Scottish Natural Heritage
Research Report No. 984

Assessing lamprey populations in Scottish rivers using eDNA: proof of concept

Scottish Natural Heritage
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RESEARCH REPORT

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Assessing lamprey populations in Scottish rivers using eDNA: proof of concept

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RESEARCH REPORT

Summary

Assessing lamprey populations in Scottish rivers using eDNA: proof of concept

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Keywords

Environmental DNA (eDNA); qPCR (quantitative polymerase chain reaction); brook lamprey; river lamprey; sea lamprey; *Lampetra planeri*; *Lampetra fluviatilis*; *Petromyzon marinus*; monitoring; molecular biomonitoring; Article 17; Habitats Directive; Special Areas of Conservation (SAC).

Background

Scottish rivers support three species of lamprey: brook lamprey *Lampetra planeri*, river lamprey *L. fluviatilis*, and sea lamprey *Petromyzon marinus*. Five Scottish rivers are designated as Special Areas of Conservation (SAC) for one or more of the lamprey species and they are assessed every six years, although national assessments of undesignated watercourses are also required to provide data for Habitats Directive *Article 17* reports.

Lampreys are generally assessed using conventional handheld electrofishing apparatus. However, this technique is time consuming, costly, and sometimes yields low quality data. To establish distribution and abundance of the species, environmental DNA (eDNA) based surveys could represent a cost-effective and more accurate form of distribution data collection as shown by recent work on sea lamprey eDNA in Ireland (Gustavson *et al*., 2015). This report summarises the state-of-the-art in the practice, temporal aspects, and cost-effective approaches to explore how a national lamprey distribution survey could be undertaken using the collection and analysis of eDNA.

Main findings

- DNA sequences for *L. planeri* (776 sequences) and *L. fluviatilis* (243 sequences) were present in GenBank and have been investigated as potential targets for qPCR assay design.
- In agreement with previous studies, lamprey species (*L. fluviatilis* and *L. planeri*) were found to be too genetically similar to be discriminated using the available genetic information.
- A potential qPCR approach for detection of the *fluviatilis-planeri* complex that would complement the assay available for sea lamprey (Gustavson *et al*., 2015) is suggested, but a potential collaboration with Guillaume Evanno of the French National Institute for Agricultural Research [INRA], which has recently identified up to 40 (currently unavailable) single-nucleotide polymorphisms (SNPs) that could be used to discriminate between the *L. planeri* and *L. fluviatilis* ecotypes is highlighted.

- A period of three months is estimated for qPCR assay optimization.
- eDNA sampling should be undertaken 15, 50 and 100 m downstream of lamprey habitat.
- Both water and sediment samples should be collected in triplicate and from at least three locations along a river.
- Contamination can be minimized by using sterile equipment, cleaning the equipment between sampling points, and including blank samples.
- Sampling should be performed when the probability of detecting lamprey is highest, hence during migration and spawning seasons, and when the water body is in its normal hydrodynamic state.
- Several preservation methods are available, but the storage of filtered samples in Longmire buffer and sediments in sterile Falcon tubes at cold temperatures is recommended.
- Field validation is highly recommended in order to test the qPCR and other emerging protocols empirically in natural settings.
- Costings and suggestions for the best approaches for achieving economies of scale are discussed.

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1. INTRODUCTION

1.1 Lamprey distribution in Scottish catchments

The lampreys (family *Petromyzonidae*) belong to an ancient order of vertebrates, the Agnathans or *jawless fishes*. Lampreys occur in temperate waters in both the northern and southern hemisphere. There are 39 recognised species (Potter, 1980) of which three occur in the UK (Maitland, 2003). All three UK lamprey species, namely sea lamprey (*Petromyzon marinus* L.), river lamprey (*Lampetra fluviatilis* (L.)), and brook lamprey (*L. planeri* (Bloch)) are listed on Annex IIa of the Habitats Directive and Appendix III of the Bern Convention. The river lamprey is also listed on Annex Va of the Habitats Directive and Schedule 3 of The Conservation (Natural Habitats &c.) Regulations 1994. Annex II of the Habitats Directive lists species of community interest, the conservation of which requires the designation of Special Areas of Conservation (SACs). A number of rivers throughout the UK, known to support sustainable populations of lamprey, have now been designated as SACs. In the UK there are 19 SACs designated for sea lamprey, 17 for river lamprey and 17 for brook lamprey (McLeod *et al*., 2002). In Scotland, the network of lamprey SACs comprises the rivers Tay, Teith, Tweed, Spey and the Endrick Water (see Table 1); the last three sites are also designated as Sites of Special Scientific Interest (SSSI) for lamprey.

Table 1. Scottish river SACs and SSSIs designed for their lamprey populations.

River	SSSI	SAC
Endrick Water	*L. planeri* *L. fluviatilis**	*L. planeri* *L. fluviatilis*
River Spey	*P. marinus*	*P. marinus*
River Tay		*L. planeri* *L. fluviatilis* *P. marinus*
River Teith		*L. planeri* *L. fluviatilis* *P. marinus*
River Tweed	*L. planeri* *L. fluviatilis* *P. marinus*	*L. planeri* *L. fluviatilis* *P. marinus*

*The Endrick Water supports two morphologically distinct forms of river lamprey. One which is truly andromous and migrates to sea before returning to freshwater to spawn and and another which migrates to Loch Lomond and completes the adult phase of its lifecycle before returning to the Endrick Water SAC to spawn.

The Habitats Directive (Article 11) explicitly requires Member States to implement surveillance of the conservation status of habitats and species of Community Interest. In the UK the condition of special interest features, such as lamprey spp. is determined through a six-yearly programme of Site Condition Monitoring [SCM]. The primary aim of this assessment is to establish whether each of the special features present within a designated site is likely to maintain itself under its current management regime.

