Natural England Commissioned Report NECR290

eDNA monitoring for migratory fish assemblages

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Foreword

Natural England commission a range of reports from external contractors to provide evidence and advice to assist us in delivering our duties. The views in this report are those of the authors and do not necessarily represent those of Natural England.

Background

DNA based applications have the potential to significantly change how we monitor biodiversity and which species and taxa we monitor. These techniques may provide cheaper alternatives to existing species monitoring, an ability to detect species that we do not currently monitor effectively and the potential to develop new measures of habitat and ecosystem quality.

Natural England has been supporting the development of DNA techniques for a number of years. The use of environmental DNA (eDNA) to determine the presence or absence of great crested newts in ponds is now a standard tool for developers and consultants.

There are still significant limitations to the use of this technology in others areas and in 2017/18 Natural England worked with NatureMetrics to prove the concept of using eDNA for monitoring migratory fish species in the Rivers Wye, Froome and Tamar. The results show huge potential for the use of eDNA to monitor the presence of not only migratory fish, but whole fish assemblages.

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Further information
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Introduction

All migratory fish in England (twaite and allis shad, Atlantic salmon, sea trout, European eel, smelt, river and sea lamprey) are protected under a variety of legislative drivers including the Eel Regulations (2009), the Salmon and Freshwater Fisheries Act (1975), the Marine and Coastal Access Act (2009) and the Conservation of Habitats and Species Regulations (2017) in sites where they are designated features. Yet outside of salmonids, most are relatively poorly understood, despite being among the most sensitive of species to anthropogenic impacts on river and marine habitats. Many of them serve as indicator species for the health of a river system. Conserving the homing ranges of migratory fish is a key aim for Natural England and is of particular importance in light of barriers to these migrations (e.g. weirs). Moreover, the temporal distribution of various life stages of the migrating fish has an important effect on their likely survival (e.g. salmon overwintering in the lower parts of the Tamar may have different survival rates to those that do not).

Migratory fish are usually monitored at different life stages and include non-invasive methods (ie. visual surveys) and invasive methods (electrofishing, trapping). These range in quantitativeness and only the more invasive methods can be used to gauge abundance (e.g. electro-fishing with depletion sampling within a rigid quadrat framework or fish counters). These methods are not always possible, especially in saline environments or where rivers are too wide to sample. They can also be expensive and time-consuming, will bias towards bigger fish (or adults) and can potentially harm the fish. Moreover, traditional survey methods for migratory fish can be very condition and timing dependent. For example, high river levels can result in a year's survey window being missed.

There is a growing body of literature that suggests that molecular techniques offer a viable alternative method for surveying fish communities and circumvent some of the issues associated with more conventional techniques: eDNA methods (environmental DNA) are non-invasive, can be performed in any water system regardless of salinity, are not limited by the size of the water body, and are cost-effective, easily performed, and require no expensive or specialist equipment to sample. As such, eDNA could potentially provide a convenient method for determining the distribution of spawning migratory species within a river system. eDNA metabarcoding could potentially be a much more reliable method than other types of survey for these species and therefore would contribute to the delivery of the Conservation Strategy (Natural England, 2016).

Methods

Field sampling

eDNA water samples were collected by Natural England staff from three river systems with good historic fish assemblage data and confirmed presence of migratory fish species: River Tamar, River Wye and River Frome (Figure 1). Details of historic data from each river system are shown in Table 1.

Within the Tamar and Frome river system, a total of three sampling stations were selected, with 5 replicate samples taken at each station (Upper, Middle and Lower; n = 15; see Figure 2). Within the Wye river system, a set of 5 replicate estuarine and riverine samples were selected (n = 10; see Figure 2). For each replicate sample, a total of 1 L of river water was collected into a Whirlpak bag from 20 subsamples. After ensuring the water (the subsamples) was adequately mixed within the bag, a 50 mL syringe was used to plunge the water through a filter. This was repeated until the filter was clogged with sediment or the sample bag was empty. A preservative (Longmire's solution) was added and the filter outlet was capped and sealed in a plastic bag and posted to the NatureMetrics eDNA laboratory for analysis.

