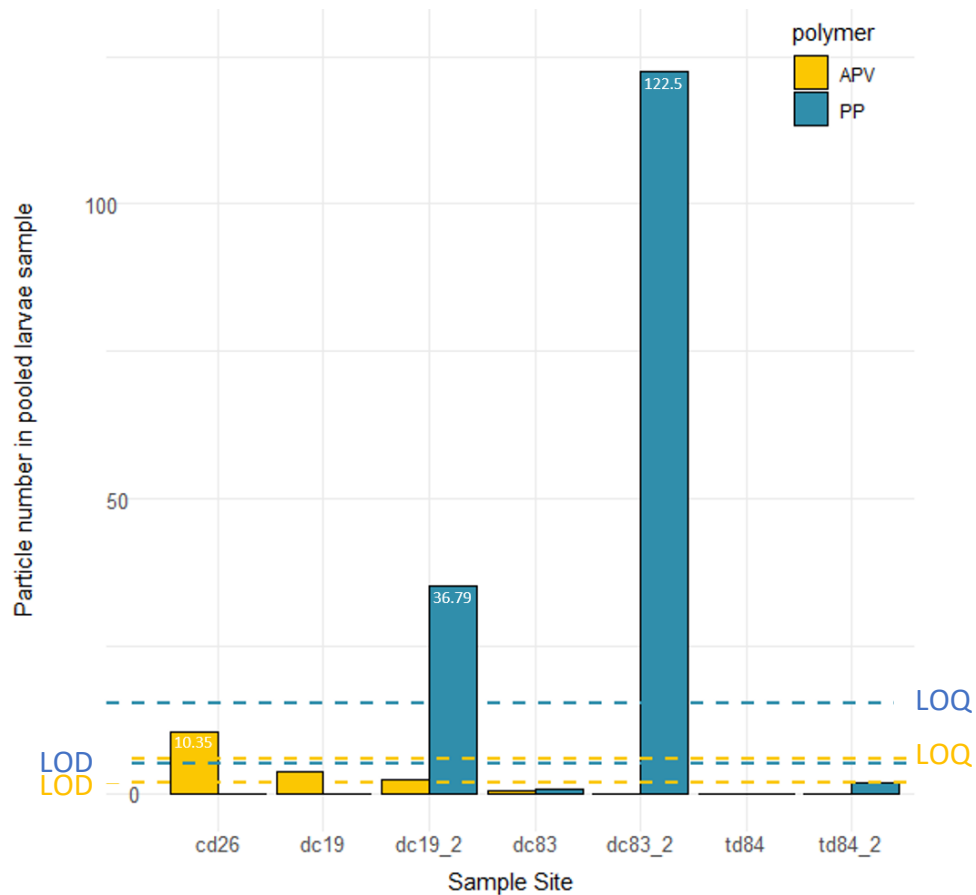


Figure 2.5 Blank corrected particle number of microplastics (APV and PP) detected in pooled *Lampetra* larvae samples collected from four sites. Dashed lines representing detection (LOD, yellow) and limit of quantification (LOQ, blue).

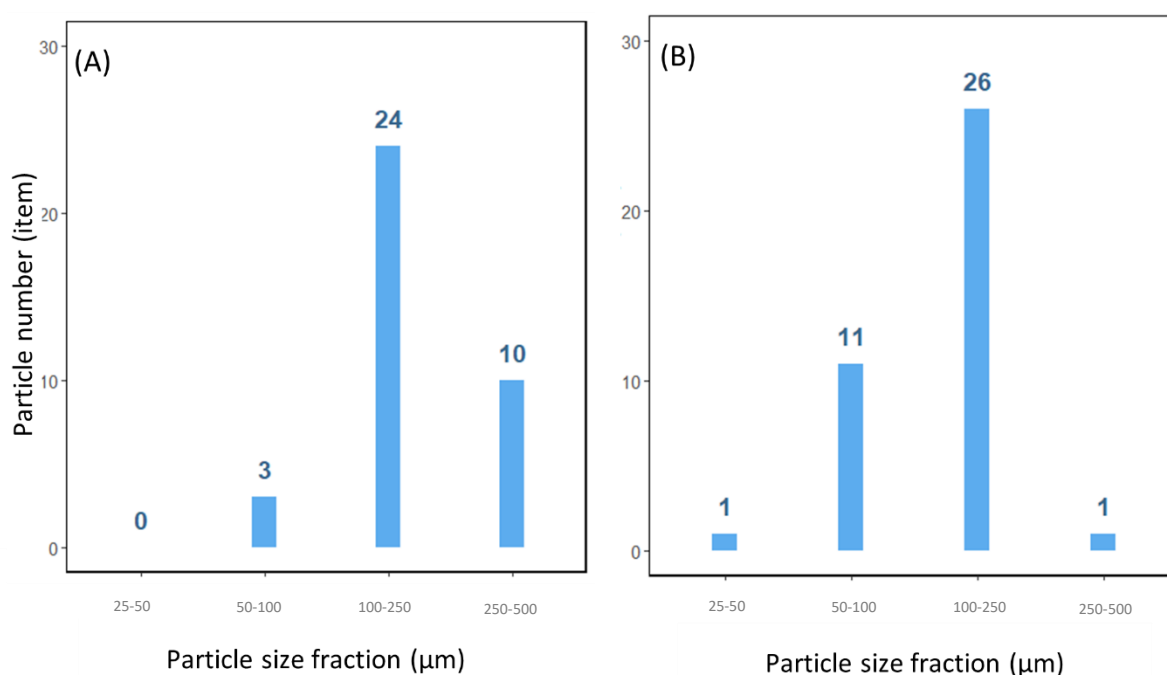


Figure 2.6 Microplastic abundance within size distributions in sediment (A) and lamprey larvae (B).

A total of seven plastic particles (2 APV, 5 PP) were identified across three procedural blanks following the biota sample preparation and six (4 APV, 2 PP) within the sediment procedural blanks. A total of one polymer (APV) was identified in the atmospheric control sample (Appendix A Figure 1). The particles identified in the sediment blank, matched the size distribution of the sediment field samples with the highest frequency of particles within the 100-250 µm fraction (Figure 2.6A). The size distribution of plastic contamination in the blanks from the biota workflow was dissimilar to the *Lampetra* field samples, with the highest frequency of particles in the 25-50 µm size fraction (Figure 2.6B).

2.3.4 Identify and address any issues which arise from processing samples of sediment and lamprey

These pilot studies allowed identification of issues which arise from processing samples of sediment and lamprey. With regards to method development to isolate microplastic particles, it was possible to reduce organic matter within samples to allow microplastics to be identified, quantified and analysed building on previous literature. However, the pilot data highlighted the need to optimise the sample volume deposited onto silver filters for analyses. The volume of organic material on silver filters in the initial data run may have led to particles being

masked or leading to particles to be identified as a 'mass' of particles, as opposed to identifying each particle as a separate identity.

Therefore, further method optimisation is needed to reduce organic material, with additional Fenton's reaction and optimised conditions during enzymatic digestion (time, temperature, pH). The increase in sand particles in dc83 could be because of the heavier sand particles remaining at the bottom of the sample vial, reflecting the difficulty of depositing a representative sample from all fractions of the sample (shaking inefficient at mixing sample fractions). This methodological limitation could also explain the increase in polypropylene, in both dc19_2 and dc83_2 (Fig.5.A). However, this does not align with the polymer's density, as polypropylene falls within the light fraction and can float, with a specific gravity of 0.9 g/mL (Kedzierski *et al.* 2017).

The overall increase in the number of particles, both natural and synthetic, identified following an additional Fenton's reaction and with less material deposited on the filter, suggests the uFTIR is better able to identify separate particles when excess organic material has been removed. However, attributing this to either an additional Fenton's reaction or less sample deposited is difficult to conclude. It is likely that both sample preparation optimisation is needed and a decrease in sample deposition on the silver filter will aid in identifying more synthetic particles present in the samples.

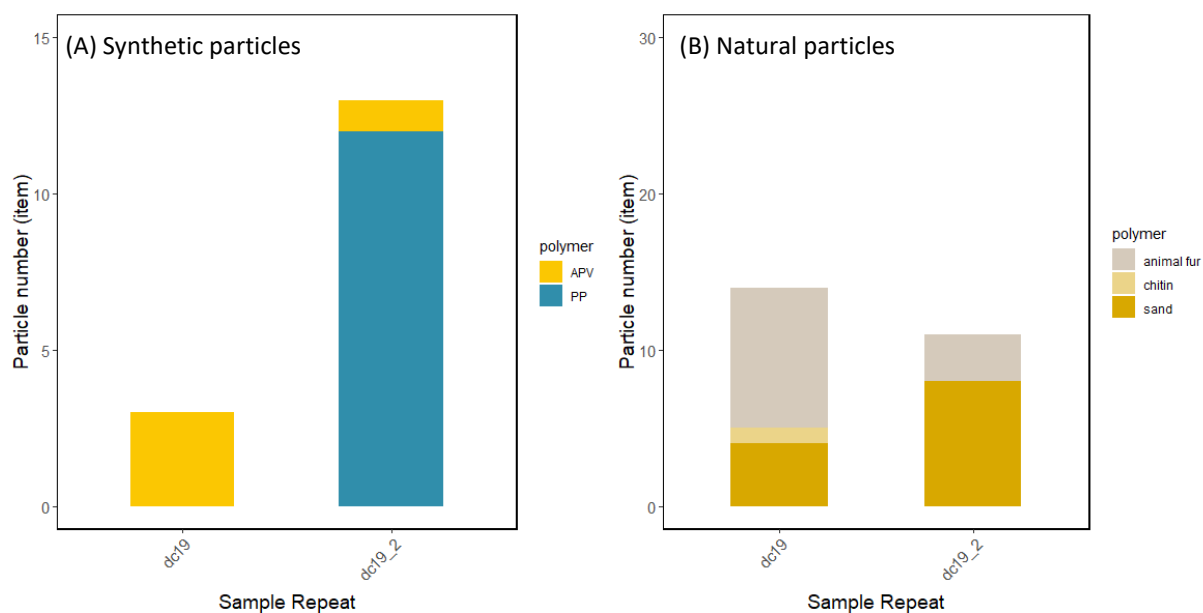


Figure 2.7 Comparison of first and second deposition of lamprey larvae samples from River Carron (dc19 and dc19_2) showing raw particle number counts of synthetic (A) and natural (B) particles counts detected on the silver filter. An increase in polypropylene (blue, A) and decreased in natural particles (B) is apparent in the second deposition (dc19_2).

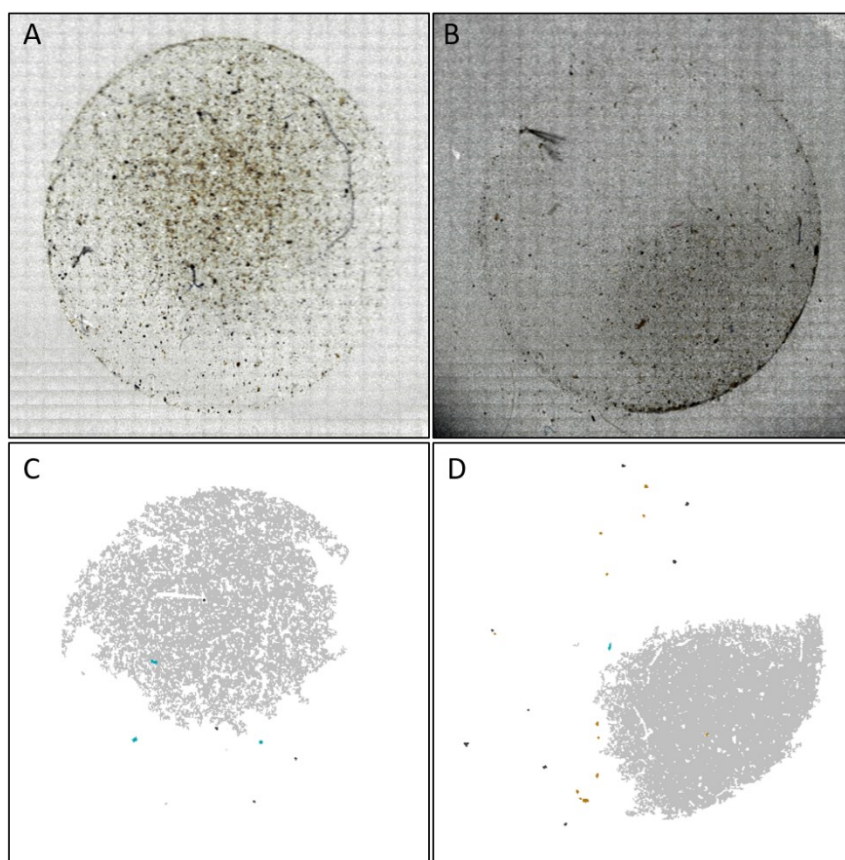


Figure 2.8 Image (A, B) and particle map (C, D) showing particles before and after a Fenton's reaction within of sub-sample dc19 onto silver filters. (A, C) Prior to additional Fenton's reaction with 67.38% of the sample deposited. (B, D) dc19_2 following an additional Fenton's reaction (24h) with the aim of removing organic matter and 32.62% sub-sample added to the filter.

Sediment samples were visually characterised by numerous ubiquitous black particles (Fig.2.9). Recent studies report large concentrations of black particles in deep sea sediment, which the authors presumed to consist of coal (Bergmann *et al.*, 2017). These particles are highlighted as a concern for two reasons, firstly it is often not possible to identify black material via uFTIR, as materials such as carbon black, causes disruptive fluorescence phenomena upon irradiation, resulting in almost complete absorption of infrared light (Eisentraut *et al.*, 2018; Wagner *et al.*, 2018; Järlskog *et al.*, 2020). Secondly, due to the abundance of black particles it is feasible that microplastic particles in the samples could have been masked during measurements. Therefore, it was important to identify this material for future studies to understand it's chemical nature and how it can be isolated from samples undergoing μ FTIR analyses.

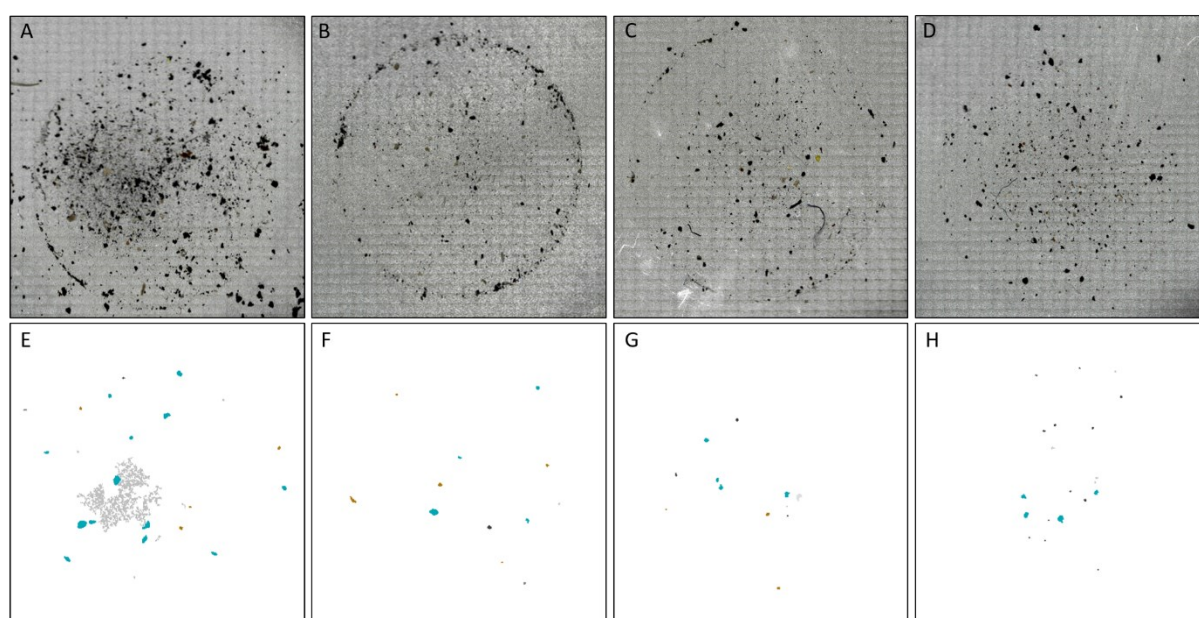


Figure 2.9 Black particle abundance in sediment samples deposited onto silver filters; (A)(E) cd26, 43.61% sample deposited. (B) (F) dc19, 43.76% deposited. (C), (G) dc83, 52.11% deposited. (D), (H) td84, 40.59% deposited.

Therefore, a sub-sample of black particles were first run FTIR-ATR to gain additional spectra, which could suggest a synthetic origin. However, the match from the spectra database was <20%, resulting in a very weak identification as a polyaromatic hydrocarbon. The larger fraction of this black material was stored in ethanol for further analysis via SEM/EDS. SEM/EDS and optical microscopy has been used to screen for microplastics retained from fish guts (Wang *et al.*, 2017). The analysis does not allow determination of whether these are of natural or synthetic carbon nature but gives information on whether the particles are carbon based and traces of other elements, such as zinc, which could suggest tyre wear particles. To

gain some further insight into the black particles, a sub-sample recovered from the sediment samples were analysed using SEM-EDS, to gain an insight into the morphological and elemental composition of the particles. SEM/EDS allows for particles to be screened in a relatively short time and characteristics of particle surfaces, such as cracks and pigments, can indicate microplastics through elemental composition signatures (Wang *et al.*, 2017). Samples were mounted on a double-sided adhesive carbon tab, on an aluminium SEM stub. The results gained suggest that the particles in the sediment samples were coal or black carbon, with a high carbon peak (Appendix A Figure 3), however images revealed different structures and elemental data across particles (Appendix A Figure 3). Considering the morphological features of the particles (Fig.2.8) and comparing this to other SEM images, some of the particles could be TWP (Wagner *et al.*, 2018; Appendix A Figure 4). However, it was not possible in this study to determine the origin of the black particles, largely attributed to disruption of studies during the covid restrictions and lack of access to suitable analysis equipment such as GCMS.

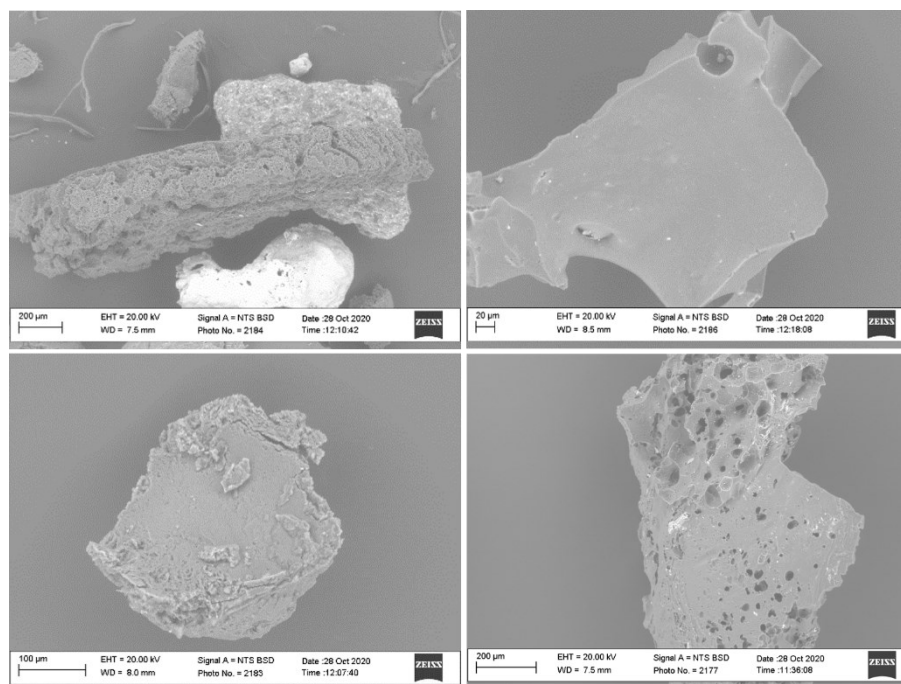


Figure 2.10 Optical images of black particles recovered from sediment samples, complexity of structures suggest both organic plant or animal residues and synthetic based particles.

Road runoff accumulates particulates associated with vehicle traffic, such as brake dust, exhaust emissions, and tyre and road wear particles (Panko *et al.*, 2013). During high rainfall events or atmospheric dispersal, such pollutants can wash into receiving water bodies, such as adjacent freshwater streams and rivers. It is therefore possible that the black material in

sediment samples could be synthetic in nature, such as tyre wear particles, given the proximity of sites to urban and heavy traffic. However, it was not possible to conclude this from the data collected and future research is needed. Alternative techniques such as PY-GC-MS have been shown to successfully quantify and identify black material from environmental samples, indicating large quantities of tyre wear particles (Unice *et al.*, 2013; Wagner *et al.*, 2018).

2.4 Methodological recommendations

2.4.1 Evolution of methodologies: optimisation of methods for future studies based on these findings

Methodological considerations moving forward for chapter three aims to remove the number of steps in the sample processing to reduce microplastics losses and contamination. The SOP for chapter three is adopted as an alternative technique to enzymatic digestion (Fig.2.9; Loder *et al.*, 2017) for biota based on Lopez-Rosales *et al.* (2021), adapted for lamprey larvae to reflect an increased inorganic tissue and complexity compared to plankton samples. This technique was chosen over enzymatic digestion due to reduce number of filtration steps, (reduce microplastic losses and contamination; Lopez-Rosales *et al.*, 2021) economic and workload considerations. Adaptations to the protocol include an increase concentration of SDS from 5%-10%, based on suggestions from Loder *et al.* (2017), an additional 40ml 30% H₂O₂ was added and left for another 24hrs to complete oxidation, due to lipids being visible in preliminary sample (Fig.2.9). The final concentration of hydrogen peroxide was increased from 15%-20% to reflect the need to remove more organic matter discovered in the test samples analysed via μ FTIR.

Concern is high within microplastic research for false positives and the loss of microplastics from samples during multiple sample filtration steps, leading to under-reporting the true quantify of plastic particles present within samples (Hersmen *et al.*, 2018). This is particularly apparent for samples with a high number of processing steps, with concern that the biota workflow could have presented a high number of occasions for particles to be lost. Therefore, future work needs to include more robust spiked recoveries with suitable polymer types and particles, along with reduced filtration steps to prevent microplastic losses in the preparation

stages. Future studies will implement appropriate microplastic spiking procedures in the form of matched matrix positive controls (Fig.2.9).

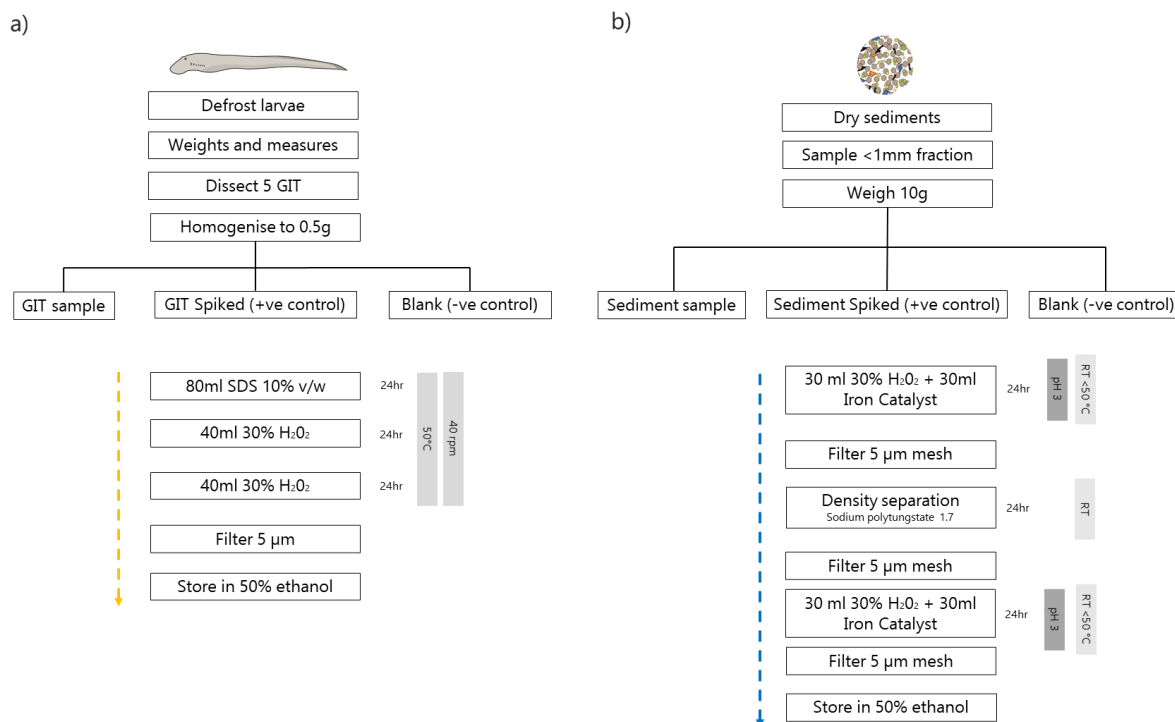


Figure 2.11 Flowchart of optimised methods used to prepare and purify lamprey (a) and sediment (b) samples, for quantification of microplastic particles using μ FTIR. Lamprey tissue methods developed from oxidative method (López-Rosales *et al.*, 2021).

2.5 Conclusion

Although microplastic ingestion by freshwater biota has been widely documented, one of the key barriers to studying the microplastic load in freshwater organisms is the lack of a standardised methods to extract and quantify microplastics from biological samples. Without standard methods and routine reporting of microplastic particle recovery efficiencies across a range of polymer types, the relative hazard of microplastic particles cannot be compared temporally or geographically and estimates of microplastic burdens in wildlife cannot be accurately made. To resolve this, here we developed a series of methodological recommendations to identify an efficient protocol for the extraction and quantification of a range of microplastics from lamprey larvae (*Lampetra*) gastrointestinal tract.

The aim of this pilot was to identify suitable methods to enable purification, and to obtain data on the presence of microplastic particles in riverine sediment and larval *Lampetra*. This study is the first documentation of microplastics in larval lamprey and it shows that microplastics are present, in detectable numbers in *Lampetra* larvae and associated

sediments. The variation in microplastic abundance, size distribution and polymer composition between biota and sediment samples was relatively small, and no significant correlation between microplastic abundance in sediments and *Lampetra* was detected. It was not possible to directly link plastic particle ingestion to individual fish, rather pooled samples of larvae from each site. It is important to also note that the low numbers of sediment and *Lampetra* sampled might introduce some uncertainty in data interpretation and the results should be handled with care. Due to the limited sample number from both matrices, characteristics and conclusions cannot be drawn about each site, but instead handled as group or non-specific to location. It is also important to highlight that many microplastics, less than 25 µm, could be present within the samples, but have not been quantified. This is because of <25 µm requiring much greater machine effort, which would take several days.

Consideration and development of both contamination controls and blank sample procedures led to informed suggestions for future studies to obtain robust results. These specifically include the use of laminar flow hoods, as opposed to fume hoods to reduce contamination in samples. It is recognised that undertaking sample preparation under a fume hood is not ideal, given that ambient unfiltered air flows into the fume hood and across the work surface, when the front sash is open. Data suggests that the use of a laboratory fume hood reduces contamination of samples by approximately 50%, however it does not fully prevent it (Wesch *et al.*, 2017). Although only one microplastic particle (Acrylate) was identified in the atmospheric control, it is likely others were masked due to the number of natural particles on the filter (Appendix A Fig.1).

With regards to the number of particles present in the blank samples, it is important to consider this at all steps and to use LOD/LOQs to give realistic estimates of microplastics in the samples. The risk of sample contamination presents a significant problem within microplastics research, and it is unclear whether the contaminating microplastics within samples were due to the air, reagents or processing procedure. However, as the same polymer types were identified in the blanks and in the samples, it can be assumed that some of this was contamination. Future methods need to adopt suitable clean-air devices with particle filtration, such as biosafety cabinets, to sufficiently reduce airborne contamination, thereby reducing the limit of detection, limit of quantification and error rates within the data (Wesch *et al.*, 2017).

Chapter Three - From the Environment into the Biomass: Microplastic Uptake in a Protected Lamprey Species

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Author comments: FRB, CB and EL jointly developed the project concept. Field data collection was carried out by FRB and CB with laboratory work carried out by FRB, with guidance from RCross, RCox and GA. The manuscript was prepared by FRB, with comments from CB, EL, RCox and GA. The paper was published in the journal *Environmental Pollution*, received media attention from the BBC (February 2023), was presented at The Lamprey Conference (Institute of Fisheries Management and Natural England) in York (February 2023) and SETAC as a platform session (April 2023).

3.1 Abstract

The relationship between the ubiquitous presence of microplastics in the environment and exposure of biota needs to be better understood, particularly for vulnerable species and their habitats. In this study, we address the presence of microplastics in the riverine habitat of a threatened lamprey species (*Lampetra sp.*), both in habitats with protective interventions in place (designated as Special Areas of Conservation), and those without these protective interventions. By sampling both riverbed sediments and larval lamprey, we provide a direct comparison of the microplastic loadings in both, and insights into how knowledge of sediment loadings might predict biological uptake. Microplastic particles, analysed using micro-Fourier transform infrared (μ FTIR) spectroscopy, were detected in all samples of lamprey larvae and paired sediment, ranging in abundance from 1.00 to 27.47 particles g^{-1} in dry lamprey gastrointestinal tract (GIT) tissue, and 0.40 to 105.41 particles g^{-1} in dry sediment. The most urbanised catchment exhibited the highest average microplastic particle count in both

lamprey and sediment. Across sites, the microplastic abundance in lamprey GIT tissue was not correlated with that of the surrounding sediment, suggesting that either specific polymer types are retained or other factors such as larvae residence time within sediment patches may influence biological uptake. The most encountered polymer types in lamprey from their immediate habitat were polyurethane, polyamide, and cellulose acetate. To the best of our knowledge, this is the first study to document microplastic contamination of larval lamprey in-situ, contributing another potential stressor to the population status of a vulnerable species. This highlights where further research on the impacts of plastic contamination of freshwater environments is needed to aid conservation management of this ecologically important species.

3.2 Introduction

Microplastic contamination occurs in almost every ecosystem on earth, from Everest (Napper *et al.*, 2020) to the deepest part of the world's ocean (Peng *et al.*, 2018). However, the consequences of the presence of microplastics for these ecosystems are not elucidated. In particular, whether protected vulnerable species are exposed is a pertinent question when trying to understand the consequences of plastics in our natural environment. The accumulation of microplastics in river sediments is widespread and increased occurrence is associated with urban hydrometric areas (Woodward *et al.*, 2021). Sources of microplastics to rivers include effluent from wastewater treatment plants (Murphy *et al.*, 2016; Woodward *et al.*, 2021), run-off from agricultural soils (Nizzetto *et al.*, 2016) and debris from roads in the form of tyre wear particles (Knight *et al.*, 2020). Higher loadings of microplastic contamination have been observed in sediments compared to the water column (Bondelind *et al.*, 2020), particularly areas with high deposition and low water velocity (Corcoran *et al.*, 2019; Vincent and Hoellein, 2021). The consequences of this deposition for benthic species in-situ is not well understood and whether contamination in biota reflects this deposition needs to be investigated.

Lamprey larvae inhabit depositional freshwater sediments for up to seven years, after which they undergo metamorphosis into adult forms, which are either parasitic or reproduce without feeding. Lamprey larvae are detritivores, feeding on substrate and deposited organic matter, including fungi and microorganisms that exist within the biofilm complex (Limm and Power, 2011). Food particle selection is passive and primarily thought to be based on size of

the larvae's oral ciliae, with small particles trapped in mucus networks within the pharynx, which is constantly swallowed (Moore and Mallatt, 1980).

The larval stage of all lamprey species acts as a key component at the base of the freshwater food chain, representing a large proportion of the biomass in streams (Beamish and Youson, 1987), and forming a key food source to predators (Close *et al.*, 2002). They are widely distributed in temperate freshwaters across the globe. Lamprey larvae are important burrowing ecosystem engineers; oxygenating sediments, contributing to nutrient cycling, increasing microbial composition and habitat variability, which in turn generate suitable habitats for other species (Shirakawa *et al.*, 2013). Lampreys face a range of anthropogenic stressors across their natural range, with the most significant threats to population status identified as barriers to migration, water pollution, and riverine habitat degradation (Close *et al.*, 2002). These have led to population declines and subsequent legislation to protect vulnerable populations of lamprey species, most notably throughout Europe and North America (Maitland *et al.*, 2015).

Plastic contamination has been identified in 257 species of freshwater fishes, with concentrations varying between taxa, life stages, trophic guild and importantly feeding strategy (Horton *et al.*, 2018; Azizi *et al.*, 2021; Galafassi *et al.*, 2021). The detritivores fish *Prochilodus lineatus* has one of the highest recorded ingestion rates of microplastics, at 40.88 microplastic items per individual on average. This high microplastic ingestion is suggested to be a result of the species feeding strategy (Blettler *et al.*, 2019). Equivalent to lamprey larvae, the diet of detritivores fish consists of organic matter and algae, which more closely reflect the morphology of microplastic particles compared to other food sources (Zheng *et al.*, 2019).

Therefore, the vulnerability of lamprey larvae to microplastic contamination could be high considering their lengthy benthic residence times, depositional habitat selection and feeding strategy. Their foraging and bioturbating in the benthos of depositional riverine habitats, may increase encounter rates with microplastic contamination hotspots when compared to rheophilic fish species. As microplastic ingestion causes internal blockages and injury to the digestive tract of fish, microplastics could act as stressor to lamprey (de Sá *et al.*, 2015; Hamed *et al.*, 2020; Dimitriadi *et al.*, 2021). Furthermore, recent research has highlighted the need to establish whether this novel contaminant poses a toxic risk to lamprey populations (Madenjian *et al.*, 2021). However, to the best of our knowledge, no research has investigated

the in-situ microplastic exposure risk and importantly, the levels of contamination in larval lamprey.

In this study, we investigate the co-occurrence and particle characteristics of microplastics isolated from lamprey larvae gastrointestinal tract (GIT) tissue and those in the immediate sedimentary environment at riverine locations selected from two Special Areas of Conservation (SACs) and two urban hydrometric areas in Southern and Central Scotland, UK, representing a range of microplastic exposure risk. We addressed the following objectives: (1) To investigate whether the microplastic contamination in lamprey reflects the contamination in sediments they inhabit, both in terms of particle numbers but also polymer types; (2) To assess whether there is a difference between contamination at sites with protective intervention.

3.3 Material and Methods

3.3.1 Site selection

Sampling took place at 18 locations along seven streams, within four hydrometric areas in Central and Southern Scotland in the United Kingdom (UK) in September 2020 (Appendix B Figure 1, Table 1). Two of these systems (River Teith and River Tweed) are designated under the EU Habitats and Species Directive as Special Areas of Conservation (SACs; Habitats Directive, 1992: Council Directive 92/43/EEC of 21 May 1992, on the conservation of natural habitats and of wild fauna and flora) with lamprey species being one of the principal designating features. The other two systems (River Clyde and Water of Leith) flow through two of the largest urban areas in Scotland. Final sampling sites within each hydrometric area were selected, noting sites with potential upstream microplastic inputs into the system, such as wastewater effluents and urban land cover (Appendix B Figure 1, Table 1, 2).

3.3.2 Lamprey larvae sampling

Ten lamprey larvae were collected from each site (180 sampled in total, median length 112.0 mm $\pm$ 15.6 mm, median wet weight 1.97 g $\pm$ 0.86 g; Appendix B Table 4). This sample size was determined following consultations considering the existing typical larval densities encountered during previous surveys of the selected sampled rivers, the inherent patchiness in sediment composition and corresponding anticipated wide variation in lamprey encounter potential. A pragmatic approach was agreed in determining sample size, and the number of

individuals removed were not considered to have a detrimental effect on population status. Sampling was undertaken using back-pack electric fishing equipment with smooth direct current (DC) at a 180-Hz frequency, from a 1-3 m² patch of fine sediment, with characteristics typical of lamprey larvae habitat (Torgersen and Close, 2004). The samples may have been composed of both resident brook (*L. planeri*) and river (*L. fluviatilis*) forms due to the inability to distinguish between them in the field. Individual larvae that were smaller than 70mm total length were omitted from sampling due to difficulties with gastrointestinal tract tissue removal. Lamprey larvae were euthanised by an overdose of Tricaine methanesulfonate (MS-222; Matthews and Varga, 2012). Larvae from each sampling location were aggregated, wrapped in aluminium foil and snap-frozen in a liquid nitrogen dry-shipper for transport back to the laboratory. Tissue was stored at -80 °C freezer until dissection and all subsequent work was carried out under a laminar flow hood to minimise airborne microplastic contamination (Hermesen *et al.*, 2018).

3.3.3 Lamprey larvae dissection

Once defrosted, the weight (± 0.1 g) and length (± 0.1 mm) of each lamprey larvae were recorded, and the exterior thoroughly rinsed with filtered Milli-Q water to remove any external microplastic contamination. The gastrointestinal tract (GIT) from each lamprey larvae was then removed and separately combined with the faeces on clean labelled foil and transferred to the -80 °C freezer (Appendix B Figure 2a-e). Non-destructive methods to identify microplastic ingestion in biota, such as faecal sampling (Pérez-Guevara *et al.*, 2021) would be the preferred sampling method, particularly notable for protected species. However, such methods were considered impractical for documenting microplastic uptake in this case due to the small size of lamprey larvae and impracticalities of collecting faecal samples.

The samples consisted of lamprey larvae pooled from each site, rather than each sample representing an individual larva. Pooling samples diminishes replication and individual larval representation within sites, thereby limiting the ability to assess variation at the individual level. However, considering the chosen analysis method and the resulting constraints of time and cost efficiency, pooling samples emerged as the most pragmatic approach. This strategy facilitated the analysis of a greater number of samples while emphasising the representation

of sites, thereby offering valuable insights into overarching patterns and trends in microplastic contamination within lamprey populations across diverse habitats

To prevent cross-contamination between dissections, all metal implements were washed thoroughly with Milli-Q water three times. For sample preparation, the tissue was thawed and homogenized by carefully slicing and mixing it with a scalpel knife. The weights of the individual subsamples were adjusted proportionally to the wet weights of the larvae to avoid bias towards larger individuals. From each of the five individual gastrointestinal (GIT) samples, a proportional subsample of tissue (approximately 0.20 grams) was taken to create a pooled sample weighing 1 gram (wet weight). This pooled sample was then homogenized and divided into halves, with each half weighing 0.5 gram. This specific weight of tissue was determined through preliminary experiments (see Appendix B Figure 3) aimed at identifying the optimal tissue volume for analysis using micro-Fourier Transform Infrared Microscopy (μ FTIR) with a Perkin Elmer Spotlight 400 FT-IR Microscope. Subsequently, each 0.5 gram sample was placed in clean Erlenmeyer flasks that had been rinsed three times with Milli-Q water and covered with foil. These samples were randomly assigned to a processing batch, with 14 samples per batch. Additionally, each batch included one negative control (blank) and one positive control (matched matrix spiked sample with 50 μ l spiking stock, see Appendix B Figure 11).

3.3.4 Oxidative method to reduce complex organic matrix of lamprey larvae gastrointestinal tract

A two-step chemical process based on López-Rosales *et al.* (2021) was undertaken for lamprey samples. All liquids for both lamprey and sediment preparation were filtered prior to use with a glass fibre filter to prevent contamination from reagents (ϕ 90 mm, 1.2 μ m/0.7 μ m, Fisherbrand MF 200/300). Each 0.5 g pooled lamprey tissue sample was combined with 80ml 10% sodium dodecyl sulphate (SDS) in an Erlenmeyer flask (Appendix B Figure 2f) and incubated (40rpm) at 50°C for 24h (Appendix B Figure 2g). Following this 40ml of 30% H₂O₂ was added to each sample and incubated (shaking incubator) at 50 °C for 24h. After 24 hr another 40ml of 30% H₂O₂ was added and incubated again at 50 °C for 24h (Appendix B Figure 2i). To achieve the final sample, H₂O₂ was removed by filtration and the final isolated microplastics were stored in 50% ethanol for analysis.

3.3.5 Sediment collection and microplastic extraction

Directly following the collection of lamprey larvae at each site, three riverbed sediment samples were also collected. The sediment surface was sampled in all cases to approximately 10 cm depth using a stainless-steel scoop, collected to fill a 700ml glass jar, ensuring minimal excess water was retained. Site features, such as depositional areas, sediment type and visible plastic contamination were recorded (Appendix B Table 1, 2, 3). Sediments were stored in darkness at 4 °C before being oven dried at 40 °C for 72 hours and sieved with a 1 mm metal mesh to remove larger stones and organic matter. As with lamprey GIT samples, all subsequent work was carried out under a laminar flow hood to minimise airborne contamination (Hermesen *et al.*, 2018). Dried sediments were sub-sampled from the <1 mm size fraction, composing of a homogenised sample from each site, to form a 10 g sample and placed in a glass beaker (Fig.2.9). All filtering steps were completed using a 5 µm filter and rinsed using filtered Milli-Q water.

Each homogenised sediment sample was randomly assigned to a processing batch (n=8 per batch) and processed through a series of chemical and physical processes to isolate the microplastics within the samples. All batches were accompanied by both a positive and negative control, in the form of a spiked matched matrix (100 µl spiking stock, Appendix B Figure 10) and a blank sample, respectively. Each 10 g sediment sample was transferred to a 100 ml measuring cylinder and placed in an ice-bath. A Fenton's reaction was undertaken on each sample, with an additional 10 ml of H₂O₂ added until the reaction was complete (Fig.2.9). The spent reagents were filtered, before transferring the sample to a density separation in the same measuring cylinder with sodium polytungstate (SPT), made up to 1.7 g cm⁻³ (Fig.2.9). Samples were left quiescent for a minimum of 20 hours to allow density separation. Following a period of physical separation, the SPT supernatant from each sample was filtered, and the sample was decanted into a sample beaker and stored. The remaining sample (30-40 ml) was resuspended, a homogenous profile of solids was made, and a second floatation was undertaken for a minimum of 20 hours. A final Fenton's reaction was undertaken on filtered SPT supernatant samples, together with the previous day's supernatant (Fig.2.9). Once complete each sample was filtered and transferred into 50 % ethanol for storage and separation into coarse (>198 µm) and fine (<198 µm) fractions.

3.3.6 Deposition of samples for analysis via µFTIR

A small filter unit was assembled, consisting of a small glass Buchner flask, glass filter holder, and a silicone washer (10 mm internal diameter, 20 mm external diameter, 3 mm thick). The sample was shaken to homogenise and immediately pipetted (glass) and deposited on the silver filter (Sterlitech, silver membrane filters, 3.0 μm , 25 mm). Care was taken to ensure that particles did not appear as a mat, with an insufficient spread between particles for FTIR to distinguish between them. Where possible, the whole sample was deposited on the silver filters, but for samples in which substantial material was present after processing, a subsample was deposited. For those samples in which a subsample was deposited, the volume deposited was calculated to allow the total number of particles per g solids to be back calculated. Preliminary optimisation of sample processing allowed 100% of biota samples to be deposited onto the silver filter for analysis (Appendix B Figure 3). Smaller volumes of sediment samples were deposited as result of higher concentrations of black particles in both the fine and coarse fraction causing particle overload on the filter. Therefore, the average volume of the sample deposited onto the silver filter for sediments was equal to $53.7\% \pm 34\%$. To minimise subsampling errors, the whole deposition area was analysed for all samples, as it is only edge-cases which are additionally impacted by inhomogeneous distribution of particles on filters (Brandt *et al.*, 2021). For those samples in which the whole sample was deposited, the vial was washed three times with filtered deionised water, and this was deposited again onto the filter. The silver filters with deposited sample were then transferred to a labelled glass slide, secured in place, and placed in a slide holder, prior to transfer to the μFTIR . All samples were covered and held in paper card boxes until analysis.

3.3.7 Spectral analysis of microplastics 25-500 μm using micro-FTIR imaging

Samples were analysed for plastic and natural particle number, composition, size (25-500 μm) and mass using micro-Fourier Transform infrared (μFTIR , Perkin Elmer, Spotlight 400 FT-IR Microscope) spectroscopy. The silver filter, onto which the samples were deposited, reflects infrared (IR) light enabling the light to pass through the particles and be reflected through the particles to the FTIR detector. Groups of spectra are collated via raster mapping to produce a spectral map, which is compared with a database to identify polymer types. The wavenumber range used was 700 - 4000 cm^{-1} . The results of four scans were combined and interpreted as the final result. The resulting IR maps were analysed using Purity Microplastic Finder (pMPF), an automated spectra comparison software using machine learning alongside a

polymer reference spectra database (Hufnagl *et al.*, 2019). This approach enabled human bias to be excluded from particle identification.

3.3.8 Procedural blanks (negative control), resolution and limit of detection (LOD)

Given the importance of accurately quantifying microplastic particles in the lamprey GIT and sediment samples, blank corrections were run, alongside calculating the limit of detection (LOD) and limit of quantification (LOQ) for each microplastic polymer type (Dawson, 2022). Procedural blank results (n=3 per matrix) were used to calculate LODs and LOQs for sediment and biota samples, separately for each studied polymer. The LOD was defined as 3.3 x the standard deviation of the blank. This gives 95% confidence that any detected value >LOD is not a false positive result. The LOQ was defined as 10 x the standard deviation of the blank. All microplastic particle numbers reported here are blank corrected values, which fall above the LOD for the given polymer type. Data is blank corrected for each specific polymer, to avoid inflation of MP concentrations from the unavoidable baseline contamination of some polymers that cannot be entirely prevented during the extensive extraction procedures needed for these complex samples (Johnson *et al.*, 2020; Horton *et al.*, 2021). Whilst there is no standardised approach to interpreting blank data, its importance has been highlighted as essential for QA/QC (Koelmans *et al.*, 2019). In addition, LOD/LOQ approaches have been recently proposed as the most promising candidates for preventing overestimation of particle concentrations in a systematic assessment of 51 different blank assessment approaches identified in the literature on a dummy set of blank data (Dawson *et al.*, 2022).