Article 17 of the Habitats Directive differs from SCM in that it requires Member States to report on the conservation status of all species and habitats on its Annexes at a national level every six years. It also differs from Site Condition Monitoring in that the reporting format set out by the European Commission requires each Member State to provide information on specific parameters: (range, population, habitat for species and future prospects). Article 17 also asks Member States to provide longer-term trends for range, population, area, and habitat for each species. A national survey was last conducted in 2003/2004 (Ecological Research Associates, 2017). During the survey, 141 catchments throughout mainland

Research Associates, 2017). During the survey, 141 catchments throughout mainland Scotland, Orkney, Shetland, and the Western Isles were surveyed by electrofishing for larval lamprey.

The 2003/2004 survey confirmed lamprey presence in most rivers with previous records and identified 17 catchments with previously unknown populations. In total, lampreys were identified in 65 river catchments: sea lamprey were present in 13 catchments, river lamprey in 20, and brook lamprey in 51. Sea lamprey was the encountered species (Ecological Research Associates, 2017).

1.2 Traditional survey techniques

To date lamprey population condition assessments have focussed on the larval phase of the species which spend several years buried in fine sediment, often along channel margins. This phase is relatively easy to survey using conventional handheld electrofishing apparatus. However, electrofishing-based surveys are time consuming and costly and, because they are often undertaken in the autumn, may be hampered by inclement weather and high river levels. Data collected outwith optimal sampling periods may therefore be low quality.. Additionally, lamprey populations are likely to vary from one year to another as a result of, for example, changes in the quantity and quality of their habitat as a consequence of high flows and consequent sediment transport. There is also now a consensus forming amongst lamprey experts that relatively low numbers *of P. marinus* larvae are caught during electrofishing surveys because they may prefer sediment in deeper parts of the channel that cannot be reached safely by surveyors. The University of Stirling is currently investigating the use of airlift and suction pump samplers for lamprey surveys in deeper water (Bull *et al*., 2018). These techniques may yield better results, but their use for site assessments is also likely to be time consuming and costly, as well as being weather and flow dependent. A relatively new and promising approach for monitoring the distribution of aquatic species is the analysis of environmental DNA (eDNA) (Ficetola *et al.*, 2008; Foote *et al.*, 2012; Bohmann *et al*., 2014).

1.3 eDNA

Environmental DNA is DNA that has been released by an organism into the environment via faeces, hair, urine, skin, gametes, etc. (Bohmann *et al.*, 2014). Forms of eDNA can be extracted from environmental samples such as water and soil without having to isolate the target organism. Once released into the environment, eDNA may be transformed or degraded by biotic and abiotic factors or it may persist, becoming adsorbed in organic or inorganic particles (Levy-Booth *et al.*, 2007). Aquaria experiments have demonstrated that species start to release DNA just after their placement in a container (Thomsen *et al.*, 2012), and its persistence varies from few hours (Dell'Anno & Corinaldesi, 2004) to weeks (Dejan *et al.*, 2011) depending on the environmental conditions and the method used for DNA detection (Pilliod *et al.*, 2014).

Due to the higher chance of detection, especially for elusive species or species occurring at low densities, less effort is needed to detect a species using eDNA when compared with coventional sampling techniques. As it is unnecessary to catch species to detect their presence, the method is non-invasive and does not damage habitats (Deiner & Altermatt, 2014; Deiner *et al.*, 2016). One disadvantage of eDNA methods is the difficulty of relating the amount of eDNA with the density or biomass of the target species. However, a novel alternative to estimating population sizes from genetic sequence data generated from eDNA has recently been described (Sigsgaard *et al.*, 2016; Creer & Seymour, 2017) and is discussed below.

1.4 Using qPCR of DNA taxonomy markers for species detection

DNA barcoding is a taxonomic method that uses short, standardised, diagnostic genetic markers that can be used to successfully identify a large proportion of the world's species (Hebert *et al.*, 2003a). The same style of barcoding can be used to identify species from eDNA (Bohmann *et al*., 2014). The most commonly used marker for species identification by means of DNA barcoding is a fragment of the mitochondrial gene, cytochrome oxidase subunit 1 (COI) (Hebert *et al.*, 2003b; Ratnasingham & Hebert, 2007), but other markers are frequently used for molecular taxonomy depending on the taxa investigated (Avise, 1994; Beebee & Rowe, 2007). Accordingly, Gustavson *et al.* (2015) recently designed species-specific primers for the COI region of sea lampreys in order to augment detection of this organism from eDNA sources.

After sample collection, the DNA is extracted and analysed using Polymerase Chain Reaction (PCR) or quantitative PCR (qPCR) using specific oligonucleotide systems that target the molecular marker. For eDNA, qPCR is generally preferable to conventional PCR, as it is generally more specific—especially when using a TaqMan® approach—and more sensitive (Pilliod *et al.*, 2014). There are two approaches to performing qPCR assays. The first technique is the non-specific SYBR® Green which, similarly to conventional PCR, includes only two primers, forward and reverse. The second method is the TaqMan® which includes the primer set in conjunction with a hybridization probe that binds specifically to the target DNA strand, realising fluorescence upon amplification, hence generating a signal only when the target species DNA is present.

1.5 Objective of this report

The primary objective of this report is to address a range of questions posed by Scottish Natural Heritage about the development and feasibility of eDNA surveys for assessing the three species of lamprey that are found in Scotland.

2. RECOMMENDATIONS

2.1 Molecular assay design for lamprey detection from eDNA

2.1.1 Data availability and strategies for species discrimination

Question: Are primers and sequences available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) which will allow the reliable detection of a) brook lamprey and b) river lamprey, and will eDNA be able to differentiate between the larvae of brook and river lamprey? Gustavson *et al.* (2015) have demonstrated that sea lamprey may be detected for using eDNA, but what further development is needed to do the same for brook lamprey and river lamprey?