This protocol was used for all the sampling stations apart from the estuary sampling station at River Wye, where the samples were collected by the EA and filtered in the office later by NE staff.

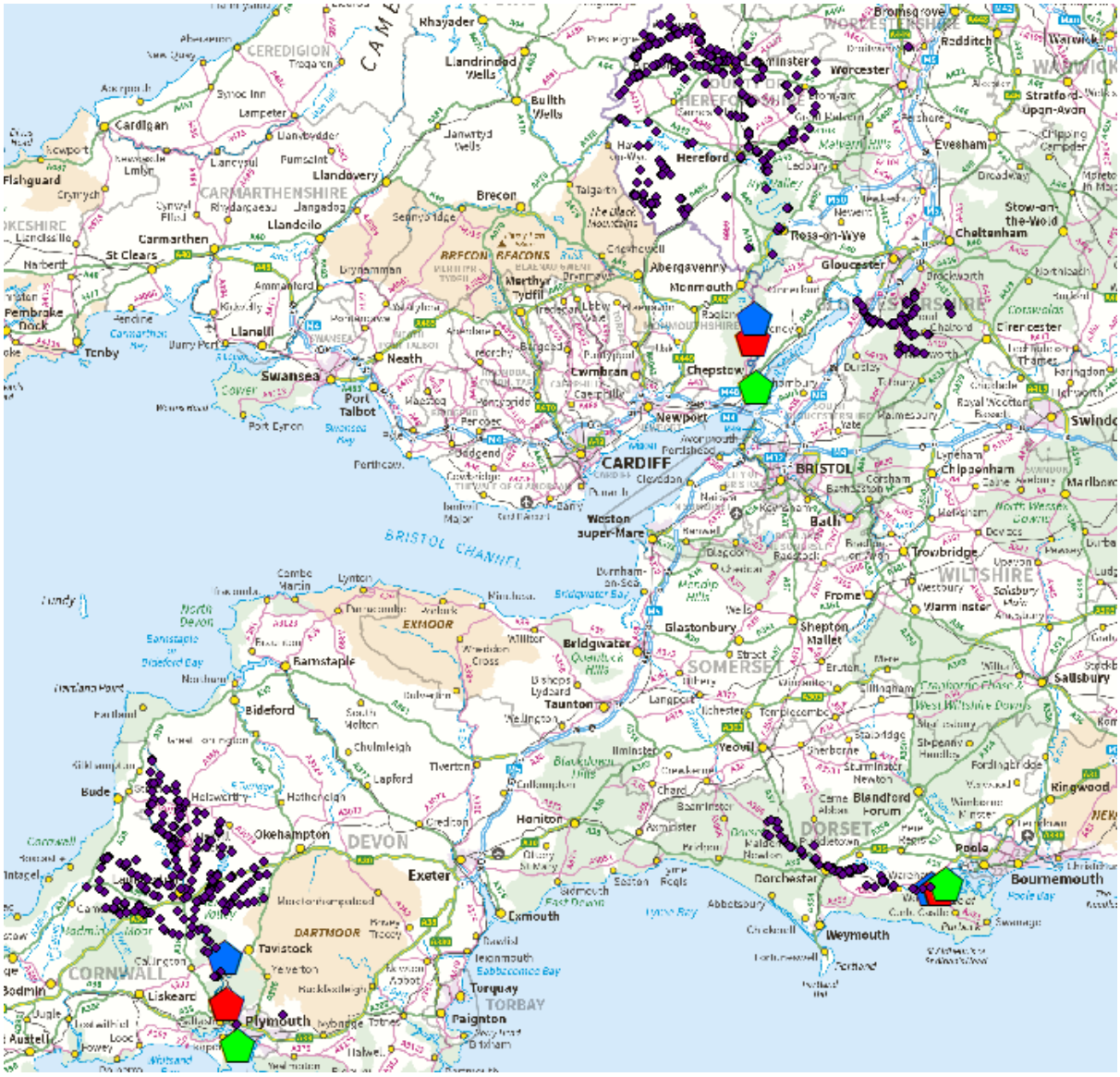

Figure 1: Map of migratory fish eDNA sampling sites. Pentagons represent the eDNA sampling locations (lower = green, mid = red, and upper = blue), while the purple diamonds represent Environment Agency survey locations. The northernmost locations correspond to the River Wye, easternmost correspond to the River Frome, and westernmost correspond to the River Tamar. Note that no Environment Agency survey data were available for the River Wye,

Table 1: Historic records of migratory fish species within River Tamar, River Wye, and River Frome. The data source and collection year are given in brackets.

River system	Data source	Year	Migratory species present
Tamar	Environment Agency. Freshwater Fish Counts for all Species, all Areas and all Years.	1984 - 2017	*Anguilla anguilla* (European eel), *Salmo salar (*Atlantic salmon), *Salmo trutta* (brown trout), *Lampetra planeri* (brook lamprey)
Wye	APEM report - Rivers Wye and Usk Baseline and Flow Impact Monitoring 2012	2012	*Salmo salar*, *Petromyzon marinus* (sea lamprey), *Anguilla anguilla*, *Salmo trutta*
Frome	Environment Agency. Freshwater Fish Counts for all Species, all Areas and all Years.	2002 - 2018	*Salmo salar*, *Petromyzon marinus*, *Anguilla anguilla*, *Salmo trutta*, *Lampetra fluviatilis* (river lamprey), *Lampetra planeri*

Figure 2: Sampling locations within the Tamar (a), the Wye (b), and the Frome (c).

eDNA analysis