3.3.9 Spike recovery (positive control)

Positive controls, in the form of spike recoveries, were used to assess the recovery efficiency of the extraction procedures for both biota and sediment processing. For each workflow (biota and sediment), three replicates of a single matched matrix sample were separately spiked with a dispersion (Appendix B Figure 10) containing a known concentration of irregular fragments of polyamide (PA, 1.13 g cm⁻³, 63-90 µm, dry powder produced in house through cryo-milling and cascade filtration 3,000 MP/mL, CoV 11%) and polystyrene (PS, 0.96-1.05 g cm⁻³, ~45 µm liquid dispersion, Polysciences Europe GmbH, Germany, 840 MP/mL, CoV 14%). Each spiked sample was processed as if they were biota or sediment samples, with one positive control accompanying each batch of samples to accurately reflect each sample batch

processing steps. The recoveries were calculated as a percentage of the total particles added to the sample and the coefficient of variance was calculated to determine the consistency of recovery. No quantitative correction based on spike recoveries is currently possible due to standard and certified reference materials being unavailable, compounded by a lack of understanding of the role of size, polymer, shape, colour etc. on recovery. However, the qualitative assessment of recovery executed in this study still represents a higher standard of quality assurance on this front than most studies to-date (Koelmans *et al.*, 2019).

3.3.10 Data analysis

Data presented are from the identification of microplastics via μ FTIR data followed by automated data analysis (pMPF). Statistical analyses were performed on data firstly corrected for contamination found in procedural blanks, and secondly those data points which fell above the limit of detection (LOD) for the given polymer type (Appendix B Table 5). All results reported are therefore based on these corrected data and expressed average value $\pm$ standard deviation, unless otherwise stated. The normality of the data was checked with observations of the residuals, followed by the Shapiro-Wilk test. Microplastic particle count, sizes and polymer types in lamprey larvae among sites and hydrometric areas was compared using Kruskal-Wallis analysis with *kruskal.test()* using the R statistical program (version 4.1.2; RStudio Team, 2020). The same test was used to compare sediment microplastic particle count, sizes and polymer types among sites and hydrometric areas. To identify which sites were significantly different from one another, pairwise post-tests were conducted between sites with *kwAllPairsNemenyiTest()* in 'PMCMRplus' in R. The uptake factor represents the overall ratio of particles categorised by polymer type, taken up from sediment to lamprey biomass. Specifically, this is the count of each microplastic polymer per gram of overall dry lamprey biomass divided by the count of each microplastic polymer per gram of overall dry sediment, thereby providing an estimate of the overall ratio for uptake for each polymer type.

Since the microplastic particle count data were not normally distributed, the relationship between abundance and polymer composition of microplastic particles at each site in lamprey gastrointestinal tract tissue and sediment was analysed using Spearman's rank correlation coefficient. Spearman's rank correlation coefficient was also used to test the relationship between pooled lamprey larvae wet weight and microplastic particle count in the lamprey samples.

3.4 Results

3.4.1 Microplastic contamination in lamprey

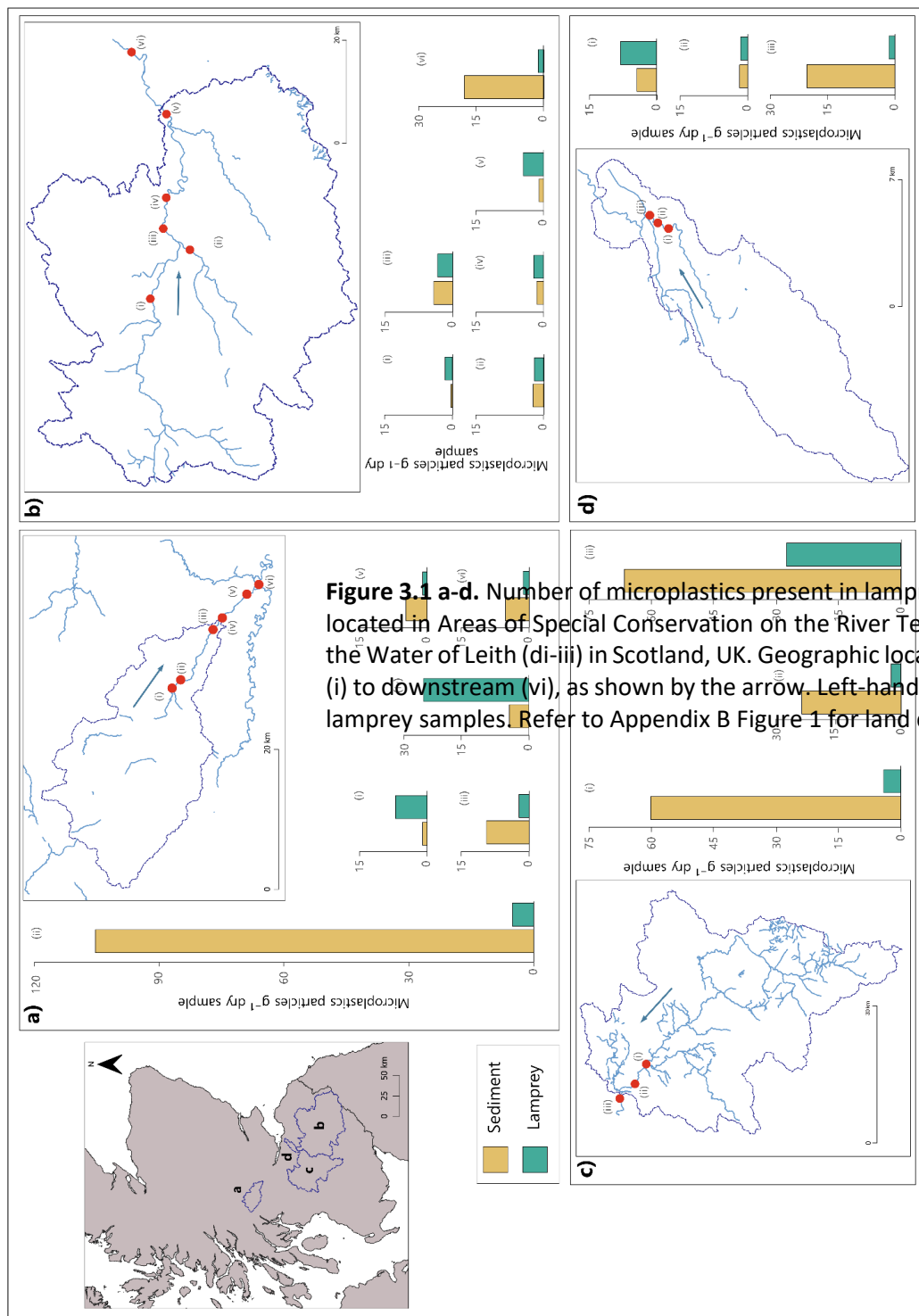
Microplastic contamination of gastrointestinal tract (GIT; nearest 0.1 g wet mass, median GIT $0.32 \text{ g} \pm (\text{SD}) 0.13 \text{ g}$) tissue from lamprey larvae was identified, at all sampling locations (Fig.1). Microplastic particle counts were converted from wet to dry weight of lamprey larvae GIT, assuming that the dry weight of lamprey larvae is 20% of wet weight (Mallatt, 1982). Microplastic particle abundance ranged from 1 particle g^{-1} dry GIT (River Tweed, Fig 3.1bvi) to 27.47 particles g^{-1} dry GIT (River Clyde, Fig. 3.1ciii) and the average values did not differ significantly between the eighteen sites or four hydrometric areas ($p > 0.05$). The average number of microplastic particles identified in pooled lamprey GIT samples across all sites was 5.84 ± 7.57 particles g^{-1} dry GIT tissue (Fig. 3.1). No significant relationship was observed between the pooled body weight or pooled body length of lamprey larvae and microplastic particle abundance ($p > 0.05$).

The average microplastic count for the rivers located within Special Areas of Conservation (River Teith, Fig.3.1a; River Tweed, Fig.3.1b), was 7.14 ± 8.45 particles g^{-1} dry GIT and 2.72 ± 1.21 particles g^{-1} dry GIT, respectively. Those rivers which flow through areas with a higher urbanised landscape (River Clyde, Fig.3.1c; Water of Leith, Fig.3.1d) had an average microplastic count of 11.29 ± 11.46 and 4.00 ± 3.35 particles g^{-1} dry GIT, respectively. The average microplastic count from larvae collected in hydrometric areas without SAC status was higher than those collected from SAC sites. An average microplastic count of 4.93 ± 4.83 particles g^{-1} dry GIT was recorded for SAC rivers and 7.64 ± 7.41 particles g^{-1} dry GIT recorded for urban hydrometric areas however this was not significantly different ($p > 0.05$).

As each pooled GIT sample was composed of a proportional mass from five lamprey larvae, it was possible to estimate the microplastic particle abundance within an individual lamprey's GIT. The number of estimated microplastic particles per individual larvae GIT tissue (wet weight) ranged from 0.51 to 36.81, with an average number of estimated microplastic particles load per lamprey larvae (GIT wet tissue) for all samples from all hydrometric areas of 5.45 ± 9.36 . Across all sites sampled, the highest estimated microplastic load per individual lamprey larvae was 36.81 particles, from a site on the River Clyde which was 300 metres downstream from an outflow from a large Water Treatment Plant (Fig.3.1ciii). The lowest

estimated microplastic load per individual was 0.51 particles, at a site on the River Teith, located in a Special Area of Conservation (Fig.3.1avi).

As lamprey larvae inhabit and feed directly from the sediment, it is possible to estimate the uptake for each polymer type from sediment to lamprey larvae. The uptake factor is the ratio of the concentration of microplastic polymers in lamprey biomass to the sediment concentrations. Polyurethane (PU) has the highest uptake factor in lamprey larvae, followed by cellulose acetate (CA) and polyamide (PA) (Fig.3.3)



3.4.2 Microplastic contamination in sediment

All sediment samples collected from the immediate habitat of lamprey larvae contained microplastic particles, with concentrations ranging from 0.40 to 105.41 particles g^{-1} dry sediment, with an average of 18.89 ± 20.31 particles g^{-1} dry sediment (Fig.3.1). The average length and width of particles detected in sediment samples was $71.5 \pm 22.7 \mu\text{m}$ and $46.4 \pm 13.8 \mu\text{m}$, respectively.

The average microplastic count for the rivers located with Special Areas of Conservation (River Teith, Fig.3.1a; River Tweed, Fig.3.1b), was 22.04 ± 37.38 particles g^{-1} dry sediment and 4.89 ± 6.48 particles g^{-1} dry sediment, respectively. Those rivers which flow through areas with a higher urbanised landscape and without protective SAC status, the River Clyde (Fig.3.1c), and the Water of Leith (Fig.3.1d) had an average microplastic count of 50.16 ± 18.74 and 9.32 ± 8.47 particles g^{-1} dry sediment, respectively. The average microplastic count from sediment collected in hydrometric areas with higher urban land cover was higher than those collected from SACs, with an average of 13.47 ± 21.93 particles g^{-1} dry sediment for SACs (Tweed and Teith) and 29.74 ± 13.61 particles g^{-1} dry sediment for urban hydrometric areas (Clyde and WoL). However, the difference was only significant between the rivers with the highest (River Clyde) and lowest (River Tweed) concentrations ($p = 0.02$).

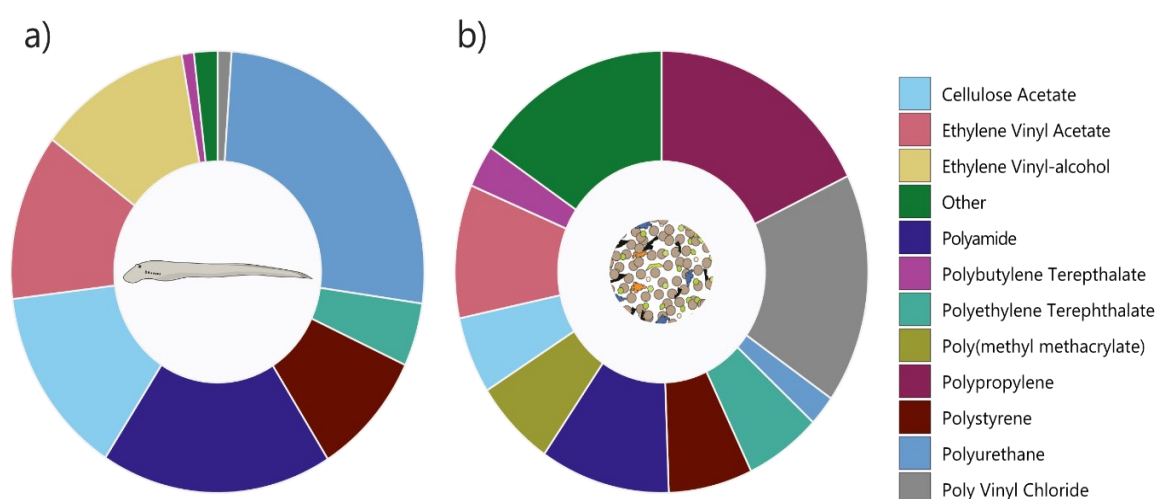


Figure 3.2 Proportions of microplastics polymers present in (a) lamprey gastrointestinal tract tissues (b) surface 10 cm of sediment from 18 riverine sampling sites on the River Tweed, River Teith, Water of Leith and River Clyde in central and southern Scotland, UK. Polymer types included in 'other' category: pe, abs, pc, pom, pan, ppsu, psu, si and pla (refer to full polymer names in main text and Appendix B).

3.4.3 Co-occurrence of microplastic contamination in lamprey and paired sediment samples

There was no significant correlation between the number of microplastic particles in the sediment samples and the corresponding lamprey larvae GIT samples ($p > 0.05$, Fig.3.1). Nonetheless, the average number of microplastic particles across sites was highest in the River Clyde for both sediment and lamprey larvae (Fig.3.1c).

Nineteen different types of microplastic polymers were identified above the limit of detection (LOD) in sediment and lamprey larvae samples; Fig.3.2a, b, Appendix B Figures 3-7). A total of eleven different polymers were identified in lamprey GIT including polyurethane (PU; 26.21%, $0.05\text{-}17\text{ g cm}^{-3}$), polyamide (PA; 18.08 %, $1.13\text{-}1.15\text{ g cm}^{-3}$) and cellulose acetate (CA; 13.90%, $1.54\text{-}1.63\text{ g cm}^{-3}$). Nineteen different polymers were identified in sediment including polypropylene (PP; 17.86%, $0.9\text{-}0.95\text{ g cm}^{-3}$), polyvinyl chloride (PVC;16.84%, 1.38 g cm^{-3}) and PA (10.16%). Microplastics polymer types were not found to be similar, or evenly distributed between sediment and lamprey samples at each site, i.e., different ratios of polymer types were observed between the paired sediment and lamprey samples (Fig.2a, b). For example, PVC made up significantly more of the microplastic particles found in sediment than in lamprey samples at the sites sampled ($p = 0.0002$; PVC biota; 1.08 % of total; sediment 16.84 % of total, Fig.3.2, Appendix B Figures 3-5). We also found microplastic polymers of both high (cellulose acetate; $1.54\text{-}1.63\text{ g cm}^{-3}$) and low density (ethylene vinyl acetate; $0.93\text{-}0.95\text{ g cm}^{-3}$) in both lamprey larvae and sediment. No differences were found in either the average length or width of microplastics present in the lamprey GIT and the surrounding sediment ($p > 0.05$; Appendix B Table 6).

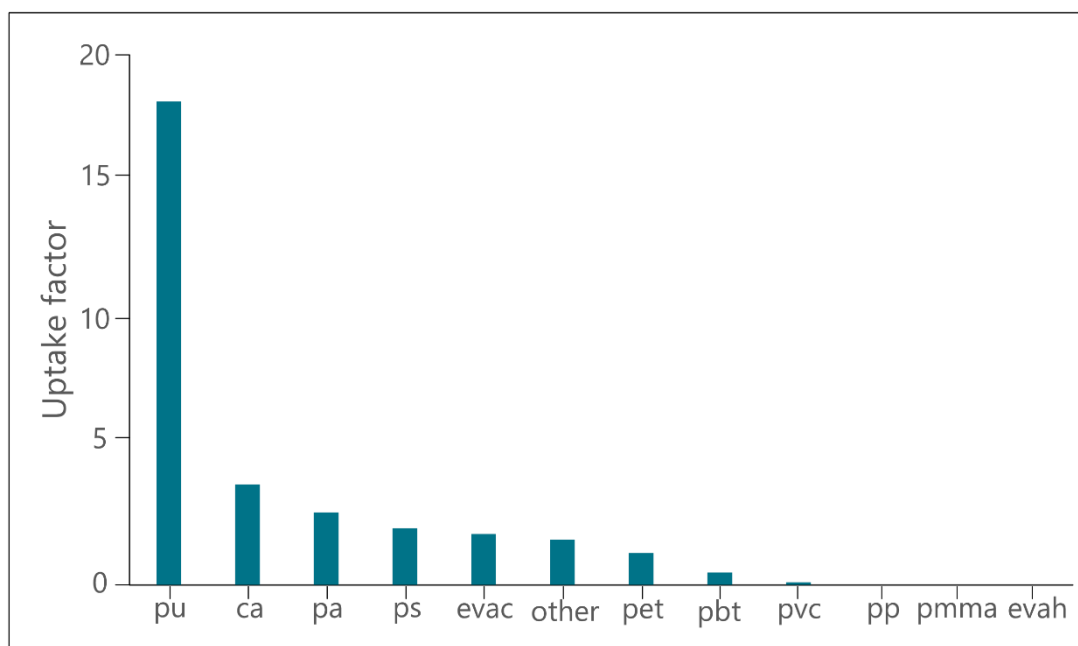


Figure 3.3 Uptake factor of microplastics polymers into lamprey larvae gastrointestinal tract tissue from immediate sedimentary environment. Polymer types include, polyurethane (pu), cellulose acetate (ca), polyamide (pa), polystyrene (ps), ethylene vinyl acetate (evac), polyethylene terephthalate (pet), polybutylene terephthalate (pbt), polyvinyl chloride (pvc), polypropylene (pp), poly (methyl methacrylate) (pmma) and ethylene vinyl-alcohol (evah). Polymer types included in 'other' include pe, abs, pc, pom, pan, ppsu, psu, si and pla (refer to full polymer names in main text and Appendix B).

3.3.4 Matched matrix spike recoveries (positive controls)

To validate the workflow and methods used to isolate microplastic particles from both lamprey and sediment samples matched matrix spike recoveries were used. Both polymers were consistently detected in all spike recovery samples, indicating that these particles could be recovered from the extraction process and still identified from their spectra in the final analysis. The average recovery rate for polyamide from sediment positive controls was 14.4 ± 8.2 (SD) % recovery and 53.4 ± 30.4 (SD) % recovery for polystyrene. The average recovery rate of spiked particles from lamprey positive controls was 48.9 ± 8.2 (SD) % recovery for polyamide and 69.6 ± 4.9 (SD) % recovery for polystyrene. As spikes consisted of a dispersion of the two polymers, the coefficient of variance (CoV) in the recovered samples can be compared to the CoV in the stocks as a qualitative check on consistency in recovery from the sediment and tissue matrices. Recovery from the lamprey tissues was extremely consistent with the expected variation from the spiked stocks, with CoV for polyamide and polystyrene

of 16.8 and 7.1% as compared with 11 and 14% in the stock itself. These results therefore seem very reproducible based on recovery of these known standards. Recovery from sediments was less consistent, with CoV of ~50% for both polymers, indicating that the complex sediment matrix may increase variability in the data. Whilst quantitative correction of the data based on these recoveries is not possible, they demonstrate that these microplastics could be recovered from both matrices, with the best recovery and reproducibility observed from the lamprey tissues.

3.5 Discussion

Studies investigating paired environmental contamination and microplastic in freshwater fish are lacking (Collard *et al.*, 2019), and this current study reveals the disparity between microplastic occurrence in the fish and that of their surrounding habitat. This study is the first to elucidate the occurrence of microplastic particles in different lamprey habitats as well as in lamprey larvae themselves. Microplastics were identified in lamprey and sediment at all riverine sampling sites, and all contained common polymer types used in plastic packaging through to construction (Appendix B 6, 7). However, microplastic contamination in lamprey did not reflect the contamination in sediments they inhabit, both in terms of particle numbers and polymer types (Fig.1, Appendix B 3-5). The estimated microplastic abundance ranged between 0.51 – 36.81 microplastic particles per individual lamprey larvae, which closely reflects the range of 0 - 40.88 items per individual reported in other freshwater fishes (Azizi *et al.*, 2021). Microplastics have been documented in many freshwater fish species, however there are key knowledge gaps on whether protected vulnerable species are exposed. This study suggests that threatened lamprey species residing in areas with protective interventions have on average lower microplastic contamination, than those without these protective interventions.

The River Clyde and Water of Leith are considered to have poor water classification and flow through two of the largest urban areas in Scotland, Glasgow, and Edinburgh, respectively (Appendix B Table 2). Lamprey larvae reside in these rivers, however no protective measures are in place for the conservation of this threatened species. These sites were expected to have the highest potential microplastic contamination, with high run-off, major roads, large populations, and industry in the surrounding catchment. This was evident in both lamprey and sediment samples from the River Clyde, exhibiting the highest microplastic count on

average across sites (Fig.1c). However, the Water of Leith in comparison to the Clyde had a low microplastic count (Fig.1d). This disparity is potentially due to the differences in wastewater effluent received by these water bodies (Appendix B Table 1). Effluent from wastewater treatment plants are critical sources of microplastic contamination to river systems (Murphy *et al.*, 2016). The River Clyde receives inputs from two major secondary wastewater treatment plants, with an estimated 65 million microplastics released every day from one wastewater treatment plant on the River Clyde (Murphy *et al.*, 2016). In fact, the site with the highest pollution for lamprey larvae was 300 metres downstream of Daldowie wastewater treatment plant (WWTP) (Fig.1ciii, Appendix B Table 1). The Water of Leith (WoL) on the other hand, according to the Scottish Pollutant Release Inventory has no major wastewater releases. The microplastics detected at the WoL sites could originate from combined sewage overflows (CSOs), of which the Water of Leith Conservation Trust has identified approximately 54 on the river (see SI). Therefore, the greater volume of wastewater emitted to the river system and greater size of the catchment of the River Clyde, compared to the Water of Leith could explain the differences in number of microplastic particles identified for both lamprey and sediment samples.

The sites within the Special Areas of Conservation (SACs), River Tweed and the River Teith, and are classified as having moderate river status. Despite protective status and rural setting, microplastic particles were detected in sediment and lamprey samples at all sites. The variability of microplastics detected on the River Teith (i.e., high levels at site aii in Fig.1) could be a result of local-scale processes, such as local pollution sources, which have been identified as important factors in predicting microplastic pollution (Dikareva and Simon, 2019; Appendix B Table 3). Furthermore, the microplastics within SAC sites could originate from the wastewater treatment plants on these river systems, as ten of the twelve sampling sites were between 120m to 9.6 km downstream from wastewater treatment plants (Appendix B Table 1).

Despite the close and repeated interactions between larval lamprey and their surrounding riverine sediments the results presented here suggest that the probability of microplastic ingestion is not always dependant on the levels and composition in the surrounding sediments. For example, differences in the microplastic polymer compositions, suggest that the uptake of microplastic by larvae may not be directly proportional to the availability in the

surrounding sediment. This result may be explained by larval mobility and transient feeding between sediment patches (White and Harvey, 2003). The gut passage time of lamprey larvae typically ranges from 54 to 70 hours, therefore sampled gut contents of larvae provide a relatively short snapshot of the dietary composition (Evans *et al.*, 2019). The higher occurrence of polyurethane, polyamide, and cellulose acetate in lamprey GIT compared to the sediment, could also be explained by these polymers having a higher uptake rate in lamprey larvae. The gut passage time and uptake rate or retention of specific polymer types (Lahive *et al.*, 2022), as well as the movement of larvae between patches, could therefore explain the differences in microplastic polymer proportions and microplastic loading between sediment and lamprey samples from each site. However, further investigation into how local scale hydrodynamics and spatial feeding affect microplastics identified in lamprey larvae is required. These results align with the findings from previous studies which have collected paired environmental samples, concluding that samples differ in microplastic contamination patterns for the studied compartments (McNeish *et al.*, 2018; Scott *et al.*, 2019; Yuan *et al.*, 2019).

Microplastic contamination in the environment has a wider size continuum than the fraction that is available to organisms that ingest microplastic i.e., many of the microplastic particle sizes present are too large to be ingested by organisms (Koelman *et al.*, 2020; Sørensen *et al.*, 2020). The physiological limits of ingestion for lamprey larvae are based on two factors: (1) the size of the mouth parts (oral hood); (2) the size of the oral cillae on the inner surface of the oral hood (Moore and Mallatt, 1980). Based on the oral hood length in brook lamprey larvae (*Ichthyomyzon greeleyi*) being $3.4 \pm 0.3\%$ of total length (Beamish and Austin, 1985), the median oral hood length for the larvae sampled in this study is 3.81 mm. However, the oesophagus of lamprey larvae of this size is approximately 620 μm (Beamish and Austin, 1985; *Ichthyomyzon greeleyi*), suggesting that particles larger than this would be expelled through the gills or oral hood (Limm and Power, 2011). In the current study the size of microplastics detected in larvae were between 35-103 μm , however natural particles within the range of 5-340 μm have been found within the guts of wild lamprey larvae (Moore and Mallatt, 1980). The narrow size range suggests that this was the size of microplastics available, which is shown by the matched particle size of microplastics in the surrounding sediment.

The presence of microplastics within all lamprey larvae samples demonstrates that microplastics are ingested by this species, a pre-requisite for internal tissue damage (Usman *et al.*, 2021) and/or food dilution (Koelmans *et al.*, 2020; Amariei *et al.*, 2022). Decreased nutritional value of food (“food dilution”) due to microplastic ingestion, is a commonly proposed effect mechanism for toxicity of microplastics (Koelmans *et al.*, 2020; Rauchschalbe *et al.*, 2021). Although the impacts of microplastic ingestion were outside the scope of this study, we were able to estimate whether the number of microplastic particles ingested by lamprey could contribute to food dilution. Individual lamprey sampled in this study were estimated to have between 0.51 – 36.81 microplastic particles in their gut at the point of capture. Based on the Corey shape factor distribution (Kooi and Koelmans, 2019), it is possible to calculate the simplified ellipsoid volume of the microplastic particles within the intestine of a lamprey larvae (Koelmans *et al.*, 2020, Appendix B Table 7, 8). Using measurements of the anterior and posterior intestine of *Lampetra planeri* larvae (Hilliard *et al.*, 1983), we estimated the percentage of intestine occupied by microplastic particles. The average percentage of intestine taken up by microplastics in lamprey larvae across all sites was 0.01 %, with a maximum of 0.13 % for those larvae sampled from the most contaminated site on the River Clyde. This estimation suggests that the total volume of microplastics within the lamprey larvae intestine is very low compared to the total cavity volume of the gut.

Therefore, although ubiquitously available and ingested, as food dilution is the most common toxicity mechanism for microplastic ingestion, the risks of microplastic contamination to lamprey larvae sampled in this study are low. Furthermore, as larvae are continuously exposed to natural organic particles, it could be anticipated that physical damage from microplastics particles is negligible. However, microplastics may be retained or bioaccumulated in the gut (Alomar *et al.*, 2021; Lahive *et al.*, 2022) posing an ecotoxicological risk through their inherent particulate properties or leaching of polymer additives (Paluselli *et al.*, 2018). Whether the presence of microplastics in the gut will result in internal tissue damage or specific exposure risk is not within the scope of this study and remains to be elucidated. So, although it is clear microplastics are available to and ingested by lamprey larvae, the consequence of this is not clear. Thus, ecotoxicological studies investigating the effects of microplastic ingestion on lamprey larvae are needed.

3.6 Conclusions

To conserve and manage threatened lamprey populations, it is essential to first understand the stressors placed upon them. Here we provide the first *in-situ* robust measurements of microplastic abundance and characteristics in lamprey larvae. Lamprey larvae from all sites, including Special Areas of Conservation, ingested microplastic particles, and these differed in microplastic contamination patterns from the sediment background contamination. Together with effect data, our characterisation of the exposure will support the risk assessment for microplastics in rivers and will contribute to the conservation management of this threatened freshwater fish. This paper also highlights the need for further investigation of the extent of microplastic contamination in lamprey larvae and importantly what the consequences of ingestion could be for physiological processes and overall lamprey larvae health.

Chapter Four - Does Age of Lamprey Larvae have an Impact on Microplastic Ingestion or Accumulation?

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4.1 Abstract

Microplastics are pervasive pollutants occurring in sedimentary habitats, therefore it is vital to understand their interactions with organisms such as lamprey larvae. Lamprey larvae are protected ecosystem engineers, that undergo an extended life stage burrowing and feeding in sediment. This study investigates age-dependent microplastic ingestion patterns in lamprey larvae from the River Teith, Scotland, with implications for conservation monitoring. Results suggest that all lamprey larvae, from ages classes 0+ to >1+, ingest microplastics, displaying distinctive polymer type patterns across age classes. The size of the microplastics ingested by lamprey larvae was not correlated with lamprey body length and corresponding age. Polymer-specific variations suggest differences in micro-habitat exposure between larger and smaller larvae, emphasizing the importance of considering microplastic exposure in conservation strategies. The study contributes further baseline data for assessing the ecological risk of microplastics in Special Areas of Conservation in Scotland, emphasizing lampreys' role as bio-indicators for river health. The findings underscore the need for standardized sampling methodologies, highlighting the importance of rigorous procedures for accurate assessments. In conclusion, this research explores microplastic ingestion by lamprey larvae, offering insights into age-related variations and contributing to the conservation management of this ecologically significant species.

4.2 Introduction

Microplastics affect the ecological functioning and structure of sedimentary habitats, therefore understanding the interactions of microplastics with sediment-dwelling freshwater organisms is essential for assessment of their environmental risks (Green *et al.*, 2017). As previously discussed, lamprey larvae are important ecosystem engineers (Beamish and Youson, 1987; Close *et al.*, 2002; Shirakawa *et al.*, 2013). They have a relatively long larval stage, burrowing into freshwater sediments for up to seven years, in which microplastic hotspots can occur (Corcoran *et al.*, 2019; Bondelind *et al.*, 2020; Vincent and Hoellein, 2021). Lamprey begin feeding once hatched, consuming deposited organic matter within the biofilm complex of sediments (Limm and Power, 2011).

Lamprey larvae populations are routinely sampled to monitor their conservation status, therefore understanding whether it is necessary to consider microplastics sampling during these campaigns is beneficial. Favourable conservation status for lamprey species is determined when two or more length (age) cohorts are abundant within the sampled populations (Cowx, Harvey, Noble, & Nunn, 2010; Harvey & Cowx, 2003; Nunn *et al.*, 2017). Different age cohorts of lamprey occupy different micro-habitats (Sugiyama and Goto, 2002; Aronsuu and Virkkala, 2014), therefore they may be exposed to microplastic contamination through varying routes. For instance, young first-feeding larvae may be exposed to microplastics residing in temporary, peripheral habitats. While older larvae might accumulate microplastics from depositional habitats that accumulate in the environment over time. Monitoring across larvae ages may help us to understand these exposure dynamics and contribute to Special Areas of Conservation site condition monitoring.

Ingestion is considered the primary pathway of microplastics exposure for fish (Su *et al.*, 2019) and is a pre-requisite for internal tissue damage (Usman *et al.*, 2021) and food dilution (Koelmans *et al.*, 2020; Amariei *et al.*, 2022). Ingestion patterns of microplastics are often related to life history traits, including body size and trophic level (Parker *et al.*, 2021). It is currently not known whether microplastic ingestion is correlated with age class or corresponding body length in lamprey larvae, however recent research in marine habitats suggests elder fish ingest both higher numbers of microplastics and a greater range of polymer types (Ding *et al.*, 2023). In contrast no correlation was observed between cod and saithe fish length and microplastic ingestion (de Vries *et al.*, 2020). In the freshwater

environment, larger female roach (*Rutilus rutilus*) have been shown to ingest higher number of microplastics (Horton *et al.*, 2018).

The different life stages of aquatic organisms have varying sensitivities to environmental contaminants (Cope *et al.*, 2008; Vardy *et al.*, 2013). Early life stages, such as larvae, are often more vulnerable to the negative effects of pollutants due to their developing organs and internal systems (Mohammed, 2013). Monitoring pollutants across ages can help identify potential impacts on growth, development, and survival. Furthermore, concentrations of environmental pollutants can vary within the age classes of fish including bioaccumulation of heavy metals (Farkas *et al.*, 2001; Suhareva, 2020) and uptake of microplastics (Ding *et al.*, 2023). The exposure can have deleterious effects with chronic water pollution altering the maturation of individuals, adjusting the fecundity of populations and maintenance of populations sizes (Moiseenko, 2002). Furthermore, increased mortality of older age classes from toxic load can change population structure (Moiseenko, 2002).

Determining the age structure of a fish population by assessing the abundance of each size class present is a sensitive indicator of population state and can indicate impacts (Moiseenko, 2002). Populations with an impaired length distribution (i.e. lack of recruitment of younger age classes into older) can signify a population under threat (Harvey & Cowx, 2003). Local environmental variability and differences in habitat suitability can be influenced by anthropogenic stressors and thus drive fluctuations in the frequency and strength of recruitment into older age classes (Shepard *et al.*, 2019). If pollutants are affecting specific age classes disproportionately, it can lead to imbalances in population structure and reduce the overall reproductive capacity of the population.

Given the ubiquitous presence of microplastics in river sediments (Woodward *et al.*, 2021) and many freshwater organisms, including lamprey larvae (Rendell-Bhatti *et al.*, 2023), it is important to consider whether ingestion of microplastics is correlated with age in these threatened populations. Understanding the composition of microplastics and whether such contaminants are present in higher concentrations in older age classes of lamprey larvae could aid in the monitoring of populations. As a protected species which are known to ingest microplastics it is fundamental for assessment of their environmental risks (Nunes *et al.*, 2023).

Here, we analysed the variation of microplastic particle count, mass, size and polymer type in lamprey larvae across a range of ages from the River Teith, Scotland. This study provides further insights into the influence of age on microplastic ingestion of fish and contributes baseline data to assess the ecological risk of microplastics in Special Areas of Conservation in Scotland.

4.3 Material and Methods

4.3.1 Lamprey larvae sampling

A total of 80 lamprey larvae of increasing body length were collected from one site on the River Teith (56.175836, -4.039641), in central Scotland in the United Kingdom (UK) between December 2022 and January 2023 (Fig. 4.1). This river is designated under the EU Habitats and Species Directive as a Special Area of Conservation (SACs; Habitats Directive, 1992: Council Directive 92/43/EEC) on the basis that the river supports all three native UK lamprey species. A pragmatic approach was agreed in determining sample size, and the number of individuals removed were not considered to have a detrimental effect on population status.

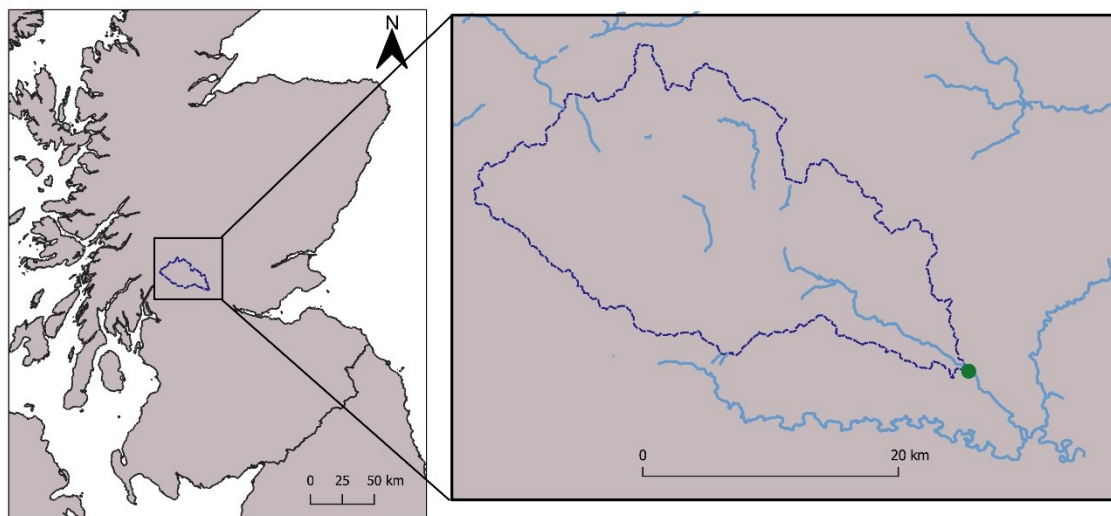


Figure 4.1. Sampling location of lamprey larvae on the River Teith, Scotland (green dot). This site corresponds to site (a)iv in Chapter 3, in which both lamprey larvae and sediment samples were analysed.

Lamprey larvae select and occupy different habitat and substrates depending on their size (Sugiyama and Goto, 2002; Aronsuu and Virkkala, 2014). Younger 0+ age classes (~21 – 40 mm) were collected from the peripheral fine sediment patches of the river, whereas older 1+ larvae (40-80+ mm) were collected from coarser sediment located in deeper water. Sampling

was carried out using Electracatch back-pack electric fishing equipment, fitted with a 30cm diameter anode ring with smooth direct current (DC) at a 180-Hz frequency, from a 1-3 m² patch of fine sediment, with characteristics typical of lamprey larvae habitat (Torgersen and Close, 2004). The samples may have been composed of both resident brook (*Lampetra*) and river (*L. fluviatilis*) forms due to the inability to distinguish between *Lampetra* ammocoetes in the field. Directly following collection, lamprey larvae were euthanised by an overdose of Tricaine methanesulfonate (MS-222; Matthews and Varga, 2012). Larvae from each size class were aggregated, wrapped in aluminium foil and snap-frozen in a liquid nitrogen dry-shipper for transport back to the laboratory. Tissue was stored at -80 °C freezer until dissection and all subsequent work was carried out under a laminar flow hood to minimise airborne microplastic contamination (Hermsen *et al.*, 2018).

4.3.2 Lamprey larvae sample preparation

Once defrosted, biometrics of each lamprey larvae were recorded site (ranging from 0+ to >1+), and the exterior thoroughly rinsed with filtered Milli-Q water to remove any external microplastic contamination. Each sample was composed of two lamprey larvae which were size matched in body length, with a 3 mm increment between each sample, giving a range from 21 – 108 mm (n=80 total, sex determination was not possible). Two size-matched lamprey per sample were used to gain a larger sample size of individuals, without overloading the filter for analysis. This approach was chosen based on understandings gained from Chapter 2 on the volume of tissue which can be effectively prepared for analysis. The larvae were split into three separate age cohorts, based on total body length (mm), as applied to the conservation management assessment protocol for lamprey larvae populations in UK rivers (Nunn *et al.*, 2008). Specifically, these were age 0+ (20-40mm), 1+ (40-70mm) and >1+ (>70mm). It is not possible to reliably separate older age cohorts > 1+, due to the high degree of overlap in length of older age classes, as a result of slowing in growth (Hardisty, 2006). To discern age groups larger than 1 year during field surveys (Nunn *et al.*, 2008; Pinder, Harrison, & Britton, 2017) would require destructive sampling as it relies on the examination of statoliths banding pattern (Meeuwig & Bayer, 2005; Volk, 1986).

4.3.3 Isolation of microplastics

Microplastic isolation from the whole gastrointestinal tract (GIT) of lamprey larvae was performed following our previous method (Chapter 3) Each lamprey sample was assigned to a processing batch (n=11 per batch), alongside one negative control (blank) per batch of samples. Briefly, the procedure was run as follows: (i) The GIT tract of each lamprey was dissected out, weighed and underwent tissue maceration with 10% sodium dodecyl sulphate (SDS). (ii) Reduction in organic material within the sample was achieved with 30% H₂O₂ at 90 rpm at 50°C. (iii) To achieve the final sample, SDS and H₂O₂ was removed by vacuum-filtration through a 5µm stainless-steel filter and the final isolated microplastics were stored in 50% ethanol for analysis. Strict procedures were followed with regards to contamination control.

4.3.4 Spectral analysis of microplastics 25-500µm using micro-FTIR imaging

Samples were prepared and analysed using micro-Fourier Transform infrared (µFTIR, Perkin Elmer, Spotlight 400 FT-IR Microscope) spectroscopy, as in *Chapter 3, section 3.3.6 and section 3.3.7* and Rendell-Bhatti *et al.* (2022). Briefly, samples were deposited onto a silver filter (Sterlitech, silver membrane filters, 3.0 µm, 25 mm). The silver filters with deposited sample were then transferred to a labelled glass slide, secured in place, and placed in a slide holder, prior to transfer to the µFTIR. All samples were covered and held in paper card boxes until analysis. Samples were analysed for plastic and natural particle number, composition, size (25-500µm) and mass using µFTIR, and analysed using Purity Microplastic Finder (pMPF), an automated spectra comparison software using machine learning alongside a polymer reference spectra database (Hufnagl *et al.*, 2019). This approach enabled human bias to be excluded from particle identification. Procedural blanks and detection limits were calculated as in Chapter 3, section 3.3.8, with three procedural blanks run alongside each processing batch, to enable calculation of the limit of detection (LOD) and limit of quantification (LOQ).

4.3.5 Spike recovery (positive control)

Positive controls, in the form of spike recoveries, were used to assess the recovery efficiency of the extraction procedures. In contrast to previous methods of using a set of positive controls, here each sample was directly spiked with a known count of yellow stained milled polyvinyl chloride (PVC) fragments between 90-150 µm (average particle count = 25, min = 18, max = 45). Once a known number of particles is added to each sample, it is possible to

count them back directly in the map to give a more comprehensive understanding of recovery rates across all samples. This more rigorous positive control gives the benefit that the particles can be localise individually in the microplastic map overlay capability in the Purency software used. Therefore, microplastic particle maps and spectra were used to visually check samples and manual particle edits to identify particles were undertaken where needed (Appendix C Figure 5). Both auto and manual particle edits are reported in the results. And discussed in chapter 3, no quantitative correction based on spike recoveries is currently possible due to standard and certified reference materials being unavailable, compounded by a lack of understanding of the role of size, polymer, shape, colour etc. on recovery. However, the qualitative assessment of recovery executed in this study represents a higher standard of quality assurance than the majority of studies to-date (Koelmans *et al.*, 2019) and builds on our methods in Chapter 3.

4.3.6 Data analysis

Data presented are from the identification of microplastics via μ FTIR data followed by automated data analysis (pMPF). Statistical analyses were performed on data firstly corrected for contamination found in procedural blanks. All results reported are therefore based on these corrected data and expressed average value $\pm$ standard deviation, unless otherwise stated. The spiked PVC particles were subtracted from the reported values for each sample and were not considered in the microplastic polymer or size characteristics. The normality of the data was checked with observations of the residuals, followed by the Shapiro-Wilk test. Since the microplastic particle count data were not normally distributed, the relationship between abundance, size and polymer composition of microplastic particles for each sample was analysed using Spearman's rank correlation coefficient.

The abundances of microplastics in the GIT of lamprey larvae are expressed as both the number of microplastics per individual (count per individual) and the number of microplastics per gram based on the dry weight of the digestive tract (count per 0.1 gram). Microplastic particle counts were converted from wet to dry weight of lamprey larvae GIT, assuming that the dry weight of lamprey larvae is 20% of wet weight (Mallatt, 1982). This was calculated firstly to allow comparison with Chapter 3, which considers count per 1 gram of dry GIT weight. Secondly, this was converted to 0.1g because this is more biologically relevant for the discussion here as individual lamprey GIT dry weight varied between 0.039 – 0.250 g.

4.4 Results

4.4.1 Lamprey larvae biometric data

Lamprey larvae body length and average lamprey body weight was positively correlated with average wet gastrointestinal (GIT) weight (g) ($R = 0.97$, $p < 0.01$; $R = 0.96$, $p < 0.01$; Fig.4.2).

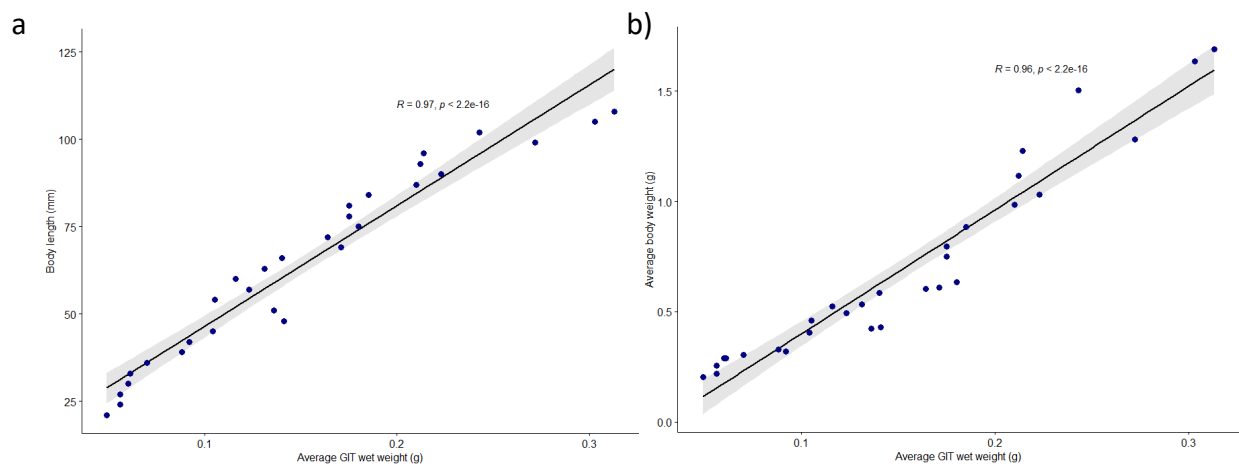


Figure 4.2 Positive correlational relationship between (a) lamprey body length (mm) and average wet gastrointestinal (GIT) weight (g) ($R = 0.97$, $p < 0.01$) and (b) average lamprey body weight (g) and average GIT wet weight (g) ($R = 0.96$, $p < 0.01$) Therefore, both body length and average GIT wet weight can be used interchangeably.