Answer: Currently (March 2017) there are 243 nuclear and mitochondrial DNA sequences of *L. fluviatilis* and 776 of *L. planeri* in the National Centre for Biotechnology Information (NCBI) GenBank. Such a comparatively large collection of sequences represents a good coverage of the genetic variability of these species, thus facilitating an investigation into the feasability of primer design for species detection.

The detection of target organisms using eDNA has proven effective for a wide range of taxa (Ficetola *et al.*, 2008; Jerde *et al.*, 2011; Dejean *et al.*, 2012; Takahara *et al.*, 2012) including *P. marinus* (Gustavson *et al.*, 2015). However, the relatively short length of the DNA sequence typically targeted for barcoding (648 base pairs (bp)) combined with shorter fragments currently targeted for eDNA analysis (ca. 100–300 bp) can reduce the taxonomic resolution of this approach, and it may not therefore be feasible to distinguish closely related species.

Species discrimination can be further confounded by the demographic history of closely related taxa, for example if there is a historical, recent, or ongoing introgression, as is the case between river and brook lamprey. Phylogenetic studies based on both mtDNA and nuclear DNA showed a lack of species-specific characters in these paired-species (Schreiber & Engelhorn, 1998; Docker *et al.*, 1999; Espanhol *et al.*, 2007; Blank *et al.*, 2008; Pereira *et al.*, 2011; Hume, 2013), suggesting that river and brook lamprey are best characterised as ecotypes of a single species. The phylogenetic relationship between the two lamprey species across the UK were also examined by Hume (2013), and no species-specific differences were found.

A recent genomics study on lamprey populations in France by Rougemont *et al.* (2017) inferred that *L. fluviatilis* and *L. planeri* were historically genetically isolated before gene flow was re-established relatively recently. This demographic history would be consistent with a period of geographic isolation followed by secondary contact (Barton & Hewitt, 1985). As a result of this recent gene flow, Rougemont *et al.* (2017) found that there were very few differences between the two ecotypes across most of the genome. However, they did identify 40 polymorphic sites in the genome which were diagnostic and could correctly assign a genotyped individual to either river or brook lamprey. The authors believe that these polymorphisms may be restricted to a small region of the genome which is not transmitted between the ecotypes despite neutral gene flow, and could possibly be linked to some intrinsic reproductive barrier. Unfortunately, at the time of writing, the genomic coordinates of these diagnostic polymorphic sites are not publicly available. It was not therefore possible to design an eDNA assay based around them. However, the authors of the above study are designing an SNP array comprised of these markers that will be made publicly available. Environmental DNA studies typically target mitochondrial DNA markers as there are several thousand more copies of the mitochondrial genome per cell compared with the nuclear genome. It is believed that no previous published study has targeted SNPs from the nuclear genome for an eDNA study. Therefore, although the 40 polymorphic markers from the Rougemont study are the only known markers able to distinguish *L.*

fluviatilis and *L. planeri*, it is uncertain whether they could be used in an eDNA study due to low copy numbers. Nevertheless, in order to be able to combine the use of eDNA technology to try to discriminate between *L. fluviatis* and *L. planeri*, the development and deployment of such an array would be the intuitive next step.

Despite extensive evidence of the occurrence of macrobial eDNA, a fundamental understanding of what eDNA is, especially in aquatic environments, is still lacking. The state-of-the-art relies on the fact that eDNA can be obtained by precipitating DNA from small volumes of water samples (Ficetola *et al.*, 2008), but also by using a variety of filter sizes (≥ 0.22 µm) to recover eDNA from the water column (Rees *et al.*, 2014). Such protocols lead to a working hypothesis that aqueous environmental DNA (i.e. eDNA extracted from water samples) is either derived from cellular organellar (e.g. mitochondria) or extracellular sources. Initial sequential mesocosm filtration experiments suggest that the largest concentrations of aqueous eDNA correspond to the eukaryotic cell size fraction (i.e. 1–10 µm) (Turner *et al.*, 2014; Wilcox *et al.*, 2015) and not organellar forms (i.e. ca. 100 nm for the mitochondrial nucleoid) (Turner *et al.*, 2014; Gilkerson *et al.*, 2013). Therefore, contemporary aqueous eDNA may be available in the genomic state, at least until natural degradation processes liberate intracellular material into the environment. Once this is either confirmed or refuted by additional studies, it will greatly inform strategies for the acquisition (e.g. filtering, replication, sample volumes and spatial sampling strategies) and preservation (Renshaw *et al*., 2015) of eDNA and could be a fruitful avenue for future research.

Despite the above challenges, all of the mtDNA and nuclear sequences available in GenBank were collected and analysed to find regions which could potentially discriminate the two species/ecotypes and thus that could be targeted for primer design. However, in agreement with previous studies, no species-specific markers were found; the two lamprey forms are genetically too similar to be discriminated using these forms of markers, essentially derived from a single maternally inherited genomic locus (i.e. mtDNA) (see Figure 1).

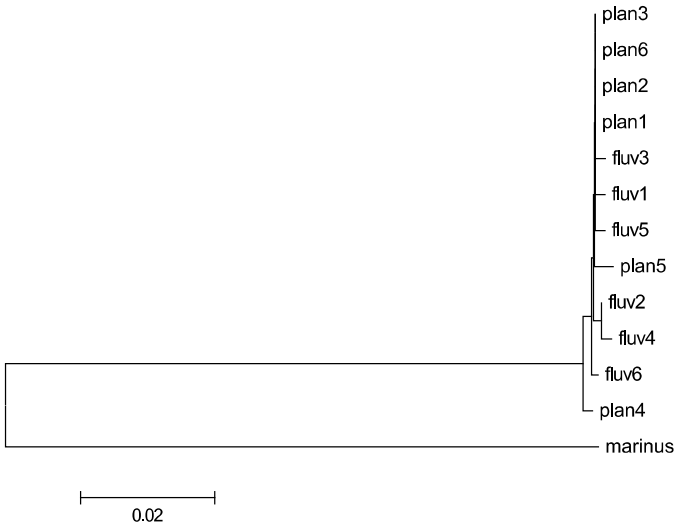

Figure 1. Phylogenetic Neighbor-Joining tree of L. fluviatilis ('fluv'), L. planeri ('plan') and P. marinus ('marinus') based on the ATPase 6 mitochondrial gene sequences from Hume (2013). While the sea lamprey is clearly separated from the two Lampetra *species, brook and river lamprey are nested within the same clade making it difficult, if not impossible, to genetically distinguish the two forms based on mtDNA gene data alone.*