DNA from each filter was extracted using a commercial DNA extraction kit with a protocol modified to increase DNA yields. DNA was purified to remove PCR inhibitors using a commercial purification kit. Purified DNAs were amplified with 12 replicate PCRs for a ~230 bp hypervariable region of the 12S rRNA gene to target fish as part of the 'eDNA survey - Fish' pipeline. The primers used were the MiFish primers (Miya et al. 2015. R. Soc. Open Sci. 2(7): 150088). PCRs comprised 1X Phusion Green Mastermix, 0.3 µM of each primer, 1 mM of $MgCl_2$, 2 µL of DNA, and up to volume with H_2O. PCR cycle conditions were 95°C for 3 minutes, 45 cycles of 98°C for 20 seconds, 65°C for 15 seconds, 72°C for 15 seconds, and a final elongation of 72°C for 5 minutes.

Lamprey (Hyperoartia, herein lamprey) and ray-finned fish (Actinopterygii, herein fish) were analysed separately because the assay 12S rRNA gene is significantly different, the primers typically used for the fish do not bind well with the orthologous region in lamprey. A bespoke analogous lamprey assay was designed and tested on these samples (see 'Lamprey eDNA assay'). It should be noted that these fish community analyses do not include lamprey data. All PCRs were performed in the presence of both a negative control and a positive control sample (mock community with a known composition). Amplification success was determined by gel electrophoresis. PCR replicates were pooled and purified, and sequencing adapters were added. Success was determined by gel electrophoresis. Amplicons were purified and checked by gel electrophoresis, these were then quantified using a Qubit high sensitivity kit (Table 2) according to the manufacturer's protocol. All purified index PCRs were pooled into a final library with equal concentrations. The final library was sequenced using an Illumina MiSeq V2 kit at 15 pM with a 10% PhiX spike in.