4.4.2 Microplastics in Lamprey larvae

Microplastics were found in all 80 lamprey larvae samples at abundances ranging from 0.50 to 412.50 particles per individual (average 35.39 ± 85.73 particles per individual, GIT wet weight) and from 0.23 to 121.55 particles per 0.1 gram of dry GIT tissue (average 19.51 ± 32.10). We observed no statistically significant relationship between average microplastic count (Fig.4.3a, $R = 0.22$, $p = 0.24$) or average microplastic mass (Fig.4.3b, $R = 0.008$, $p = 0.97$) and lamprey GIT weight.

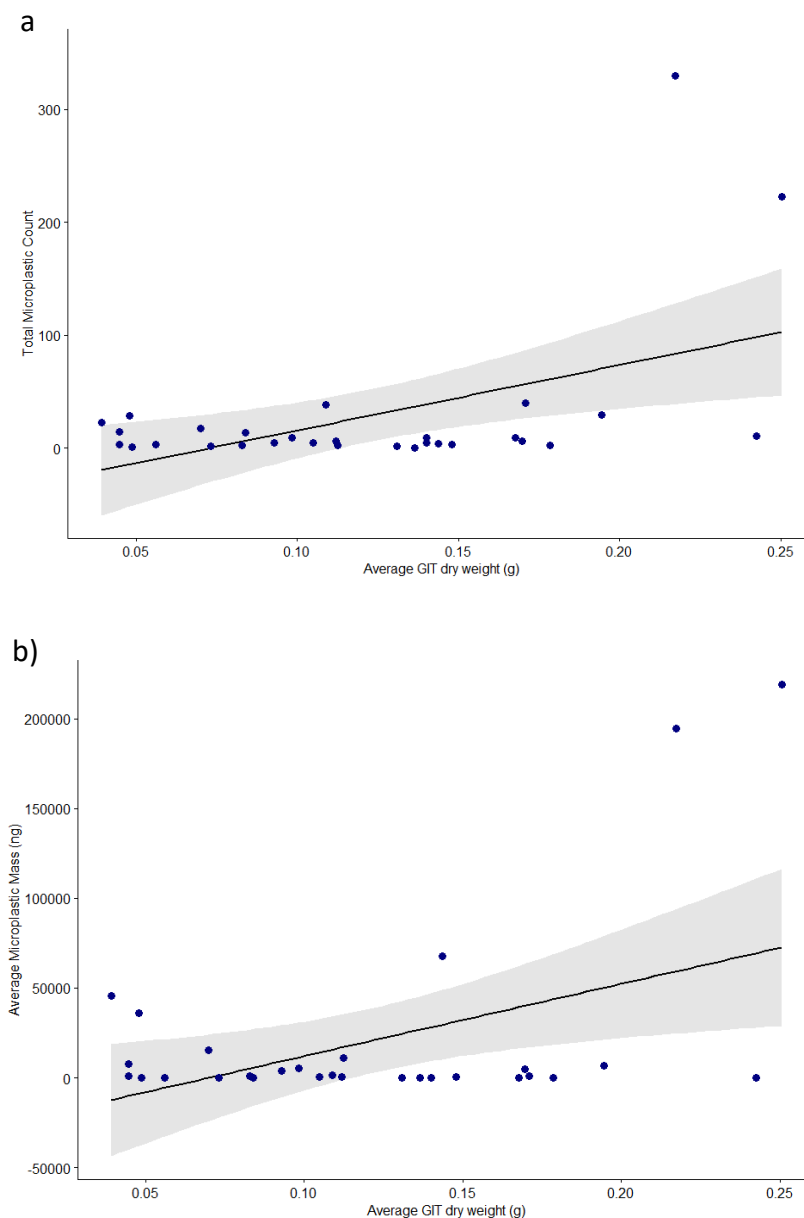


Figure 4.3 No correlational relationship between (a) total microplastic count and average dry gastrointestinal (GIT) weight (g) ($R = 0.22$, $p = 0.24$) or (b) average microplastic mass (ng) and average GIT dry weight (g) ($R = 0.008$, $p = 0.97$).

The average length of the detected microplastics ranged between 2 – 80 μm and average width ranged between 1 – 50 μm . We classified the microplastics into four length size categories, i.e., <10, 10 to 20, 20 to 30 and >30 μm . The most common size range was 20- 30 μm , followed by >30 μm . The size of the microplastics ingested by lamprey larvae was not related to lamprey body length or GIT weight (Figure 4.4a,b). The median particle length and width ingested across all lamprey larvae was $52.00 \pm 14.91 \mu\text{m}$ and $35.00 \pm 13.00 \mu\text{m}$, respectively.

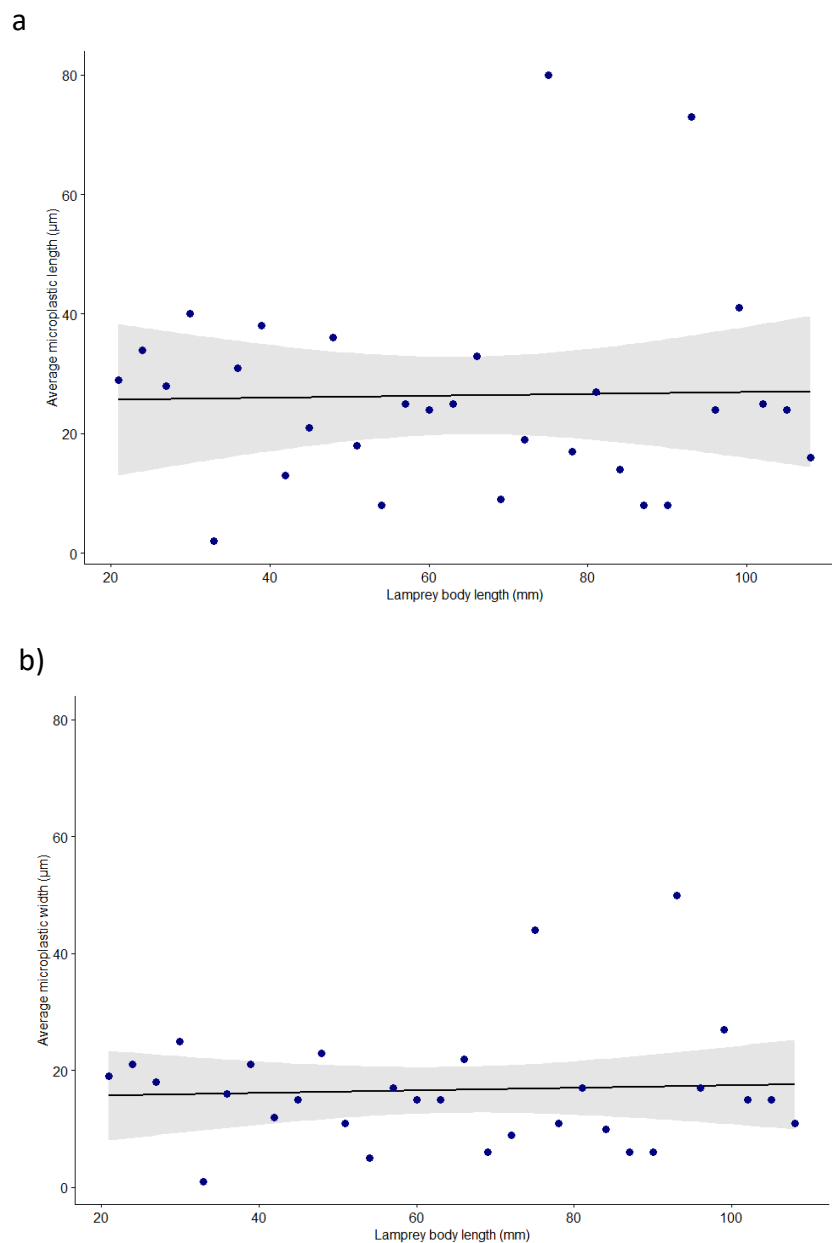


Figure 4.4 No correlational relationship between (a) average microplastic length and lamprey body length ($R = -0.16$, $p = 0.39$) or (b) average microplastic width and lamprey body length ($R = -0.15$, $p = 0.44$).

With regards to the microplastic polymer composition observed in the lamprey larvae, a total of 21 polymers were detected across the samples following blank correction (Fig.4.5), 15 of which were above the limit of detection (LOD) and three of which were above the limit of quantification (LOQ, polymers: ABS, EVAc, EVAh). There were no EVAc particles detected above the limit of quantification in larvae below 96 mm. However, between 96-108 mm (n =7) the average EVAc particle count above the LOQ is 195.43, with the highest observed count in larvae with a body length of 99 mm with 759 particles per two size matched larvae (Appendix C, Figure 1).

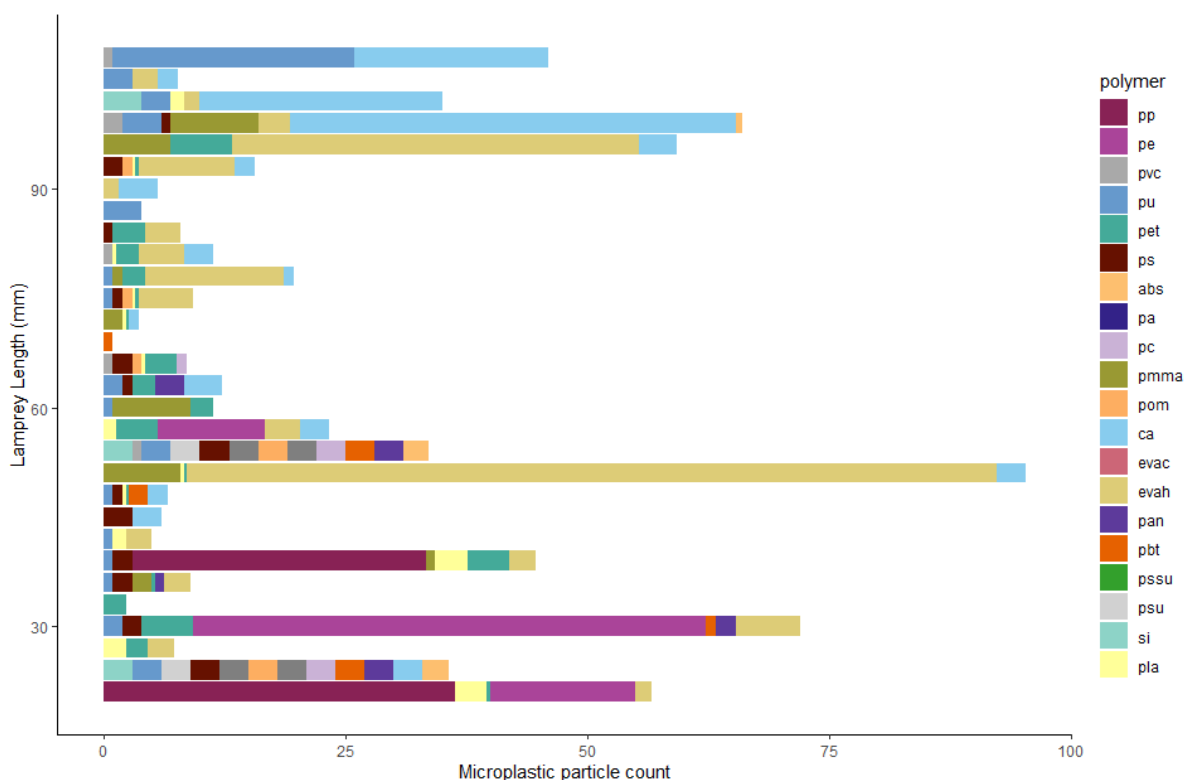


Figure 4.5 Microplastics count of each polymer type with increasing lamprey length (mm). EVAc removed from all samples to show patterns in other polymer types, due to high levels in larvae 96 -108mm. Figure in Appendix C shows patterns with EVAc retained.

4.4.3. Variation of microplastic characteristics considering lamprey larvae age classes

Microplastics were observed in all three age groups of lamprey larvae (Fig4.6). However, the microplastic polymers detected were distinct for each age class, with age class 1 (21-39 mm) being characterised by polyethylene and polypropylene (Fig 4.6a). Age class 2 (42-69 mm) was characterised by EVAh and age class 3 (72-108 mm) was characterised substantially by EVAc (Fig 4.6b,c).

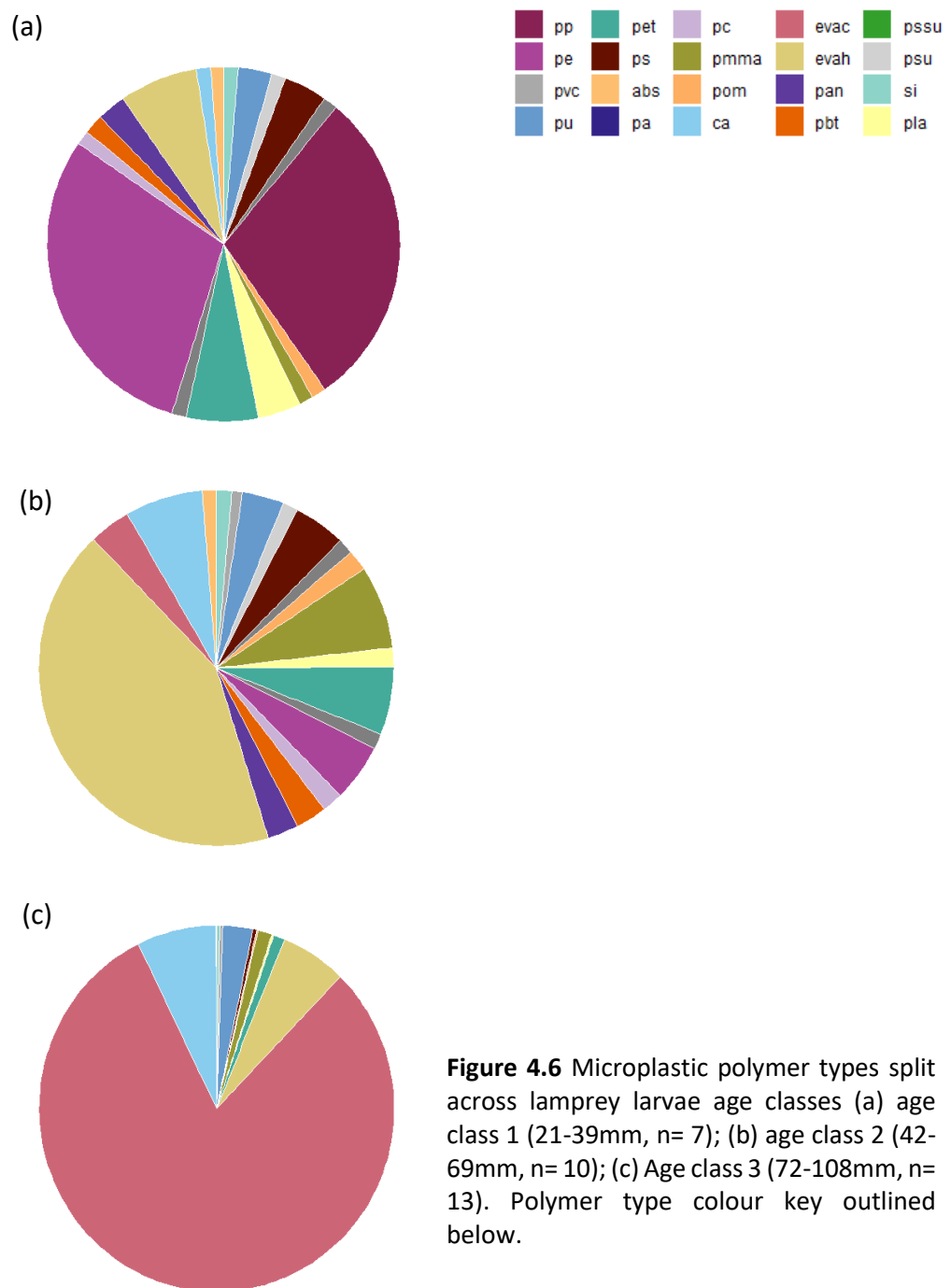


Figure 4.6 Microplastic polymer types split across lamprey larvae age classes (a) age class 1 (21-39mm, n= 7); (b) age class 2 (42-69mm, n= 10); (c) Age class 3 (72-108mm, n= 13). Polymer type colour key outlined below.

4.4.4 Spike recoveries (positive controls)

To validate the workflow and methods used to isolate microplastic particles from lamprey samples, spike recoveries were adopted in all samples. Spiked PVC particles were consistently detected in all samples, indicating that these particles could be recovered from the extraction process and still identified from their spectra in the final analysis from larvae ranging from 21 – 108 mm in body length. The average number of PVC particles spiked to samples was 25 per sample and of these we had an average recovery rate of $37.34\% \pm 19.90\%$ based on Purency's auto particle detection. This increased by 3.22% when manual particle edits were considered, achieving an overall average rate of recovery across samples of $40.56\% \pm 22.23\%$. There was a statistically significant decrease in the percentage recovery rate of spiked PVC particles with increasing lamprey larvae length (Fig.4.7).

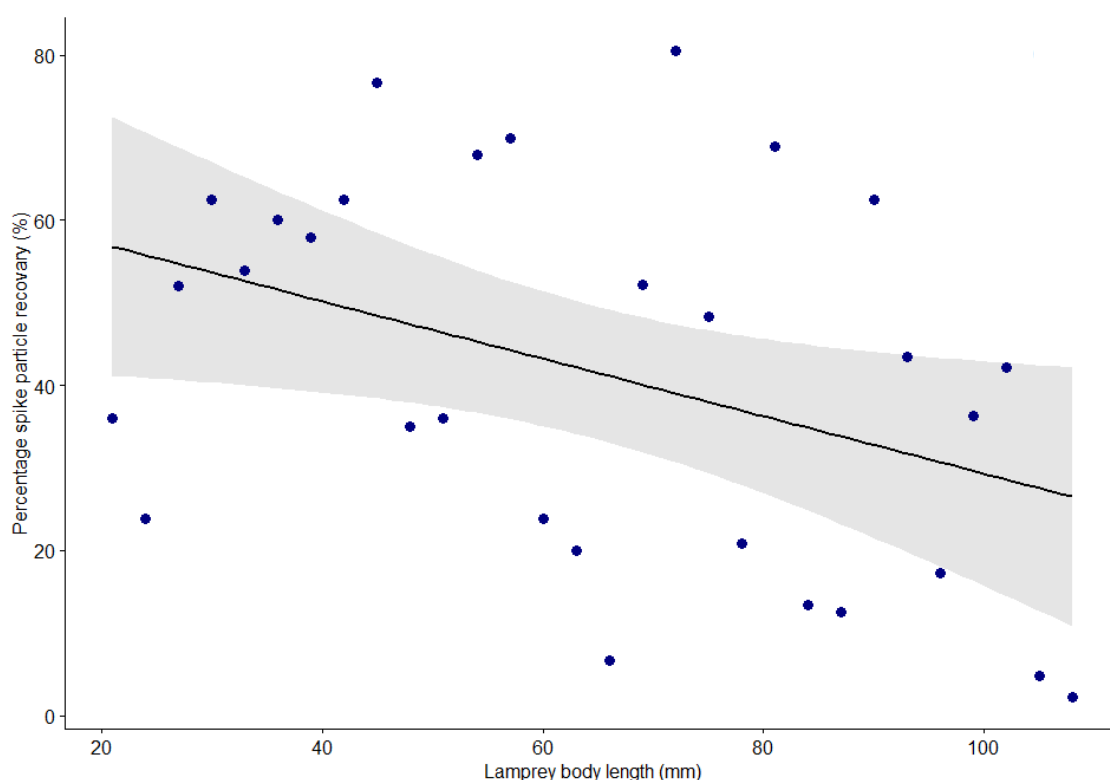


Figure 4.7 Percentage recovery of spiked PVC particles decreases with increasing lamprey larvae length (mm) ($R^2=-0.38$, $p = 0.039$).

4.5 Discussion

Current understanding of microplastic ingestion by different age cohorts of fish is very limited. However, understanding this contributes to the protection of species under threat and contributes understanding to conservation management efforts. The framework often used for monitoring larval lamprey populations identifies three principal metrics to monitor and assess conservation status: level of occurrence in a catchment, density at sampling sites, and an indication of population (demographic) structure (Harvey and Cowx, 2003; Shephard *et al.*, 2019). As lampreys are often considered a bio-indicator species for the health of aquatic ecosystems (Mustonen and Tossavainen, 2018), this data can provide valuable insights into the overall condition of rivers and streams. The abundance or absence of lamprey larvae may also signal issues such as habitat degradation, pollution, or changes in water quality (Lucas *et al.*, 2021).

Here, our results add insight into microplastic ingestion in *Lampetra* larvae residing in a Special Area of Conservation. Our data suggest that all lamprey larvae ingest microplastics from 0+ to >1+ (21-108 mm body length) and the patterns in microplastic polymer type are distinctive between lamprey larvae age classes (Fig.4.5). Although we can tentatively consider the data with regards to age classes, it is not biologically accurate to split the lamprey larvae into categorised age classes without an accurate population analysis. This is due to the difficulties in discerning lamprey age classes based on body length alone (Nunn *et al.*, 2008; Pinder, Harrison, & Britton, 2017). Instead, it is best to consider this data with regards to an overall relationship of increasing body length and microplastic characteristics.

We observed no accumulation of microplastics in lamprey larvae with increasing body length and subsequent age (Fig.4.3, 4.4). This observation could reflect the gut passage time and lack of retention of microplastics in the gut for long periods of time (Roch *et al.*, 2021). Although the time taken for elimination of microplastics varies, previous research on fish suggests anywhere between 12 - 72 hours for most particles to be evacuated (Mazurais *et al.*, 2015; Grigorakis *et al.*, 2017; Cong *et al.*, 2019; Hoang & Felix-Kim, 2020). Furthermore, lampreys display an intolerance of pollution and habitat disturbance (Mustonen and Tossavainen, 2018), therefore accumulative exposure could be reduced by larvae moving patches in which high microplastic contamination occurs.

Physiological limits of ingestion dictate the microplastic particle size range available to lamprey larvae (Rendell-Bhatti *et al.*, 2023; Chapter 3). Subsequent gut transition time varies depending on ability to excrete microplastics, presence and quality of food, size of organism and complexity of gut morphology (Porter *et al.*, 2023). We would expect with increasing lamprey larvae size, microplastic size available to ingest would also increase, due to increasing size of mouth parts and oral cillae (Moore and Mallatt, 1980). However, we saw no relationship between microplastic length and width, and increasing larvae body length. The oral hood length in brook lamprey larvae (*Ichthyomyzon greeleyi*) is considered to be $3.4 \pm 0.3\%$ of total body length (Beamish and Austin, 1985). Therefore, the lowest size of larvae sampled would have an oral hood size of approximately 714 μm . However, we also need to consider the oesophagus size of larvae in the smallest size range, as although particles less than 714 μm may be able to enter the oral hood, these will be expelled through the gills or oral hood if they are not small enough to enter the oesophagus (Limm and Power, 2011). As the oesophagus size of a lamprey larvae is estimated to be approximately 0.17% of the oral hood length (Beamish and Austin, 1985; *I. greeleyi*), we predict that the smallest larvae (21 mm in body length) sampled in this study can ingest particles of up to approximately 121.38 μm .

Therefore, as the microplastic particle size range falls within the size range that all larvae can physically ingest, the lack of differentiation in microplastic size ingested by the different ages of lamprey larvae could be explained by what is available in the environment. The narrow range of microplastic sizes ingested by lamprey larvae in this study (1-80 μm) was similar to that of larvae sampled in Chapter 3 (25-103 μm). The smaller range (1-25 μm) detected in this study, could be a result of the broader size of lamprey larvae sampled. This size range is of particular concern due to the ability of these microplastics sizes to be retained, translocate (Roch *et al.*, 2021) and to disturb intestine function (Shang *et al.*, 2020).

Considering the differences in synthetic polymers ingested by lamprey larvae, one key observation from the data is the increase in the microplastic count of the polymer EVAc with increasing lamprey length (Fig.4.6). Although EVAc is present in the smaller larvae, it is not in the high concentrations observed in the lamprey with longer body lengths. Considering why it might be that larger individuals ingest or accumulate more EVAc, when compared to smaller larvae could be a function of differences in habitat selection and use by different age classes.

Larger lamprey larvae often inhabit deeper deposited sediment, whereas smaller first-feeding larvae (0+) occupy ephemeral, peripheral edge habitats (Sugiyama and Goto, 2002; Aronsuu and Virkkala, 2014). The differences in polymer uptake between age classes may reflect different micro-habitat selection and the resulting differences in microplastic polymer exposure with increasing sediment depth (Dai *et al.*, 2018; He *et al.*, 2021; Cera *et al.*, 2022; Yuan *et al.*, 2023).

Research indicates that microplastic abundance can both decrease or increase with depth of sediment, however much of this research considers lakes and marine systems and may not be applicable to riverine systems (Willis *et al.*, 2017; Li *et al.*, 2020). Insights from river sediments suggest microplastic concentration increases from shallow to deeper layers (Niu *et al.*, 2021). Furthermore, the higher levels of organic matter in deeper habitats, may not only attract larger lamprey larvae (Aronsuu and Virkkala, 2014), but also may determine microplastic concentrations (Nel, Dalu and Wasserman, 2018; He *et al.*, 2021).

An alternative explanation could be that the deeper habitats which are occupied by the larger larvae could have experienced local contamination, which was not present in the more temporary habitats (Dikareva and Simon, 2019). Another consideration is that we do not know how long each lamprey larvae has occupied a specific patch for or where they have been feeding prior to being sampled (White and Harvey, 2003). The larger larvae could have been displaced from a site with high EVAc contamination or equally the site could be contaminated with EVAc and the smaller larvae have recently drifted into the area. This would explain the presence of EVAc in some of the smaller larvae, but the considerably higher numbers present in the larger larvae.

This is a plausible explanation as the background contamination in sediment at this site collected in 2020, was characterised by microplastic composed of EVAc, with 5.03 particles of EVAc per gram of dry sediment (Rendell-Bhatti *et al.*, 2023). However, no EVAc was recovered from larvae collected in September 2020 from this site. Microplastic abundance in sediment and larvae is shown to vary between seasons (Nel, Dalu and Wasserman, 2018). Therefore, as the previous sampling campaign was in September 2020 this could reflect the differences in larvae from the same site across temporal variation. Furthermore, as Chapter 3 highlighted, microplastic ingestion by lamprey larvae is not always dependant on the levels and composition in the surrounding sediment (Rendell-Bhatti *et al.*, 2023). Reasons for this are

discussed in Chapter 3, however briefly this could result from variable gut passage time, polymer retention, lamprey drift between patches and local scale hydrodynamics (White and Harvey, 2003; Evans *et al.*, 2019; Lahive *et al.*, 2022).

With further consideration of the methodologies and the evolution of these methods from both Chapter 2 and 3, the spiking procedure adopted in this chapter provide a more rigorous assessment of microplastic recoveries from samples. Spiking all samples was necessary here because the volume of GIT within each sample was different, therefore the isolation and recovery of microplastics from each sample could vary. As such, we observed here that indeed the recovery of spiked PVC particles decreases with increasing lamprey larvae length or GIT weight (Fig. 4.7). This is an important consideration for assessing how we standardise microplastic samples and their analysis. For example, we could be underestimating the numbers of microplastics in larger individuals, if in those samples we recover fewer particles compared to those samples with less tissue.

As a listed species on the European Habitats Directive there is a requirement for a 6-year reporting process on the conservation of *Lampetra* larvae (Habitats Directive, 1992: Council Directive 92/43/EEC). However, post-Brexit this requirement no longer exists in the UK. Despite this, the Scottish Government announced that, it would maintain or exceed European standards of environmental protection, and this is now confirmed in the Continuity Scotland Act 2021. To deliver this protection in Scotland, a new independent body Environmental Standards Scotland (ESS) aims to monitor ecosystems and ensure that the legislative framework effectively encourages actions with positive environmental outcomes. Therefore, it is certain that Scotland will continue to monitor SAC sites with potential integration of new technologies such as eDNA to explore where monitoring can be improved (Deiner, Yamanaka, Bernatchez, 2021; Pascher, Savara and Jungmeier, 2022). Although further investigation is needed, these data can contribute to our understanding of lamprey abundance patterns and monitoring across Scotland SAC sites. Although microplastics may be ingested ubiquitously across age classes, the differences in polymer types could reflect difference in habitat use and residence.

Chapter Five - Behavioural Toxicity of Tyre Wear Particles to Lamprey Larvae

5.1 Abstract

Lamprey larvae, despite their ecological significance, remain understudied in terms of their response to microplastic contamination. In this study, we investigated microplastic avoidance and habitat selection behaviours in lamprey larvae, focusing on their interactions with polyvinyl chloride (PVC) microplastics and tyre wear particles. Our findings suggest that lamprey larvae do not exhibit avoidance behaviour towards PVC microplastics, as indicated by method development studies. This lack of avoidance behaviour raises concerns regarding increased microplastic ingestion and its potential deleterious effects on individual lamprey larvae. Exposure tank experiments revealed a significant increase in the number of burrowing attempts by lamprey larvae into tyre wear particle-contaminated sediment. While hesitation in burrowing was observed, the exact mechanisms underlying this behaviour remain to be elucidated. Place preference tests demonstrated a faster locomotion rate in lamprey larvae exposed to tyre wear particles, suggesting an altered response to contaminated sediments. However, these differences were not statistically significant compared to control conditions, emphasising the need for further investigation.

Despite the insights gained, this study has limitations, including the lack of consideration for individual personality traits in lamprey larvae and challenges in fine-scale locomotion behaviour analysis. Future research using advanced tracking software such as DeepLabCut could provide a more detailed understanding of lamprey behaviour in response to microplastic contamination. Our study contributes to the broader understanding of how anthropogenic stressors, such as microplastics, may impact habitat selection and behaviour in lamprey larvae. Further research is warranted to assess the significance of altered locomotion behaviour and its potential implications for lamprey.

5.2 Introduction

The behavioural effects elicited in response to microplastic contamination are essential to understand and allow us to holistically assess their ecological impact (Porter *et al.*, 2023). Behavioural studies can inform subsequent research and contribute to the setting of conservation priorities, particularly for protected species and habitats (Anbumani and Kakkar, 2018). Research has found ubiquitous microplastic contamination across protected and

vulnerable aquatic habitats and species (Kutralam-Muniasamy *et al.*, 2021; Nunes *et al.*, 2023; Rendell-Bhatti *et al.*, 2023). Recent research suggests that whitefish (*Coregonus lavaretus*) larvae do not avoid microplastics when present alongside food intake (Huuskonen *et al.*, 2020). A lack of microplastic avoidance behaviour in larvae exposed to high levels of microplastic contamination could lead to negative effects on organism health (Foley *et al.*, 2018), because of internal tissue damage (Usman *et al.*, 2021) and/or food dilution (Koelmans *et al.*, 2020; Amariei *et al.*, 2022).

With advances in technology, measurements of animal behaviour are becoming more sensitive and integrative as a measure of response to exposure in the assessment of microplastic contamination (Chen *et al.*, 2020; Chen *et al.*, 2022; Huang *et al.*, 2023). However, the use of behavioural tests in microplastic toxicity analysis presents several challenges, particularly the lack of consideration and protocols for non-model species. As the sensitivity of behavioural endpoints can vary widely, it is important to gather data on responses to emerging contaminants of concern across a wider range of species (Stienbarger *et al.*, 2021). Therefore, to provide a greater understanding of how species outside of the typical models, such as zebra fish, may be affected by microplastic contamination we consider the behavioural effects of microplastics on lamprey larvae.

A key behavioural characteristic of lamprey larvae is residing in and burrowing into freshwater sediment. Burrowing behaviour enables larvae to avoid predation, whilst feeding in the water column or on surrounding sediment (Hardisty, 2006). Burrowing behaviour is triggered by negative phototaxis (Rovainen, 1980), therefore larvae are largely active within the hours of darkness (Quintella *et al.*, 2005; Dauble, Moursund and Bleich, 2006; Zvezdin *et al.*, 2018). When no suitable substrate is available, larva exhibit positive thigmotaxis, whereby individuals rest on their sides (Tummers *et al.*, 2016). As lamprey larvae burrow within sediment, only emerging when stressed (Yamazaki, 2006), this presents challenges for detailing behaviour *in-situ*.

A handful of studies suggest that lamprey larvae behaviour is sensitive to a multitude of anthropogenic stressors, including metal contamination (Myllynen *et al.*, 1997), persistent organic pollutants (Soimasuo *et al.*, 2004) and pesticides (Andersen *et al.*, 2010). The concentrations of these contaminants in lamprey in the environment are considered below lethal levels (Madenjian *et al.*, 2020). However, organisms that are not adapted to the

increased energetic costs associated with compensation for anthropogenic stressors must compensate for the physiological and biochemical effects that contaminants provoke (Rudneva, 2013). Although essential at all life stages, proper metabolic function is particularly important for larvae as this life stage serves as a bottleneck for survival (Beamish *et al.*, 2004). Contaminants can elicit alterations to an animal's metabolic rate which impacts life history traits, such as growth, behaviour, reproduction and feeding (Meador, Yeh and Gallagher, 2018; Yeh, Meador and Lunsman, 2021; Ball *et al.*, 2023). This has further implications for responses to climate change, as higher temperatures may increase the rate of uptake of pollutants via changes in ventilation rate, due to increased metabolic rate and decrease in oxygen solubility (Kennedy and Walsh, 1997; Schiedek *et al.*, 2007).

Given the absence of studies addressing the sub-lethal effects or avoidance of microplastic contamination on lamprey, it is essential to draw insights from research on other environmental contaminants to understand whether contaminants could elicit a behavioural response in this group. There is limited documentation of sublethal effects of contaminants on larvae, with only two studies directly addressing this: 1. Lamprey larvae exposed to contaminated sediments (dioxins and furans) elicit increased locomotion and ventilation time, although neither difference was statistically different from the control (Salmelin *et al.*, 2016). 2. Larvae display aversion behaviour to contaminated sediments (mercury, pesticides, PAHs and PCBs), evidenced as protracted burrowing time and re-emergence from the sediments when compared to those exposed to uncontaminated control sediments (Unrein *et al.* 2016). Furthermore, these authors reported larvae expelling sediment from their buccal cavity and remaining in the open for extended periods of time, from anecdotal video observations. These experiments suggest that there could be important effects of environmental contaminants on lamprey larvae burrowing and feeding behaviour, however the evidence presented thus far remains inconclusive.

One approach that can be utilised is to monitor the locomotor activity of individuals in the laboratory, by quantifying speed and distance moved, following exposure to microplastic contamination. Other fish species exposed to microplastics have exhibited altered swimming and movement patterns, such as hyperactivity (Chen *et al.*, 2020; Yu *et al.*, 2022), static decreased swimming activity (Santos *et al.*, 2021), reduced speed and distance moved (Sun *et al.*, 2021), and altered escape response to predators (Naninge *et al.*, 2021). These changes

could be related to stress or impaired sensory perception caused by the presence of microplastics in their systems. However, some studies also report no modified activity of fish in response to microplastic exposure (Cormier *et al.*, 2021).

To obtain reliable measurements, it is essential to adopt suitable technology to streamline data collection and reduce experimental noise. The advances in technology and deep-learning software have led to a range of open-source tools for analysing animal behaviour (Kwok, 2019). Many researchers have found key successes in recording fish behaviours in experimental systems (Jolles, 2021). Across a broad range of biological research fields, many have adopted Raspberry Pis (RPis) as a low-cost computer which is built upon open-source software. Using componentry such as this has enabled the ability to acquire highly detailed individual based movements using custom-written tracking software (Jolles *et al.*, 2018; Jolles *et al.* 2019). Furthermore, adopting RPis in behavioural research has led to fully automated high-throughput behavioural assay system, using automated image analysis techniques to quantify rheotaxis behaviour of zebrafish (Todd *et al.*, 2016). However, tracking software for wildlife and aquatic organisms typically focuses on more common and well-studied species, such as zebrafish (*Danio rerio*; Todd *et al.*, 2016), three-spined sticklebacks (*Gasterosteus aculeatus*; Jolles *et al.*, 2019) and guppies (*Poecilia reticulata*; Walter and Couzin, 2021). As such, there are currently no specific tracking software solutions designed exclusively for lamprey larvae.

The focus of this chapter is a series of method development studies with the purpose of identifying ecologically relevant behavioural endpoints for larval lamprey in a laboratory setting. Lamprey larvae are known to change locations when stressed (Yamazaki, 2006), therefore it is essential to detect stressors within the laboratory which may initiate re-establishment of burrows. Firstly, we needed to describe a range of typical lamprey behaviours observed in a laboratory setting. Secondly, we needed to determine whether we can track lamprey movement through tracking technology. Thirdly, we needed to understand whether larvae select or avoid different habitats such as sediment depth, sediment grain size or microplastic exposure. These preliminary experiments determined what conditions allow us to monitor behaviour of lamprey larvae in a microplastic exposure experiment, and secondly allow us to define a positive control, i.e. a behaviour which we can reliably compare against. This approach is then applied to investigate the behavioural response of lamprey

larvae to tyre wear spiked sediment and the response to a place-preference test following exposure for 14-days to the tyre wear particles. The first methods and materials of this chapter outlines the preliminary experiments methods and results. This is then followed by a second material and methods sections which considers the place preference tests.

5.3 Preliminary experiments: Materials, Methods & Results

5.3.1 Lamprey larvae collection, husbandry and aquaria set-up

Brook lamprey larvae (*Lampetra planeri*, n=64 80-110 mm body length) were collected from the wild between in April 2021 by electrofishing from the Endrick Water (Glasgow, Scotland). This site was chosen as it is above the known upstream limit for river lampreys (Potts of Gartness), therefore removing any chance of including river lampreys in the sample. A pragmatic approach was agreed in determining sample size, and the number of individuals removed were not considered to have a detrimental effect on population status. Those larvae used for observational experiments were returned to their habitat and released at the site of capture. Sampling was undertaken using Electracatch back-pack electric fishing equipment, fitted with a 30cm diameter anode ring with smooth direct current (DC) at a 180-Hz frequency, from a 1-3 m² patch of fine sediment, with characteristics typical of lamprey larvae habitat (Torgersen and Close, 2004). Lamprey were transported back to the University of Glasgow's Scottish Centre for Ecology and Natural Environment in river water and sediment and held in a flow-through holding tank (200 Litre) with natural river sediment for four days. This was undertaken to check the health status of the animals by confirming that no mortality had occurred and that no oother signs of ill health or disease were observed within the larvae during the settling period. A total of 16 tanks with 25L volume were used for the experiments were set up in a separate room to the holding tank, each set up in groups of four on small shelving units (Fig. 5.1). The experimental tanks were static, water was replaced every 3 days and an air stone provided oxygen for each tank.

5.3.2 System Overview and Componentry for experimental tanks

To effectively record lamprey larvae behaviour and responses with minimal disturbance, a video surveillance system was set up adopting the use of RPis and infrared cameras (Fig.5.1, 5.2). The cameras and RPis were mounted on a wooden beam (2.95m, Fig. 5.1) using custom 3D-printed mounts to enable airflow and reduce over-heating in the Raspberry Pi. Cameras

were fixed to the underside of the wooden beam using heavy duty Velcro strips (Fig.5.2). The preliminary experiments adopted the software Motion Eye OS to record, monitor and upload the media files. This easy-to-use interface allows the RPis to be programmed and once triggered by movement, to record lamprey behaviour during both day and night. The system also allows remote access to files via google drive and stores the files on the micro-SD card. Tests prior to these live experiments were conducted on a fake a lamprey larva to establish the sensitivity of the motion detection software and these parameters were used for the observational experiment.

As a result of needing to streamline the programming of the RPis to ensure effective data collection and uploading to minimise data loss, we used this work towards producing an application which allowed ease of recording during the experiments. Building upon challenges met with Motion Eye OS discussed below, we developed a low-cost lamprey behaviour surveillance system, along with a friendly user interface (*LamPi*). *LamPi* is an image-based tracking application designed to track single animals in aquatic laboratory studies. A user-friendly interface allows those with no coding skills to use the application effectively. Videos were filmed using Raspberry Pi Night Vision (5 MP) cameras, at 1296 x 972 resolution, 30 frames/second, using the user interface LamPi (Fig. 5.2).

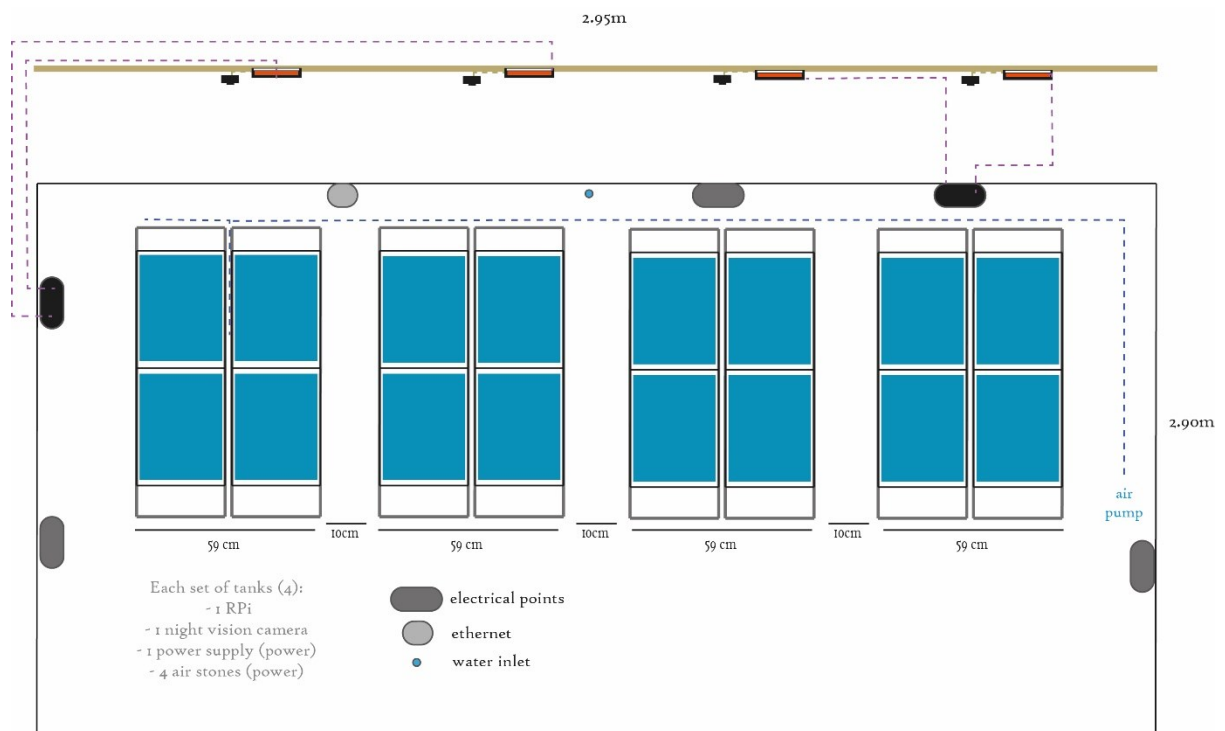


Figure 5.1 Experimental tank set up depicting four sets of four separate tanks with air stones powered by air pump, electrical points for Raspberry Pis set up and water inlet to allow tank water changes.



Figure 5.2 Set up of laboratory surveillance system with Raspberry Pi. RPi camera positioned above the tank to capture video images of the movement of larvae above the sediment, during both day and night.

5.3.3 Preliminary experiment 1: Burrowing and emergence of lamprey larvae from sediment in aquaria

The aim of this experiment was to monitor emergence rate and establishment of burrows of lamprey larvae in our experimental set-up. Here emergence behaviour is defined as the emergence from sediment burrows into the fluvial water. We set out to 1) understand movement patterns of lamprey larvae, namely burrowing, whilst in sediment of aquaria tanks, and 2) monitor larvae emergence behaviour through aerial video footage to determine movement patterns above the sediment. Experiments to track the level of lamprey larvae movement took place over a 7-day period. A total of 8 larvae were transferred from the holding tank to the experimental tanks, with one lamprey placed in each tank. To address our first aim and visualise burrows, different coloured sediments were layered in the aquaria (Fig 5.3a). This was set up to enable lamprey larvae burrows to be visualised on the underside of the tank (Fig.5.3b, c). Lamprey larvae were also monitored from an aerial view using the RPi set up and night vision cameras, to address our second aim (Fig.5.3a).