Table 2. Candidate qPCR probe-based assay for the simultaneous detection of both river and brook lampreys from eDNA

	Gene	Size	Forward Primer	Reverse Primer	Probe
1	COI	84	GCCTTCCCACGTATAAACAACA	TGCTTCAACTCCTGCGGAA	ACTTCCACCCTCACTCCTTC
2	COI	72	TTAGCGCCCCAGATATAGCC	GAAGGAGTGAGGGTGGAAGT	TCCCACGTATAAACAACATAAGCT
3	COI	79	CCAATAATACTTAGCGCCCCAG	GGAGTGAGGGTGGAAGTAGT	GCCTTCCCACGTATAAACAACA
4	ATPase6	90	GCCTTAGCCCACTTATTACCAG	AGGTCGGATGAAAAGGCTAAT	CACCCCAATTGCACTCATCC
5	ATPase6	76	CCAGAAGGCACCCCAATTG	CAATAGGTCGGATGAAAAGGCT	ACTCATCCCTATACTTGTTATTATCGA
6	ATPase8	69	AAACCCAAACAACCCACCTG	TGGTTGGGGATTTAAATTGGTCA	ACCTGACCATGACACTAGCA

Based on this knowledge of the state-of-the-art of the available genetic resources for the *Lampetra* species, it is suggested that one immediate option is to design a qPCR assay for detection of the *fluviatilis-planeri* complex. This possibility was tested *in silico* and potential candidate assays are given in Table 2. These would need to be fully trialled to investigate their efficacy and specificity. In order to detect sea lamprey and river/brook lamprey in Scottish rivers, currently the only possible option is to combine the sea lamprey specific assay from Gustavson *et al.* 2015 with the suggested *fluviatilis-planeri* assay.

The two assays can be performed in a duplex qPCR reaction. Multiplex qPCR is a simple, efficient and cost effective solution for overcoming the challenges of limited samples and costly analysis. Multiplex qPCR enables the amplification of more than one target DNA sequence in a single reaction using probes that bind to different target sequences and each with distinct fluorescent dye making it possible to use less samples and to detect multiple species simultaneously.

The procedure to test and optimize qPCR assays includes three steps: 1) assay design and *in silico* test; 2) *in vitro* test; and 3) *in situ* test. The following description is for standard qPCR, which is the most commonly used and established approach. Alternatively, if the platform is available, digital droplet PCR, an emerging technique, allows for increased sensitivity while accepting the same assay design guidelines as qPCR.

1) A panel of candidate assays is developed based on the sequences available in Genbank. The online primer design software Primer3 (http://primer3.ut.ee/) (Untergasser *et al.,* 2012) has proved to be highly efficient for designing qPCR probes and is one of the most widely used. Specificity of the candidate assays is assessed *in silico* using another freely available online program, PrimerBlast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), which tests the primer pairs against all known sequences in GenBank.

2) During the *in vitro* step, DNA is extracted from tissue samples (e.g. dorsal fin clips) collected from a few different specimens of the target species, preferably from different populations to include geographic genetic variation (see Hume 2013 for lamprey population genetic structure in Scottish catchments). Tissue samples from all three species should be extracted and tested simultaneously to assess the specificity of the primers. The minimum amount of the target DNA sequence that can be detected in a sample using the designed assay—the limit of detection (LOD)—should be estimated by running a dilution series of a known amount of DNA, with several PCR replicates per concentration.

3) eDNA samples should ideally be collected at three sites: one where the target species are known to be present at high densities; one where they are known to be present at low densities; and one where they are absent. The analysis should be carried out using these samples (water and sediment, see below) to test the reliability of the qPCR assay and the sampling methods in natural conditions. If the designed assay does not pass all three tests (*in silico*, *in vitro*, and *in situ*) a new assay should be designed and tested (see Table 2 for multiple candidate assays).

Quantitative PCR can be used to estimate the amount of DNA in a sample by comparing it with standards of known DNA yield and concentration. However, this then provides an estimate of the biomass of DNA in an ecosystem, and there are many variables that could account for variation in the biomass of DNA that may not accord with the biomass of the target species, in this case lampreys. Confounding variables include local environmental factors such as endogenous nucleases, hydrolysis, UV radiation and bacterial action. Biotic factors, such as whether the animals have been feeding and thus defecating, or whether an animal has died and is thus shedding cellular DNA into the environment, will also strongly influence eDNA concentrations in the biosphere.

A novel alternative to estimating biomass from eDNA yield is to estimate effective population size directly from genetic sequence data generated from eDNA (e.g. Sigsgaard *et al.* 2016; Creer & Seymour, 2017). The effective population size is an estimate of the number of breeding individuals in an idealised population (a population of constant size, in which all individuals inter-breed randomly). The effective population size (Ne) is typically lower than the census size (N), and while the assumption of an idealised population is a mathematical necessity and is rarely if ever applicable to wild populations, Ne is widely considered a useful estimator for conservation monitoring (Allendorf & Luikart, 2009). When calculating effective population size it is important to consider the genetic region used to assess genetic diversity among populations as different regions of the genome undergo different rates of mutation. However, once the species-specific mutation rate (μ) of a genetic marker is known, then the genetic diversity (π) of that marker can be used to estimate effective population size using the formula, Ne = π/4μ. Typically, the most common genetic region used to design primers is the mitochondria, which is maternally inherited, meaning effective population sizes are specifically assessing the number of effective females, such that the Ne female = π/2μ.