Sequence data was processed using a custom bioinformatics pipeline for quality filtering, dereplication, and taxonomic assignment. After filtering, taxa were identified by comparing those sequences to our own curated reference database and supplemented with sequences from the GenBank reference database. The presented species-level identification is the top hit on the databases based on species identity. If multiple reference sequences match equally to the query sequence then all of those references are reported in the table. Note that unidentified or misidentified taxa can result from incomplete or incorrect reference databases, and missing taxa can result from low quality DNA, environmental contaminants, or overrepresentation of certain species, which physically dominate the sample. Contaminant DNA sequences were removed from the dataset, these included human, cow, and pig, which are common environmental, laboratory, and/or food contaminants.

Table 2. Volume of water filtered and the resultant concentration of purified index PCRs.

NM ID	Sample ID	Volume filtered	DNA (ng/µl)	Index (ng/µl)
2903	Lower 1 (TAMAR)	1000 ml	0.732	6.42
2914	Lower 2 (TAMAR)	1000 ml	2.79	5.76
2912	Lower 3 (TAMAR)	1000 ml	2.22	5.62
2907	Lower 4 (TAMAR)	1000 ml	1.33	11.8
2900	Lower 5 (TAMAR)	1000 ml	2.45	6.94
2901	Mid 1 (TAMAR)	1000 ml	0.796	4.3
2906	Mid 2 (TAMAR)	1000 ml	1.09	5.38
2911	Mid 3 (TAMAR)	1000 ml	4	5.94
2902	Mid 4 (TAMAR)	1000 ml	1.18	5.42
2904	Mid 5 (TAMAR)	1000 ml	1.5	5.98
2908	Upper 1 (TAMAR)	1000 ml	0.143	4.94
2909	Upper 2 (TAMAR)	1000 ml	0.177	4.18
2905	Upper 3 (TAMAR)	1000 ml	0.204	5.38
2910	Upper 4 (TAMAR)	1000 ml	1.04	6.54
2913	Upper 5 (TAMAR)	1000 ml	4.32	5.64
2968	1 Wye Estuary	200 ml	5.38	10.4
2966	2 Wye Estuary	200 ml	7.6	11.2
2971	3 Wye Estuary	200 ml	5.7	12
2967	4 Wye Estuary	200 ml	9.88	10.8
2965	5 Wye Estuary	200 ml	11.6	11.2
2969	6 Wye - Brockweir	1300 ml	3.5	12
2972	7 Wye - Brockweir	1400 ml	6.42	11
2974	8 Wye - Bigsweir	950 ml	4.16	12
2970	9 Wye - Bigsweir	1000 ml	7.34	11.2
2973	10 Wye - Bigsweir	600 ml	5.38	11
2993	River Frome Estuary 1	840 ml	10	10.4
2995	River Frome Estuary 2	600 ml	>12	10.6
2996	River Frome Estuary 3	840 ml	11.2	11.4
2997	River Frome Estuary 4	900 ml	>12	12
2998	River Frome Estuary 5	960 ml	>12	12
2988	River Frome Middle site 1	990 ml	>12	11.2
2989	River Frome Middle site 2	1020 ml	>12	11.8
2990	River Frome Middle site 3	720 ml	11.8	12
2991	River Frome Middle site 4	720 ml	>12	12
2992	River Frome Middle site 5	1260 ml	>12	12
2994	River Frome Upper site 1	1080 ml	9.72	8.76
2999	River Frome Upper site 2	1020 ml	10.6	12
3000	River Frome Upper site 3	960 ml	>12	11.6
3001	River Frome Upper site 4	960 ml	12	10.8
3002	River Frome Upper site 5	800 ml	>12	11.2