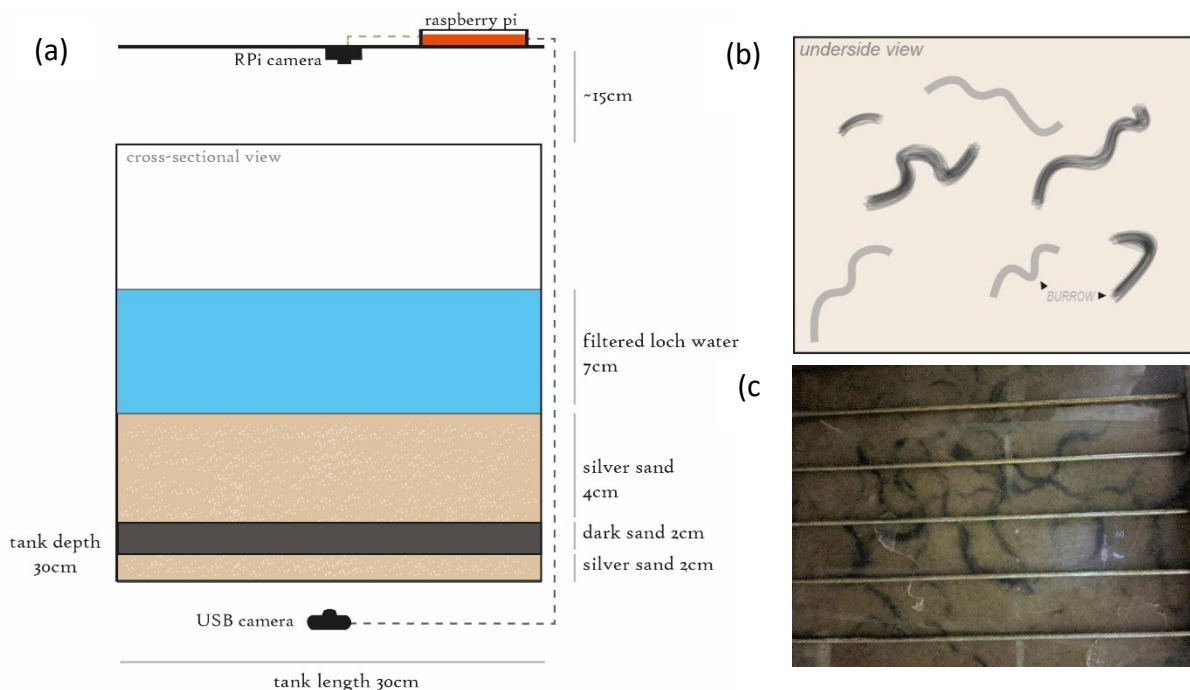


Figure 5.3 (a) Aquaria set up: glass 25L tank, 35 x 30 cm. Substrate depth total, 8cm; water depth, 7cm; 15cm from top of tank to position of the Raspberry Pi. RPi camera positioned above the tank to capture video images of the movement of larvae above the sediment, during both day and night. USB camera positioned below the tank to capture still images of the burrowing patterns, as depicted in (b) and an example image provided by John Hume (c).

The underside of each tank was photographed every two days to check for movement patterns in the form of the darker burrows. Following one week from entering the observational tanks, no emergence of larvae at night or day was detected using the night vision cameras and very little burrowing movement from underneath was observed. Images of the underside of each tank revealed evidence of some larvae movement, with up to six burrows established in tank 3 (Figure 5.4, Table 5.1).



Figure 5.4 Tank set up and underside image showing burrowing experiments to monitor movement of lamprey larvae in experimental tank units.

Table 5.1 Number of identifiable burrows from the underside of the tank during burrowing experiment to determine general movement patterns of lamprey larvae in experimental tanks.

Tank	Number of identifiable burrows			
	29/04/2021	31/04/2021	02/04/2021	05/04/2021
1	0	1	1	2
2	0	0	1	1
3	0	3	4	6
4	0	0	0	1
5	0	0	1	1
6	0	2	2	3
7	0	1	1	2
8	0	0	0	3

Learnings from these observational experiments was that the movement of lamprey larvae in aquaria tanks is infrequent over a 7-day period and that larvae are relatively sessile. The burrows were inconsistent and broken in places, suggesting that it was difficult to use sand colour change to record burrowing behaviour reliably. There was no emergence of lamprey larvae from sediment detected in the 7-day period, which suggests that either the larvae remained buried or that the movement did not trigger the software thereby not recording the emergence. We cannot be certain whether the lamprey larvae did not emerge from the sediment or whether, the motion detection settings in Motion Eye OS were not set correctly to pick up larvae movement. The lack of movement from larvae could suggest that static experimental conditions could result in the larvae remaining burrowed (Dawson, Quintella and Almeida, 2015). To address this, a continuous recording was set up during the experiment to determine whether the lamprey larvae were moving, and the software was not picking it up. However, unfortunately the software had an issue and after one recording (five minutes) stopped the recording. Therefore, it was decided for any future experiments to swap software back to RPi IOS to allow customisation of the filming.

During experiments, when movement occurred it was during the night, however the rate of emergence was less than anticipated based on the literature. Emergence is predicated to aid in downstream migration during darkness to avoid predation (Pavlov *et al.*, 2014), therefore due to the static nature of the experimental units, there may have been a lack of signals to promote emergence. Additionally, the movement from holding tanks to experimental units may have encouraged larvae to remain burrowed for a longer duration due to the disruptive handling and environment.

5.3.4 Preliminary experiment 2: Documenting behavioural response to sediment depth and sediment grain size

The central question of these experiments was to determine whether choice exists for lamprey larvae in an experimental set up between two habitats of different quality with regards to sediment depth and sediment grain size. If the larvae are presented with two habitat options of different quality, will larvae move to the more desirable habitat. For the depth profile experiment, a deeper sediment habitat was considered more desirable to allow complete submersion and protection (Quintella *et al.*, 2007). For the sediment grain size experiment, a finer grain size was considered more desirable to allow construction of burrows

(Docker, 2015; Sugiyama and Goto, 2002). Therefore, it was predicted that if placed in a shallow depth of sediment, larvae will move to the deeper habitat patch during darkness.

To determine whether larvae exhibit a choice to move from an undesirable habitat, a choice experiment was set up with two distinct habitats. Patch 1 consisted of a lower volume of sand, equating to 5cm depth, whereas Patch 2 consisted of sediment volume at 2.5 cm depth (Fig.5.5). A total of eight lamprey larvae were placed in individual tanks and monitored for six days. The depth profile place preference tests revealed seven of the eight larvae moving from Patch 2 (shallow) to Patch 1 (deep). Results are presented in the supplementary material.

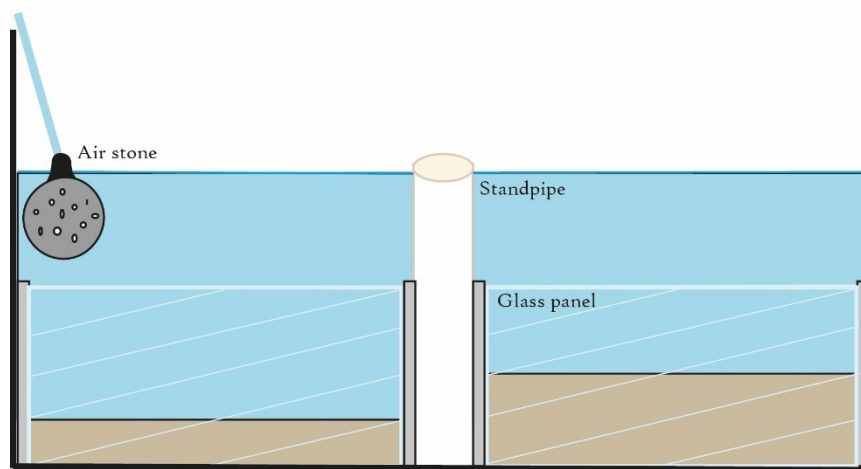


Figure 5.5 Depth profile place preference set up. Patch 1 on the right consisted of 2.5 kg of sand, equating to 5cm depth, whereas Patch 2 on the left, consisted of 1.25kg at 2.5 cm depth.

The aim of the second choice experiment was to monitor whether lamprey larvae exhibit an avoidance response to sediment grain size, when two habitats of different sediment grain size are present. As above, eight larvae were placed into individual tanks with two partially adjoining patches of the same sediment depth. The patches consisted of Patch 1 'fine' sand (<250 μm) and Patch 2 'coarse' sand (1-2 mm). There was no detectable behavioural response to these choice tests, in which larvae were initially forced into an 'undesirable' patch, defined by the coarse substrate (1-2 mm grain size). Following 48 hours of choice between the coarse and fine substrate, all larvae remained in the patch in which they burrowed. After seven days following a choice between coarse and fine substrate, all larvae remained in the patch in which they burrowed.

5.3.5 Preliminary experiment 3: Avoidance behaviour of Microplastic Polyvinyl chloride (PVC)

As we had evidence from the depth profile experiments that lamprey larvae do move if the habitat is deemed undesirable, avoidance experiments with polyvinyl chloride (PVC, 0.5% w/w) were designed to assess whether microplastics elicit an avoidance response. PVC was selected as the polymer type because it is the second most common polymer type found in sediments in which lamprey larvae reside (Chapter 3, Figure 3.2) and has been shown to exhibit high toxicity to freshwater organisms, due to its higher additive chemical content (Capolupo *et al.*, 2020; Rendell-Bhatti *et al.*, 2021). The aim here was to observe whether *Lampetra* larvae exhibit an avoidance behaviour when exposed to sediment spiked with microplastics. The hypothesis was that larvae will preferentially burrowing in 'clean' sand (control) opposed to contaminated (synthetic polymer) sand in a choice experiment with equal availability of distinct sediment types. If larvae do not avoid contaminated patches and remain burrowed in the microplastic patch no response would be recorded.

The test principle was to assess whether organisms distribute at random between uncontaminated and contaminated sediment, or whether they avoid contaminated sediment. The assays were performed under a dual-choice design, where half of each static tank was filled with either aquaria sand or microplastic spiked aquaria sand (Fig. 5.6). In each replicate tank, one lamprey larva was placed in either the control patch or the microplastic spiked patch. This method was undertaken to control for initial patch selection, due to the response of larval lamprey to burrow into sediment (Rovainen, 1980).

This exposure was set up to observe the behavioural response of lamprey larva to a short term (seven days) exposure of a choice experiment, in which an individual animal has a choice between two distinct patches in a 289 x 338 mm aquaria tank. Each patch (289mm x 169mm) is composed of 150-250 µm fine natural aquaria sand, with the addition of 0.5% (w/w) polyvinyl chloride particles (PVC; 100-150µm), density 1.38 g/cm³) to one patch. The two patches were separated by removable glass partitions. Each sediment patch was filled with 1.8kg sand, to approximately a 5cm depth with 7cm loch water to larvae allow movement between patches via emergence. A total of 16 tanks, each with two randomly placed distinct patches, were run simultaneously, equating to 16 individual larvae in total and eight individuals per treatment (Fig.5.7). Each larva was released into the corresponding patch and burrowing time was recorded. Throughout the experiment, larvae emergence behaviour and

movement (emergence rate and re-establishment of burrows) was monitored via continuous interval recording from an aerial view using a Raspberry Pi interface (*LamPi*). Daily location of larvae was recorded by identifying larvae burrows manually via images of the underneath the tank. Following the seven-day exposure larvae were dispatched via MS222 and frozen at -80°C.

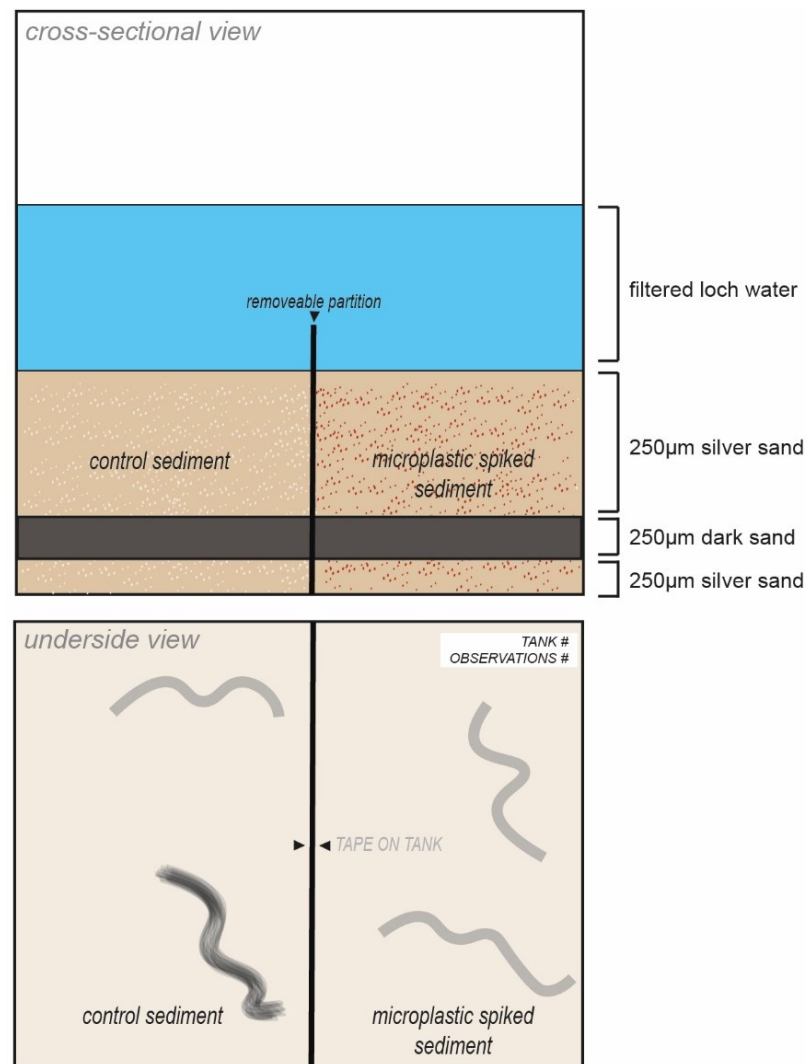


Figure 5.6 Schematic diagram of microplastic exposure observational tanks, showing a cross-sectional and underside view of the aquaria. Removeable partition separates sediment types and can be visualised by tape on the underside of tank. Black lines on the underside view represent the burrowing patterns of larvae. Control sediment; 250-micron sand. Treatment; microplastic spiked 250-micron sand at environmentally realistic concentrations.

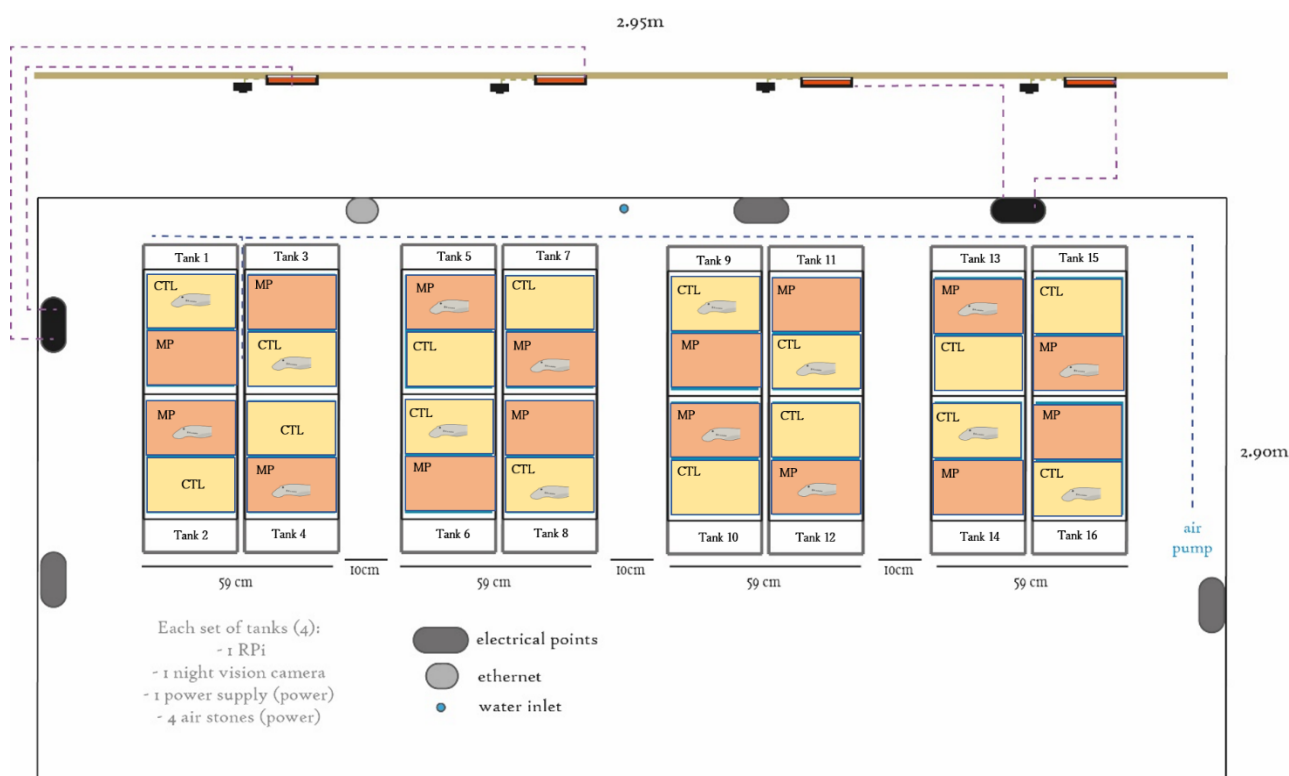


Figure 5.7 Schematic of experimental design for place preference tests with microplastic particles (0.5% (w/w) polyvinyl chloride particles (PVC; 100-150 μ m), density 1.38 g/cm³). Orange patches indicate microplastic (MP) patches and yellow patches indicate control (CTL) patches. Lamprey head indicates which patch each larva was placed into at the start of the place preference test.

The results of the place preference test with microplastic exposure (PVC, 0.5% w/w) indicated no avoidance response to microplastic contamination within a patch. A total of three individuals placed into control patches had moved into the microplastic patch after seven days, and all remaining larva remained burrowed in the patch they were placed in (Table 5.2). Furthermore, there was no significant interaction between burrowing time and substrate type (microplastic spike and control, $F_{1,14}=2.5466$, $p=0.1329$) on entry to the tank. Larvae burrowing performance was also not explained by larvae weight ($F_{1,14}=0.0259$, $p=0.8744$) or length ($F_{1,14}=0.073$, $p=0.7909$).

Table 5.2 Results from the microplastic place preference tests including lamprey morphometrics, start and end patch, burrowing time into patch and patch location after 24-168 hours.

Tank	Larva length (mm)	Larva weight (g)	Start patch	End patch	Burrowing time (Seconds)	24hr patch	48hr patch	72hr patch	168hr patch
1	100.00	1.57	C	MP	121.50	C	C	C	MP
2	140.00	3.78	MP	MP	27.60	MP	MP	MP	MP
3	130.00	3.03	C	MP	10.20	C	C	C	MP
4	145.00	4.44	MP	MP	235.00	MP	MP	MP	MP
5	130.00	3.39	C	C	8.00	C	C	C	C
6	110.00	2.16	MP	MP	80.00	MP	MP	MP	MP
7	130.00	3.33	MP	MP	48.00	MP	MP	MP	MP
8	130.00	2.74	C	C	11.00	C	C	C	C
9	120.00	2.69	C	MP	64.00	C	C	C	MP
10	140.00	3.48	MP	MP	76.00	MP	MP	MP	MP
11	130.00	2.86	C	C	7.30	C	C	C	C
12	135.00	3.78	MP	MP	46.70	MP	MP	MP	MP
13	140.00	4.00	MP	MP	31.00	MP	MP	MP	MP
14	130.00	3.16	C	C	15.00	C	C	C	C
15	125.00	2.97	MP	MP	13.00	MP	MP	MP	MP
16	140.00	3.08	C	C	75.00	C	C	C	C

5.4 Material and Methods: place preference tests

In response to the preliminary experiments, the following experiment was designed to document whether exposure to microplastic contamination leads to a change in behaviour, using the reliable positive control of burrowing behaviour in lamprey larvae. The experiments had two aims:

- 1) identify whether lamprey larvae respond in their burrowing behaviour to introduction to sediment spiked with tyre wear particles
- 2) identify whether following 14 days of exposure in sediment spiked with tyre wear particles, lamprey larvae exhibit differences in their burrowing behaviour in a place preference test

The first aim will allow us to determine whether lamprey larvae respond differently in their burrowing behaviour to microplastic contaminated sediment. This includes both the number of burrowing attempts and the time taken to burrow into contaminated sediment when compared to the control sediment with no contamination.

The second aim will allow us to determine whether exposure to tyre wear particles for 14-days in the exposure tanks elicits a behavioural response in larva when presented with a place preference test. Based on previous literature this could either be more erratic locomotion and faster completion of the place preference test (reference) or slower reaction times and therefor completion of the test.

Therefore, the central question to this chapter was: Does prior exposure to microplastic contamination at environmentally relevant concentrations (0.5% w/w) for 14 days, alter behavioural performance of larval lamprey (*Lampetra planeri*), measured as time to burrow and burrowing response in a place preference test?

5.4.1 Lamprey larvae collection, husbandry and aquaria set-up

Brook lamprey larvae (*Lampetra*, n=114, 80-110 mm body length) were collected from the wild in September 2021 by electrofishing from the Endrick Water (Glasgow, Scotland). This site and sampling methods were as per *section 5.2.1*. Lamprey were transported back to the University of Glasgow's Scottish Centre for Ecology and Natural Environment (SCENE) in river water and sediment and held in a flow-through holding tank (200 Litre) with natural river

sediment for four days. This was undertaken to check health status of the animals by confirming that no mortality or other signs of ill health or disease were observed within the larvae during the settling period.

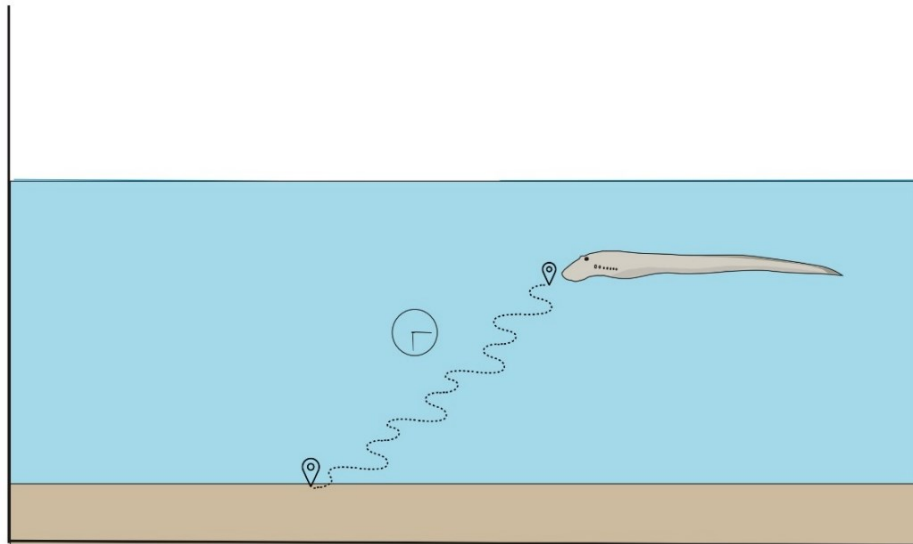


Figure 5.8 Tank set up to recording burrowing time of larval lamprey in a simple burrowing experiment.

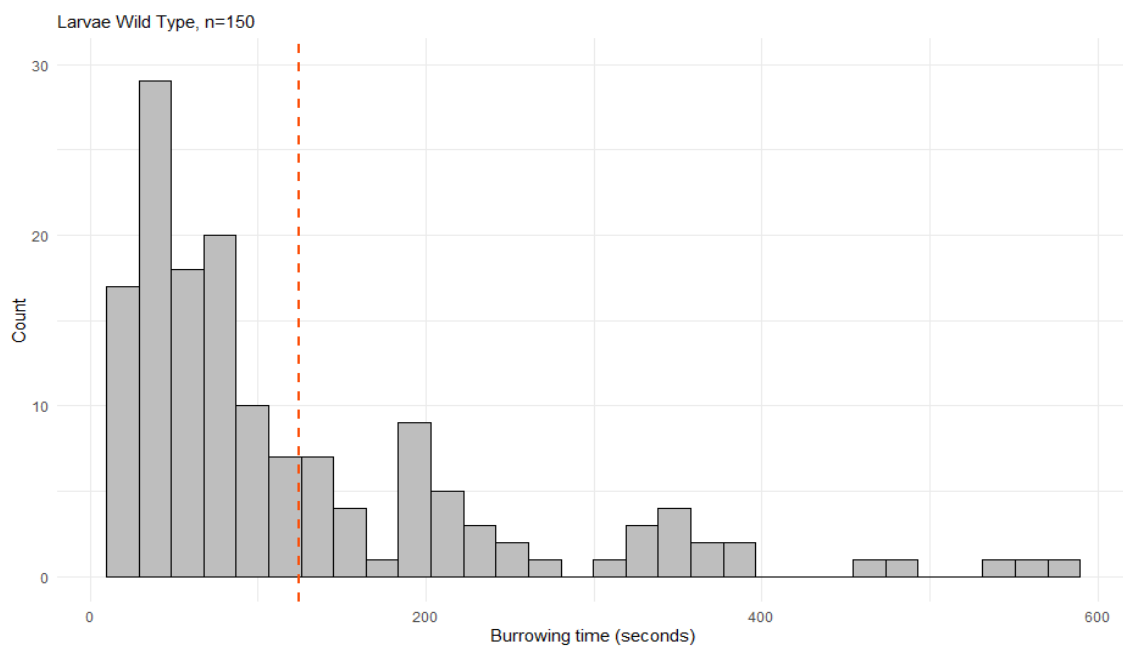


Figure 5.9 Distribution of total burrowing time of larval lamprey across wild type population (n=150). Red line indicates the mean of 117.8 seconds.

5.4.2 Identifying a positive control: Burrowing behaviour

The preliminary experiments identified larva burrowing behaviour to be consistent and strong, therefore it was decided that this could reliably be used as a positive control or endpoint for the microplastic experiments. Burrowing time here is defined as the time taken (seconds) between larvae being released into the water to complete submersion in sediment (Fig.5.8). The burrowing time of 150 lamprey larvae were recorded burrowing into clean sediment using a set-up as depicted in Figure 5.8. The mean time to find and burrow in the sediment, was 117.8 seconds within the wild-type population (n=150; Fig.5.9). The following experiments were designed with this acting as a benchmark and positive control for the behaviours observed.

5.4.3 Exposure material: Tyre Wear Particles

Recycled end of life tyres in the form of fine rubber crumb (<1 mm) (Tyre Wear Particles, TWP) were purchased (Tread X tyres) and dry sieved through a series of metal mesh sizes; 500 µm, 250 µm, 125 µm and 90 µm, rotating at 4000 rpm. As the TWP used were recycled end of life tyres, they were therefore considered to potentially include road associated contaminants. Each size fraction of TWP was weighed, and the 125-250 µm size class was chosen for the exposure experiments, to match the size of the sediment particles used in the tanks (150-250 µm). A concentration of 0.5% w/w, reflecting significant plastic contamination in the environment, was chosen for the tyre wear exposures (Luo *et al.*, 2021). A total of 100g 125-250µm TWP was hand-mixed mixed with 20kg damp aquaria sediment until an even spread was reached. Loch water was added to the tanks under a slow flow, as not to disturb the heterogeneity of the TWP.

5.4.4 Exposure tanks and burrowing performance into contaminated sediment

Following the four-day settling period, larvae were individually removed from the holding tank using a hand net and transferred to the adjacent exposure tanks one at a time. Lamprey were housed in four static 80 litres tanks ($n=28$ per tank, $n=57$ per treatment) with 20kg fine silver aquaria sand (~6cm depth) and 30 litres aerated loch water (~13cm depth, ~18°C, pH 6.5-7). Both male and female larvae were used in the exposure and ranged from 80-130 mm in length. Time to complete burrowing (complete body submersion in sediment) and burrowing rejection count were recorded on introduction of a sub-sample of 40 larvae from each treatment to the exposure tanks. Burrowing rejection count is defined as the number of failed attempts to burrow in the sediment, this includes contacting the sediment in a head-down movement. All larvae remained in the exposure tanks for a total of 14 days for acclimation prior to place preference tests. Larvae were kept on a 16:8 light/dark photoperiod at 18°C. Temperature, pH, oxygen, nitrite (NO_3^-) and total ammonia ($\text{NH}_3/\text{NH}_4^+$) were monitored every three days (Fig.5.10).

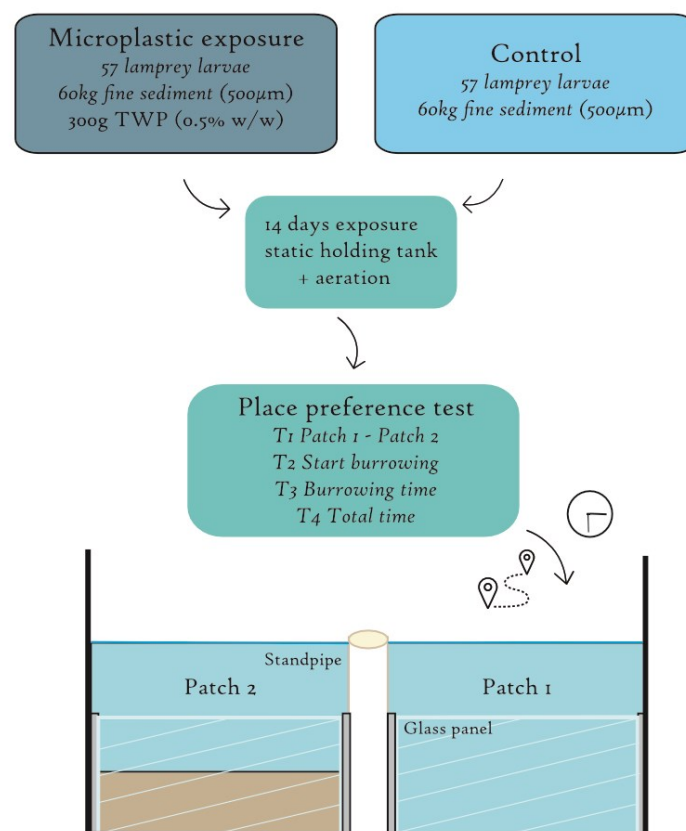


Figure 5.10 Experimental design for place preference test including schematic set up design. Two distinct patches, patch 1 in which the lamprey is introduced with no sediment and patch 2 with sediment. Patches were separated by a glass panel with space for the lamprey to swim between patches.

5.4.5 Place preference tests: Behavioural response following microplastic exposure

Following 14 days of exposure, larvae were removed from the exposure tanks via a hand net and transferred in groups of four, to the place preference tanks. Four adjacent tanks (289 x 338 mm), each with two distinct partially adjoining patches (289mm x 169mm) were used in the place preference tests. A glass partition partially separated each patch (289mm x 169mm, as in Fig 5.10). Access to the sediment patch via 60mm space above the glass panel to allow movement between patches. Patch 2 was composed of 150-250 μm fine natural aquaria sand (Fig. 5.10; 5.11). Avoidance studies, using dual choice design, often place the animal on the border line separating the two patch types to ensure the starting position does not influence the experiment outcome (Ryder *et al.*, 2004; Aldaya *et al.*, 2006; Antunes *et al.*, 2007; Marques *et al.*, 2009; Pereira *et al.*, 2009; Szczybelski *et al.*, 2018). Therefore, one larva per tank was placed in the centre of the tank and its behaviour was filmed via the user interface LamPi (Fig 5.2). The observer left the experimental room for the duration of the test. Once all larvae had burrowed, larvae were removed from the sediment, dispatched under schedule 1 methods and frozen at -70°C . This was repeated for all tanks, alternating which tanks larvae

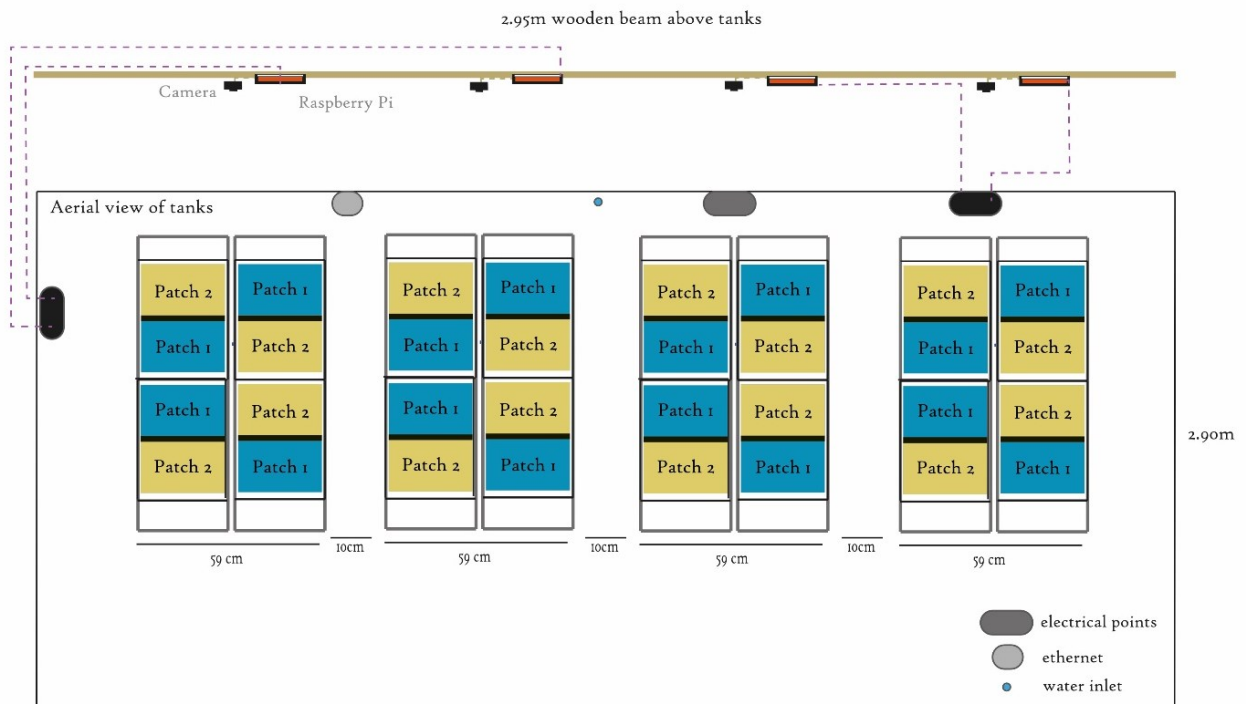


Figure 5.11 Place preference test experimental set up from an aerial view. Each Raspberry Pi monitors four adjacent tanks (289 x 338 mm) tanks each with two distinct partially adjoining patches (289mm x 169mm): Patch 1 loch water only and Patch 2 with sediment. Each patch separated by a glass panel as depicted in fig. 5.1. Access to the sediment patch via 60mm space above the glass panel to allow movement between patches.

were retrieved from for the place preference tests. Preliminary research (Fig.5.8) indicates that the burrowing behaviour of larval lamprey is strong, with the mean time to find and burrow sediment, being 117.8 seconds within the wild-type population (n=150). Therefore, the total time (seconds) for lamprey to complete the place preference test, i.e. burrow, with complete submersion into sediment, was recorded from the videos. The timing, in seconds, of the behaviours were broken down into four stages; (T1) time to reach Patch 2 (sediment) from Patch 1 (no sediment), (T2) time to start burrowing once in Patch 2, time to start and complete burrowing (T3) and total time from start to end of place preference test (T4) (Fig.5.12)

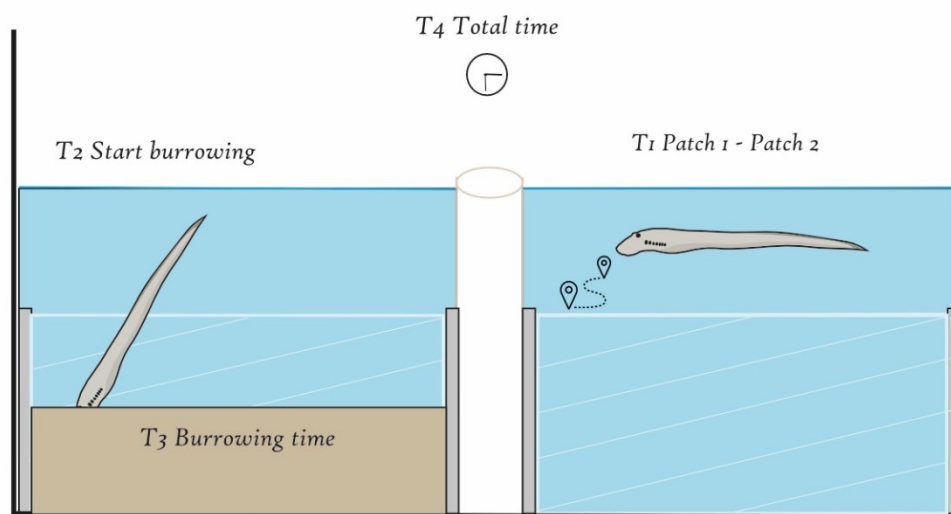


Figure 5.12 Stages of the place preference test. The timing of the behaviours was broken down into four stages; (T1) time to reach Patch 2 (sediment) from Patch 1 (no sediment), (T2) time to start burrowing once in patch 2, time to start and complete burrowing (T3) and total time from start to end of place preference test (T4).

5.4.6 Statistical and Video Analysis

Video footage from the place preference tests were used to record individual lamprey behaviour. Specifically, the time in seconds, to reach patch 2 (sediment) from patch 1 (no sediment) (T1), time to start burrowing in patch 2 (T2) and time to complete burrowing (T3), which resulted in a total time to complete the place preference test (T4). All data analyses were carried out on R version 4.1.2 (01 November 2021). To describe changes in burrowing behaviour into exposure tanks and within the place preference test, Gamma generalised linear models (Gamma GLM) were adopted. Gamma models were employed where data were not normally distributed upon analysis of model's distribution of the residuals. Burrowing times were logged for density plots to compare treatments. EthoVision® XT 15 software (Noldus information technology, Wageningen, Netherlands) is an integrated system for automatic recording of activity, movement, and interactions of animals (Noldus, Spink and Tegelenbosch, 2001). As Ethovision can be configured to track a range of small laboratory animals and has been used in a variety of ecotoxicology studies, we determined this software to be the most suitable for our setup and species (Varshney *et al.*, 2022, 2023; Chang *et al.*, 2023; Rejo *et al.*, 2023). The video recordings from the place preference tests were analysed using EthoVision to assess the distance moved, velocity and speed and duration of time spent in zones.

5.5 Results

5.5.1 Burrowing performance into 14-day exposure tanks

Burrowing time for both control and microplastic tanks ranged between 5.75 and 217.60 seconds (n=80, Fig.5.13). The mean time taken to burrow for control larvae (n=40, Fig.5.13b) was 45.59 ± 50.59 seconds, and the mean for larvae burrowing in the microplastic tank was 62.40 ± 44.93 seconds (n=40, Fig.5.13b). Although there was a trend for longer time to burrowing in the microplastics treatment, no significant effect of microplastic presence on burrowing time was found, controlling for exposure tank and larvae length (Gamma GLM, $t_{79}=1.85$, $p=0.068$). The number of times burrowing was rejected was significantly different between the control and the microplastic exposure tanks, controlling for exposure tank and larvae length (Fig.5.14; Gamma GLM, $t_{79}=2.86$, $p=0.0055$). The mean number of rejections was 4.5 for control and 9.75 for microplastic exposed larvae. Temperature (17.3-18.2), pH (6.5-7),

oxygen (97-98.8), nitrite (NO_3^-) and total ammonia ($\text{NH}_3/\text{NH}_4^+$) remained stable in the exposure tanks.

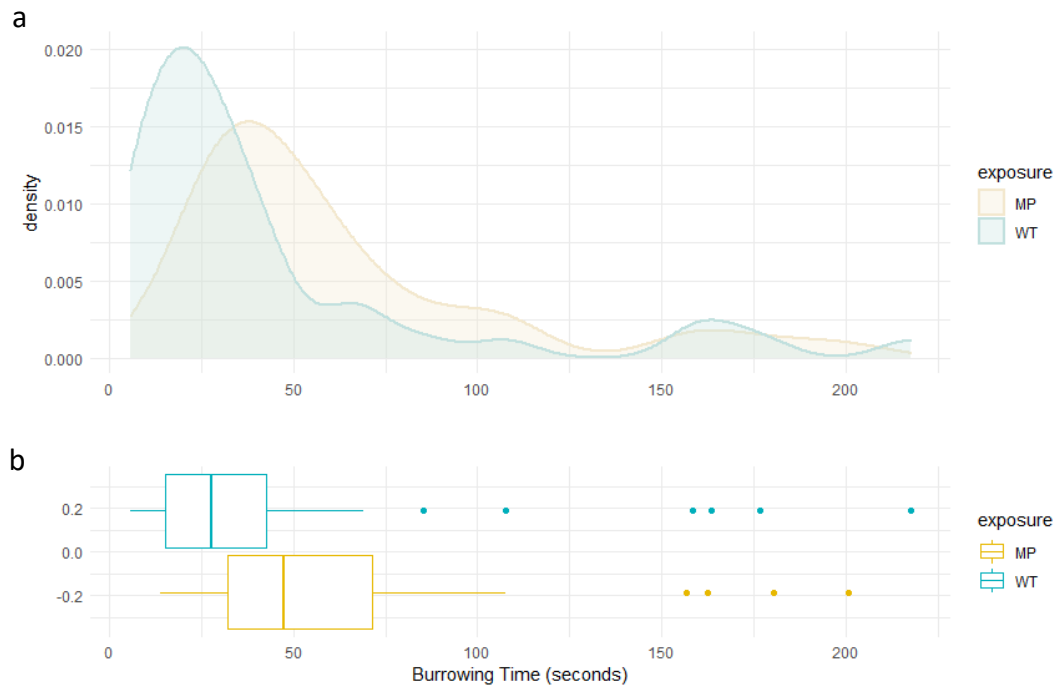


Figure 5.13 (a) Density plot and (b) box plot showing the differences in burrowing times, in seconds, of lamprey larvae into either control (WT, blue) aquaria sand or 0.5% tyre wear particles (MP, yellow) aquarium sand.

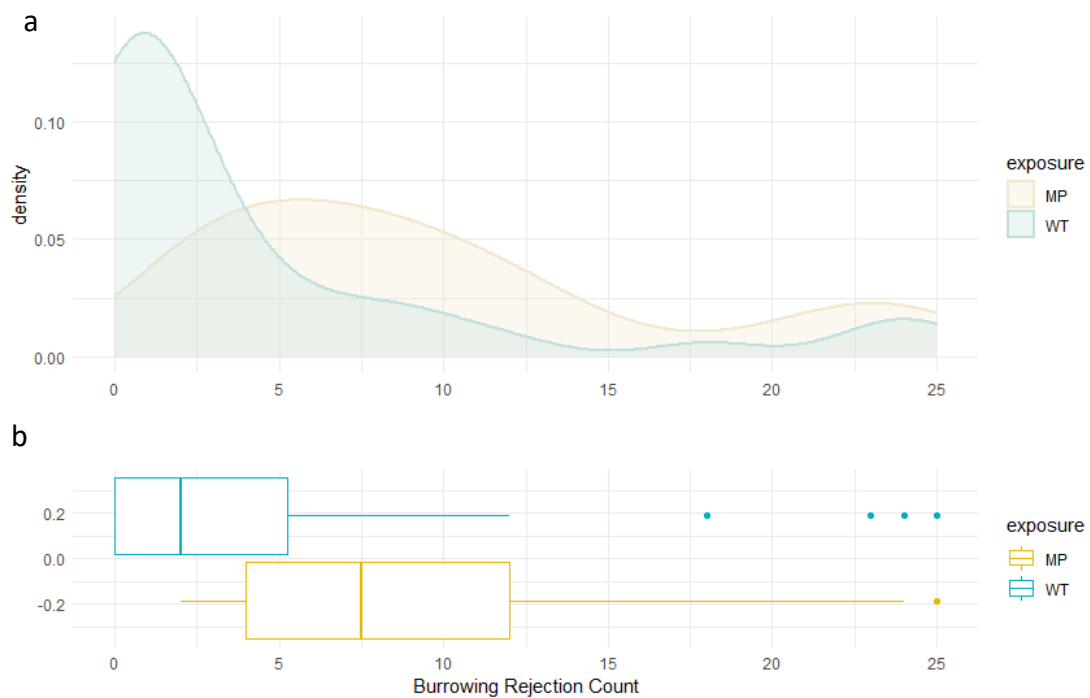


Figure 5.14 (a) Density plot and (b) box plot to visualise the burrowing rejection count of lamprey larvae into either control (WT) aquaria sand or 0.5% tyre wear particles (MP) aquarium sand.

5.5.2 Place preference tests following tyre wear exposure

Following 14-days in exposure tanks, lamprey larvae from each treatment were transferred to a place preference test, as described in section 5.2.4 (Fig.5.10; Fig.5.15). The average time for lamprey larvae exposed to tyre wear particles to complete all stages of the place preference test was faster than that of the control larvae, however there was no significant effect of exposure conditions on total time for lamprey to complete place preference test (Gamma GLM, $t_{113}=1.28$, $p>0.05$; Table 5.3, T4). No observable difference was found when burrowing behaviours were broken down into components: time to reach Patch 2 (Gamma GLM, $t_{113}=1.15$, $p>0.05$; Table 5.3, T1), time to start burrowing (Gamma GLM, $t_{113}=0.08$, $p>0.05$; Table 5.3, T2) and time to complete burrowing (Gamma GLM, $t_{113}=2.31$, $p=0.02$; Table 5.3, T3).

Table 5.3 Time for lamprey larvae exposed to either control (WT) treatment or tyre wear particles (MP) for 14 days to complete each behaviour in the place preference test.