In a recent, landmark study, Sigsgaard *et al*. (2016) sequenced mitochondrial DNA control region sequences of whale sharks from sea water samples collected at the Al Shaheen oil field offshore of Qatar in the Arabian Gulf. As a proof of concept, they estimated the number of effective females from the nucleotide diversity of their sequence data. The authors were clear that this was a first step in using eDNA to estimate population genetics, and that the estimate was likely to be a coarse one. However, this does highlight the potential for monitoring population size, and other population metrics (e.g. genetic differentiation between locations) through eDNA collection and sequencing.

2.1.2 Time and costs for assay optimization

Note that VAT has not been included in the following costs.

Question: If these primers and sequences are not available, what would it cost to develop and optimise appropriate primers and how long would that take?

Answer: Sequences are available and accessible. Designing, testing and optimizing the qPCR assay could take up to three months depending on the skills and experience of the person performing the procedure.

Designing a qPCR assay could be done either by a development company, e.g. PrimerDesign (www.primerdesign.co.uk), which may charge around £870 for a proprietary species detection kit, or in-house using freely available software tools (see section 2.1.1). Designed primers and probes can be ordered online from companies, e.g. Eurofins Genomics (https://www.eurofinsgenomics.eu/) offers a cheap and efficient service. The cost from Eurofins for a fluorescently-labelled probe qPCR probe is £180 and for primer pairs £14; the yield (i.e. amount synthesised) is generally high.

For eDNA studies, the slightly more expensive TaqMan method is preferred over the SYBR® Green because its higher specificity makes it superior for detection of target species. However, costs and time are drastically reduced by using the duplex qPCR design. For testing and optimization of the qPCR assay, one TaqMan® Genotyping Master Mix will cost £68; the number of reactions depends on the reaction volumes.

Additional costs include the DNA extraction kit (£159 for 50 extractions), plasticware, laboratory consumables, sampling equipment, etc. (see section 2.2.7).

2.2 Survey strategies for assessment of lamprey populations in Scottish rivers

2.2.1 Spatial factors and sampling design

Question: What spatial factors should be taken into account, e.g. how frequently along the length of a river should samples be taken?

Answer: The success of the method depends heavily on the chance of finding DNA of the target species in a sample. Methods should be adjusted to suit the species and their habitat. It is suggested that sites are selected using the same criteria as those used for conventional lamprey surveys in Scottish rivers. Optimal habitat, where high abundances of individuals are expected, should be prioritised; areas of sub-optimal habitat should be used in the absence of optimal habitat. Habitat definitions are given in Harvey & Cowx (2003).

Although studies exist that show invertebrate eDNA can be transported up to 12 km (Deiner & Altermatt, 2014), empirical studies suggest that, in order to maximize the possibility of detecting eDNA, water samples should be collected as close as possible to areas downstream of selected optimal or sub-optimal habitat and that the maximum distance downstream should be 250 m (Jane *et al.*, 2015). Sampling in the vicinity of estuaries, where eDNA could be too degraded and could be easily dispersed (hydrodynamically or physicochemically) by tidal processes, is not recommended. In general, sampling downstream of optimal or sub-optimal habitat where lampreys are expected to occur and avoiding sampling upstream of natural or artificial obstacles that may prevent lamprey migration (e.g. dams, waterfalls) would be ideal.

The frequency of sampling along a river will depend upon its characteristics, but in general a minimum of three sampling localitions should be used. More intensive surveys are recommended. Sampling in areas where the abundance of lampreys is known to be low or in unexplored areas where there are no previous records is also recommended.

2.2.2 Sampling methods and risks of contamination

Question: How should samples be taken, what equipment should be used, and what are the key sources of contamination?

Answer: A growing number of published papers have explored how to optimize capturing eukaryotic eDNA from the aquatic environment, e.g. Turner *et al.*, 2014; Deiner *et al.*, 2015. Initial approaches to sampling eDNA entailed collecting small volumes of water (e.g. 15–50 mL) that were subsequently preserved in ethanol and the DNA extracted using sodium acetate/ethanol centrifugation/precipitation (Ficetola *et al.*, 2008). Presently, the standard used for great crested newt population monitoring utilizes this small volume sampling approach (Biggs *et al.*, 2015). Sampling larger water volumes, particularly taking into account the spatial extent of the sampling area, is expected to decrease the chance of false negatives (Reese *et al*. 2014), however the effect of sample volume on species detection has yet to be tested comprehensively.

A recent survey of the contributors to the EU COST Action project *DNAquanet* (http://dnaqua.net/) revealed that the vast majority of eDNA practitioners have moved away from the analysis of small volumes and ethanol precipitation approaches for species detection because more effective measures have been identified. Accordingly, as the field of aquatic eDNA analysis has matured, the community has moved towards filtering larger volumes of water. The method has therefore evolved into the collection large volume water samples and transportation to the laboratory for filtration. The collection and transportation of water samples has several disadvantages. For example, large volumes of water need to be transported on ice to the laboratory which is inconvenient if sampling sites cannot be reached in a vehicle or if the sampling location is distant from the laboratory. This method

also requires more storage space and, withhout appropriate preservation, the eDNA signal degrades. A number of researchers now routinely filter samples on-site which reduces the volumes of water that need to be transported and minimises the risk of contamination.