Lamprey primer design

The primers we use to target fish species were modified to target lamprey-species. The primers target a ~230 bp hypervariable region of the 12S rRNA gene and are able to differentiate sea lamprey from brook and river lamprey but not brook from river lamprey. The primers were redesigned *in silico* based on our modified MiFish primers (Miya et al. 2015. R. Soc. Open Sci. 2(7): 150088) and bind perfectly to the river lamprey (*Lampetra fluviatilis*: accession Y18683.1) and sea lamprey (*Petromyzon marinus*: accession U11880.1) mitochondrial genomes (NM_Lamprey_F: 5' - GCT GGT AAA CCT CGT GCC AGC - 3' and NM_Lamprey_R: 5' - CAT AGC GGG GTA TCT AAT CCC GGT TTG - 3'). These primers also bind to other species of lamprey (Pouched lamprey, Least brook lamprey, American brook lamprey, Korean lamprey, silver lamprey, northern brook lamprey, Arctic lamprey, and Asiatic brook Lamprey), but these are all non-native. The primers have at least 3 mismatches to any species (closest matches were rainbow trout *Oncorhynchus mykiss* and common whitefish *Coregonus lavaretus*) likely to be found in the UK.

The primers were tested *in vitro* using natural eDNA samples known to contain lamprey. These samples were used to optimise the PCR conditions. Purified DNAs were amplified for a ~230 bp hypervariable region of the 12S rRNA gene to target lamprey. All PCRs were performed in the presence of a negative control and a positive eDNA sample known to contain lamprey. Amplification success was determined by gel electrophoresis.

Results

DNA yields were high enough to proceed with the 'eDNA survey - Fish' pipeline (Table 2). DNA concentrations were lower for the River Tamar samples (average = 1.6 ng/µl) compared to the River Wye (6.7 ng/µl) or the River Frome (>11.6 ng/µl) samples, this was expected because the method used to extract DNA from the Tamar filters has since been improved to further increase the concentration of DNA. The volume of water filtered for the Wye Estuary samples was five times less (200 ml) than the other samples which averaged 969 ml.

PCR reactions were consistently successful for all 40 samples. Electrophoresis bands were strong, of the expected size and no repeat PCRs were necessary. All 40 samples were successfully indexed, and no repeat reactions were necessary. All amplicons were successfully purified and were of high yield (Table 2).

The MiSeq paired-end sequencing of the 40 samples yielded 4,551,632 reads, of which 78% passed our internal quality filter. Both negative and positive controls performed as expected. Very few sequences were discarded prior to dereplication, which is indicative of high-quality data with minimal PCR and sequencing errors. A total of 610,018 high-quality unique sequences were generated and used for taxonomic assignment.

Sample composition

Of the 8 recognised migratory fish species known in the UK (twaite and allis shad, Atlantic salmon, trout, European eel, smelt, river and sea lamprey), we are able to detect Atlantic salmon, trout, and European eel using our eDNA survey - Fish' pipeline, and also lamprey

using our ‘lamprey eDNA’ assay. Here we discuss the ray-finned fish first and later the lamprey (‘lamprey eDNA assay’).

A total of 42 taxa were detected across the 40 samples (excluding non-metazoan and contaminant taxa), of which 34 could be identified to species level, the remainder were identified to the lowest taxonomic level (discussed in Table 10). These 42 taxa belong to 13 orders (Anguilliformes, Atheriniformes, Clupeiformes, Cypriniformes, Esociformes, Gadiformes, Gasterosteiformes, Mugiliformes, Osmeriformes, Perciformes, Pleuronectiformes, Salmoniformes, and Scorpaeniformes), 20 families (Anguillidae, Atherinidae, Carangidae, Clupeidae, Cottidae, Cyprinidae, Esocidae, Gadidae, Gasterosteidae, Gobiidae, Labridae, Lotidae, Moronidae, Mugilidae, Nemacheilidae, Osmeridae, Percidae, Pleuronectidae, Salmonidae, and Scombridae), and 41 genera. The diversity is summarised in Table 3. The three different sites (Rivers Tamar, Wye, and Frome) had different species richness and composition and will be discussed in turn.