Exposure	Behaviour (average time in seconds)			
	Reach patch 2 (T1)	Start burrowing once in P2 (T2)	Burrowing (T3)	Complete place preference test (T4)
Control (n=57)	130.46 ± 178.77	11.49 ± 38.70	21.19 ± 12.93	163.14 ± 180.59
Tyre Wear (n=57)	94.95 ± 140.52	9.06 ± 23.41	18.97 ± 11.41	122.98 ± 140.26

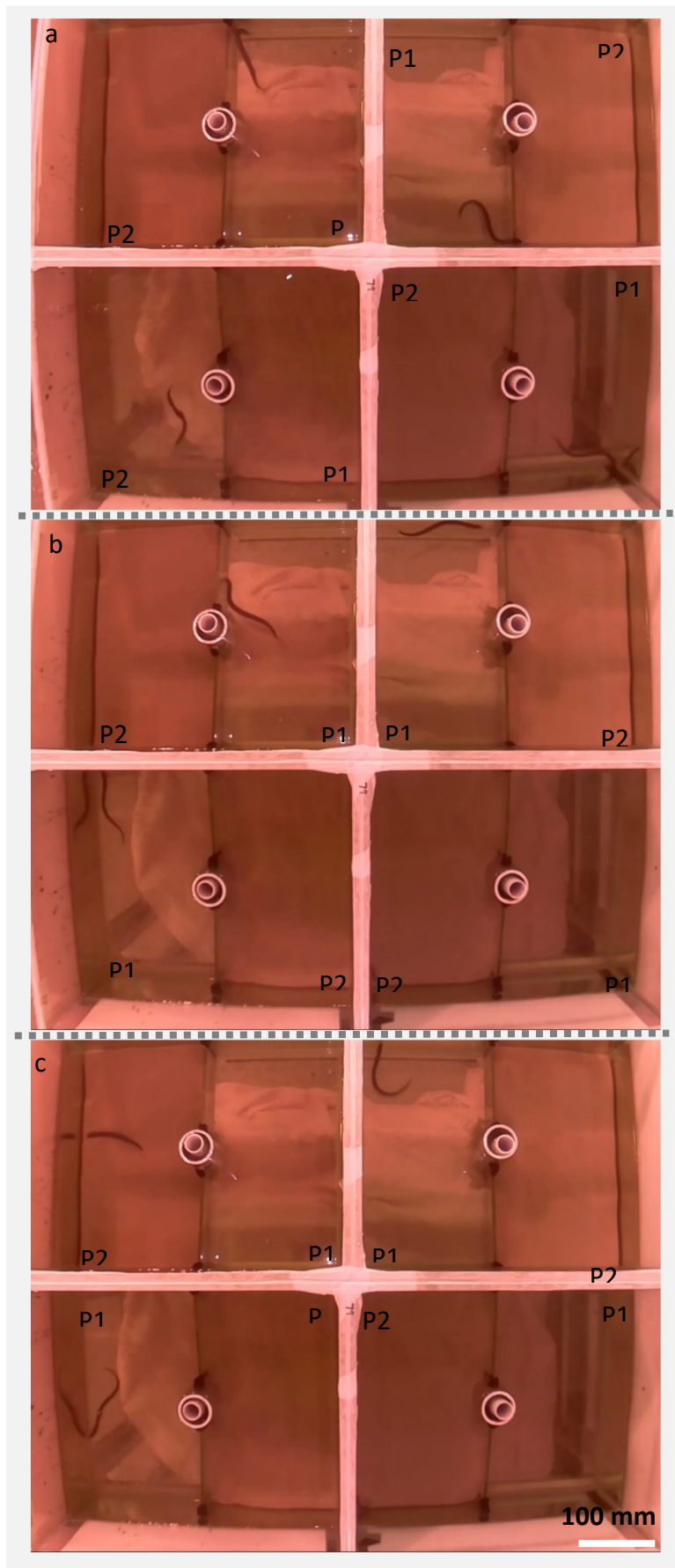


Figure 5.15 Place preference test set up from aerial Raspberry Pi view. Four separate tanks, each with Patch 1 (P1, no sediment) and Patch 2 (P2, sediment) and one larva in each tank. Space for lamprey to move between patches available above glass panel (60mm). Panel a-c show a series of photographs from one set of four tanks through time. (a) start of test with all larvae in patch 1 (T1) (b) 55 seconds, three larvae remain in Patch 1, bottom right tank larva has burrowed and completed place preference test (T4). (c) 76 seconds, two larvae remain in patch 1, top left tank larva is burrowing into Patch 2 (T3). Scale bar = 100mm.

5.5.3 Video analysis: Assessing locomotor activity from place preference tests

The differences in timing to complete the place preference test between control and treatment groups were not statistically different, however it is important to consider that timings of each stage do not provide high resolution data on the specifics of lamprey behaviour. For example, the timings cannot provide information on the fine-scale locomotor activity of the larvae, such as speed and total distance moved which could indicate hyperactivity. Therefore, a random subsample of videos was run through the software Ethovision to determine whether the movement of lamprey exposed to tyre wear particles was different from that of the control group (Fig.5.16).

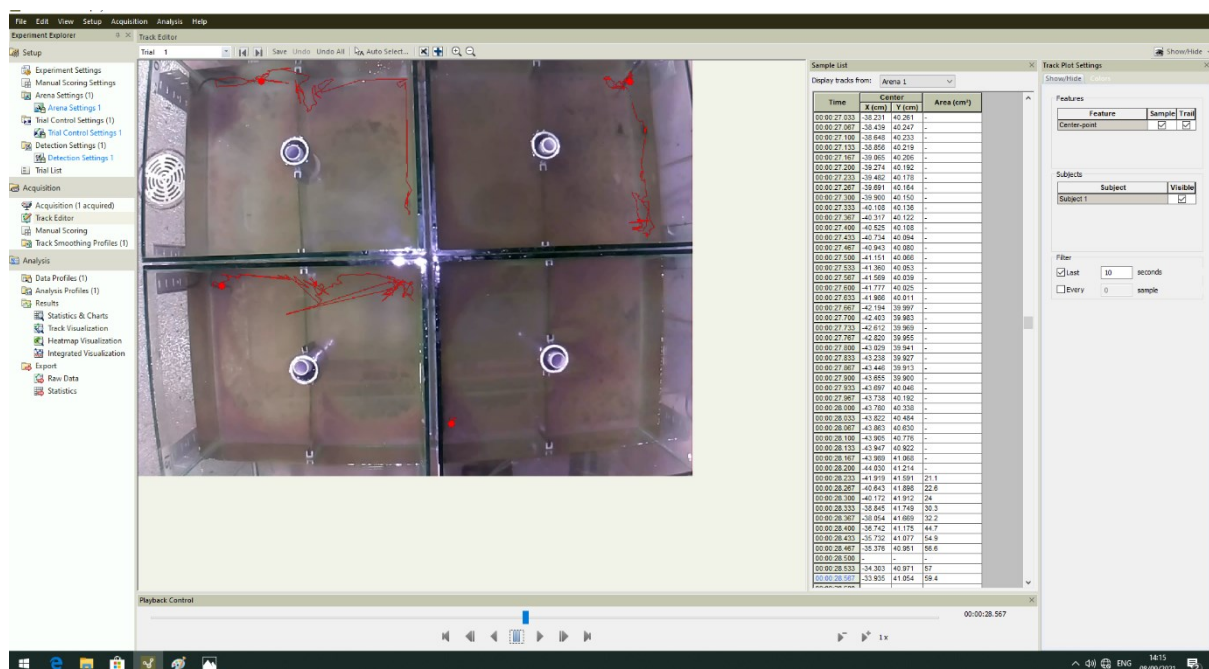


Figure 5.16 Tracking software Ethovision showing experimental set-up with four individual tanks and lamprey. Red lines highlight the tracking which Ethovision has determined for each lamprey based on movement. Table to the right provides a timestamp for the area, however due to the hypervariability of the lamprey this was inconsistent.

The videos were analysed to collect data on the speed, distance moved, and time spent in patches for both control and microplastic exposed larvae. The resulting tracks were extremely erratic and not consistent with the lamprey movement in the video (Fig.5.16). The software estimates the centre of mass of the pixels occupied for the animal being tracked, which for a fish with an elongated, cylindrical body form, such as a lamprey, is hypervariable and often not even on the animal itself. To overcome the issues of lamprey geometry and movement, it was necessary to manually edit the tracking for each time stamp. This is a highly time intensive

process and despite efforts it was not possible to process the number of videos needed to draw a reliable data set. Unfortunately, the tracking acquired through Ethovision was not successful because traditional software applications are designed for model fish. Following this other traditional fish tracking software such including idtracker (Pérez-Escudero *et al.*, 2014) and ToxTrac (Rodriguez *et al.*, 2018) were assessed for the suitability of analysis of the videos. However, each of these tracking software's presented similar same issues with the geometry of the lamprey creating problems for analysis. Moving forward to investigate other options, the open-source tracking system TRex was used to test tracking the lamprey movement (Couzin and Walter, 2021). To increase tracking success, each video was cropped and masked using OpenCV in Python, to reduce background noise and anything interfering with the tacking (Fig 5.17). Unfortunately, this was not enough to enable the tracking to accurately follow the lamprey. Further problems were encountered with the software, as the tracking picked up areas in which the same lamprey had been previously, possibly from substrate disturbance (Fig 5.17).



Figure 5.17 Trex open-source tracking software with a split lamprey in red and green in the centre. The substrate disturbance from the larva's previous position in the tank was being picked up by the software to identify multiple animals. The black shapes show the mask created in OpenCV is visible to reduce background noise.

After seeking advice from the software's innovators Couzin and Walter (2021), it was possible to fine-tune some parameters, such as preferencing the number of individuals to track and

specifying blob sizes of individuals. However, as with other tracking software's, TRex is parameterised for model animal species, therefore due to the length and movement of lamprey it was not possible to collect fine-scale locomotion data. Despite efforts of custom labelling and training to fine-tune the tracking, larva remained broken into two 'fish' as opposed to one. These issues proved insurmountable, and it was not possible to successfully reconnect the segments that were identified as individual fish or track the larva.

5.6 Discussion

5.6.1 Method development and learnings from preliminary experiments

Little is known about microplastic avoidance or micro-habitat selection in lamprey larvae. The learnings taken from the method development studies conducted in this chapter suggest that lamprey larvae do not avoid polyvinyl chloride microplastics particles. The place preference tests suggest that larvae could in fact move into contaminated patches, however this response could be random and needs to be explored further. Therefore, if larvae are not actively avoiding patches with high microplastic contamination, this could result in increased chance of microplastic ingestion and deleterious effects on individuals (Foley *et al.*, 2018). Furthermore, this is pertinent for the learnings from previous chapters, which suggests ubiquitous uptake of microplastic contamination across different environments. The prevalence of microplastics in freshwater environments (Zandaryaa, 2021) alongside a lack of avoidance behaviour, could result in ecological consequences for lamprey larvae populations (Ma *et al.*, 2020).

5.6.2 Exposure tank burrowing behaviour

Whilst this study suggested no significant differences between the time taken for larvae to burrow into control and tyre wear exposure tanks, there was a significant increase in the number of times lamprey larvae attempted to burrow into tyre wear- particle-contaminated sediment. Hesitation or repeated attempts at burrowing is evident for lamprey larvae exposed to tyre wear particles in sediment. Therefore, although larvae may not avoid microplastic contamination entirely, individuals may have spent a longer time investigating the microplastic environment before deciding to burrow. This might have occurred due to detection of novel physical and chemical characteristics of tyre wear particles, such as synthetic tyre constituents, compared to the uncontaminated sand (Klößner *et al.*, 2020; Beji

et al., 2023). Indeed, other studies have reported benthic fauna, specifically the brittlestar (*Amphiura filiformis*) exhibiting impaired burrowing in response to microplastic contamination (Coppock *et al.*, 2021). However, the actual mechanism behind this behaviour has not yet been determined.

Microplastics can have a negative impact on fish and their behaviour, even when not ingested. For example, adherence of microplastics to larva gills and skin may alter oxygen consumption and ion regulation triggering respiratory stress, thereby influencing behaviour (Abdel-Tawwab *et al.*, 2019; Watts *et al.*, 2016). Furthermore, tyre wear particles may present a physical or chemical risk to the larvae burrowing, leading to an increase in the number of burrowing attempts. Heavy metals and organics within tyre wear particles could have resulted in failed burrow attempts (Klößner *et al.*, 2020). As the tyre wear particles size matched that of the sediment grain size (125-250µm), it is unlikely that the size elicited the increased burrowing attempts. However, microplastics can reduce sediment porosity and as lamprey larvae rely on the texture and porosity of sediment to burrow effectively, this could have resulted in failed burrowing attempts (Green *et al.*, 2016).

5.6.3 Place preference tests

The results from the place preference tests indicate faster locomotion rate at all stages of initiating burrowing behaviour for lamprey larvae exposed to tyre wear particles, compared to the larvae exposed to uncontaminated sediments. This finding aligns with previous research into lamprey larvae response to contaminated sediment (Salmelin *et al.*, 2016; Unrein *et al.* 2016) and akin to these previous studies the differences were not statistically different from the control. In contrast to this finding, previous research on other biota has reported decreased burrowing speed of a bivalve mollusc (*Scrobicularia plana*), and no change in response from a polychaete worm, ragworm (*Hediste diversicolor*) exposed to a range of tyre particle concentrations (Garrard, Spicer and Thompson, 2022).

5.6.4 Study limitations and recommendations for future work

The main data presented in this chapter are a result of an evolution of methods and ideas accumulating to gain an understanding of whether exposure to microplastic contamination has behavioural implications with regards to microplastic avoidance in lamprey larvae. The preliminary studies identified burrowing behaviour as the most suitable positive control

which could be reliably recorded. Burrowing response is also identified the most characteristic behavioural pattern of lamprey larvae (Quintella *et al.*, 2007). Burrowing rate is deemed as a suitable end point for monitoring the behavioural response to tyre wear particles (Garrard, Spicer and Thompson, 2022). Therefore, burrowing responses within a place preference test were adopted as a behavioural endpoint to assess lamprey larvae following microplastic exposure to tyre wear particles. The burrowing behaviour of lamprey larvae into the exposure tanks was also monitored.

Individual-level variation, such as personality traits, may influence responses to microplastic contamination, as seen in gilt-head seabream (Nananga *et al.*, 2021). Animal personalities, observed as consistent individual differences in behaviour, have been demonstrated across fish species. Recent research into animal personalities highlights the importance of individual variation in ecological and environmental challenges (Sih, Bell & Johnson 2004; Reale *et al.*, 2007; Overli *et al.*, 2007; Huntingford *et al.* 2010; Silva *et al.*, 2010; Martins *et al.* 2011; Castanheira *et al.*, 2013a, b; Rey *et al.*, 2013; Herrera *et al.*, 2014). Animal personality traits are categorised into three distinct behavioural profiles, 'proactive (active coping or bold)', 'intermediate' (more flexible individuals), and 'reactive' (passive coping or shy), each showing distinct differences for the same measured variables, such as risk taking and exploration, aggression or feeding (Cerqueira *et al.*, 2016; Rey *et al.* 2013; Castanheira *et al.* 2015). Therefore, a key limitation in this study is that individual personality types are not considered. For example, a movement away from or towards a patch could be down to a more proactive individual, characterised by increased foraging activity and therefore movement towards a novel patch. On the other hand, reactive individuals can be characterised by immobility and avoidance behaviour under aversive events (Castanheira *et al.*, 2015). Without the capacity to assign personality types to individual larvae, it is impossible to disentangle the factors to determine the response rate in the place preference tests.

With regards to the analysis of fine-scale locomotion behaviours of lamprey larvae in the place preference, improvements of the initial experimental set up could have aided the results. For example, using sand with increased contrast and providing higher illumination from at least 2 sides, would eliminate shadows in the substratum would help the software to track each individual larva. Key learnings from this experience are to adopt a physical fix within the experimental set up first, followed by software if needed. By testing the system with the

tracking software prior to running the final experiments and taking care of any issues, such as lighting and substratum contrast, would have aided the results we were looking for. As this was not undertaken here, it was necessary to adopt software fixes which are laborious and time consuming. With regards to using these videos with future software, TRex 2.0 would potentially solve these issues as it has the ability for deep machine learning based segmentation, however this was not available at the time of video analysis.

Behavioural ecology research often requires characterisation of the frequency and timing of movement, to provide a comprehensive understanding of species' behaviour and ecology. Automated methods of monitoring threatened species provide a cost-effective way to track behavioural changes of individuals to emerging contaminants of concern. However, for non-model species such as lamprey it is difficult to collect fine-scale data on locomotion as software is not designed for the hypervariable movement of a lamprey body. Following conversations with other fish behaviour labs (Killen lab, Glasgow University) it was suggested that DeepLabCut could be a suitable application for lamprey, as it can locate and track specific positions on animals after being trained. The open-source software package, DeepLabCut, allows users to train a computational model to track animal postures in videos (Mathis *et al.*, 2018). If more time had been allowed, this is the software which would have investigated further to quantify whether there were significant differences in the fine-scale locomotor activity of the lamprey exposed to tyre wear particles and the control group.

Given the lack of research conducted on behavioural indicators for lamprey larvae, there is a clear gap in our understanding and a need for research that allows for strong inference about how anthropogenic stressors, such as microplastics, could affect habitat selection by lamprey larvae. Here we looked at both avoidance behaviour based on the presence of tyre wear particles, and how exposure to tyre wear particles might influence burrowing behaviour. Despite not observing significant effects, these novel understandings contribute to our wider knowledge on lamprey conservation. Further studies are needed to discern whether the increased locomotion rate observed here is significant. Impaired or altered locomotion behaviour can have a significant impact on both lamprey larvae vulnerability as a prey and through feeding efficiency in patches, thereby influencing survival (Salerno *et al.*, 2020).

5.7 Conclusions

Studying the behavioural toxicity of microplastics on protected organisms can provide a basis for assessing ecological risk and conservation management. Many pollutants in the freshwater environment elicit an integrated stress response in fish, in addition to their toxic effect at the cellular and tissue level (Rudneva, 2013). Documenting behavioural changes is an indication of an immediate and important response to an environmental stressor (Wong and Candolin, 2015). The body of research presented in this chapter developed a means by which to test on a protected species and to detect behavioural changes and preferences in lamprey larvae. This is not well documented across lamprey species; with only two previous studies we are aware of investigating the behavioural effects of contamination on lamprey larvae (Salmelin *et al.*, 2016; Unrein *et al.* 2016) and no previous research we are aware of investigating the effects of microplastics on lamprey. Therefore, extending our knowledge on how to conduct experiments with lamprey larvae is valuable for scientific, ecological and conservation understanding.

The experiments were devised in response to the time and resources available during the pandemic, and as a result do not yield fully comprehensive results. Nevertheless, this chapter contributes to our understanding of lamprey behavioural responses elicited from exposure to tyre wear particles. The work highlights that although no statistical differences were found between those larvae exposed to tyre wear particles and the control group, there could be finer-scale locomotion behaviour which is not documented through simple video analysis. The chapter highlights the need for software which can consider non-model aquatic organisms such as lamprey larvae. With improved machine learning and artificial intelligence embedded into animal tracking software, perhaps analysing locomotion of organisms such as lamprey will be easier in the future.

Chapter Six - Developmental Toxicity of Recycled Tyre Wear Microplastics to Lamprey Larvae *Lampetra planeri*

6.1 Abstract

Whilst research on the occurrence of microplastic in freshwater environments has been increasing in recent years, the consequences of the presence of microplastics for these ecosystems are not elucidated. Particularly, whether the early developmental stages of protected vulnerable species are impacted is a pertinent question when trying to understand the consequences of plastics in our natural environment. Untreated road run-off, is a significant source of tyre wear particles and associated contaminants to the freshwater environment, often exceeding the lethal concentration of some chemicals to aquatic biota. The impacts of tyre wear particle contamination on early developmental life stages are understudied, and yet the consequences of this sensitive period being disrupted, could have important ecological consequences. Tyre wear particles are a substantial proportion of microplastics reported in the aquatic environment and they can leach a range of chemical compounds, which have been shown to elicit toxic effects. Lamprey species in Europe are undergoing population declines, due to a multitude of stressors. To conserve and manage threatened populations, it is essential to first understand the stressors placed upon them. To the best of our knowledge, this is the first study to date to provide a morphological characterisation of the developmental defects that leachates from tyre wear particles elicit in lamprey embryos. Here we use recycled tyre wear particles to generate a microplastic leachate in which embryos were cultured. Preliminary data generated here suggest that exposure to leachates, following 10-day incubation of tyre wear particles at 0.5 % filtered to 65µm, elicits consistent and severe developmental abnormalities in brook lamprey embryos (*L.planeri*). The phenotype observed in the microplastic treatment suggests that compounds in the leachate elicit weakening of the vitelline membrane, causing the vitellus (yolk) to leak out. This study documents developmental abnormalities in larval lamprey which were exposed to leachates of tyre wear particles, demonstrating the potential contribution of this stressor to the population status of this vulnerable species. This work highlights that further research on the ecotoxicological impacts of tyre wear particles is needed to aid our understanding of this complex contaminate.

6.2 Introduction

The early life stages of a developing embryo and larva is the most sensitive stage in the life cycle of fish and invertebrates (von Westernhagen, 1988). This increased vulnerability in sensitivity in early life stages compared to their adult counterparts is suggested to be a result of a multitude of factors, primarily the criticality of developing organs (Mohammed, 2013). Disruption to important developmental stages present potential consequences for recruitment into adult life stages and population level impacts (Downes *et al.*, 2021). Therefore, as aquatic embryos exhibit a higher sensitivity and are often exposed to contaminant from fertilisation it is ecologically important to research these early life stages (Johnson, Carew and Sloman, 2007).

Lamprey, due to their evolutionary significance, have been instrumental in elucidating the origin and development of the vertebrate body plan (Boorman and Shimeld, 2002; Jimenez-Guri and Pujades, 2011; Wotton *et al.*, 2011; Green and Bronner, 2014; Green *et al.*, 2017). Therefore, well-established protocols for gamete fertilisation and embryo incubation are available (Maine *et al.*, 2017; Lampman *et al.*, 2020). Given their high conservation status in many areas, efforts have been directed towards understanding embryo development (Nikitina *et al.*, 2009; Lampman *et al.*, 2016) and mitigating lamprey population declines through the development of embryo and larvae culture methods (Vikstrom, 2002; Meeuwig *et al.*, 2005; Lampman *et al.*, 2016).

Lampetra planeri is a non-parasitic species of lamprey that remains in freshwater habitats after undergoing metamorphosis. The optimal spawning habitat for these lampreys is characterized by fast-flowing water and gravel substrate (Malmqvist, 1983). All lampreys undergo external fertilisation, engaging in the excavation of nest depressions in sediment to deposit their eggs. This provides both shelter and substrates for egg adhesion (Yorke and McMillan, 1979). In their natural environment, the adhesion of lamprey embryos to virtually any available surface offers several advantages. This adhesive quality not only aids in embedding the embryos within the substrates, but also serves to separate individual eggs, thus preventing potential mortality due to fungal growth (Smith and Marsden, 2009). However, along with other aquatic species this external fertilisation process presents vulnerabilities to developing embryos from surrounding aquatic pollutants (Shi *et al.*, 2022).

For example, exposure of microplastics and triclosan to the broadcast spawning bivalve *Tegillarca granosa* leads to suppressed fertilisation success (Lu *et al.*, 2023).

While research into the prevalence of microplastics in freshwater environments has received increased attention in recent years, our understanding of the ecological ramifications of their presence remains incomplete (Zandaryaa, 2021). Notably, an essential aspect in comprehending the impact of plastics on aquatic communities is whether the early developmental stages of protected and vulnerable species are affected. Microplastics have been shown to present ecotoxicological risks for early life stages of several aquatic organisms (Cormier *et al.*, 2021; DeMarco *et al.*, 2022; Lu *et al.*, 2023). Examples include impairment of growth (Park and Kim, 2022), developmental toxicity (Rendell-Bhatti *et al.*, 2021; Paganos *et al.*, 2023) and neurotoxicity (Xiong *et al.*, 2022). Furthermore, many studies highlight the increased sensitivity to microplastics in earlier life stages, compared to juveniles and adults (Prokić *et al.*, 2021).

Untreated road runoff contributes of microplastics to the aquatic environment, specifically Tyre Wear Particles (TWP) and associated contaminants (Knight *et al.*, 2020). In some cases, these pollutants surpass lethal concentrations for certain aquatic biota (Spromberg *et al.*, 2016; McIntyre *et al.*, 2021). There is a scarcity of studies investigating the effects of tyre wear particle contamination on aquatic life, particularly on early developmental life stages and little is known about the potential ecological implications. Research has demonstrated that chemical leachates from TWP can induce toxic effects in freshwater biota (McIntyre *et al.*, 2021; Tian *et al.*, 2021; Chibwe *et al.*, 2022; Wang *et al.*, 2023). Indeed, studies have reported adverse consequences associated with TWP leachates, including inhibition of phytoplankton growth (Page *et al.*, 2022), morphological alterations in zebrafish larvae (Varshney *et al.*, 2022), and an increased incidence of severe embryonic deformities in fathead minnows (Chibwe *et al.*, 2022). The multifaceted impacts of TWPs underscore the urgency of investigating and comprehending their ecological ramifications within aquatic ecosystems, (Delaeter *et al.*, 2022).

The primary objective of this chapter is to investigate and monitor the development of *Lampetra* embryos and larvae when exposed to environmentally relevant tyre wear particles and their leachate. The research design utilises recycled tyre wear particles to generate a microplastic leachate in which the lamprey embryos were then cultured and monitored,

enabling an examination of the developmental consequences of this exposure. The specific aims of this study were to answer the following questions: (1) does toxicity vary with leaching time of tyre wear particles (3-day and 10-day incubation); and (2) does particle size in the leachate exposure effect toxicity (leachate filtered to <0.7 µm and <65 µm).

6.3 Materials and Methods

6.3.1 *Generation of tyre wear particle leachate*

Tyre wear particles were used to obtain leachates. Recycled end-of-life tyres in the form of fine rubber crumb (<1 mm) (TWP) were purchased from Tread X tyres (UK) and dry sieved through a series of metal mesh sizes to collect the desired size fractions. The rubber crumb used to prepare exposure leachate was in the size range of 25-250 µm. Stock TWP leachate was prepared without agitation at a concentration of 0.5% w/v TWP mixed with natural freshwater directly from Loch Lomond. The leachate remained static at room temperature (15°C) on a natural photoperiod for 10 days. The stock solution was filtered 63 µm and stored at 4°C until gametes were available. It is important to note that filtering to 63µm does not remove microplastics <63 µm, nanoplastics or metal(oxide) nanoparticles. This step was completed to gain an understanding of the effects of fine tyre wear particles (<63 µm) on lamprey larvae. This was a preliminary study to determine whether effects can be observed from fine tyre wear particles and future experiments were planned with a more complex design system, including smaller filtrate sizes (Please see Appendix D Figure 2).

The exposure concentration was chosen because has been observed to cause a negative effect in fathead minnow embryos (Wik and Dave, 2005; Kolomijeca *et al.*, 2020; Chibwe *et al.*, 2021) and is close to environmentally relevant concentrations (Luo *et al.*, 2021). Water parameters including temperature and pH were monitored at the start and end of the leaching process. Control loch water was treated in parallel to the TWP leachate without the addition of the TWP.

Heavy metals have been shown to affect various developmental process in fish, leading to morphological abnormalities, delay in hatching and reduced viability (Jezierska et al., 2009). Tyre wear particles are a known as a key source of zinc to the environment (Councell et al., 2004). Therefore, we analysed the levels of trace zinc in each treatment by ICP-MS. Acidified aqueous solutions were analysed directly on the Perkin Elmer ICP-MS using standard conditions. However, unfortunately due to COVID restrictions these were analysed two years after collection and equipment failure disrupted storage conditions of the samples. This lead to the samples being invalid for interpretation.

6.3.2 Animal husbandry and embryo exposure

Adult lamprey (*Lampetra*; one male, one female) were collected using lamprey traps situated in the Endrick Water (Glasgow, Scotland). Lamprey were transported back to the University of Glasgow's Scottish Centre for Ecology and Natural Environment in river water and held in a flow-through holding tank. On the day of capture, individuals were dispatched under licence using a Schedule 1 method allowed within the Animals (Scientific Procedures) Act 1986. Gametes were immediately hand-stripped from the gravid male and female lamprey following methods as outlined in the appendix. If ova quality was visually assessed as being viable milt was collected and 10-20 μ l added per 200 eggs. Eggs were batch fertilised in a 95mm diameter petri dish and then divided into labelled treatment beakers, thereby reducing variation in fertilisation success between treatments and replicates. Gametes were carefully hand stirred for 10 seconds and left to stand for nine minutes to allow fertilisation. Gametes were then rinsed several times with loch water to prevent later egg mortality, as per (Lampman *et al.*, 2020). Approximately 60 eggs per replicate were transferred to separate clean labelled beakers with either loch water (wildtype x 3) or loch water with 0.5 % TWP (<65 μ m) as specified above (treatment x 3). Beakers were securely placed in temperature control room at 10-12°C in darkness. Larvae that are reared in static cultures have shown improved survival and growth compared with those that are raised in recirculating systems (Lampman *et al.*, 2020). Culture water was replaced every week, dead embryos removed every two days and shells removed once larvae had hatched.

6.3.3 Index of microplastic impact and statistical analysis

To quantitatively assess toxicity of tyre wear particle exposure, we classified embryos obtained from each treatment based on deviation from wildtype phenotype. The wildtype phenotype is that of the control and the typical form expressed in the wild population. Live images of each replicate were assessed from 19 hours post fertilisation (hpf) to 381 hpf to monitor development. Embryos and larvae were to be classified by morphological deviation from the wildtype in a set of continuous categories to reflect the increasing developmental delays and other morphological malformations. However, due to the consistent phenotype observed in the tyre wear exposure, it was considered best to categorise as a percentage of embryos exhibiting normal (category 0) and abnormal (category 1) phenotypes. Methods available to test for differences between wildtype embryo morphology and those exposed to tyre wear particles were limited by the small number of replicate groups in this study. Statistical comparisons were carried out using two proportion z-tests, and a chi-squared tests. Probit analysis was undertaken on the data for both embryos exposed to tyre wear particles and the control (wild type) to determine the median effect time (ET50), which is the time taken for 50 % of the population to be affected.

6.4 Results

6.4.1 Morphological assessment of *Lampetra* development

To evaluate the effects from TWP to *Lampetra* embryos, the embryos were classified according to the degree of morphological alternation and developmental delay. This allowed a quantitative measure of severity of the possible impact of the TWP to be established. Embryos were imaged and assessed at 19, 45, 120, 168, 222, 288 and 381 hours post fertilisation (hpf), which corresponds to key developmental time points in wildtype embryos (Table 6.1).

Table 6.1 Development of wildtype (control) larvae times by stage, including description of phenotype.

Stage	Description	Date	time	Hours post fertilisation	Days post fertilisation
0	Egg	11/05/2022	16:00	0	0
16-cell	16-cell	12/05/2022	11:00	19	0
morula	Morula	13/05/2022	13:00	45	1.9
8	Blastula	16/02/2022	10:00	120	5
9	Neurula	18/05/2022	10:00	168	7
12	Head protrusion	20/05/2022	16:00	222	9.25
12	Hatching	24/05/2022	16:00	288	12
14	newly hatched	26/05/2022	13:00	381	16
15	Eye spots	02/06/2022	13:00	549	23
16	Velum beating	06/06/2022	10:00	642	27

A consistent developmental abnormality was observed in the treatment embryos (Fig 6.1,6.2,6.4), therefore it was decided to only use two categories, normal (category 0) and abnormal (category 1) phenotype. This was also determined due to the lack of literature to define abnormalities and lack of expertise in classifying abnormalities in lamprey embryos. The abnormal, category 1, phenotype consisted of embryos which had ‘bubbling’ or fragmentation of cells around the embryo (Fig 6.1,6.2,6.4). This abnormal phenotype became consistently apparent in the exposed embryos from 120 hpf (5 days post fertilisation; Fig 6.3M-O).

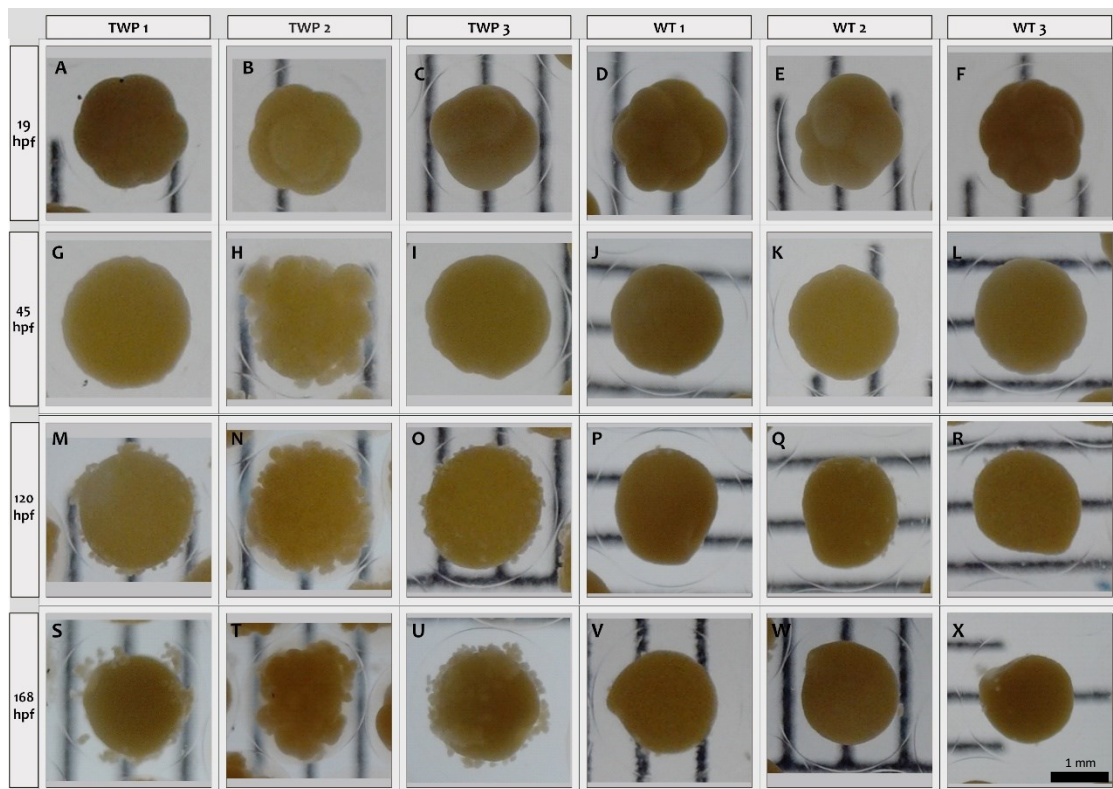


Figure 6.1 Phenotypic comparison of wildtype and treatment *Lampetra* embryos from 19 - 168 hours post fertilisation (hpf). Scale bar = 1 mm.

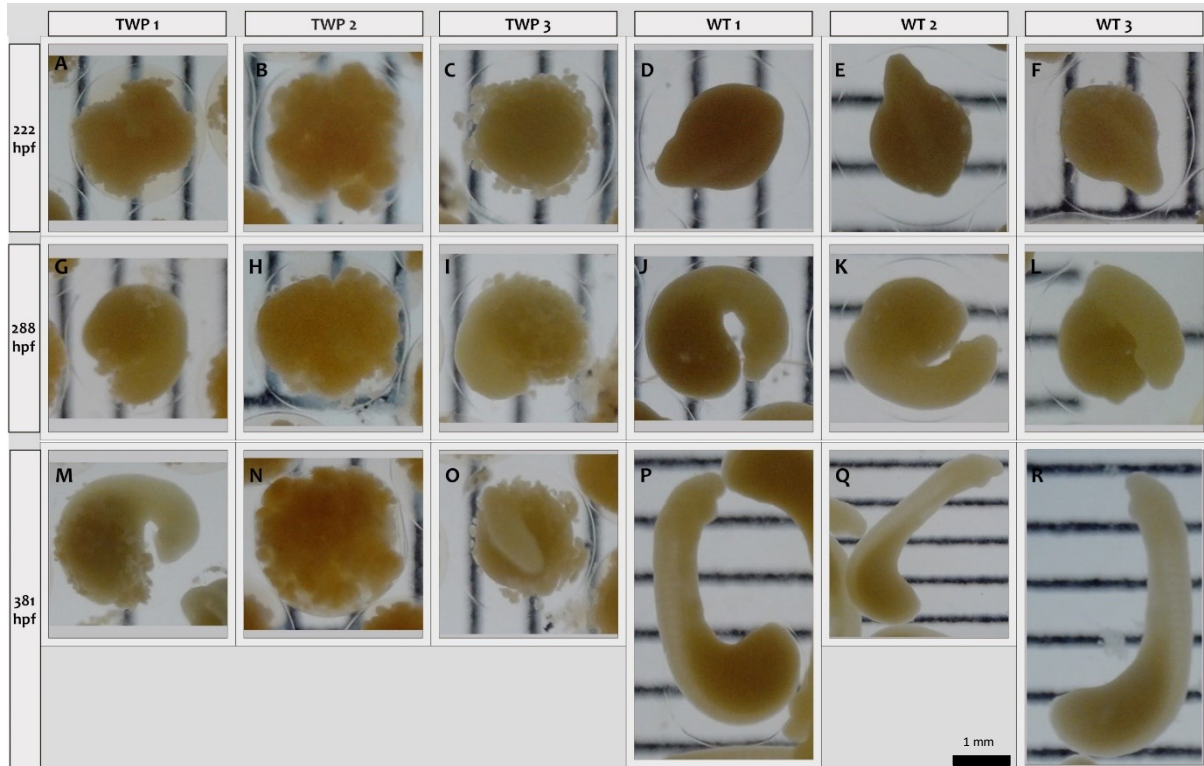


Figure 6.2 Phenotypic comparison of wildtype and treatment *Lampetra* embryos from 222 – 381 hours post fertilisation (hpf). Scale bar = 1 mm.

At 19 hpf (Fig 6.1A), most wildtype (n=180) and TWP treated (n=190) embryos were classified as normal, with a clear 16-cell formed embryo (category 0; Table 6.1, Fig 6.1A-F). This observation was consistent between replicates in both wildtype and tyre wear exposed embryos (chi-squared test, $p>0.05$). At 45 hpf wildtype embryos were normal, exhibiting a morula phenotype which was consistent between replicates (chi-squared test, $p=0.471$, Fig 6.1J-I, Table 6.1). However, at this stage many of the tyre wear-exposed embryos began to exhibit an abnormal phenotype (category 1 = 46.3%, Fig 6.1G-I, Fig 6.3). This was particularly evident in replicate two (TWP 2) at 45 hpf showing a statistically higher number of abnormal embryos compared to replicate one and three (Fig 6.1H, chi-squared test, $p<0.01$). At 120 hpf an average of 6.1% of the wildtype embryos across replicates were classified as undergoing abnormal development (Fig 6.3), whereas 93.7% of the exposed embryos were considered abnormal (Fig 6.1M-O, Fig 6.3). Both phenotypes were consistent between replicates within groups (chi-squared test, $p>0.05$). Between 168 hpf and 381 hpf, the phenotype of the embryos exposed to tyre wear particles was dominated by category 1 embryos, increasing from 94.6% to 100% abnormal (Fig 6.3, Fig 6.4). This was consistent between replicates, although replicate one was statistically different from replicate two and three, this was not due to a difference in phenotype, but due to the removal of dead embryos thereby reducing the observations from approximately 60 to 30 embryos (chi-squared test, $p<0.01$).

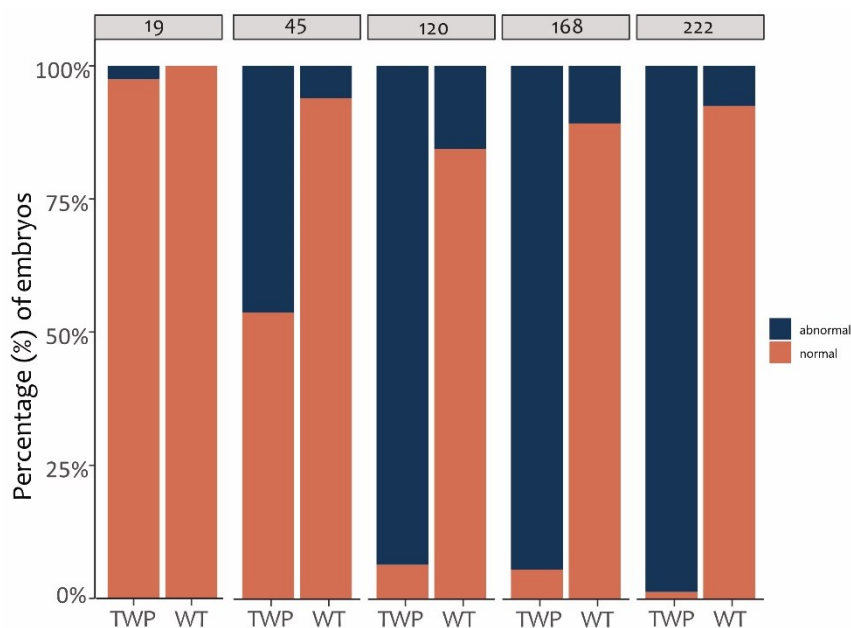


Figure 6.3 Effect of tyre wear particle (TWP) and leachates on the development of *Lampetra*, with unexposed or wildtype embryos (WT) as a control, from 19 - 222 hours post fertilisation (hpf). Average percentage of three replicates (n=190 WT, n=180 MP) embryos and larva classified as abnormal when phenotype varied from wildtype phenotype of *Lampetra*.

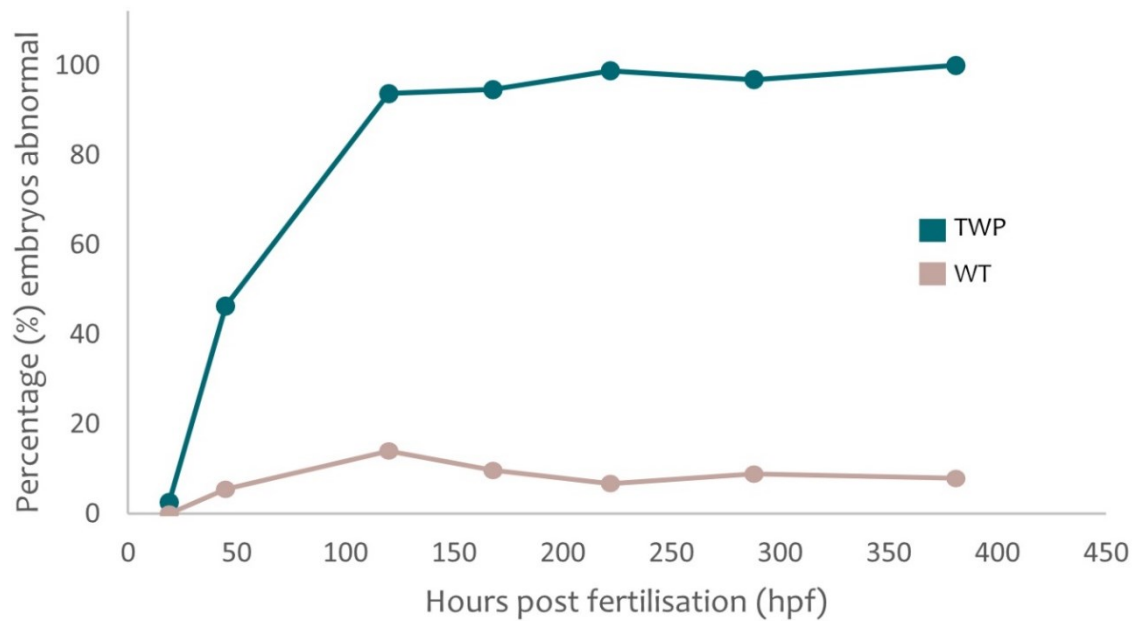


Figure 6.4 Percentage of lamprey embryos exhibiting an abnormal phenotype for those exposed to tyre wear particles (TWP) and control embryos (wildtype, WT). ET50 corresponds to 55 hpf in TWP treatment and 513 hpf in the control.

Those embryos exposed to tyre wear particles showed a consistent and severe phenotype, which resembled excessive abnormal or fragmented cell growth around the embryos (Fig 6.1,6.2,6.4). This anomalous phenotype was significantly higher than the control (wildtype) for all analysed time points, apart from 19 hpf in which a higher number of embryos were considered normal (two proportion z-test, $p < 0.001$). The median effect time (ET50) corresponds to the time taken for 50% of individuals to be affected. For lamprey embryos exposed to TWP the ET50 was 55 hpf (Fig.6.4), whereas in the control embryos this approximately 10 times longer at 513 hpf. In contrast to this abnormal phenotype, most wildtype embryos showed consistent and normal growth from 5 – 381 hpf, with the highest abnormal phenotype expressed in 15.6% of embryos at 5 days post fertilisation (120 hpf; Fig.6.3). Phenotypes observed within wildtype group replicates were consistent between replicates across all time points observed (chi-squared test, $p > 0.05$). In terms of egg abnormality in the wildtype treatment, this is within what would be expected (typically 10-25%, Lampman *et al.*, 2021). This egg loss is often a result of fertilising eggs mechanically, which is open to some eggs failing to contact sperm in time (e.g., insufficient mixing, clumping of ova, presence of mucus/blood/other waterborne contaminants).

6.5 Discussion

The morphological comparisons of embryos indicates that exposure of *Lampetra* to particles and leachates resulting from a 10-day incubation of tyre wear particles (TWP) at a concentration of 0.5% consistently induced developmental defects. In contrast, embryos exposed solely to loch water exhibited normal wildtype development. These results show that the observed abnormalities are attributable to exposure to tyre wear particles and their leachates.