Based on the results of the different tested methodologies, and past experience of transporting water (Bista *et al.*, 2017), the use of ≥ 1 litre water samples combined with 0.22–1.0 µm Sterivex filters (Millipore, Bedford, MA, USA) is suggested for the optimal capture of eDNA. Sterivex filters are column filters encased in a glass tube with female luer inlets and male nipple or male luer outlets. It is recommended that filters with male luer outlets are used to allow both ends of a filter to be capped with screw-on luer caps after filtering a sample. The preservation of samples for transportation to a laboratory can be accomplished by either freezing the filter, or by adding 1.5 mL of Longmire's buffer (Longmire *et al.*, 1997) to the filter unit after expelling all of the water from it and sealing the ends of the unit with luer caps. Alternatively, a qualitative filter paper membrane, (e.g. cellulose nitrate, glass fiber, polycarbonate tracketched) may be used with appropriate filtering apparatus holders (e.g. disposable filter holders with a funnel) (see Deiner *et al.*; 2014; Wilcox *et al.*, 2015; Bista *et al.*, 2017 for examples). Filters are preserved by placing them into 2 mL microcentrifuge tubes with 900 µL of Longmire's buffer to completely immerse the filter paper. However, using external filters with disposable filter holders instead of encased filter columns (e.g. Sterivex filters) increases the risk of cross contamination (Spens *et al.*, 2016).

As lamprey spend most of their life as larvae buried in silt/sand, taking both sediment and water samples should be considered. In aquatic systems, DNA concentrations in sediment can be three to four orders of magnitude higher than those present in the water column (Corinaldesi *et al.*, 2005). Turner *et al.* (2015) showed that fish eDNA was indeed highly concentrated in aquatic sediment. However, as eDNA does not degrade as rapidly in soil and sediment as it does in water, dispersion from its original source may be limited. This suggests that samples need to be taken close to the place where organisms released their DNA. Sediment samples should be collected by scooping material from the top 2–5 cm of the river bed, placing then in sterile Falcon tubes and storing them in sterile plastic bags on dry ice or by using another cooling system (e.g. immersion the sample in liquid nitrogen, putting them in a freezer).

Samples may be contaminated during fieldwork or in the laboratory. Recent work on eDNA has focussed on the need to develop efficient methods for the successful collection of freshwater samples in the field whilst minimizing the risk of cross-contamination. When visiting multiple sites, human error is generally the main source of cross-contamination. The use of new gloves and bottles for each site visited is now standard practice. All equipment, including footwear, should be cleaned with 10% bleach solution followed by several rinses with deionized water or, if large volumes of deionised water are not available, copious volumes of native water prior to entering the water body. In the laboratory, blank samples (e.g. deionised water) of the same volume as the experimental samples should pass through the assessment procedure to identify whether contamination has occurred. Blank and experimental samples should be processed in exactly the same way.

As eDNA is likely to be present in only trace amounts, clean laboratory facilities that are dedicated to the analysis of eDNA are essential. Herder *et al.* (2014) describe the range of workflows associated with the analysis of macrobial eDNA. This comprehensive guidance emphasises the explicit use of separate preprocessing, clean laboratory and post PCR analysis workflows and the need for positive and negative controls, to yield reliable eDNA analytical data. Staff at the Molecular Ecology and Fisheries Genetics Laboratory, University of Bangor, have adapted an ancient DNA (aDNA) laboratory and adhere to many of the processes developed for the field of aDNA, in order to reduce the risk of contamination. Note that in this report considerations of contamination are restricted to qPCR analyses, since a

number of other factors will need to be considered for meta-barcoding style analyses (Deiner *et al.*, 2017.

2.2.3 Sampling size

Question: How many water samples should be taken at each sampling point and should multiple samples be pooled or aggregated?

Answer: To enable efficient statistical analysis, triplicate water and sediment samples should be collected as a minimum. Sampling should begin in the lowest reach of a stream or river and proceed upstream. Triplicate, replicate, sampling is the minimum required to calculate standard deviation (i.e. variation) per site, which allows for an assessment of how similar the results from the different replicates are. Accurately measuring data variation within and among sites is essential for reliably determining differences among data points. Additional replicate sampling will allow for a more accurate assessment of the variation among samples and more statistical power when performing analyses.

In all population surveys, there is a possibility that the species of interest is undetected due to a range of factors including the low abundance of the target organism, or limitations in the effectiveness of sampling methods employed. Some species may be cryptic and limit their movements to areas which may not be adequately sampled, e.g. crayfish in burrows. It may also be that some habitats are only used for short periods of the target organism's life cycle and sampling outside of these periods may yield a negative result. Furthermore, some organisms may not routinely slough sufficient volumes of eDNA and may be more detectable at certain periods in their life cycle when eDNA production is more abundant, e.g. during a spawning season. The highly standardized methodology and cost-effectiveness of eDNA-based survey methods allows high sampling replication and so powerful statistical methods, such as occupancy modelling, to assess detection probabilities. Occupancy models follow the basic formula $E(C) = N * p$, where $E(C)$ is the expected number of individuals at a given site, N is the actual number of individuals at a given site and p is the probability of detection. Detection probababilties, within and among sites, can be calculated from replicate eDNA-based sampling data, allowing occupancy models to be used to gauge the likelihood of a target species being present at a site, which inturn allows for optimization of sampling methods and proxy rank abundances across sampling sites to assess difference in population sizes across space (Mackenzie *et al.*, 2002; Schimidt *et al.*, 2013). By analysing positive eDNA detection results across multiple lamprey sampling sites, detection probabilities, in their simplest form, can be used to illustrate the probability of detecting lamprey using eDNA at a site. Detection probabilities can also be used to assess optimal sampling strategies by modelling the probability of detection relative to the number of replicate samples taken (e.g. Schmidt *et al.*, 2013). Additionally, occupancy models may be used to estimate detection probabilities across lamprey sampling sites by calculating detection probabilities per site and ranking the probabilities across sites.

Consideration should therefore be given to survey design and ensuring that sampling effort is sufficient for the collection of adequate data to allow the calculation of detection probabilities. If they are to be used effectively, occupancy models have three key requirements. First, the number of sampling points used to calculate each probability should be large as small sample sizes will lead to poor optimization of the parameters and possibly spurious results. Schmidt *et al.* (2013) recommends using $N = 20$ to adequately account for variation, which may not be logistically reasonable when assessing within-site variation. Second, the heterogeneity of sampling methods will affect occupancy model outputs, meaning any variation in sampling methodology or timing may adversely affect the model parameters. This can be avoided by taking samples at the same time, ensuring water sample volumes are adequate for filtering, and standardizing the sample method. The third of the key requirements concerns the belief that occupancy models generally assume that

sites are closed and that measurements taken are spatially and temporally independent. It is also important to consider that occupancy models are assessed using presence/absence data, which can be extracted from qPCR data, but do not replace genuine quantitative ecological data.

2.2.4 Optimum sampling time

Question: When is the best period during a year to collect water samples to determine whether brook, river or sea lamprey are present, and can a single sampling event be used to assess whether all three are present?

Answer: In order to maximize the probability of detecting lamprey DNA, sampling should focus upon the migration and spawning periods when populations at the sampling sites are expected to be in at their greatest abundances. In the UK, river and brook lampreys start migrating upstream in autumn, from October to December. Spawning in British rivers starts when the water temperature reaches 10–11°C, usually in March and April (Morris & Maitland, 1987). After spawning, all lampreys die (Larsen, 1980) and eDNA concentrations may be high during the period when they do. It is therefore suggested that water samples are collected during these periods; November and April could be ideal months for sampling. Lampreys metamorphose during the summer— generally between June and September— and this may be another good sampling period although it may be less effective than sampling during the main spawning period. Tables 3 and 4 illustrate the life history events and best sampling periods for brook, river and sea lamprey.

Sea lamprey have slightly different life history timings than those of brook and river lamprey. Sea lamprey spawn when the water temperature reaches at least 15°C. Their upstream migration (April to June) begins later than that for river lamprey, and they spawn from late May until end of June. Metamorphosis occurs over the summer months. Such environmental and life history variables are likely to influence detection probabilities and would need to be taken into consideration when assessing presence/absence.

August may be the best time to determine the presence of all three lamprey species using eDNA. Nevertheless, robust temporal testing and analysis would need to be undertaken in order to refute or confirm this view.

Table 3. Life history and suggested best sampling time for river and brook lampreys

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Upstream												
Spawning												
Metamorphosis												
Key sampling time												

Table 4. Life history and suggested sampling time for sea lamprey

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Upstream												
Spawning												
Metamorphosis												
Key sampling time												

2.2.5 River flow conditions necessary for sampling

Question: Is it possible or necessary to specify the best flow conditions for collecting samples?

Answer: Our increasing knowledge of the transport of aquatic eDNA in lotic systems (Deiner & Altermatt, 2014; Jane *et al.*, 2015; Deiner *et al.*, 2016) suggests that the ideal conditions for sampling eDNA are probably when the water body is in its normal hydrodynamic state. At present, this is simply an informed perspective and focuses on the detrimental processes that enhanced and also reduced flow regimes will have on the dilution and degradation of eDNA. Intuitively, enhanced flow regimes, such as those that follow excessive rainfall, should be avoided since the eDNA signal will become diluted and therefore more difficult to capture in detectable amounts. Conversely, lower flow regimes may give rise to conditions that will increase the vertical deposition of eDNA particles, or potentially increase the water temperature (e.g. in slow moving eddies/pools, heated by solar radiation). Slight increases in water temperature may also increase microbial and enzymatic activity in the water column, thereby promoting the degradation of eDNA. It is, therefore, important to avoid sampling after adverse weather such as storms, and when flow is faster and greater than normal, primarily due to diluition effects, but also as suspended material can quickly and easily clog filtration units. Similarly, avoiding sampling during periods of excessive algal blooms would be best since algal material will also clog filters rapidly in the field.

2.2.6 Sample preservation

Question: What are the advantages and disadvantages of the different ways of preserving samples? Do all samples need to be processed within eight hours?

Answer: Various methods for preventing DNA from degrading are available. Filtered samples (columns or qualitative filter papers) can be stored on dry ice. However, relying on a cold ambient temperature poses practical and logistical problems where, for instance, sampling sites are only accessible by foot, or samples need to be transported long distances. Ethanol (100%) is the most widely used DNA preservative for field-collected samples in forensic and molecular ecology, including those collected for for eDNA analyses (Goldberg *et al.*, 2013; Pilliod *et al.*, 2013). However, ethanol is classified as a hazardous chemical due to its volatility and additional consideration must be given to situations where it is transported outwith the laboratory. Licensing and disposal plans for ethanol are also required. Longmire's buffer has been shown to successfully preserve eDNA captured on filters at room temperature over a two-week period. Unlike ethanol, Longmire's buffer is not hazardous and it is comparatively inexpensive (Renshaw *et al.* 2015; Spens *et al.*, 2016). Options also exist to dry eDNA on the filters, by the addition of silica granules/pebbles.

Sediment samples can be preserved in sterile Falcon tubes and airtight plastic bags without any preservation solution or media at cold temperatures (+4°C to -80°C) until DNA extraction is performed. It has been shown that preserving sediment samples in either Ethanol or RNAlater® buffers drastically decreases nucleic acid yields at all temperatures, whereas freezing samples without any buffer gives good quality and high yield DNA up to 30 days after sampling (Rissanen *et al.*, 2010). Rissanen *et al.* (2010) also showed that the storage of samples in a phenol-chloroform-isoamyl alcohol (PCIAA) solution (25:24:1, 0.4 mL for 2 µl of sediment) at +4°C also gave good yields of high quality DNA. This does not apply to the use of PCIAA to sediments with extremely high-carbon content. However Rissanen *et al.* (op. cit.) recommend PCIAA as an alternative storage method for situations where freezing is not possible, such as those encountered during extended periods of field sampling. Further preservation options also exist, including the use of dimethyl sulfoxide and saturated salt solution. These have proved useful for the preservation of microscopic eukaryotes in sediment samples (Fonseca *et al.*, 2010; Yoder *et al.*, 2006) but remain untested for

macrobial eDNA. Table 5 summarizes the different preservation methods described in the text.