It is important to note that the mechanism of toxicity (i.e., chemicals, particles or a combination) for these morphological abnormalities cannot be determined as we do not have data on the chemical content of the tyre wear particle exposure medium. Potential toxicants in tyre wear particles include, heavy metals, organic compounds and particulate matter (Kole *et al.*, 2017). Therefore, without specific and comprehensive data on the chemical content of TWPs, it is challenging to identify which chemicals, particulates or combinations of these are responsible for observed toxic effects. Future experiments were planned to include these analyses, however unfortunately due to lack of embryo collection in subsequent years this was not possible.

We did not observe significant abnormalities in lamprey embryos exposed to TWP until the morula stage at 45 hpf (Fig.6.1, 6.3), however by 55 hours post fertilisation 50% of individual were affected (Fig. 6.4). In comparison, studies on zebra fish exposed to copper, observe the highest mortalities and abnormalities during the 'critical window' period of embryo gastrulation and segmentation (i.e., between 5 and 24 hpf) (Schilling, 2002; Johnson, Carew and Sloman, 2007). However, in the present study the developmental abnormalities could have begun to form following the observation at 19 hpf, thereby being observed in the 45 hpf time point. Therefore, future research on exposure of lamprey embryos to microplastic contamination should adopt an additional 24 hpf observation time point.

Notably, our study revealed that embryos post 45 hpf exposed to tyre wear exhibited abnormal cell division, fragmentation, and the presence of bubble-like structures surrounding the embryos. This phenotype strongly suggests that compounds associated with tyre wear particles elicited interference during the egg hardening stage, compromising the integrity of the vitelline membrane. Despite severe abnormal and slow growth, including fragmented

bodies, some embryos in the microplastic treatment managed to progress through the developmental stages (Fig 6.4). This phenomenon suggests that the compounds responsible for this phenotype did not lead to a complete developmental arrest.

Together with the chorion and the enveloping layer, the vitelline membrane presents a vital structure in which protects the developing fish embryo from chemical and physical damage (Finn, 2007). The vitelline membrane functions as a barrier to the vitellus (or yolk) and is highly impermeable to salts and water (Shelbourne, 1956). However, a weakened vitelline membrane acts as a precursor, rendering embryos less adept at maintaining their structural integrity during cell division, consequently resulting in yolk leakage and abnormal embryonic development. This weakening may further induce the precipitation of proteins such as globulin and vitellogenin, ultimately contributing to morphological malformations (Gray, 1932; van Heerden *et al.*, 1996; Cardona *et al.*, 2021). The weakening of this protective structure could also result in increased permeability and risk from external contaminants to the developing embryo (Hagedorn *et al.*, 1998).

Developmental abnormalities to the chorion and vitelline membrane have been observed in other fish embryos exposed to contaminants from fertilisation. Exposure of heavy metals to fish eggs can lead to deleterious effects, particularly when this is prior to egg hardening stage (Finn, 2007). Deleterious effects are reduced when embryos are exposed to wastewater effluent once the chorion is more developed and less permeable, i.e., four hours post fertilisation (Gellert and Heinrichsdorff, 2001). However, this exposure does not reflect the environmental conditions in which developing embryos would be exposed, with eggs laid in contaminated environments being exposed from gamete fertilisation. Indeed, zebrafish embryos exposed to copper from less than 1hpf elicit higher mortality than those exposed after 24 hpf (Johnson, Carew and Sloman, 2007).

Lamprey embryos have evolved to adhere to surfaces as an adaptive strategy to enhance their development success, particularly in fast-flowing river conditions (Moser *et al.*, 2019). However, this may present an ecotoxicological risk from microplastics in the surrounding sediment. Microplastics, characterized by their electrical charge and hydrophobic properties, are attracted to the surface of aquatic embryos, leading to adhesion and aggregation on the embryo membrane (Le Bihanic *et al.*, 2020; Zhang *et al.*, 2020; Prata *et al.*, 2022). Furthermore, pollutant transfer by direct contact of microplastics in sediments to embryos

presents a higher risk than waterborne contaminants (Le Bihanic *et al.*, 2020). Therefore, the external fertilisation process of lampreys and adherence of embryos to particles could increase lamprey vulnerabilities to microplastic contamination.

The phenotype observed in the microplastic treatment as far as we are aware has not been observed in any published literature on lamprey. However, metal exposure during fish embryonic development has been observed to cause abnormalities in the egg swelling stage, specifically a decrease in swelling (Jezierska *et al.*, 2009). This reduction in egg swelling can instigate alterations in membrane permeability, thereby leading to disruptions in the exchange of ions between the perivitelline fluid and the external environment (Peterson and Martin-Robichaud, 1982). Additionally, previous research has demonstrated that zinc can impact the physical properties of egg surfaces in fathead minnows, resulting in eggs with adhesive and brittle characteristics (Benoit and Holcombe, 1978).

Given that TWPs have been shown to release metals into the aquatic environment, triggering toxicity to aquatic organisms (Halle *et al.*, 2021), it is plausible that the observed phenotype may be attributed to the presence of such metals. Of relevance, zinc, being the most abundant heavy metal found in tyres (Marwood *et al.*, 2011), is often identified and confirmed as the primary toxicant responsible for biotic toxicity (Nelson *et al.*, 1994; Wik *et al.*, 2009; Yang *et al.*, 2022). Moreover, it is worth noting that since the exposure solution was filtered to a size of 63µm, the potential presence of metal oxides cannot be ruled out. Metal oxides have been documented to have neurotoxic effects in aquatic organisms (Truong *et al.*, 2012; Prust *et al.*, 2020). Zinc is not the only chemical contaminant of concern associated with TWP. Indeed, as discussed in Chapter 1, 6PPD-quinone and associated metabolites have been linked to fish declines in rivers (Hiki *et al.*, 2021; Varshney *et al.*, 2022; Maji *et al.*, 2023) and thus further work on TWP clearly needs to consider measurements of these chemicals to understand whether they are drivers of toxicity in this case.

Most lamprey eggs are deposited in non-stagnant areas, as flowing habitats leads to higher hatching success (Silva *et al.*, 2015). The river channel bed typically accumulates higher concentrations of microplastic particles, compared to that of the water column (Woodward *et al.*, 2021). Therefore, the exposure to deposited microplastics is likely to be lower during spawning and early embryonic development, compared to that of burrowed post-hatched larvae. However, with habitat fragmentation and flow regulation of European rivers, the

abundance and distribution of high-quality spawning habitat available for lamprey species is decreasing in many areas (Lucas and Baras, 2001; Lucas *et al.*, 2009; Silva *et al.*, 2015). This could result in lamprey spawning in sub-optimal areas that may increase the risks associated with higher densities of microplastic pollution.

Further work on developmental toxicity should include labelling of key tissue groups with antibodies against acetylated tubulin, serotonergic neurons (5-HT) and phalloidin. Abundance, connectivity, and organisation of key tissue structures were to be compared between treatment and wildtype groups (See developed standard operating procedures in Appendix D). This approach would give an understanding of whether tyre wear leachates elicit changes in key embryonic tissue structures of the brook lamprey. However, unfortunately the first season of collection of wild spawning lamprey (2022 data presented in this chapter), did not yield enough adults to conduct the original aims, with only one female and male obtained to conduct research, due to low river levels. The following season (May 2023) efforts were scaled up to increase chances of capturing adults, with more traps deployed, river surveys over multiple rivers and starting the surveys earlier. However, unfortunately 2023 was also unpredictable in lamprey spawning, due to varied rainfall and temperatures, resulting in no animals being caught in the traps. Therefore, although the original scope of aims of this project were not achieved, the work demonstrated developmental toxicity studies could be conducted using lamprey. Morphological effects were clearly observed in exposed lamprey embryos which suggest an association with TWP, however the chemical contribution could not be identified. It is advised that future work is needed to explore this association further.

6.6 Conclusions

Exposure to microplastic contamination elicits developmental toxicity across a range of aquatic biota (Malafaia *et al.*, 2020; Rendell-Bhatti *et al.*, 2021; Park and Kim; 2022; Dubey, Khan and Kushwaha, 2022; Paganos *et al.*, 2023). However, there is a consistent lack of understanding for species of protected status and performing key ecosystem functioning roles, particularly in freshwater ecosystems (Delaeter *et al.*, 2022). This study demonstrates that leachates and particles (<65µm) from recycled tyre wear elicit severe and consistent developmental abnormalities in lamprey embryos and larvae (*Lampetra*). Such abnormalities suggest a weakening of the vitelline membrane because of the compounds within the tyre wear exposure media. The study underwent a huge amount of preparation and research

energy, however unfortunately due to factors out with our control, the results presented here only offer a brief insight into the effects. It is important that these experiments are replicated, considering other factors as outlined above, to assess whether tyre wear particle present a risk to lamprey populations. Further research on the ecotoxicological impacts of tyre wear particles is needed to aid our understanding of whether this complex contaminant has a role in lamprey population declines.

Chapter Seven - General Discussion

7.1 The context of research

It is essential to better understand how novel contaminants impact freshwater systems and their functions, as they are directly or indirectly related to all the functioning of the global biomes (Steffen *et al.*, 2015). Although significant improvements have been observed in freshwater invertebrate biodiversity over the past 30 years in some UK rivers (Qu *et al.*, 2023), pressures on aquatic systems are undeniable and aquatic ecosystems receive less research attention than terrestrial ecosystems (Reid *et al.*, 2019). Furthermore, as many contaminants are not covered by the water framework directive, release into freshwater habitats will not be controlled, therefore we need to know about their contribution as stressors in freshwater habitats (Weisner *et al.*, 2022). Emerging contaminants of concern, and the multifaceted pressures on freshwater systems, are also likely to increase with increasing human population, and climate change (Nilsen *et al.*, 2019). By being able to quantify the extent of contamination and effects on specific ecosystems, we may be able to predict current and future impacts, whilst providing a buffer to already stressed biological communities. This thesis represents a small, nonetheless important, step towards understanding these processes.

Prior to understanding the risks of natural and anthropogenic stressors on lamprey populations, we must first obtain baseline information concerning the presence and effects of such stressors (Lucas *et al.*, 2021). Pollution is among the anthropogenic stressors which are attributed to lamprey population declines globally (Maitland *et al.*, 2015). The research presented in this thesis considered the larval stages of lamprey as there is limited knowledge around the protection of this life stage. Their lengthy benthic resident times places them in a particularly vulnerable position when considering microplastic contamination. Furthermore, disruption of this sensitive life stage is more likely to decrease recruitment into adult and breeding life stages, potentially leading to population effects (Murl Rolland, 2000).

Without reliable and robust research which characterises their exposure and investigates their effects, it is impossible to advise on the conservation implications of microplastic contamination for a species. However, microplastic research is a rapidly moving field (Jenkins *et al.*, 2022), and concerns have been expressed about the need to raise the publication bar

for microplastic research (Provencher *et al.*, 2020). To be able to fulfil the robust assessment of microplastic exposure, further work is required focused on producing rigorous methodologies and the generation of reproducible results. (Pérez-Guevara *et al.*, 2022). This need is reflected in the work presented in this thesis where robust, reproducible methods were first developed and refined to be suitable for the assessment of microplastics presence in both sediments and biota (Chapter 2). Implementation of controls and recovery underpinned the ability to compare sites and samples systematically and demonstrated the ubiquitous presence of microplastics at all the lamprey sites studied as part of this work (Chapter 3). Further, undertaking behavioural work with lamprey in a laboratory setting also necessitated methodology development to enable automated monitoring of their movements (Chapter 5) to ensure a thorough understanding of their behaviour.

7.2 Summary of findings

The purpose of this thesis was to expand and enhance our understanding of microplastic contamination on lamprey larvae. Specifically, this thesis fills in knowledge gaps on firstly the exposure of lamprey larvae which reside in Scottish rivers to microplastic contamination, and secondly the potential ecotoxicological and behavioural effects associated with this exposure. **Chapter 1** outlined the state of knowledge and highlighted gaps in the literature. These knowledge gaps related to insufficiency of suitable methods for quantifying microplastics in lamprey and a lack of knowledge of environmental concentrations of microplastics in lamprey larvae. Furthermore, the ecotoxicological impacts elicited from microplastic exposure were considered for other aquatic biota, but there was a clear lack of knowledge on this for lamprey larvae. These gaps relating to methods of isolation, fate of microplastics in lamprey larvae as well as methodologies for assessing ecotoxicological effects were therefore addressed in Chapters 2- 6.

Chapter 2 addressed the need for robust and reliable methods for isolating microplastics from lamprey larvae. It builds methodology and analysis to allow robust and efficient isolation of microplastics from lamprey and paired sediment samples. Sample preparation methods were developed and tested to isolate microplastics from lamprey larvae. A simple oxidative method (López-Rosales *et al.*, 2021) was found to be the optimal method for lamprey larvae. This was due to high biogenic matter removal, together with a reduction in processing steps to avoid both contamination and losses, and time and resource efficiency. This method was

recommended for the digestion of lamprey larvae for microplastic analysis, producing a clean and cost-effective sample for analysis via μ FTIR. This work showed the importance of considering trade-offs in sample preparation to optimise the recovery of microplastics from complex samples, whilst reducing losses and contamination.

Chapter 3 applies the learnings and methodologies from Chapter 2, providing a rigorous assessment of the occurrence of microplastic contamination in lamprey and paired sediment samples across a range of riverine habitats in central Scotland. It represents the first *in-situ* study to document microplastic contamination of lamprey larvae. Microplastics were documented across all samples of lamprey larvae and paired sediment, ranging in abundance from 1.00 to 27.47 particles g^{-1} in dry lamprey gastrointestinal tract (GIT) tissue, and 0.40 to 105.41 particles g^{-1} in dry sediment. The most urbanised catchment exhibited the highest average microplastic particle count in both lamprey and sediment, however no significant differences were observed between lamprey collected from protected Special Areas of Conservation catchments and lamprey collected from non-protected urban rivers. Spiking procedures were developed from Chapter 2 to include matched matrix spikes which provided us with validation of our methods.

Chapter 4 furthers our understanding of microplastic exposure and risks to lamprey by asking whether the size/ age of lamprey larvae influences the composition and count of microplastics recovered from the gastrointestinal tract. Lamprey were analysed from one riverine site within a Special Area of Conservation. The samples were based on body length and in contrast to Chapter 3, the total length of GIT was analysed from each larva. Microplastic count was not correlated to lamprey age or body length, however all larvae ingested microplastics from 0+ to >1+ (21-108 mm body length) and the patterns in microplastic polymer type were distinctive between lamprey larvae age classes. These patterns are important to consider with regards to lamprey conservation and management, particularly for those located in protected areas, such as Sites of Special Scientific Interest or Special Areas of Conservation. Monitoring pollutants in different age groups helps us understand how pollutants propagate through the food web and impact ecosystem dynamics. This information forms a platform for future research and informs conservation managers when making decisions to protect both aquatic life and ecosystems.

Chapter 5 builds on a clearer understanding that lamprey larvae are exposed to microplastic in the environment and considers how this could impact individuals. It considers the ecotoxicological effects of microplastic contamination with regards to behavioural endpoints through experimental studies. The research considers the lack of methods around toxicological testing with lamprey larvae, building knowledge through stepwise experiments to allow deeper investigation. The final experiments utilise place preference tests with lamprey larvae to understand whether microplastic exposure for 14-days impacts burrowing behaviour. This work highlighted the challenges of working with non-model organisms in experimental settings and by using tracking software adapted specifically for monitoring lamprey in tanks, gave important insights into lamprey behaviour and confirmed their low levels of movement once introduced to the tanks. This would not have been possible without this continuous monitoring approach. The chapter highlights the importance of considering non-model species that are under threat.

Chapter 6 identified developmental toxicity of microplastics (tyre wear particles) on lamprey embryos during a sensitive and important stage of the life cycle. This builds on knowledge from previous chapters on the latter stages of lamprey larvae, providing an understanding of the very early life stages of lamprey larvae. Challenges were faced in the second field season however despite this, the results offer a snapshot into the developmental toxicity of tyre wear particles to lamprey larvae and offer scope for this research to be expanded to a laboratory in which lamprey embryos can be obtained and reared reliably.

7.3 Developing methods for microplastic isolation and analysis in lamprey

The research field of microplastics is relatively new and rapidly evolving. There are constant method developments and improvements in the ability to estimate synthetic particle concentrations (both mass and number) and characteristics reliably and accurately. For simpler matrices like water, this developed quickly but for complex organic matrices there has been a need for a lot of method development, particularly to separate the microplastic particles reliably from the matrix without destroying them. At the commencement of this thesis there was a considerable lack in established robust methods for microplastic quantification in freshwater sediments and biota and no established method for isolating microplastics from lamprey larvae. However, to provide appropriate risk assessments of microplastics to lamprey populations it was essential to collect robust data on the sources,

pathways and fate of environmental contaminants. The work of Chapter 2 built upon previous methods of isolating microplastics from biota, whilst applying this to lamprey larvae and considering the background contamination in sediments. Since Chapter 3 was published (Rendell-Bhatti *et al.*, 2023), more advancements have been made in the field of methods for microplastic isolation from lamprey. However, each Chapter consists of a stepwise evolution of methods and highlights the importance of study comparison and development.

To take an example of the evolution of methods and the importance of standardisation across research we can consider the differences in sample preparation of lamprey larvae between chapters. The samples collected to assess the influence of age on microplastic ingestion in Chapter 4, are from a site previously surveyed in Chapter 3. Therefore, it is possible to compare microplastic count in the GIT of lamprey larvae to our previous data set. The median body length of larvae sampled from this site (5TEI) in September 2022 (Chapter 3) was 102 ± 11.26 mm. Therefore, if we consider larvae from Chapter 4 that fall within in the length distribution sampled in September (*i.e.* 90-108 mm), the average microplastic count was 133.25 ± 155.62 microplastics per individual (wet GIT). In contrast, larvae sampled from this site in September 2020 (Chapter 3) were estimated to have ingested approximately 10.36 ± 0.25 microplastics per individual (wet GIT). This 1186 % increase in microplastic count from larvae sampled in this data set compared to the same site in 2020 could be a result of the differences of the sampling methods of the GIT between the two data sets. In Chapter 3 a proportional representation of the homogenised GIT was sampled from each lamprey larvae to form one 0.5g sample of 10 larvae. In Chapter 4, on the other hand, we analysed 30 samples composed of the entire GIT of two size-matched larvae per sample reflecting the GIT composition in its entirety. This provides insight into the potential for underrepresentation of a sample which is not composed of the entire GIT. However, the Chapter 4 data set was collected in December 2021- January 2022, whereas Chapter 3 data was collected in September 2020. It is therefore important to note that this can equate to differences in GIT composition due to seasonal differences in temperature and subsequent feeding (Sutton and Bowen, 1994). Further research is needed to identify whether this increase in microplastic detection is a result in sampling methods or is a true reflection of the samples acquired.

Over the course of the research, analysis and software techniques also improved, more rigorous techniques were developed and as a result there was a clear improvement towards

harmonising microplastic analysis approaches to enhance study comparisons (Moses *et al.*, 2023). As such it was possible to implement new automated analysis software tools for later studies to analyse microplastic abundance and characteristics with the most up to date approach available.

As a result, there are two automated analysis tools used in this thesis, as technology advanced to analyse the infrared (IR) maps to be obtained from micro-Fourier Transform infrared (μ FTIR, Perkin Elmer, Spotlight 400 FT-IR Microscope) spectroscopy. Chapter 2 uses systematic identification of microplastics in the environment (siMPle), which is an open-source software which analyses the IR maps combining the interface of MPhunter with automated analysis pipeline for microplastic analysis (Primpke *et al.*, 2019). Chapter 3 and Chapter 4 adopts the use of Purency Microplastic Finder (pMPF), which is an automated spectra comparison software which uses machine learning alongside a polymer reference spectra database (Purency GmbH, Austria; Hufnagl *et al.*, 2019). The difference in analysis tools used for the data sets presented could impact the assessment of the data between each other and with comparisons to wider research. However, a recent comparison of siMPle and the Bayreuth Particle Finder (BPF) shows an overall good accordance between the two automated analysis tools (Moses *et al.*, 2023). This suggests that although not directly comparable, data sets analysed with different automated analysis tools can be considered against each other with some caution.

If we consider the size range of microplastics identified across the different data sets of lamprey larvae as an example, we can consider how the analysis technique used could influence the results obtained. Although collected from different sites, we can predict the size range of microplastics ingested would be similar for Chapter 2 and 3, with the median larvae length of 111 mm and 112 mm, respectively. As Chapter 4 considered a range of size classes the median larvae length was 64.5 mm, therefore perhaps less comparable. The size range of microplastics detected in lamprey larvae in the method development chapter using siMPle (Chapter 2) was between 10 – 430 μ m. For those data analysed with Purency the size range of microplastics was between 36 – 104 μ m for Chapter 3, and between 1- 80 μ m for Chapter 4. This discrepancy in the higher size range detected with siMPle (i.e. > 104 μ m) could be a result of differences in analysis technique or an artefact of the samples collected. However, it presents a consideration when standardising results from microplastics studies across

different research studies. Therefore, further research into comparing the same samples with different automated analysis techniques is needed to aid in future harmonisation and standardisation of efforts in microplastic research. Furthermore, it is important to note that as Moses *et al.* (2023) stipulate, studies on microplastic contamination that consider these smaller sizes classes should be interpreted with caution. This is because it is presently unclear how accurate results are with respect to actual occurrence of microplastic contamination in the environment, which is applicable for any current analysis method.

With regards to controls, as the research progressed, as too did the ability to run positive controls in the form of spiked samples to assess the suitability and validity of the processing methodologies. Positive controls are not currently standard practice in microplastic research as the methods are yet to be fully harmonised, agreed and adopted. Therefore, this thesis presents an evolution of positive control techniques. For example, Chapter 2 lacks positive controls whereas Chapter 3 uses a set of positive controls to allow for qualitative assessment of recovery and Chapter 4 uses a rigorous spiking technique in all samples to assess recovery of particles using fluorescent polyvinyl chloride particles. While it is recommended to correct final data for recovery rate where possible, it was not possible to correct the data of Chapters 3 and 4 for the recovery rates measured due to a qualitative assessment. This is because it is not currently possible to obtain suitable spiked plastics to cover a sufficiently wide spectrum of polymer types, sizes, and shapes. However, a qualitative guide of assessment of recovery still represents a higher standard of quality assurance than most of the studies to-date (Koelmans *et al.*, 2019). Until methods in microplastic research are improved further, recovery rate of microplastics from samples should be considered with caution when assessing the risks of microplastics. In the interim, lessons from this study and other research suggests sample preparation methods which have reduced processing and filtration steps should be considered over multi-step methods, which increase the risk of microplastic loss and contamination (Dimante-Deimantovica *et al.*, 2022).

7.4 Application of the science to conservation and management

Research on the sub-lethal effects of microplastic exposure on lamprey larvae contributes to the risk assessment for a protected and declining species. By quantifying the risk presented, it is possible to make informed decisions about the clean-up of environmental contaminants of concern within Scottish, and more broadly European fluvial systems. Research findings

presented in this thesis, allow conservation organisations and managers to build a more comprehensive picture on the level of severity microplastic pollution places on populations when considering different stages of the lamprey life cycle. For example, the severity of developmental abnormalities identified in Chapter 6 suggest that this early developmental period for lamprey larvae could be the most sensitive stage in response to microplastic contamination, and therefore should be considered further.

This research contributes to data on environmental contamination in lamprey, which could be used to evaluate the effects of environmental contamination on lamprey abundances and in turn be used toward efforts to restore native lamprey populations (Madenjian *et al.*, 2020). Our research identified microplastics in both lamprey and sediment at all survey sites, across multiple rivers in Central Scotland. It also suggests there could be ecotoxicological implications, particularly for early life stages of lamprey. With increasing load of plastic waste, it is expected microplastics polluting river environments will also increase (Lebreton and Andrady, 2019), therefore these effects may become more pertinent. Research shows that removing the input of discharges of contaminants into once highly polluted freshwater bodies, decreases the concentration of contaminants in European river lamprey larvae (Soimasuo *et al.*, 2004). Removing microplastics from riverine systems is not feasible (Gupta *et al.*, 2023), however control strategies are available for reducing inputs into riverine environments (Fältström and Anderberg, 2020). Therefore, if reducing microplastic contamination is deemed to be on the management agenda for lamprey larvae conservation, local and national legislation to regulate and reduce plastic waste into environmental systems will be key.

It is clear from this body of work that lamprey larvae in sites of both special areas of conservation and urban habitats, ingest microplastic particles from the environment. However, it is not possible to discern the extent of harm that microplastic contamination poses to lamprey larvae. Chapter 3 considers food dilution as the proposed effect mechanism for toxicity of ingested microplastics (Koelmans *et al.*, 2020; Rauchschalbe *et al.*, 2021). Here we estimate the percentage of the intestine occupied by microplastic particles, with a maximum of 0.13% in the most contaminated sites. Considering food dilution associated with ingestion is one of the most commonly hypothesised toxicity mechanisms for microplastic particles for aquatic species, the estimated risks of microplastic contamination to lamprey

larva sampled in this study are anticipated to be low. This hypothesis is largely based on short term lab-based studies on aquatic invertebrates. However, given the low movement of lamprey, as demonstrated in the tank studies, chronic exposure would be expected in sediment environments and if other mechanisms are involved in driving toxicity, it remains to be elucidated whether there are any longer-term consequences from this internal exposure. Linking measurements of stress with exposure in the field is complicated and more dedicated toxicology investigations would be needed, complicated by the licencing requirement to work with these animals in the lab. However, we note it is important to consider bioaccumulation potential (Mateos-Cardenas *et al.*, 2022), translocation of nano and micro-plastics (Ramsperger *et al.*, 2020; McIlwraith *et al.*, 2021; Clark *et al.*, 2022) and toxicity caused from leaching of polymer additives (Paluselli *et al.*, 2018).

With regards to behavioural and developmental endpoints, Chapter 5 suggests that exposure to tyre wear particles elicits no behavioural changes with regards to the place preference test used. However, the research does suggest that burrowing attempts are increased in sediments with microplastic contamination. This could result in increased energy output and have potential effects on fitness (Yin *et al.*, 2018). However, this needs to be explored in more detail to discern if there is an effect present here. The lack of behavioural avoidance response from lamprey larvae towards contaminated PVC sediment, suggests that lamprey larvae may not avoid contaminated sites to avoid predation risk. Therefore, understanding the implications and rates of ingestion are very relevant if larvae do not move from contaminated patches. The lack of an effective avoidance response by lamprey larvae could facilitate trophic transfer of microplastics into freshwater food webs (Mateos-Cardenas *et al.*, 2022). This is particularly relevant since lamprey larvae act as prey for a multitude of animals (Cochran, Leisten and Sneen, 1992; Close, Fitzpatrick and Li, 2002; Sjoberg, 2011). However, more research into the avoidance behaviour of lamprey larvae to microplastic contamination is needed to draw reliable conclusions.

Chapter 6, which adopts developmental endpoints to assess the toxicity of tyre wear particles, suggests that there are clear and consistent abnormalities in embryos exposed. However, this body of work needs to be expanded to replicate the study system across multiple clutches of eggs, a variety of exposure levels and leachate filter sizes. With research suggesting abnormalities are present in the early developmental stages of other aquatic species exposed

to microplastics (Malafaia *et al.*, 2020; Rendell-Bhatti *et al.*, 2021; Chibwe *et al.*, 2022; Park and Kim, 2022), it is highly likely that the effects we observed are relevant. It is clear from our research that microplastics are firstly available to and ingested by lamprey larvae, that the consequence of this could be detrimental with regards to developmental toxicity. Future research ideas are discussed briefly in the next section, but this is one area in which key collaborations and methodology has been developed, therefore this would present a good opportunity to apply for future funding to investigate this further.

7.5 Recommendations for future research

Microplastic research is developing rapidly, however there are still major gaps in our understanding of, particularly related to freshwater environment and protected species (Guasch *et al.*, 2022; Porter *et al.*, 2023). Some of these fundamental gaps were addressed in this thesis, whilst new gaps were identified. In terms of methodological approaches, the research presented here highlights advances in our understanding. However, it is apparent that there is a need for further refining of methods to ensure further separation of microplastics from the matrix and quantitative positive controls across a range of polymer types. This will firstly allow for larger samples to be analysed, secondly for more precise microplastic detection and thirdly obtain accurate microplastic recovery rates. The needs of both time and expense of analysing samples using μ FTIR is high, therefore, to be able to achieve more individuals per filter will allow sufficient replication for accurate quantification. As included in our research, microplastic research should consider the inclusion of recovery rate estimation and contamination control measures, at a minimum adopting measure as advised by Hersmen *et al.* (2018). Furthermore, river systems and biota-interactions are highly complex and dynamic, therefore to understand the spatial and characteristic trends of microplastics in lamprey larvae and their habitat, a repeated comprehensive approach is required to recognise trends arising both from natural environmental conditions and human-environment interactions.

Future research into the exact mechanisms of lamprey larvae feeding ecology i.e. the proportion of particle assimilation via suspended and/or deposited sources is important. This is particularly relevant for quantifying background contamination and estimating uptake from the environment. The feeding mechanism of aquatic biota has direct implications for the uptake and retention of different types of microplastics polymers, sizes and shapes (Setälä,

Norkko and Lehtiniemi, 2016; Scherer et al., 2017). Primarily lamprey larvae are considered to be suspension feeders i.e., obtaining suspended particles from the seston (Docker, 2015). However, lamprey larvae also obtain food particles from within the sediment pore water, functioning as a deposit feeder (Docker, 2015). The relative assimilation of particles via each feeding mechanism will contribute to both their nutrition and exposure to contaminants within each environmental matrix.

Chapters 3 and 4 of this thesis did not quantify the suspended microplastics which may have been ingested via suspension feeding of lamprey larvae. This was largely due to practical implications of filtering the seston (Löder and Gerdt, 2015; Campanale *et al.*, 2020) and the low residence times of microplastic in the water column, preventing the ability to determine specifically what the larvae have been feeding on in the seston. Furthermore, sediment is considered as the 'final settling tank' for microplastics (Nizzetto *et al.*, 2016), with concentrations of microplastics often higher in the benthic compared to surface water (Hoellein *et al.*, 2017; Scherer *et al.*, 2020).

However, deposited microplastic particles are largely due to their higher density as a result of abiotic and biotic characteristics (Li, Busquets and Campos, 2020), thereby this method only offers an understanding of these types of microplastics. Lighter, fiber-like microplastics which may be assimilated by lamprey larvae via the seston will be missing from our quantification. Experiments to discern the proportion of particle uptake from the two environmental compartments may include quantifying the proportion of labelled particles assimilated by feeding lamprey larvae spiked into the sediment pore compared to that of the seston. Research should consider stability of the fluorescence label or localisation methods for ingested particles, given the ability for fluorescence to dissociate from the particle, leaving a false trace (Schür *et al.*, 2019). Therefore, in order to gain a complete understanding of microplastic exposure to feeding lamprey larvae it is pertinent to firstly determine the extent to which lamprey larvae feed by these different mechanisms, and secondly sample from both the sediment and the seston.

While in this thesis it has been possible to obtain baseline information on the exposure of lamprey larvae to microplastic contamination in Scottish rivers, further ecotoxicological research using the exposure data is needed to understand if the exposure levels observed in the field translate to effects in individuals and ultimately their risks. Furthermore, future

research should focus on the role of lamprey bioturbation and microplastic transport in sediment (Bellasi *et al.*, 2020), with recent research highlighting the impacts of microplastics on other freshwater bioturbators (Wazne *et al.*, 2023). The interaction of lamprey larvae with microplastics may also influence microplastic residence times, which has been observed in filter feeders in lake ecosystems (Gilfedder *et al.*, 2023). Research highlights the need to define the mode of entry of environmental contaminants into the bodies of lamprey to confirm the assertion that the main uptake route of contaminants is dietary uptake (Madenjian *et al.*, 2020). However, undertaking microplastic depuration studies with lamprey larvae is extremely difficult, as not having the possibility of borrowing causes distress in the animals and as a protected species this presents complications.

All future research should avoid unnecessary dispatching of individuals adopting non-destructive methods, particularly for lamprey as these species are protected and experiencing population declines. Non-lethal alternatives for documenting microplastic uptake, such as faecal sampling, would have been preferred in these experiments however such methods were considered impractical this case due to the small size of lamprey larvae and impracticalities of collecting faecal samples. With advances in technology and methodologies perhaps non-destructive methods will become available in the future.

Method development for microplastic quantification in aquatic environments and biota is still an ongoing process. Whilst improvements have been made, these are yet to be fully optimised and universally applicable. For example, there are economic challenges with institutions gaining access to appropriate analysis software and hardware to use the methods we outline above (Way *et al.*, 2022). There are not yet widely accepted standards for sample collection, laboratory analysis, quality assurance or standardised reporting of results to allow cross-study comparisons. To address these challenges and subsequently reduce microplastic contamination, international collaborative research is essential (Klingelhöfer *et al.*, 2020; Zhang *et al.*, 2020).

7.6 Justification of methodologies and limitations of the study

There were several limitations and challenges associated with the research presented in this thesis. Originally the plan for this PhD research was to first characterise microplastic exposure in the environment and then use this for an ecotoxicological assessment. For example, we

would measure occurrence of microplastic contamination in paired surrounding sediments and lamprey larvae gestational tract for the most relevant size ranges for ingestion i.e. from 5 µm to 500µm. These quantifications would then have informed the effect assessments, i.e. to test particle effects for the same size distribution and boundaries, such that the exposure and effect threshold data were aligned, therefore providing a relevant and meaningful risk assessment. This is recognised as the most optimal way to assess microplastic contamination risks to biota (Koelmans *et al.*, 2020). However, unfortunately due to limitations to laboratory and fieldwork as a result of COVID-19, this approach could not be taken. Therefore, due to the time sensitive nature of the PhD I had to continue with behavioural studies of lamprey larvae, without the prior baseline data for lamprey larvae to inform the exposures.

Working with a protected species presents unique challenges, particularly for ecotoxicology studies in which stressing the animals is not ethical. In addition to this, lamprey larvae are cryptic animals with minimal research into their behaviours, particularly in laboratory environments. Research on non-model species is essential to our interpretation of ecotoxicology data (Hecker, 2018), however this also presents challenges in the form of lack of standard operating procedures and technology which is parameterised for their unique movement, as seen in Chapter 5. Therefore, a large body of this work was conducted to establish basic understandings of the species ecology and behaviour.

7.7 Conclusion

In conclusion, this thesis has provided valuable baseline information concerning the exposure of lamprey larvae to microplastic contamination from multiple rivers across Scotland. Furthermore, the research has provided knowledge of the ecotoxicological impacts of such exposure to microplastic contamination for both early developmental stages and older larval stages. Prior to this research, no data existed the presence or impacts of microplastic contamination of lamprey. As an emerging contaminant of concern, this thesis offers a foundation on which to build our knowledge and understanding microplastic contamination within the context of conservation and management of a threatened species. Lampreys often receive less attention and funding for conservation compared to more iconic and charismatic species. As a result, they may not benefit from the same level of protection and restoration efforts (Clemens *et al.*, 2021). With the decline of lamprey populations in Europe and the need to inform protection measures, future research should use the results of this thesis to focus

on identifying the potential causes of developmental defects in early life stages of lamprey larvae. Further research surveying wild populations is critical to understand whether the ubiquitous presence we see here of microplastic pollution is reflected both nationally and internationally. In addition, once the severity of the stressor is determined researchers need to collaborate with conservation and government organisations to develop a strategic plan to mitigate their effects.

Appendices

Appendix A - Developing Methods for the Isolation of Microplastics from Larval Lamprey and Paired Freshwater Sediment

Appendix A contamination control checklist

- Glass and stainless steel labware only used, apart from one yellow LDPE bottle
- Wash all labware in acid-DI water wash (45 min cycle) prior to use.
- Rinse all equipment with deionised water, followed by 35% ethanol (0.2µm MilliQ)
- Clean all surfaces 70% ethanol (0.2µm Milli-Q)
- Cotton lab coat and natural fabric clothes under it
- Rinse and check exterior of larvae
- Silver filters muffle at 350°C for 180 minutes
- Complete all sample prep under horizontal laminar flow/BSC
- Cover all beakers and dishes with aluminium foil
- 3 negative control procedural blanks

Appendix A Calculating the limit of detection and limit of quantification

Steps to carry out blank correction:

- (1) mean blank value for each polymer was subtracted from the raw count for a sample
- (2) if the corrected value was then above the LOD it was recognised as detected and if above the LOQ value it was deemed quantifiable
- (3) the resulting value was quantified by referral to the original weight of the sample used (or 10ml) and the proportion of the final sample that was transferred to the silver filter used in µFTIR analysis.

Appendix A Table 1 Literature on digestion of aquatic organisms – Investigating biomass and enzyme/chemical ratio

Treatment	Organism	Tissue	Ratio of treatment	No. plastic per biota	Author
10% KOH	Fish (7 marine species)	Esophagus, stomach, intestines	1:3 v/v	2.6% of fish sampled 0.85 ± 0.06	Foekema <i>et al.</i> (2013)
Proteinase-K	Plankton-rich seawater	0.20g dry weight	500 ug mL ⁻¹ of Proteinase-K per 0.2 g	0.27 microplastics m ⁻³	Cole <i>et al.</i> (2014)
9% NaClO	Fish (herring sardine, anchovy)	Stomach	1:3 v/v	12,10,13 (herring sardine, anchovy)	Collard <i>et al.</i> (2015)
250ml NaCl, 15% H ₂ O ₂	Fish (5 species pelagic- benthic)	GI tract	35g tissue	1.78 ± 0.97 (MP present in 28% of the 125 examined fish)	Avio <i>et al.</i> (2015)
10% KOH	Fish (21 marine species)	GI tract	1:3 v/v	Indonesia 1.4 ± 3.7 USA 0.5 ± 1.4 SD	Rochman <i>et al.</i> (2015)
10% KOH	Fish marine	Digestive tract	1:3 v/v as Foekema <i>et al.</i>	11% of 761 fish	Lusher <i>et al.</i> (2016)
500 µg/mL proteinase K 30 mL H ₂ O ₂ (30%)	<i>Salmo trutta</i>	GIT, including faecal matter, freeze fried and ground in a mortar	Not specified	68% of samples	Karlsson <i>et al.</i> (2017)
Enzymatic UEPP (SDS, protease, lipase, cellulase, H ₂ O ₂ , ZnCl ₂)	Highly concentrated plankton	UEPP be applied to multiple biota	Subsample with equal load of matrix	0.05 to 4.42 items m ⁻³ in plankton samples	Loder <i>et al.</i> (2017)
Enzymatic (SDS, protease, Chitinase and H ₂ O ₂)	Pelagic and demersal fish	Stomachs (pooled in 5) between 1-20g	Volumes of enzymes not specified, but based on Cole <i>et al.</i> 2014 and Mintenig <i>et al.</i> , 2016	Method paper	Fischer and Scholz-Bottcher. (2017)
Oxidative agents), bases and acids - concentrated and diluted (5%) + NaI separation	African catfish	1g skin + 5g minced muscle	KOH (10% w/v), H ₂ O ₂ (35%), NaOH (5 M), HCl (5% or 37%) HNO ₃ (5% or 69%), or NaClO (5%)	Methods Paper - recovery rates	Karami <i>et al.</i> (2017)
1 M KOH	Menhaden, Spotted Seatrout, Spot, Mullet, Anchovy	Digestive tracts	Approx.3 x volume of organic bio matter	Average of 26.9 ± 4.7 1.9 ± 0.1 MP fish ⁻¹ (Bay Anchovy) (MP present in 99% of fish)	Parker <i>et al.</i> (2020)
10% KOH (w/v) NaI separation	Fish <i>Neogobius melanostomus</i>	Digestive organs	10ml per gram of sample	39 MP/g	Bagheri <i>et al.</i> (2020)
10% KOH KI separation	Benthopelagic fish <i>Serranus scriba</i>	GIT + muscle at lateral line	50ml KOH per gram of tissue	3.63 ± 0.35 to 6.11 ± 0.48 items/g of tissue	Zitouni <i>et al.</i> (2020)
10% KOH ZnCl ₂ separation	Marine fish 15 species	GIT (oesophagus to anus)	1:3 organ to solution	Recovery rates	Jaafar <i>et al.</i> (2020)
30ml 10 M NaOH	Marine fish 9 species	GIT	Not specified	0.057 ± 0.019 microplastic items per fish	Baalkhuyur <i>et al.</i> (2020)
(10% KOH + 15% H ₂ O ₂) or (5% HNO ₃ +15% H ₂ O ₂)	Marine fish species <i>M. merluccius</i> <i>S. colias</i> <i>T. lyra</i>	GIT	(KOH) 3× the volume of the tissue 15 ml of 5% HNO ₃ + 15% H ₂ O ₂ mixture for each gram of tissue	2.1 ± 0.2 average microplastic items per fish	Bianchi <i>et al.</i> (2020)

Appendix A Sediment sample processing

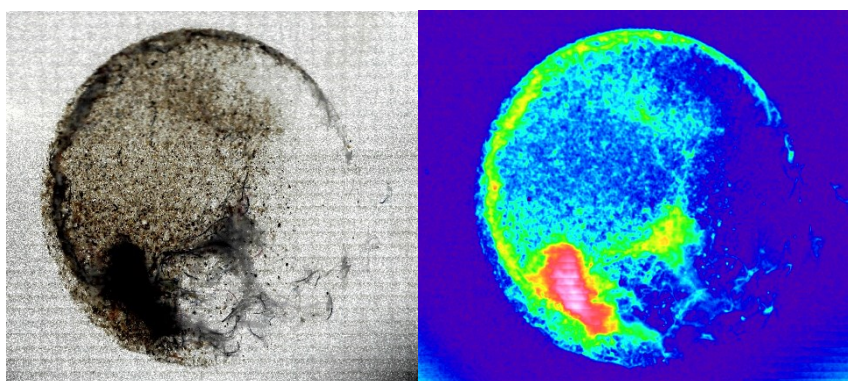
Sediments were oven dried at 40°C for 72 hours, sieved with a 1mm mesh to remove larger stones and organic matter. The <1mm size fraction was sub-sampled to 10g per site and placed in a 2L beaker. Fenton's reagent was chosen based on high efficiency of removing organic material from sediment samples, whilst preserving microplastic particles. The following Fenton's reaction protocol was based on Hurley *et al.* (2018). Fenton's reaction follows a wet peroxide oxidation with H₂O₂, with an additional iron catalyst. Briefly, the catalyst solution was composed of 20g of iron (II) sulphate heptahydrate in 1 L Milli-Q water. The catalyst solution was maintained at pH 3 using concentrated sulfuric acid. 30ml 30% H₂O₂ was added initially to 30ml catalyst solution. To continue the reaction, a further ratio of 1:1 H₂O₂ and catalyst solution was added in 5ml increments, until no further reaction (e.g. frothing) was observed. The samples were processed at room temperature and transferred to an ice bath when the temperature exceeded 40°C.

Following complete oxidation and removal of organic material, the remaining sample was decanted and vacuum-filtered onto a 10µm stainless steel mesh. The filtered sediment samples underwent density separation via pre-filtered (0.2µm) sodium polytungstate (SPT) solution at a density of 1.8 g/cm³. After the last filtration step the >10µm fraction was flushed in a 50ml beaker using pre-filtered SPT solution. The samples were then transferred to 250ml or 1000ml separatory funnels, with 125ml SPT, where they remained for 3 days.

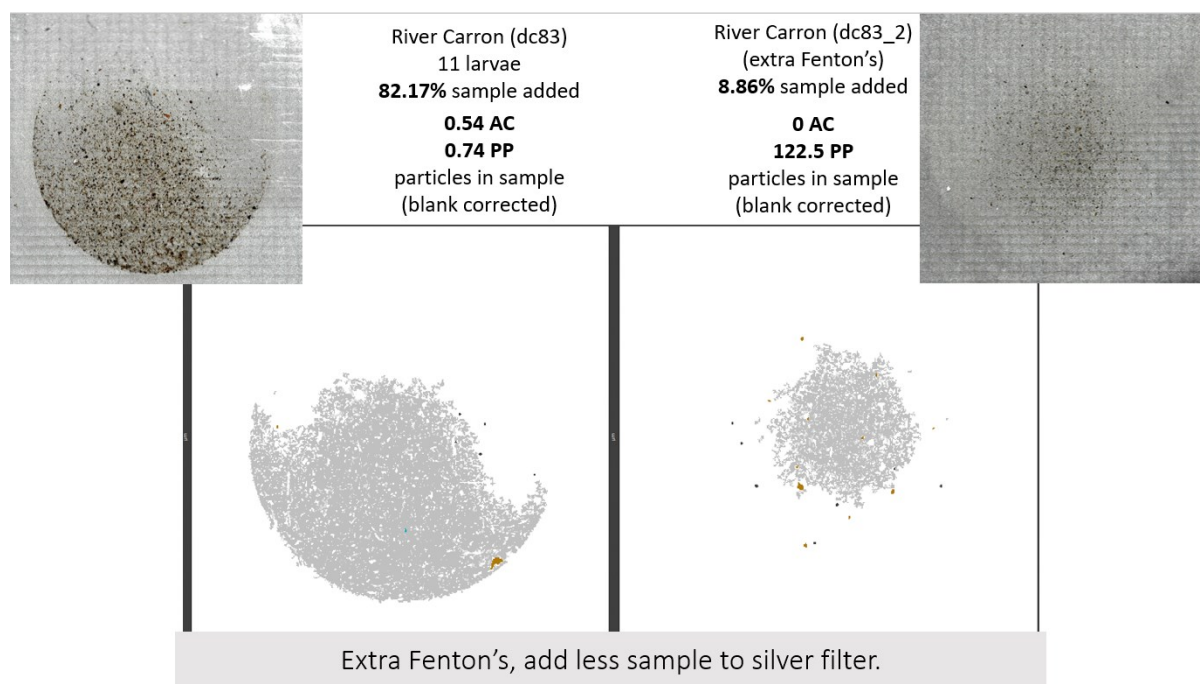
Following density separation, some organic material remained in the samples, therefore the sediment samples underwent a post-oxidation of 60ml 30% H₂O₂ at 50°C for 24h. The sediment samples were then filtered through a 10µm stainless steel mesh and the remaining material on the filter was washed off into a 50ml beaker with 50% HPLC grade pre-filtered (0.2µm) ethanol. To ensure all material was removed from the filter natural bristle brushes were used to brush off any material into the beaker. The contents of the beaker were transferred to a 10ml glass vial for sample storage and analysis. Density separation using SPT, was not successful at fractioning many black particles from the light fraction. Therefore, prior to analysis sediment samples were fractioned into >178µm and <178µm to remove larger black material from the samples to avoid masking and to further purify the samples for analysis. The larger >178µm fraction was stored in ethanol for further analysis. Sediment procedural blanks consisted of washed and muffled (350°C, 180 mins) sand.