Table 5. Possible preservation methods for water filters and sediment.

Sample type	Buffer	Temperature	Maxiumum preservation time	Hazardous
Water filter	None	-20°C	Unknown	No
	Ethanol	20°C	2 weeks	Yes
	Longmire	20°C	2 weeks	No
	Silica rocks	20°C	Unknown	No
Sediment	None	-20°C, -80°C	30 days	No
	PCIAA	+4°C to -80°C	30 days	Yes

2.2.7 eDNA lamprey survey costs

Question: How much will analysing a sample cost and how might economies of scale be achieved?

Answer: Field sampling material will include the purchase of Sterivex filter units, luer cap ends, a field peristaltic pump and silicon hosing. The peristaltic pump and silicon hosing can be substituted for syringes to save cost, while quadrupling field process time. Alternatively, the pump may also be replaced with a modified bike pump, but processing times will be doubled. The cost for Sterivex units and male/female luer caps is approximately £8.50 per sample. Peristaltic pumps range in price from £500 to £1,416; the more expnsive ones allow easier loading of silicon tubing and include the provision of an external battery for field sampling and a carrying case.

Laboratory eDNA analyses will require the use of a fully fitted eDNA laboratory/clean room workflows, and access to a qPCR thermal cycler and associated analytical software. The extraction of samples using indirect extraction methods can be performed using combinations of readily available laboratory reagents. However, for the sake of simplicity and standardization, it is assumed that proprietary DNA extraction kits will be purchased. The most common DNA extraction kit for use is the Qiagen DNAeasy Blood and Tissue kit, with Qiagen offering two kit sizes that process 50 (£159) or 250 (£716) samples. Additional proteinase K (£200/10 mL) and buffer ATL (£47.20) may be required to process samples as well. In certain instances, PCR amplification may be affected by the presence of inhibitory compounds in the DNA extract. To overcome the inhibition, proprietary cleanup kits (e.g. Qiagen, Zymo) at an approximate cost of £2–3 pound per column can be used.

Quantitative PCR will require PCR master mix, primers/probes, and DNA grade water to perform reactions. Additionally, plastics, including micropipette tips, 96/384 well plates and adhesive film will be needed. The total cost per qPCR reaction is estimated to be 80p, but will be higher using proprietary species detection kits. Each sample will require a minimum of three reactions (triplicate) and each qPCR assay will require a set of standard control samples (minimum 15 across a five fold dilution series) and an appropriate number of negative controls.

2.2.8 Field validation

Question: What, if any, field validation should be undertaken?

Answer: Given the novelty of eDNA-based methods, field validation of the planned, recommended, approach is paramount for designing a long term study program to investigate the ecological relevance of lamprey eDNA in relation to SNH's monitoring needs.

Working with SNH personnel and necessary specialists, it would be possible to identify the appropriate study system(s), spatial and temporal sampling and analytical approaches necessary to enhance the detection of the three focal lamprey species. At present, without further details/interactions, this would appear to be an academic exercise, but following the satisfactory design and testing of probes, initial considerations would include:

1. Performing experiments with lamprey mesocoms to verify sampling methods in a semi-natural environment.

2. Performing cage experiments with translocated lamprey mesocoms situated within lamprey free freshwater ecosystems to verify sampling methods in a natural environment (*sensu* Jane *et al.*, 2015).

3. Performing field tests in a variety of sites where lampreys are known to be present and abundant and comparing emergent data with physical count/abundance data. Experimental variates would include sampling method, sample volume, testing for inhibition, spatial and temporal factors, replication and physico-chemical considerations, in order to identify the most appropriate methodologies.

4. Engaging with local fishing communities to explore citizen science monitoring of lampreys, to broaden geographical and ecological coverage, whilst enhancing societal impact.

Further opportunities could include the designing of sequence-based assays (Sigsgaard *et al.*, 2016) that could be used to estimate population genetic parameters such as effective population sizes, as outlined above. Moreover, investigations into the efficacy of SNP capture arrays, or similar genome scanning approaches, could provide a route for effectively discriminating between brook and river lamprey given their recent evolutionary history and ecotype status.

3. GLOSSARY TERMS

Effective Population size (N_e) – The number of individuals (e.g. breeding individuals) in a population (N) that are needed to maintain a specified genetic quantity of the population (e.g. genetic diversity).

Environmental DNA (eDNA) - DNA captured from an environmental sample without first isolating any target organisms. Traces of DNA can be from faeces, mucus, skin cells, organelles, gametes or extracellular DNA. Environmental DNA can be sampled from modern environments (e.g., seawater, freshwater, soil or air) or ancient environments (e.g., cores from sediment, ice or permafrost).

Genetic diversity – The total variation in genetic information within or among groups.

In silico – Performed by means of computer modelling or simulations.

In situ – Assessed in the natural environment or state.

In vitro – Conducted in the laboratory, particularly outside of a living organism such as in a test tube or petri dish.

Mutation rate (μ) – The frequency of mutations, either at a given genetic region or across the entire genome, of a given individual, species or group.

Quantitative PCR (qPCR) – A molecular biology laboratory technique based on the polymerase chain reaction (PCR). Amplification of a targeted DNA template is monitored during the PCR in real time to quantitatively assess the change in DNA template abundance over time.

Single nucleotide polymorphism (SNP) – Refers to a single position variant in a DNA sequence among individual sequences.

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