Appendix A Table 2 Microplastic concentrations in sediment and *lampetra* samples

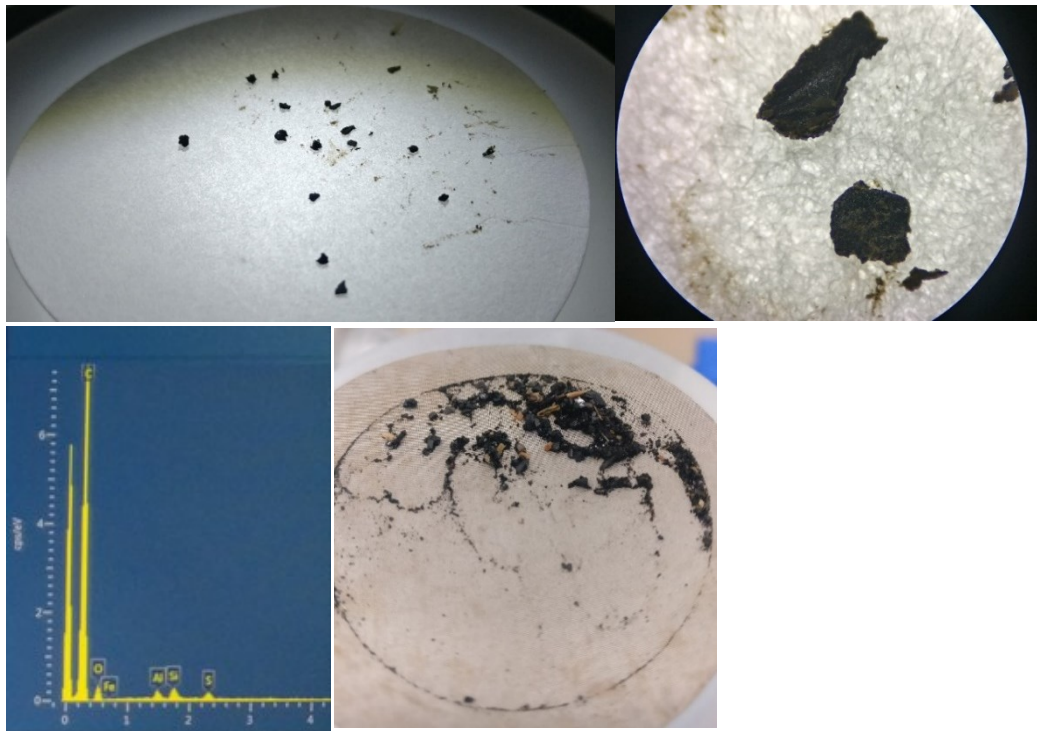
Sample Site	Matrix	Subsample volume for FTIR (%)	MP-10g sediment/pooled larvae	Compare to LOD/LOQ
cd26	sediment	43.61%	36.97	36.97 > 12.65
(NS 85273 94126)	<i>lampetra</i>	72.50%	10.35	3.86<10.35<11.69
dc19	sediment	43.76%	13.99	13.99>11.69
(NS 83576 81619)	<i>lampetra</i> _1	67.38%	3.77	3.77<3.86
	<i>lampetra</i> _2	32.62%	37.48	37.48> 11.69
dc83	sediment	52.11%	18.66	18.66>12.65
(NS 80856 83083)	<i>lampetra</i> _1	82.17%	1.28	1.28<3.86
	<i>lampetra</i> _2	8.86%	122.5	122.5>11.69
td84	sediment	40.59%	8.52	4.17<8.52<12.65
(NS 90980 96484)	<i>lampetra</i> _1	71.11%	0	0<3.86
	<i>lampetra</i> _2	28.89%	1	1<3.86



Appendix A Figure 1 Atmospheric control from fume hood showing high levels of material which could have led to masking of synthetic particles, with only 1 microplastic particles identified in the FTIR analysis.

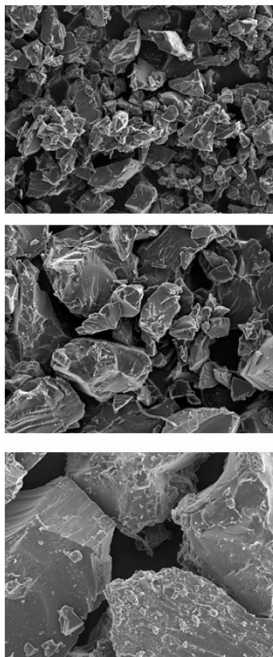


Appendix A Figure 2 Deposited lamprey samples acquired from the River Carron (dc83 and dc83_2). The second sample on the right underwent an additional Fenton's reaction prior to analysis and less of the sample was deposited (dc83_2). This was because of the high number of organic particles covering the silver filters in the first deposition of these samples.

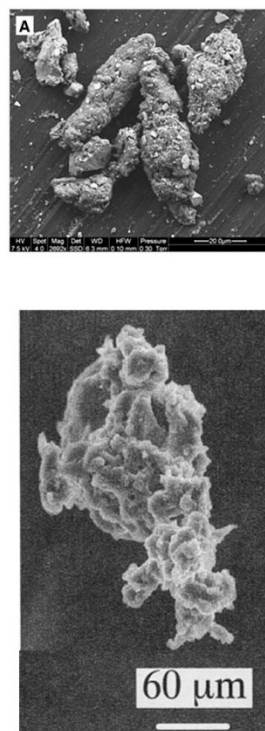


Appendix A Figure 3 Morphologies of blank particles obtained from sediment samples, including SEM/EDX elemental data.

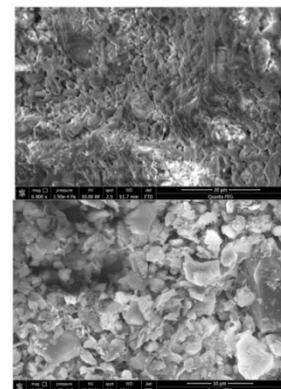
Shredded tire¹



Road simulator^{2,3}

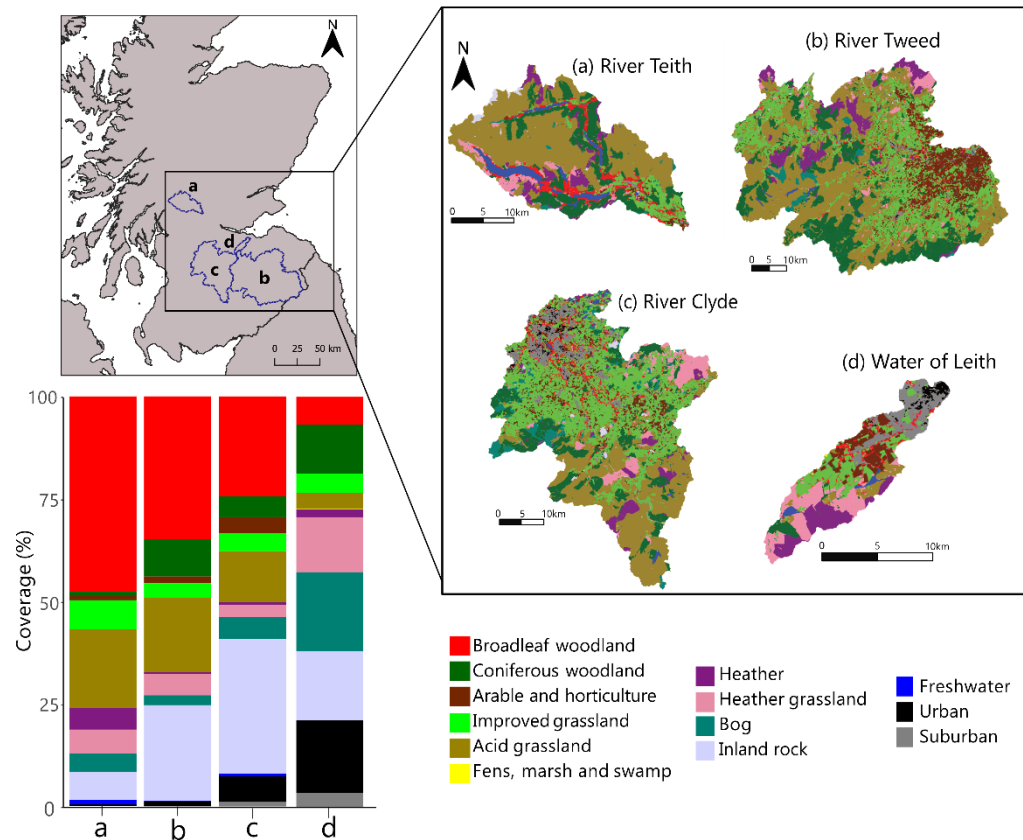


Road runoff⁴



Appendix A Figure 4 Particle morphologies of tire materials obtained under varying sampling conditions. Reprinted from Wagner, S., Hüffer, T., Klöckner, P., Wehrhahn, M., Hofmann, T., & Reemtsma, T. (2018). Tyre wear particles in the aquatic environment-a review on generation, analysis, occurrence, fate and effects. *Water Research*, **139**, 83-100.

Appendix B - From the Environment into the Biomass: Microplastic Uptake in a Protected Lamprey Species



Appendix B Figure 1 Land cover in the a) River Tweed, b) River Teith, c) River Clyde and the d) Water of Leith (WoL) hydrometric areas. Stacked bars show the percentage land cover composition of all catchments. Land cover classes and features of the maps are based on digital spatial data (GB vector LCM2015, Rowland *et al.*, 2017). Maps generated using QGIS

Appendix B Table 1. Sample location information for collection of lamprey and sediment samples in Scotland (UK), including reference to manuscript figures for sites.

Site	Site code	Fig ref.	Site description	Nearest upstream WWTP (km)	Date sampled	Hydrometric Area	Bank	Latitude	Longitude
1	1TW	2ii	Selkirk	Selkirk (0.13)	11/09/2020	Tweed	left	55.562050	-2.834250
2	2TW	2i	Peebles	Esheils (0.50)	11/09/2020	Tweed	left	55.642020	-3.157290
3	3TW	2iii	Galashiels	Galashiels (0.50)	11/09/2020	Tweed	right	55.610030	-2.762430
4	4TW	2iv	Leaderfoot	No	11/09/2020	Tweed	left	55.602390	-2.670900
5	5TW	2v	Kelso	Kelso (1.00)	27/09/2020	Tweed	left	55.609176	-2.406884
6	6TW	2vi	Coldstream	Coldstream (0.13)	27/09/2020	Tweed	left	55.663528	-2.223687
7	1TEI	1vi	Craigforth	Doune (9.6)	12/09/2020	Teith	left	56.136210	-3.970780
8	2TEI	1ii	Turnpool	Callander (0.15)	18/09/2020	Teith	left	56.236761	-4.195778
9	3TEI	1i	Meadows	No	18/09/2020	Teith	left	56.242378	-4.222150
10	4TEI	1iii	Doune Castle	Doune (0.12)	18/09/2020	Teith	right	56.181901	-4.049652
11	5TEI	1iv	Blair Drummond	Doune (1.15)	18/09/2020	Teith	right	56.175836	-4.039641
12	6TEI	1v	Blue banks	Doune (7.2)	18/09/202	Teith	left	56.149930	-3.993953
13	1WOL (iii)	4ii	Craiglockhart mid	NA	27/09/2020	Water of Leith	left	55.921346	-3.248841
14	2WOL (ii)	4iii	Craiglockhart bot	NA	27/09/2020	Water of Leith	right	55.922179	-3.248503
15	3WOL (i)	4i	Craiglockhart top	NA	27/09/2020	Water of Leith	right	55.920449	-3.249087
16	1CLY (iii)	3iii	Daldowie	Daldowie (0.30)	09/10/2020	Clyde	right	55.832815	-4.131264
17	2CLY (ii)	3i	Blantyre	Bothwell (0.50)	09/10/2020	Clyde	right	55.802675	-4.082040
18	3CLY (i)	3ii	Strathclyde	Carbarns (5.00)	09/10/2020	Clyde	left	55.783109	-4.014996

Appendix B Table 2. Information on hydrometric areas, SAC status, catchment size (km), urban land cover (including sub-urban and urban). Including pollutant run off risk and leaching potential and river status of rivers sampled. See Lily *et al.* (2018) for full explanation of classes for leaching potential of pollutants into surface waters. See Lily and Baggaley (2018) for explanation on run off risk of potential pollutants into water courses. See SEPA Water Classification Hub for River Status.

Site	Site code	Hydrometric area	SAC status	Catchment size (km)	Urban land cover (%)	Pollutant run off risk	Pollutant leaching	River status
1	1TW	Tweed	SAC	3330	1.2	Low	High (H1)	Moderate
2	2TW					High	Low (L1)	Moderate
3	3TW					Low	High (H1)	Moderate
4	4TW					Medium	Low (L)	Moderate
5	5TW					Medium	High (H1)	Moderate
6	6TW					Medium	High (H1)	Moderate
7	1TEI	Teith	SAC	517.7	0.4	Medium	High (H1)	Moderate
8	2TEI					Medium	High (H1)	Moderate
9	3TEI					Medium	High (H1)	Moderate
10	4TEI					Medium	High (H1)	Moderate
11	5TEI					Medium	High (H1)	Moderate
12	6TEI					Medium	High (H1)	Moderate
13	1WOL	Water of Leith	No	107	21.1	n/a	Urban (H)	Poor
14	2WOL					n/a	Urban (H)	Poor
15	3WOL					n/a	Urban (H)	Poor
16	1CLY	Clyde	No	1903	7.3	n/a	Urban (H)	Poor
17	2CLY					n/a	Urban (H)	Poor
18	3CLY					n/a	Urban (H)	Poor

Appendix B Table 3. Site features and visible plastic contamination present at sampling sites.

Site	Site code	Site features	Macro plastic
1	1TW	Obvious raw sewage outflow into river, industry effluent directly into river	Sewage contamination visible in water, including wet wipes and macroplastic
2	2TW	On left side of central island	Tyres in water near deposition area
3	3TW	High amounts of gravel, low areas of deposition	Not obvious
4	4TW	Confluent between two rivers	Litter (macroplastic) hanging from trees near water
5	5TW	Depositional area - downstream of WWTP discharge point	Not obvious
6	6TW	Directly below Coldstream WWTP - woody depositional area	Not obvious
7	1TEI	Woody debris, deposited sediment low	Micro and macroplastic in debris
8	2TEI	Woody debris, deep depositional area, polystyrene takeaway containers in bank hanging above	Polystyrene packaging
9	3TEI	High human use - walkers, dogs, litter, residential/picnic waste	Residential waste and litter (macroplastic)
10	4TEI	Fast following section of the river, large amount of lamprey in depositional woody area - middle section of river	Not obvious
11	5TEI	Lade off main stem of river, lamprey in woody debris on right side of the bank	Large industrial plastic bags in river
12	6TEI	Depositional area - woody	Litter (macroplastic)
13	1WOL	Below green pipe, woody debris and deposition	Not obvious
14	2WOL	Eddy by a depositional and accumulation area (plastic + sediment)	Litter in the form of plastic bottles, bags etc
15	3WOL	Marginal site over bedrock	Not obvious
16	1CLY	High water levels, 4 sites with steep banks but very good habitat and therefore larvae numbers	Obvious signs of contamination from the WWTP, litter, wet wipes, plastic bags etc.
17	2CLY	Marginal site, high sand load but little organic matter and low numbers of lampetra	Synthetic matting along the sides of the bank. Obvious signs of WWTP upstream and litter along the bank and in the river.
18	3CLY	Good depositional habitat but low organic matter, high velocity of river indicated by erosion of the bank	Urban litter, ubiquitous presence of microplastic

Appendix B Table 4. Lamprey larvae median length and weight at each site sampled.

Site	Site code	Median larvae length ($\pm$ SD)	Median larvae weight ($\pm$ SD)
1	1TW	109.05 $\pm$ 14.33	2.03 $\pm$ 0.73
2	2TW	113.5 $\pm$ 14.82	2.40 $\pm$ 0.99
3	3TW	127 $\pm$ 11.14	2.91 $\pm$ 0.66
4	4TW	118 $\pm$ 5.99	2.44 $\pm$ 0.40
5	5TW	118.5 $\pm$ 8.49	2.49 $\pm$ 0.55
6	6TW	120 $\pm$ 8.92	2.54 $\pm$ 0.43
7	1TEI	96 $\pm$ 6.12	1.25 $\pm$ 0.22
8	2TEI	108 $\pm$ 12.75	1.93 $\pm$ 0.63
9	3TEI	101 $\pm$ 14.70	1.32 $\pm$ 0.60
10	4TEI	98.5 $\pm$ 16.67	1.35 $\pm$ 0.77
11	5TEI	102 $\pm$ 11.26	1.46 $\pm$ 0.48
12	6TEI	108 $\pm$ 8.42	1.64 $\pm$ 0.40
13	1WOL	124 $\pm$ 14.81	2.70 $\pm$ 0.85
14	2WOL	111.5 $\pm$ 12.17	2.05 $\pm$ 0.70
15	3WOL	110.5 $\pm$ 12.65	1.98 $\pm$ 0.74
16	1CLY	130 $\pm$ 14.23	2.90 $\pm$ 1.10
17	2CLY	102.5 $\pm$ 18.56	1.74 $\pm$ 1.02
18	3CLY	113.5 $\pm$ 15.62	2.21 $\pm$ 0.69

Appendix B Table 5. Limit of detection (LOD) and limit of quantification (LOQ) values for given polymer type calculated from blank (negative control) samples (average calculated from three per matrix). Polymer types not shown below were not detected in the blank samples.

	Synthetic Polymer Type													
	PP	PE	PVC	PU	PET	PS	PA	PMMA	CA	EVAc	EVOh	PAN	PBT	PLA
Lamprey														
Average	40.00	14.00	0.67	0.33	0.33	0.33	0.00	14.00	0.00	1.00	0.00	3.00	0.00	0.33
Standard dev.	50.25	5.72	0.47	0.47	0.47	0.47	0.00	9.09	0.00	1.41	0.00	2.94	0.00	0.47
LOD	205.81	32.86	2.22	1.89	1.89	1.89	44.00	5.67	0.00	12.71	0.00	1.89	0.00	3.78
LOQ	542.46	71.15	5.38	5.05	5.05	5.05	104.92	15.14	0.00	32.44	0.00	5.05	0.00	10.09
Sediment														
Average	1.30	0.70	0.03	0.07	0.07	0.10	0.00	1.77	0.00	0.20	0.17	0.17	0.53	0.00
Standard dev.	0.90	0.65	0.05	0.05	0.09	0.08	0.00	2.22	0.00	0.08	0.09	0.09	0.54	0.00
LOD	2.96	2.14	0.19	0.22	0.38	0.37	9.08	0.47	0.48	0.31	2.33	0.00	0.19	0.38
LOQ	8.98	6.48	0.50	0.54	1.01	0.92	23.94	1.02	1.11	0.94	5.97	0.00	0.50	1.01

Appendix B Table 6. Microplastic size characteristics in paired sediment and lamprey larvae samples.

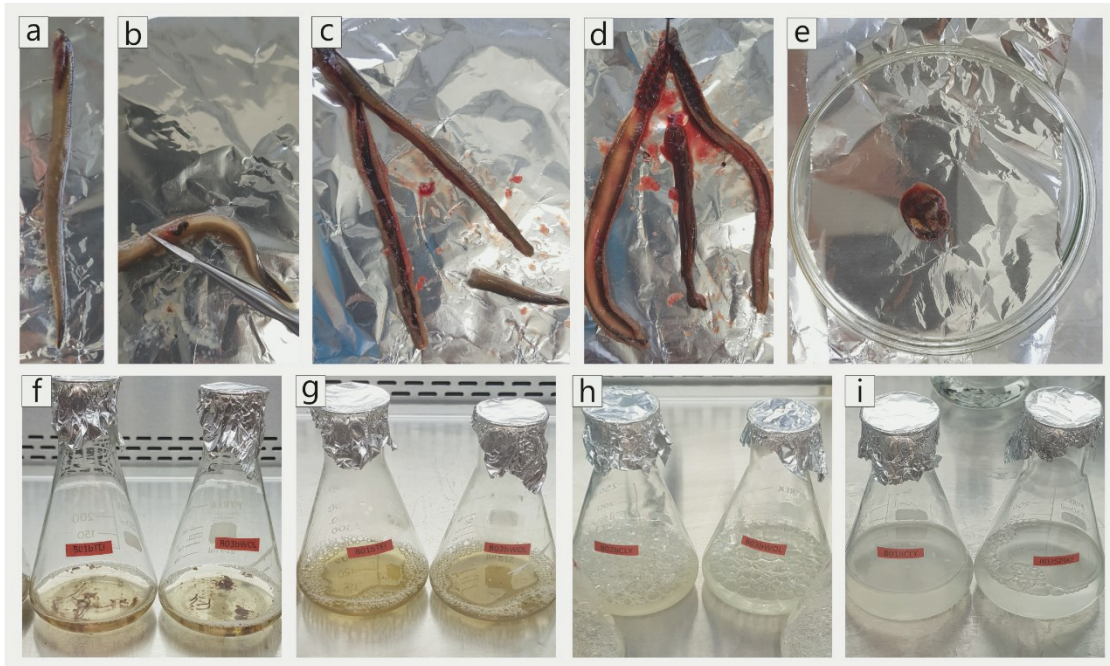
MP characteristic (um)	Sediment	Lamprey
Mean area	3695.54 ± 2343.66	3647.04 ± 1667.75
Mean length	71.54 ± 22.72	71.21 ± 16.26
Mean width	46.44 ± 13.83	47.46 ± 10.63
Min area	1971.53	2157.66
Max area	7595.27	6578.94
Min length	47.64	49.57
Max length	109.34	103.67
Min width	34.32	35.98
Max width	69.74	67.10

Appendix B Table 7. Calculations for simplified ellipsoid volume of the microplastic particles within the intestine of a lamprey larvae, based on equation 3 in Kooi and Koelmans (2019) and equation 4 in Koelmans *et al.* (2020), see further information in text below. As most particles were identified as fragments, a *W:H* ratio of 1 was assumed.

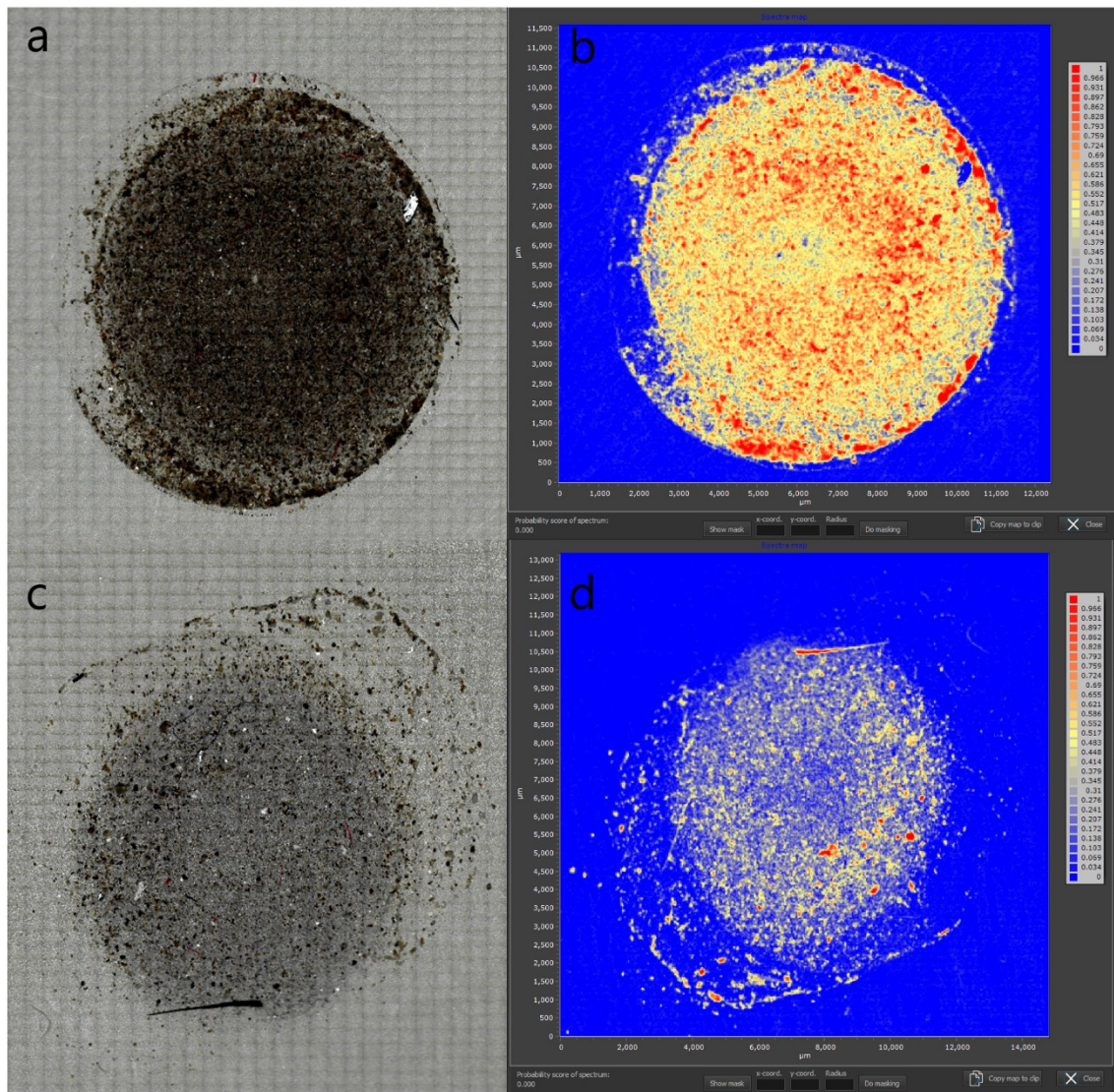
Site	CSF	Particle volume (μm ³)	Total volume of particles (μm ³)
SAC	0.798748429	85009.95336	320487.5242
URBAN	0.830060239	123740.5278	1090154.05
All sites	0.815332563	103217.3404	649237.0714

Appendix B Table 8. Calculations for space displaced from microplastic ingestion in the intestine of lamprey larvae. All values are expressed in microns (μm).

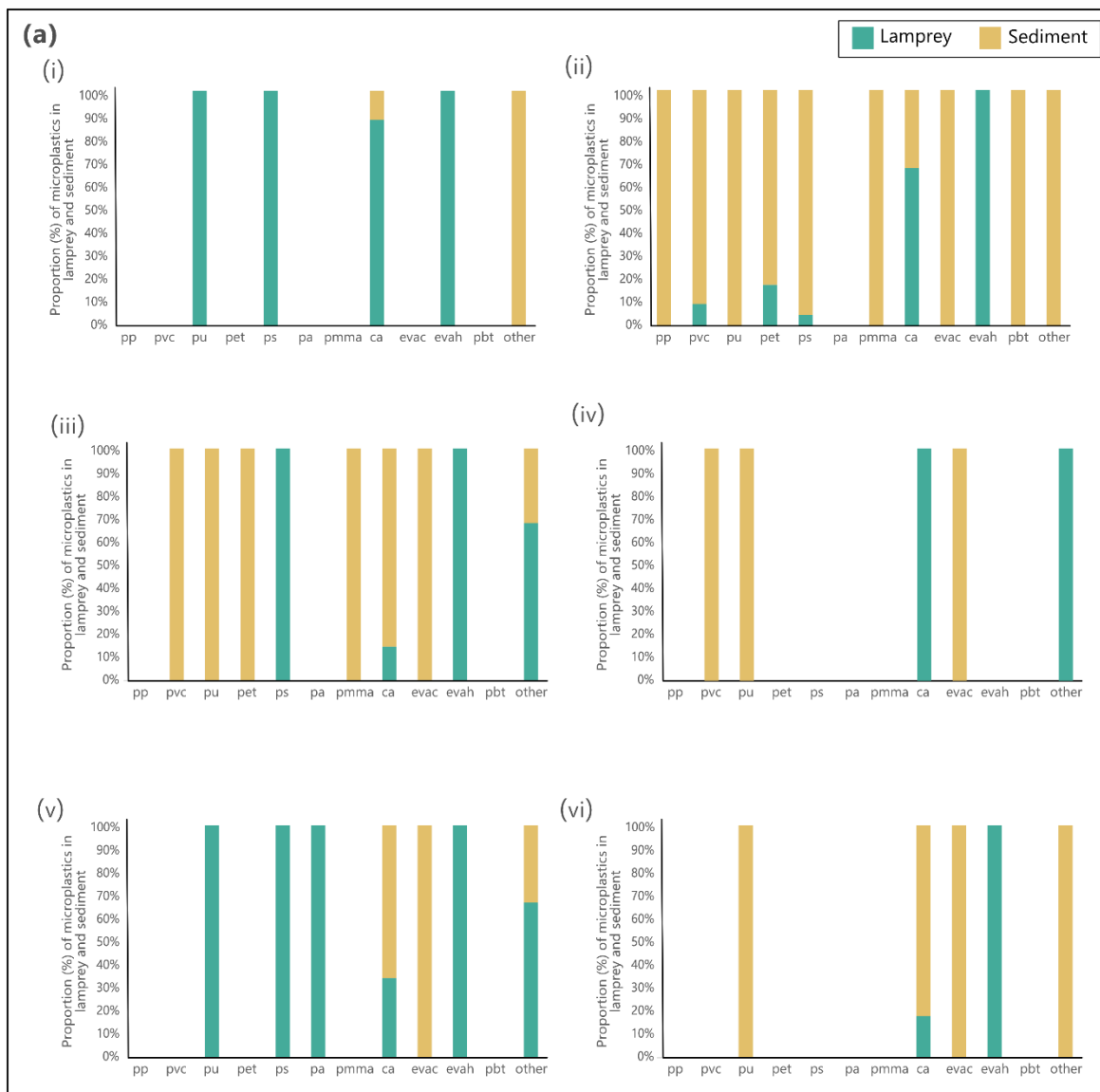
Length of intestine (μm)	Max length lamprey	Min length lamprey	Average length lamprey
SAC	56800	30400	43953
URBAN	62400	31200	46467
All sites	56800	30400	45210
Volume of intestine (μm^3)	Max length lamprey	Min length lamprey	Average length lamprey
SAC	1712280897192	4312621315	6235331652
URBAN	8852222700	4426111350	6591879510
All sites	8057792457	4312621315	6413605581
Volume remaining in intestine (μm^3)	Max length lamprey	Min length lamprey	Average length lamprey
SAC	1712280576705	4312300828	6235011164
URBAN	8851132546	4425021196	6590789356
All sites	8057143220	4311972078	6412956344
Min	1712280853837	4312577960	6235288297
Max	8847667811	4421556461	6587324621
% intestine volume displaced by microplastics	Max length lamprey	Min length lamprey	Average length lamprey
SAC	0.00002	0.00743	0.00514
URBAN	0.01232	0.02463	0.01654
All sites	0.00806	0.01506	0.01012
Min (B01TEI)	0.00000	0.00101	0.00139
Max (B01CLY)	0.05147	0.10296	0.13829



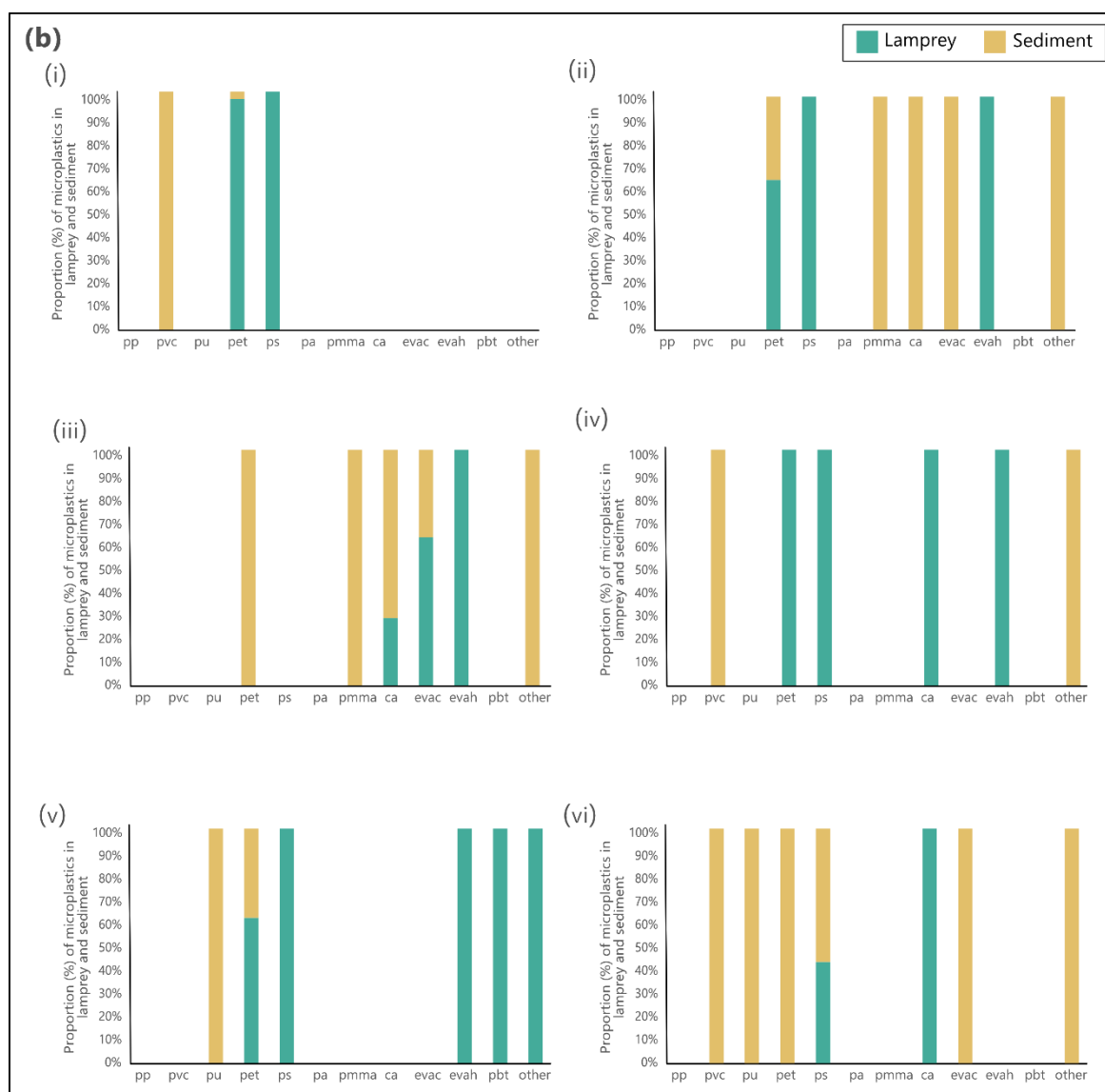
Appendix B Figure 2 a-i. Process to isolate microplastics for μ FTIR analysis, includes dissection of gastrointestinal tract (GIT) of lamprey larvae (a-e, followed by an oxidative method to reduce organic matrix (f-i). a) intact deforested larval lamprey, b) faeces removal, c) removal of caudal fin and animal cut dorsally down the centre line from eye spot, d) GIT removed intact, including tissue from the gills and mouth, e) final combined GIT tissue after dissection, f) GIT combined with sodium dodecyl sulphate, g) tissue reduction following 24 hr incubation, h) tissue reduction following 24h incubation with hydrogen peroxide (H₂O₂), i) final sample before filtration following 24h incubation with H₂O₂. Note successful reduction in dark organic particles from (f) to final sample (i).



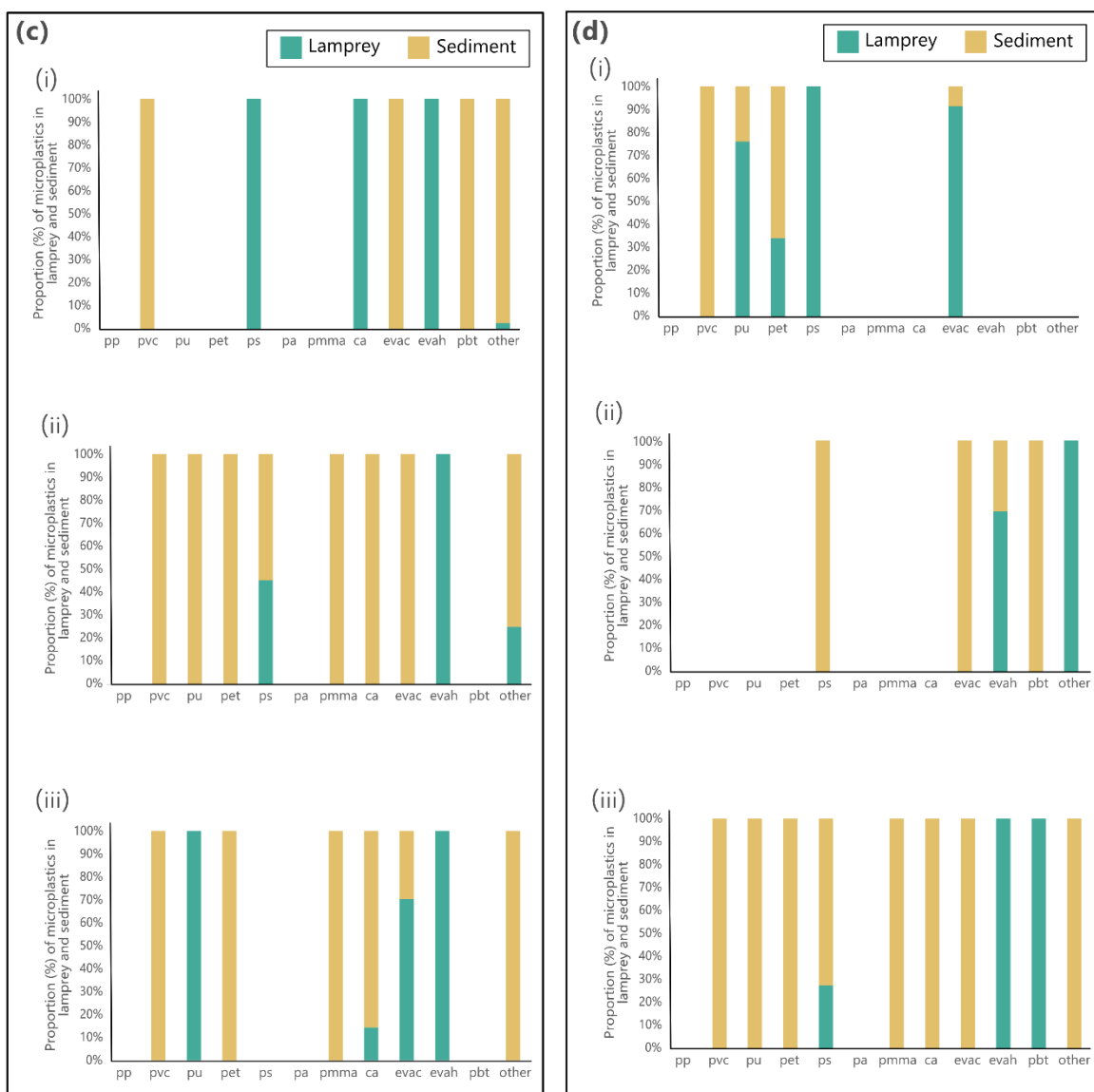
Appendix B Figure 3 Comparison of 1g wet gastrointestinal tract tissue as 100% sample deposited on silver-membrane filter (a) and in heatmap form (b), and 0.5g wet gastrointestinal tract tissue as 100% sample deposited on silver-membrane filter (c) and in heat map form (d).



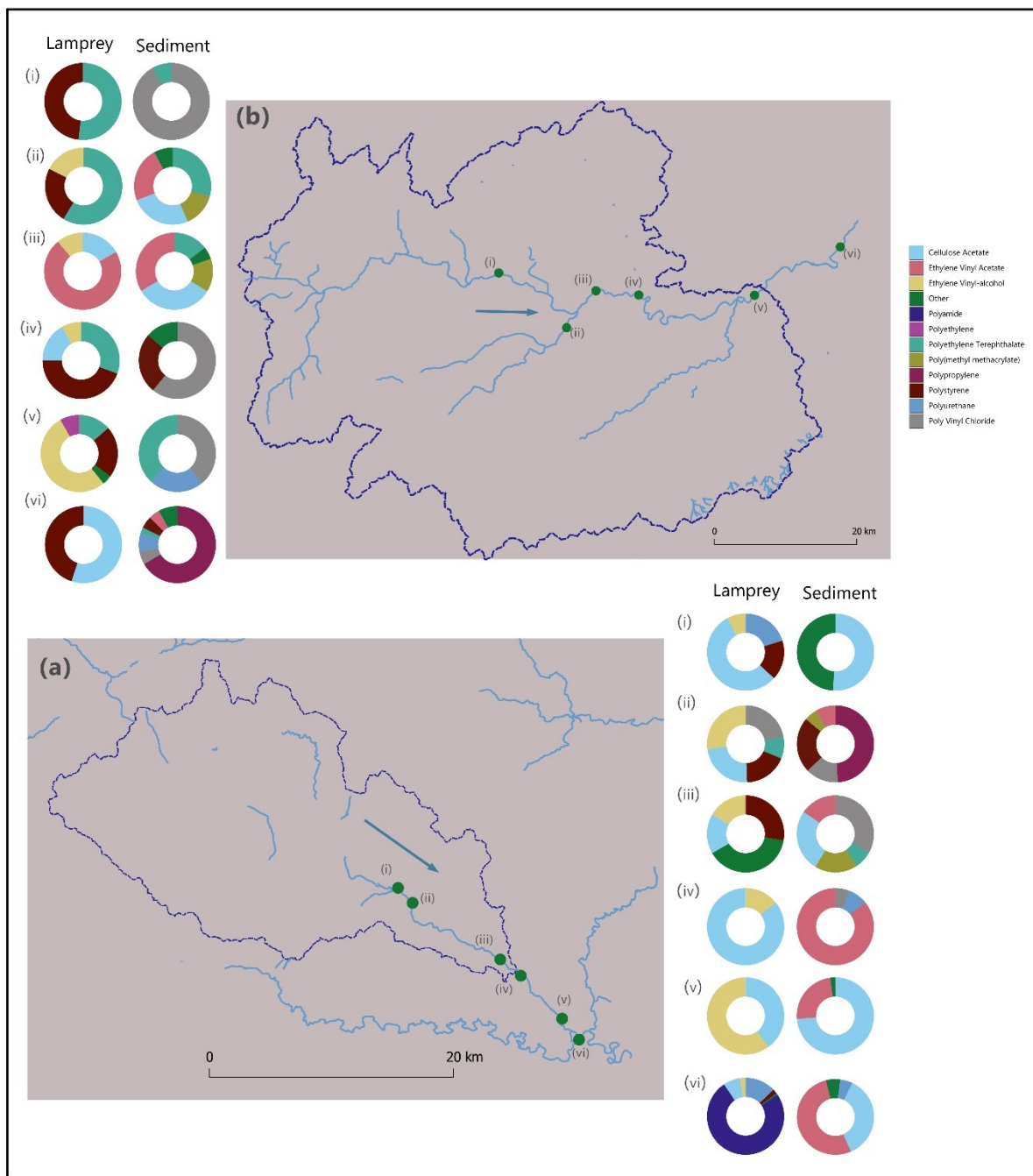
Appendix B Figure 4 Polymer types of detectable microplastics present in lamprey tissues and paired sediment samples from riverine sampling sites located in a Special Area of Conservation on the River Teith (ai-vi) in Scotland, UK. Sites are presented upstream (i) to downstream (vi). Yellow denotes sediment samples and green denotes lamprey samples. Polymer types included in 'other' include PLA, PBT, PAN and ABS (refer to full polymer names in main text and supplementary material).



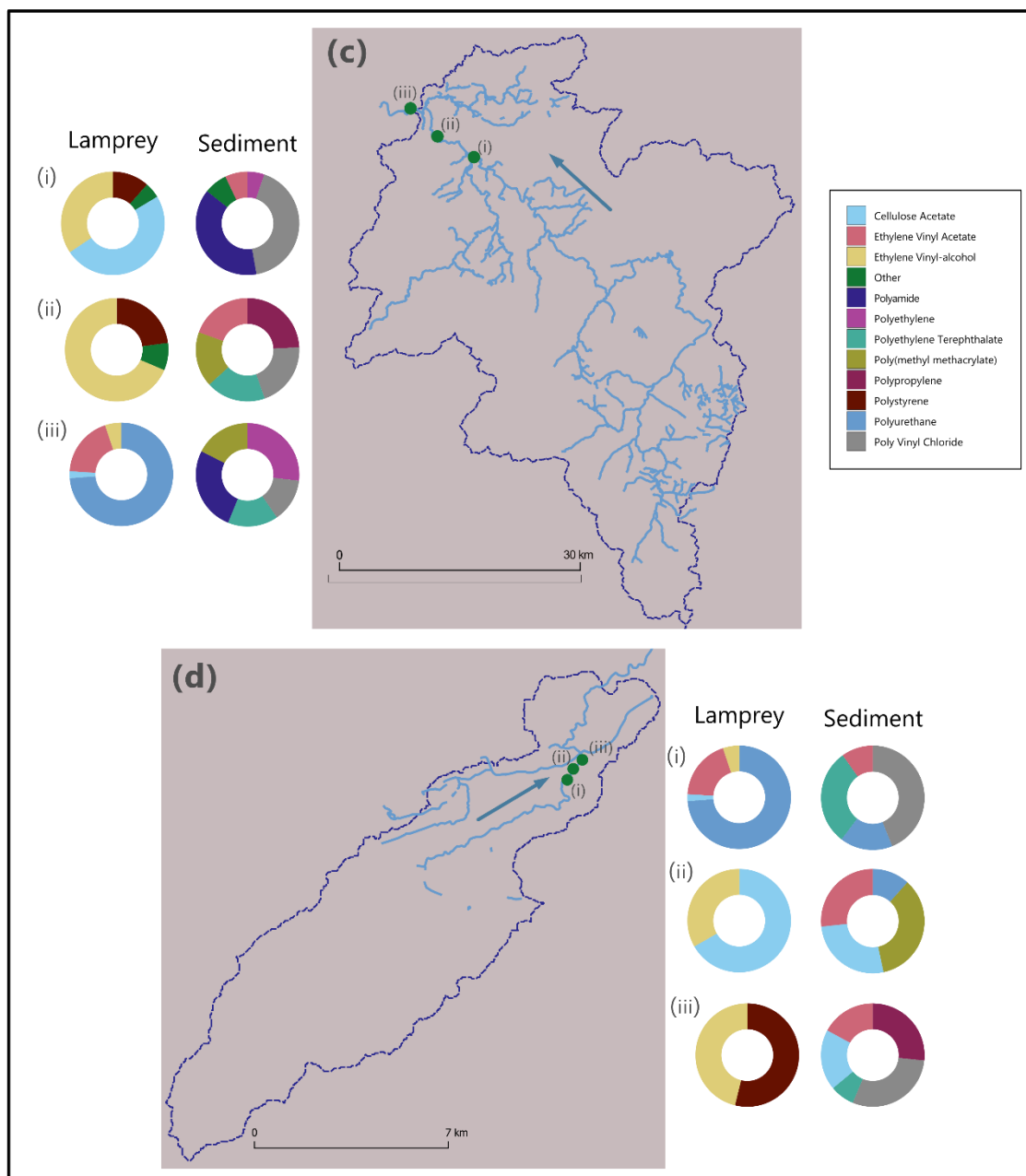
Appendix B Figure 5 Polymer types of detectable microplastics present in lamprey tissues and paired sediment samples from riverine sampling sites located in a Special Area of Conservation on the River Teith (ai-vi) in Scotland, UK. Sites are presented upstream (i) to downstream (vi). Yellow denotes sediment samples and green denotes lamprey samples. Polymer types included in 'other' include PLA, PBT, PAN and ABS (refer to full polymer names in main text and supplementary material).



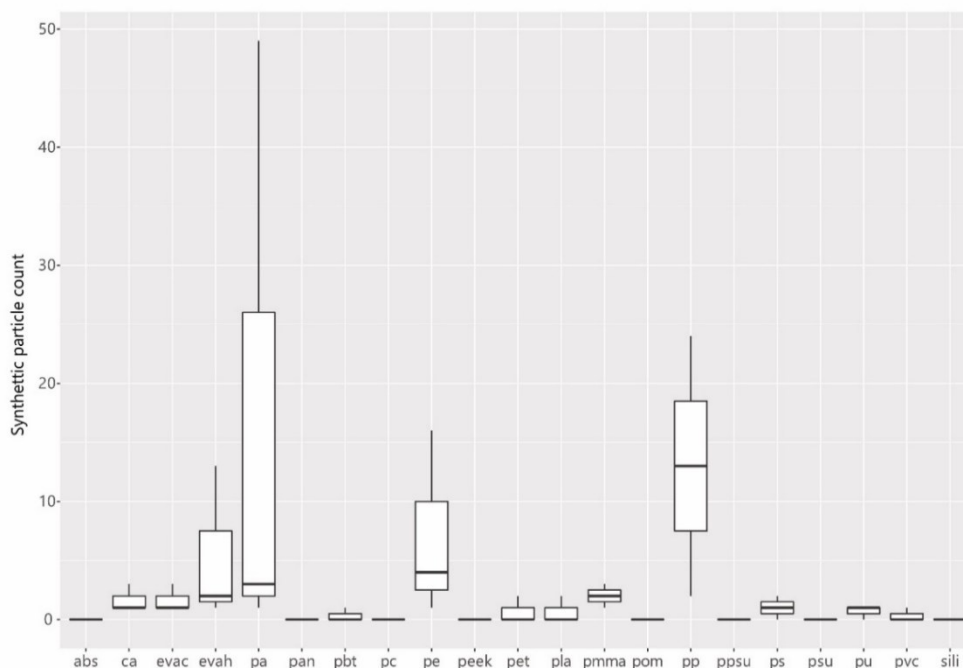
Appendix B Figure 6 Polymer types of detectable microplastics present in lamprey tissues and paired sediment samples from riverine sampling sites located on the River Clyde (ci-iii) and Water of Leith (di-iii) in central Scotland, UK. Sites are presented upstream (i) to downstream (iii). Yellow denotes sediment samples and green denotes lamprey samples. Polymer types included in 'other' include PLA, PBT, PAN and ABS (refer to full polymer names in main text and supplementary material).



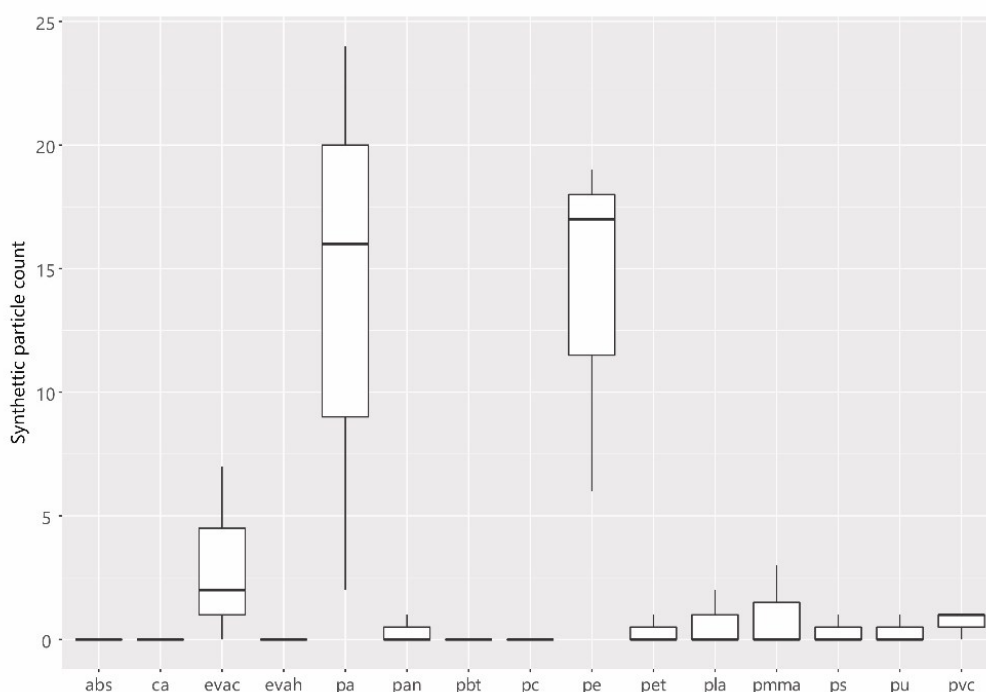
Appendix B Figure 7 Proportions of microplastic polymers present in lamprey tissues and paired sediment samples from riverine sampling sites located in Areas of Special Conservation on the River Teith (ai-vi) in Scotland, UK. Sites are presented upstream (i) to downstream (vi). Sites are presented upstream (i) to downstream (vi), as shown by direction of arrow.



Appendix B Figure 8 Proportions of microplastic polymers present in lamprey tissues and paired sediment samples from riverine sampling sites (green dots) located on the River Clyde (ci-iii) and Water of Leith (di-iii) in central Scotland, UK. Sites are presented upstream (i) to downstream (iii), as shown by direction of arrow.



Appendix B Figure 9 Box and whisker plots detailing the microplastics particle count and polymer types identified in negative controls (matrix matched blanks) ran alongside sediment samples. The average particle number for each polymer type identified in all blanks were subtracted from the number of particles identified in sediment samples to account for background contamination levels.



Appendix B Figure 10 Box and whisker plots detailing the microplastics particle count and polymer types identified in negative controls (matrix matched blanks) ran alongside lamprey samples. The average particle number for each polymer type identified in all blanks were subtracted from the number of particles identified in GIT samples to account for background contamination levels.

Further information on polymer proportions and abbreviations

Biota: ethylene vinyl acetate (EVAc; 12.18%, $0.93\text{-}0.95\text{ g cm}^{-3}$), ethylene vinyl-alcohol (EVOH; 11.99%), polystyrene (PS; 9.14%), polyethylene terephthalate (PET; 4.63 %, $1.34\text{-}1.39\text{ g cm}^{-3}$), polyvinyl chloride (PVC; 1.08%, $1.16\text{-}1.4\text{ g cm}^{-3}$), acrylonitrile butadiene and polybutylene terephthalate (ABS & PBT 0.95% each) and polycarbonate (PC; 0.19%). Sediment: EVAc (9.77%), PS (6.257%), Poly(methyl methacrylate) (PMMA; 6.40%, 1.19 g cm^{-3}), PET (5.96%), CA (5.63%), PE (5.19%), PBT (3.11%), PC (2.97%), poly lactic acid (PLA; 2.33%), , PU (2.21%), poly-acrylonitrile (PAN; 1.60%), acrylonitrile butadiene (ABS; 1.57%), polysulfone (PSU; 1.27%), poly oxyethylene (POM; 0.31%), poly phenylene sulfone (PPSU; 0.19%), and silicone (Si; 0.07%).

Further polymer abbreviations: https://www.distrupol.com/polymer_abbreviations.html

Further information on combined sewage overflows

Mapped locations of Combined Sewage Overflows (CSO's) by Forth River Trust
<https://forthrivertrust.org/sewage-outfalls-around-the-firth-of-forth/>

Further information on Table 7 and Table 8

To estimate the total volume of space that ingested microplastic particles displace within the intestine of lamprey larvae, the following methods were used. Firstly, the average internal diameter of the intestine was calculated from Hilliard, Bird and Potter (1983). This value was used to calculate the volume of the intestine, assuming a cylindrical shape and 40% of the total length of the larvae. To calculate the simplified ellipsoid volume of the microplastic particles, firstly the Corey shape factor distribution was calculated based on equation 3 from

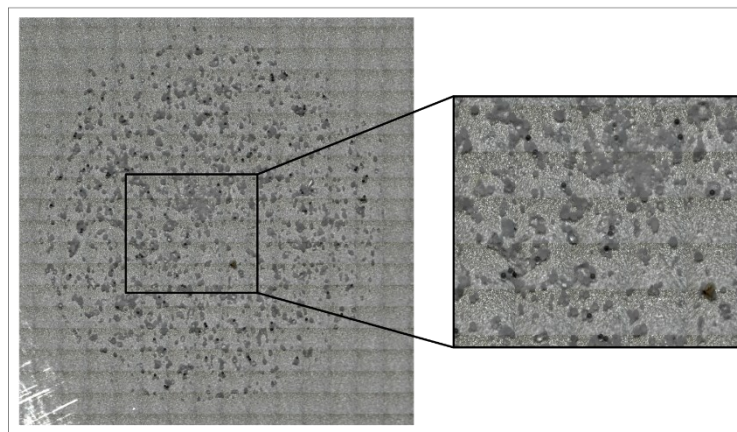
Kooi and Koelmans (2019). This was then used together with the average particle length and particle count to give a final volume of particles, using equation 4 from Koelmans *et al.* (2020).

Equation 3 (Kooi and Koelmans, 2019)

$$CSF = \frac{H}{\sqrt{LW}}$$

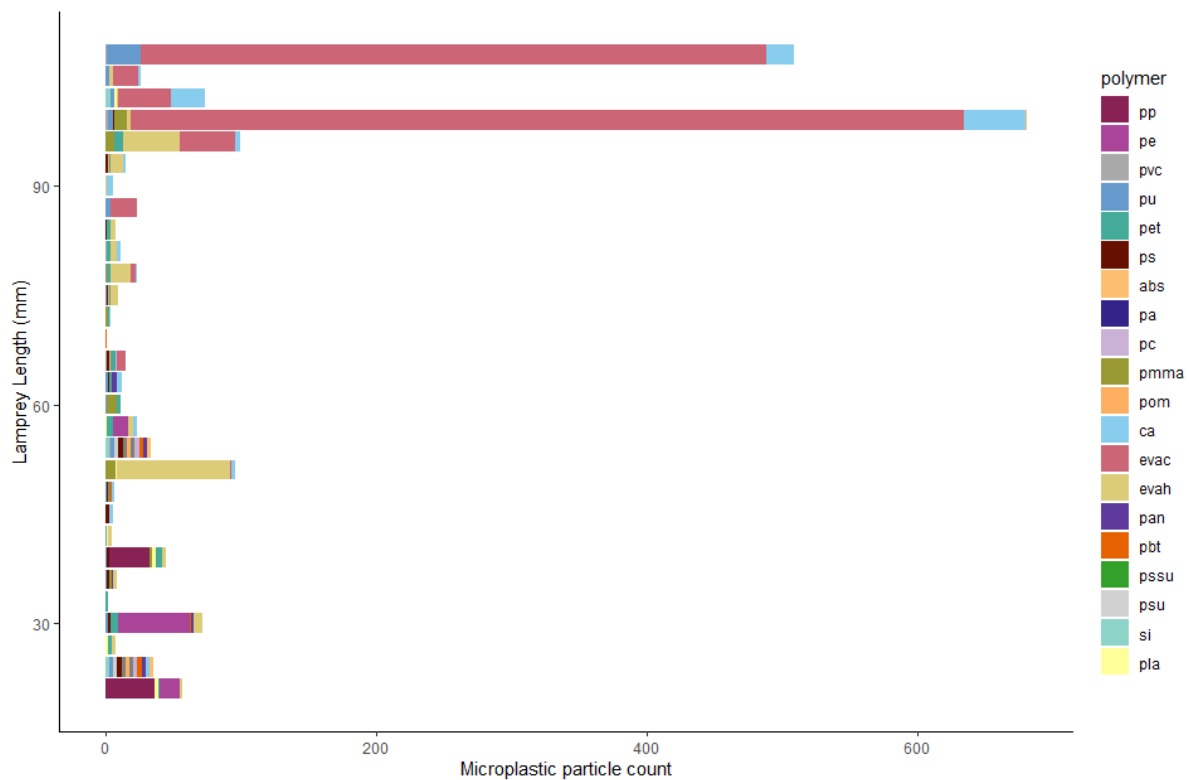
Equation 4 (Koelmans *et al.*, 2020)

$$V_{EL,i}^S = \frac{\pi}{6} L_i^3 \times CSF_i^2$$

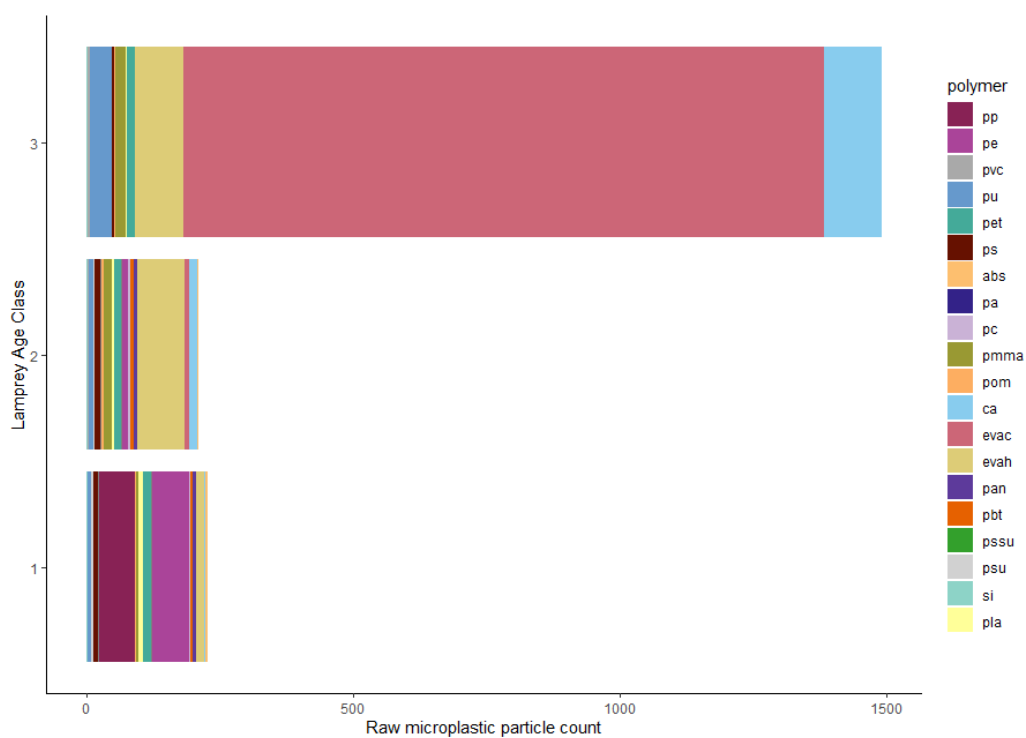


Appendix B Figure 11 Image of stock WS05 spike microplastic particles used to determine recovery rate in matched matrix samples (positive controls). Light irregular polyamide (PA) particles can be identified in contrast to the small regular dark sphere of polystyrene (PS). Refer to main text for recovery rate, density, and size of spiked particles.

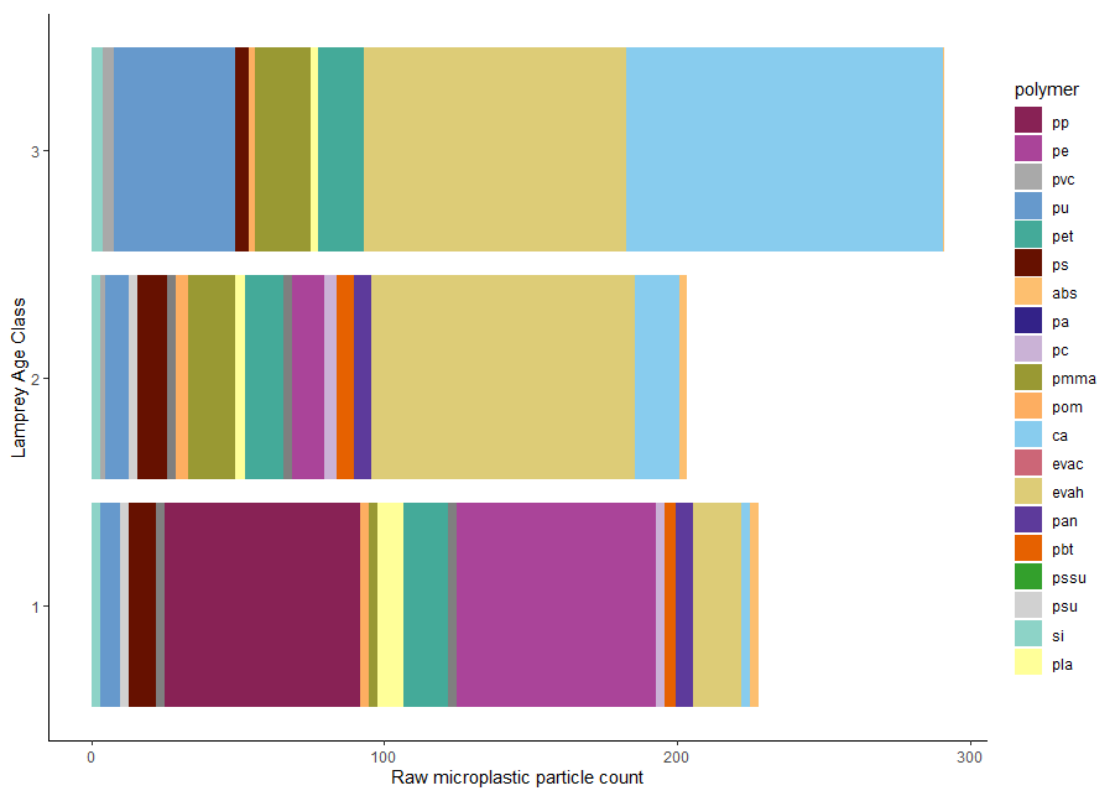
Appendix C - Does age (size) have an impact on microplastic uptake/accumulation in lamprey larvae?



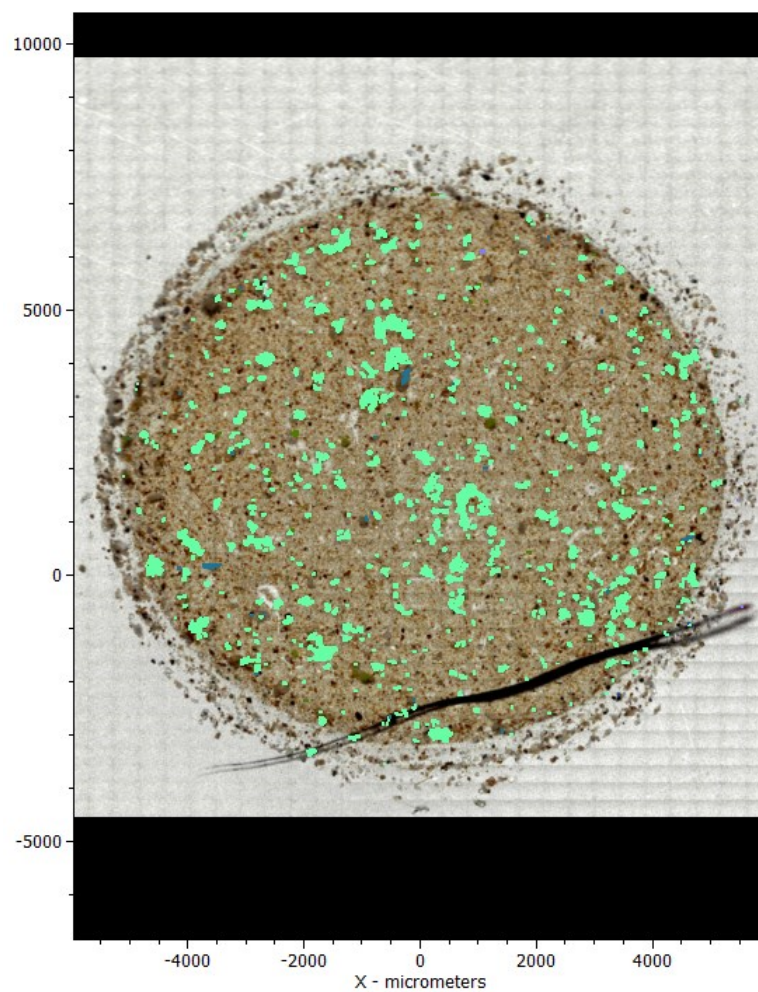
Appendix C Figure 1 Microplastics count of each polymer type with increasing lamprey length (mm). EVAc retained.



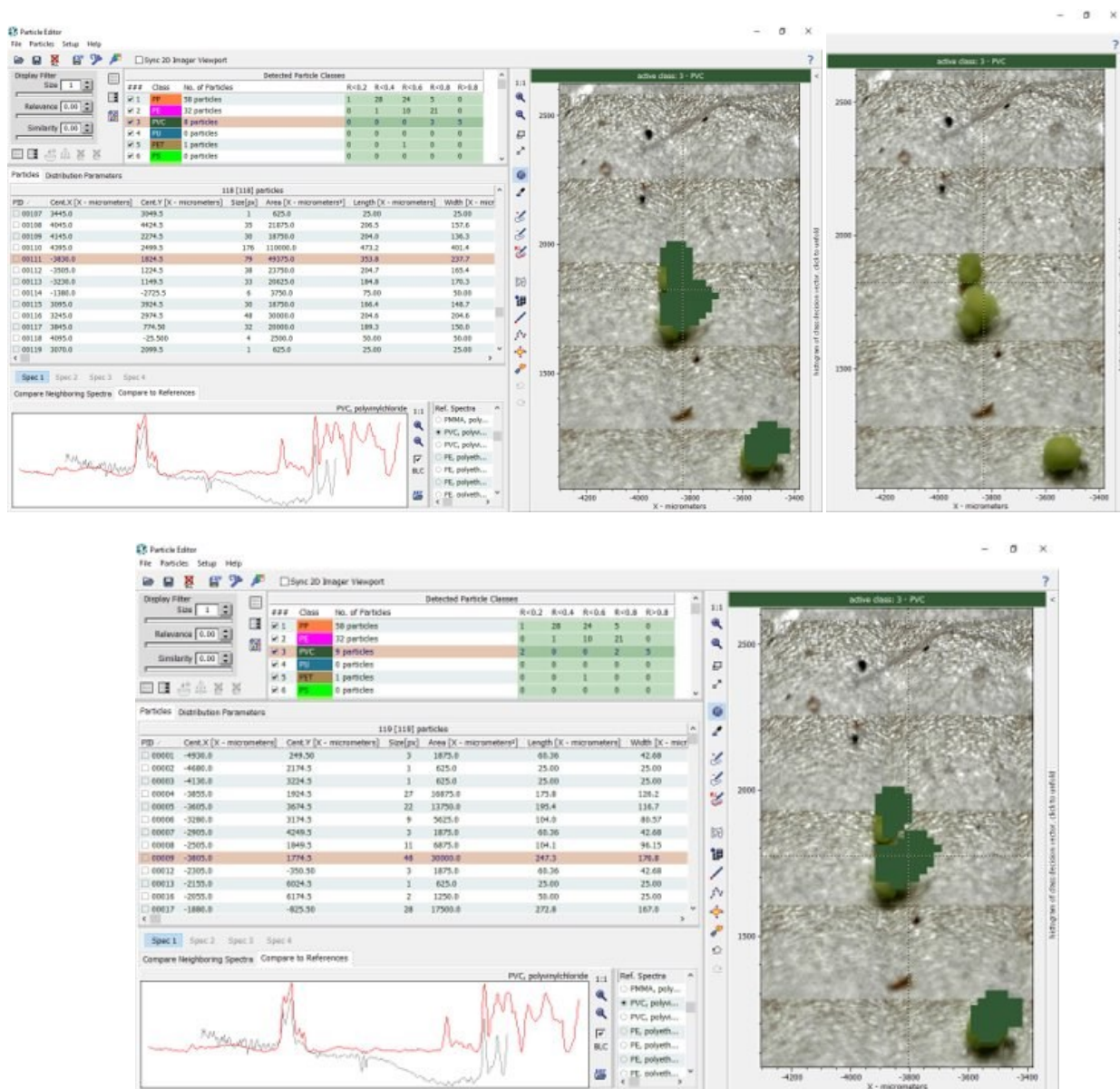
Appendix C Figure 2 Raw microplastic particle count with lamprey larvae categorised into age classes with synthetic polymer types (evac polymer retained).



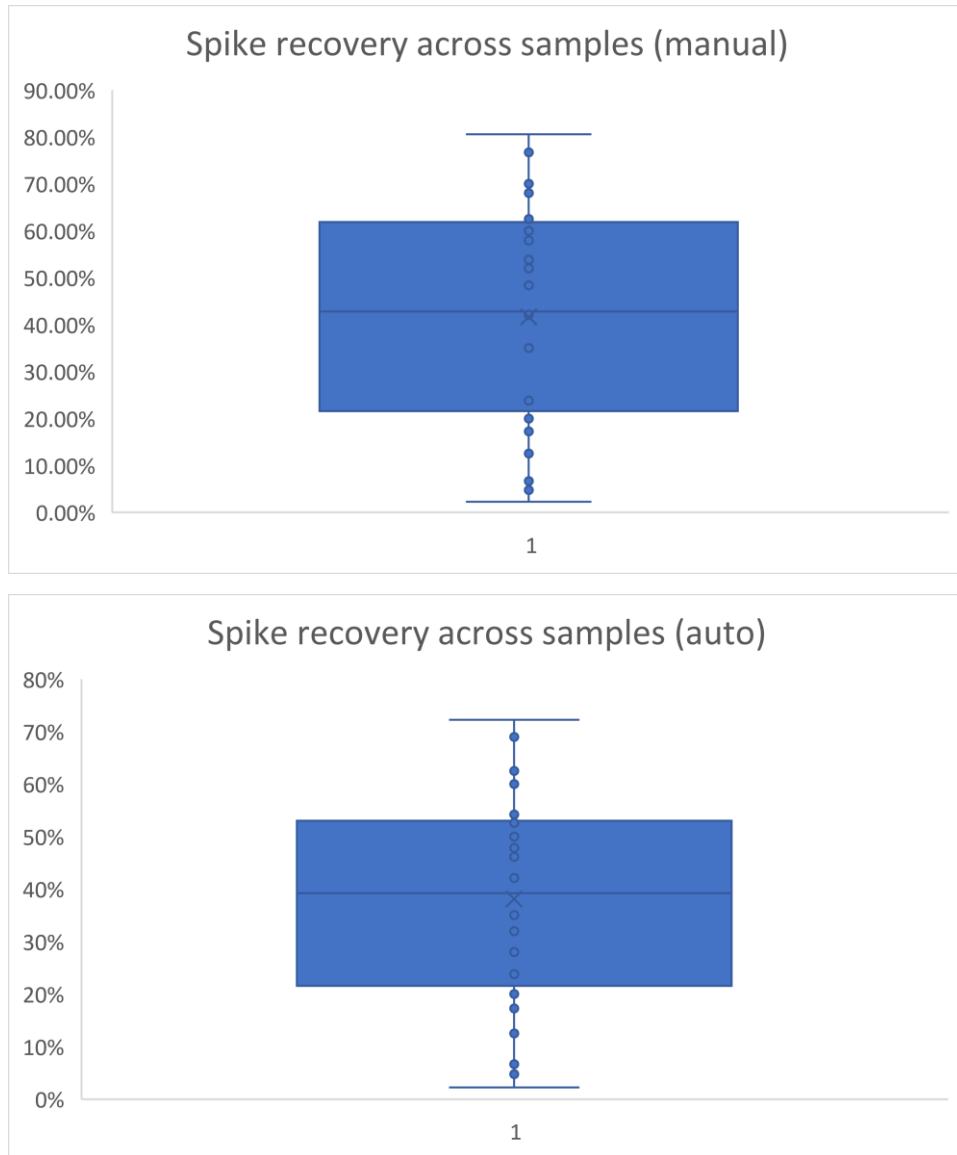
Appendix C Figure 3 Raw microplastic particle count with lamprey larvae categorised into age classes with synthetic polymer types (without polymer evac).



Appendix C Figure 4 Sample from the largest larvae of 108 mm in body length in which EVAc particles are highlighted in green on the microplastic visible map from the μ FTIR scan.

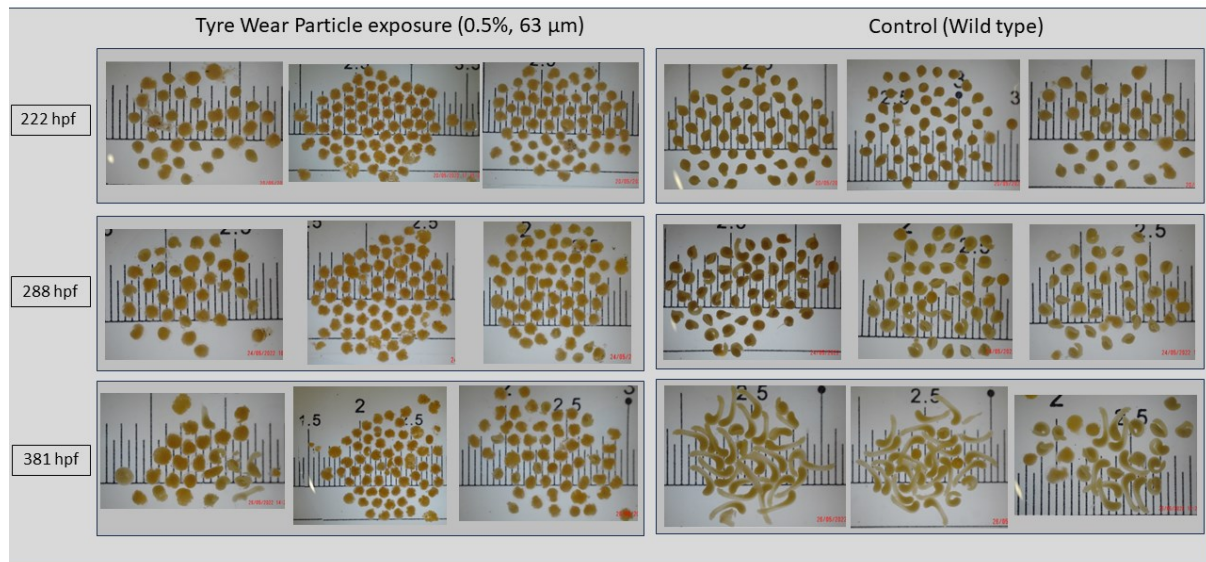


Appendix C Figure 5 Example of manual particle edits for PVC spike particles in samples. Microplastic particle maps and spectra were used to visually check samples and edit were required.

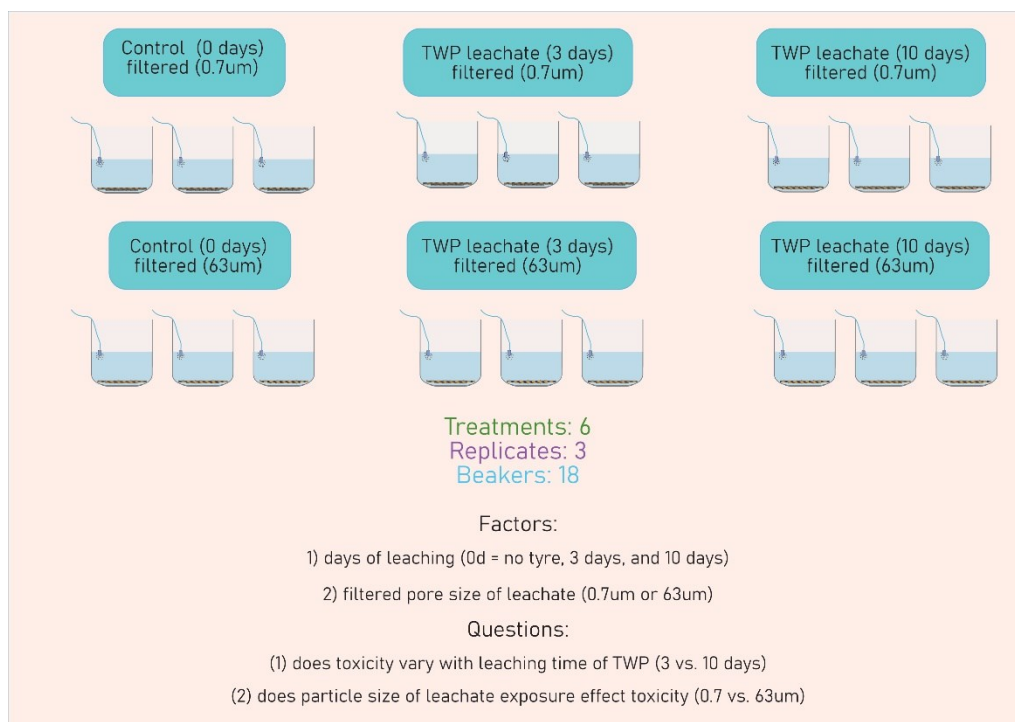


Appendix C Figure 6 Comparison of auto and manual spike recovery across samples.

Appendix D - Developmental Toxicity of Recycled Tyre Wear Microplastics to Lamprey Larvae *Lampetra planer*



Appendix D Figure 1 Phenotypic comparison of wildtype and treatment *Lampetra planeri* embryos from 222- 381 hours post fertilisation (hpf).



Appendix D Figure 2 planned experimental design for developmental toxicity experiments which did not take place due to lack of embryos.

Developmental Toxicity of Microplastic Leachates on Lamprey (*Lampetra planeri*)

Planned Standard Operating Procedures

Context: This project forms part of NERC funded IAPETUS studentship (2019-2023), work based at SCENE (Glasgow University facilities on Loch Lomond). This SOP is a workflow to observe the morphology of key developmental tissue of embryonic lamprey, specifically neural tissue, microvilli and cilia, and to compare wildtype larvae to those exposure to microplastic leachates.

Aim: The aims of this study are to determine and compare the toxicity of leachates from new and aged tyre wear particles to the development of key embryonic tissue structures of the brook lamprey (*Lampetra planeri*). The present assesses key tissue groups by labelling with antibodies against acetylated tubulin, serotonergic neurons (5-HT) and phalloidin. Abundance, connectivity and organisation of key tissue structures will be compared between treatment and control groups.

Method: Methods use wholemount immunohistochemistry on embryonic stages of the brook lamprey (*Lampetra planeri*) of both wild type (control) and microplastic exposed (treatment) embryos. Immunohistochemistry protocols based on Barreiro-Iglesias *et al.* (2008), Nikitina *et al.* (2009), Green *et al.* (2017), Yuan, York and McCauley (2018) and Suntres *et al.* (2019). Gamete collection, culture and fertilisation methods are based on Rodríguez-Muñoz & Ojanguran (2002), Meeuwing *et al.* (2005), Nikitina *et al.* (2009) and Lampman *et al.* (2021).

Antibodies:

Name	Host	Primary	Secondary	Dilution	Cost	Tissue labelling	Previous lit. (lamprey)
α -Tubulin	Mouse	Sigma, T 7451, clone 6-11B-1, AB_609894	Anti-Mouse IgG2b	1:1000 1:500*	£140 (25 μ l) + £200 (500 μ g)	Cilia	Barreiro-Iglesias <i>et al.</i> , 2008; Kusakabe <i>et al.</i> , 2020; Suntres <i>et al.</i> , 2018; *Green <i>et al.</i> , 2017
Anti-5-HT	Rabbit	Immunostar, 20080, AB_572263 Or abcam	Anti-Rabbit IgG	1:3000 1:500*	£315 (100 μ g) +£170 (500 μ g)	Serotonergic neurons	Barreiro-Iglesias <i>et al.</i> , 2008; Suntres <i>et al.</i> , 2018; *Green <i>et al.</i> , 2017
Phalloidin	-	Alexa 488 A12379	-	1:1000	£300	Actin, microvilli (cell shape)	Suntres <i>et al.</i> , 2018

Embryo exposure protocols:

Preparation of TWP leachates [methods based on Chibwe *et al.*, 2021]:

1. Incubate 5g/L (0.5%) of tyre particles in filtered loch water at 14°C in natural UV photoperiod for 10 days
2. Check water parameters
 - a. Temperature, conductivity, pH, dissolved oxygen and ammonia

Gamete collection [methods from Hume PhD thesis, Lampman *et al.*, 2021 and Meeuwing *et al.*, 2005]

1. Hand strip gametes from male and females
2. Position females over glass bowl with 1L freshwater at the same temperature as the animal
3. Force eggs out of the vent by squeezing the abdomen in a downward motion
4. Once quality of ova is determined to be good, repeat for males
5. Store any extra gametes in petri dishes to prevent air exchange of oxygen, preferably use gametes maximum of 1 hour after stripping
6. If needed milt and ova stored at 10-12 degrees for less than 6 hours

Embryo culture and fertilisation [methods based on Lampman *et al.*, 2021, Rodríguez-Muñoz & Ojanguran, 2002 and advice from Guillaume Evanno and John Hume]

Space: 2000 lamprey eggs/Litre water – 10cm min water height - temperature = 11°C - 15°C

1. Count approximately 200 eggs into separately labelled petri dishes and add 10-20 µl of milt to each dish
2. Gametes hand stirred for 10 seconds and left to stand for 9 minutes to allow fertilisation
3. Gametes rinsed several times with clean water to prevent later egg mortality
4. Transfer gametes (500 eggs per beaker) to a clean 1000ml beaker with either 500ml treated loch water (control x 6) or 500ml treated loch water with microplastic leachate (treatment x 6)
5. Beakers securely placed in a large tank with constant shallow water flow to maintain ambient loch water temperature, lighting should be darkness until larvae are in sediment when a natural photoperiod is used.
6. Replace culture water every week, remove dead embryos every 2 days and remove shells once hatched.
7. After 5 weeks (12 degrees) transfer larvae to 1L beaker with a 1-2cm sediment layer. The transfer is made when firstly the yolk reserve is fully resorbed and secondly the larvae are able to swim. Water change needs to be twice a week once larva have hatched.

Embryo Fixation

1. Remove sub-sample of approximately ~50 embryos every 2 days to monitor development and for fixation
2. Selected stages fixed with 4% paraformaldehyde 0.1 M phosphate buffer solution (PBS, pH 7.4) for further analyses (see fixation protocol below)

Time post fertilisation (tpf)	Stage	Description
78 hr	13	Blastophore
4 days	17	Neural plate
7 days	21	Head protrusion
10 days	24	Hatching**
16 days	27	Eye spots

***If fixing at stages T22, T23 and T24, remove fertilisation membranes from individual embryos with 2 pairs of sharpened forceps prior to hatching. Keeps A/P axis in same plane prior to hatching. Once embryos hatch after stage T24 this is no longer necessary since they will be straight.*

Fixation and live image counts:

Embryo fixation protocol (Nikitina *et al.*, 2009; Yuan, York and McCauley, 2018)

1. Make up 4% PFA in 1xPBS
2. Transfer ~2ml or 25 embryos in liquid from each beaker culture into 15ml falcon tube
3. Wait for embryos to sink ~5 minutes
4. Whilst wait for the embryos to sink, use a transfer pipette to remove ~20 embryos for imaging into a Eppendorf, no more than 1/3 volume of the tube
5. Remove as much loch water culture as possible without disturbing embryos
6. Add 1ml 4% PFA to falcon tube and 500µl into Eppendorf for 5 minutes
7. Remove the diluted fixative
8. Replace with fresh PFA and fix embryos for 1hr at RT or overnight at 4°C on a rocking platform
9. Wash embryos in PBST for 15 minutes. Repeat x 3 times
10. Wash embryos for 15 min each in:
 - a. 25% methanol in PBST
 - b. 50% methanol in PBST
 - c. 75% methanol in PBST
 - d. 100% methanol
11. Rinse embryos several times in methanol
12. Store at -20°C until blocking and antibody labelling

Live image counts

1. Live image counts and images on approximately 20 fixed embryos for each treatment under a light microscope obtained at specified time points to characterise embryo morphology.
2. Morphological abnormalities of embryos will be assessed under the microscope and classified on the basis of morphological features and timing of development compared to controls.

Immunohistochemistry (IHC) protocols:

Fluorescent immunohistochemistry and staining for key embryological features, namely neurogenesis (anti-serotonin), myogenesis (phalloidin and cilia (anti-acetylated tubulin) carried out on the fixed larvae.

Blocking and Antibody labelling protocol (Nikitina *et al.*, 2009; Green *et al.*, 2017)

1. Block endogenous peroxidase activity, treat embryos with 0.5% H₂O₂ in PBST(L) for 1 h at room temperature
2. Wash the embryos in PBST(L) three times for 15 min each.
3. To block nonspecific binding, incubate the embryos in 10% donkey serum in PBST(L) for 1 h at room temperature
4. Dilute the primary antibody specific for the protein of interest 1:500 in PBST(L). Incubate embryos in the diluted antibody overnight at 4°C
5. Wash five to eight times with PBST(L) for 1 h each.
6. Incubate overnight in PBST(L) at 4°C.
7. Dilute the HRP-conjugated goat anti-rabbit antibody 1:500 in PBST(L). Incubate the samples in the diluted antibody for 2 h (for embryo sections) or for 4 h (for whole-mount embryos) at room temperature
8. Remove unbound antibodies
 - i. Rinse five times in PBST(L)
 - ii. Wash overnight in PBST(L) at 4°C

Signal detection and Imaging (Nikitina *et al.*, 2009)

Signal detection

1. Dilute the DAB solution 1:20 in PBST(L)
2. Transfer the embryos to 15-mL tubes
3. Incubate in 10 mL of the diluted DAB solution for 30 min at room temperature
4. Add 3.3 µL of 30% H₂O₂ to the tube
5. Incubate for 30-45 min. Check regularly for colour development.
6. If colour does not develop after 30 min, replace the solution with fresh DAB/hydrogen peroxide mix
7. When sufficient colour formation has occurred, stop the reaction by washing the embryos in several changes of PBST(L)
8. Post-fix sections or whole-mount embryos in 4% paraformaldehyde for 20-30 min at room temperature.

Imaging

1. Rinse three to four times for 5 min each in PBST(L).
2. Process the embryos through a graded series of increasing concentrations of glycerol (25%, 50%, 75% in PBST(L)).
3. Photograph the embryos in 75% glycerol using confocal microscope

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Chapter 5 PPL and PIL:

Project Licence (PPL) number: PP0483054, Holder: Colin Ean Adams, Title: The roles of phenotype, genetics and environment on long term success in fish.

Personal Licence (PIL) number: I14436821, Holder: Flora Rendell-Bhatti, Granted: 09 Mar 2021

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