Table 3. Diversity richness among the samples

NM ID	Sample ID	Order	Family	Genus	Taxa (IDed to species)
2903	Lower 1 (TAMAR)	1	1	1	1 (1)
2914	Lower 2 (TAMAR)	1	1	1	1 (1)
2912	Lower 3 (TAMAR)	1	1	1	1 (1)
2907	Lower 4 (TAMAR)	6	10	10	11 (11)
2900	Lower 5 (TAMAR)	5	7	7	7 (7)
2901	Mid 1 (TAMAR)	2	2	2	2 (1)
2906	Mid 2 (TAMAR)	2	2	2	2 (1)
2911	Mid 3 (TAMAR)	6	6	7	7 (6)
2902	Mid 4 (TAMAR)	8	10	10	10 (9)
2904	Mid 5 (TAMAR)	1	1	1	1 (1)
2908	Upper 1 (TAMAR)	2	2	2	2 (1)
2909	Upper 2 (TAMAR)	1	1	1	1 (1)
2905	Upper 3 (TAMAR)	0	0	0	0 (0)
2910	Upper 4 (TAMAR)	4	5	5	5 (5)
2913	Upper 5 (TAMAR)	3	3	3	3 (3)
2968	1 Wye Estuary	2	2	2	2 (1)
2966	2 Wye Estuary	1	1	2	2 (1)
2971	3 Wye Estuary	1	1	3	3 (1)
2967	4 Wye Estuary	1	1	1	1 (1)
2965	5 Wye Estuary	2	2	2	2 (0)
2969	6 Wye - Brockweir	7	8	14	14 (13)
2972	7 Wye - Brockweir	9	10	20	20 (18)
2974	8 Wye - Bigsweir	8	9	18	19 (18)
2970	9 Wye - Bigsweir	4	5	11	11 (9)
2973	10 Wye - Bigsweir	6	7	13	13 (12)
2993	River Frome Estuary 1	8	11	16	17 (15)
2995	River Frome Estuary 2	10	11	16	17 (15)
2996	River Frome Estuary 3	8	9	11	12 (10)
2997	River Frome Estuary 4	9	10	15	16 (13)
2998	River Frome Estuary 5	8	11	16	17 (15)
2988	River Frome Middle site 1	9	10	15	16 (14)
2989	River Frome Middle site 2	9	10	16	17 (15)
2990	River Frome Middle site 3	7	8	8	9 (9)
2991	River Frome Middle site 4	7	8	11	12 (10)
2992	River Frome Middle site 5	6	7	12	13 (11)
2994	River Frome Upper site 1	5	6	7	8 (7)
2999	River Frome Upper site 2	5	5	8	9 (8)
3000	River Frome Upper site 3	6	7	9	10 (8)
3001	River Frome Upper site 4	6	7	9	10 (9)
3002	River Frome Upper site 5	6	7	11	12 (11)

River Tamar

Migratory fish

Sampling locations are shown in Figure 3. Among the migratory species found were Atlantic salmon, trout, smelt and eel (Figure 4). Atlantic salmon were detected in 33% of the samples (5 of the 15 replicates) with 2 incidences in the lower, 1 in the mid, and 2 in the upper stretches of the Tamar. Brown trout were detected in 20% of the samples (3 of 15; 2 in the lower Tamar and 1 in the mid Tamar). Smelt was detected in a sample in the lower Tamer but not further upstream. Eel was detected in 20% of the samples (3 of 15; 2 in the mid Tamar and 1 in the upper Tamar). The lamprey assay was positive in one sample in the Upper Tamar (Figure 5).

These eDNA data were compared to Environment Agency data from a similar area dating from 1984 to 2017 at 9 different sites in and around the eDNA sampling points (Figure 3). Across 24 surveys, over 33 years, 1262 individual fish were counted and these included 179 salmon, 875 brown trout, and 66 eels. According to the Environment Agency data for these 9 sites, no lamprey have been detected, but 5 individual brook lamprey (*Lampetra planeri*) were detected in 2016 upstream of Lamerhooe Ford (SX3982173279). Salmon were detected in 9 surveys, trout were detected in 20 surveys and eel were detected in 12 surveys. No smelt were detected with traditional survey methods.

Figure 3. Sampling locations for the eDNA samples (red) and the nine sites from which 24 different fish surveys were conducted by the Environment Agency (blue).

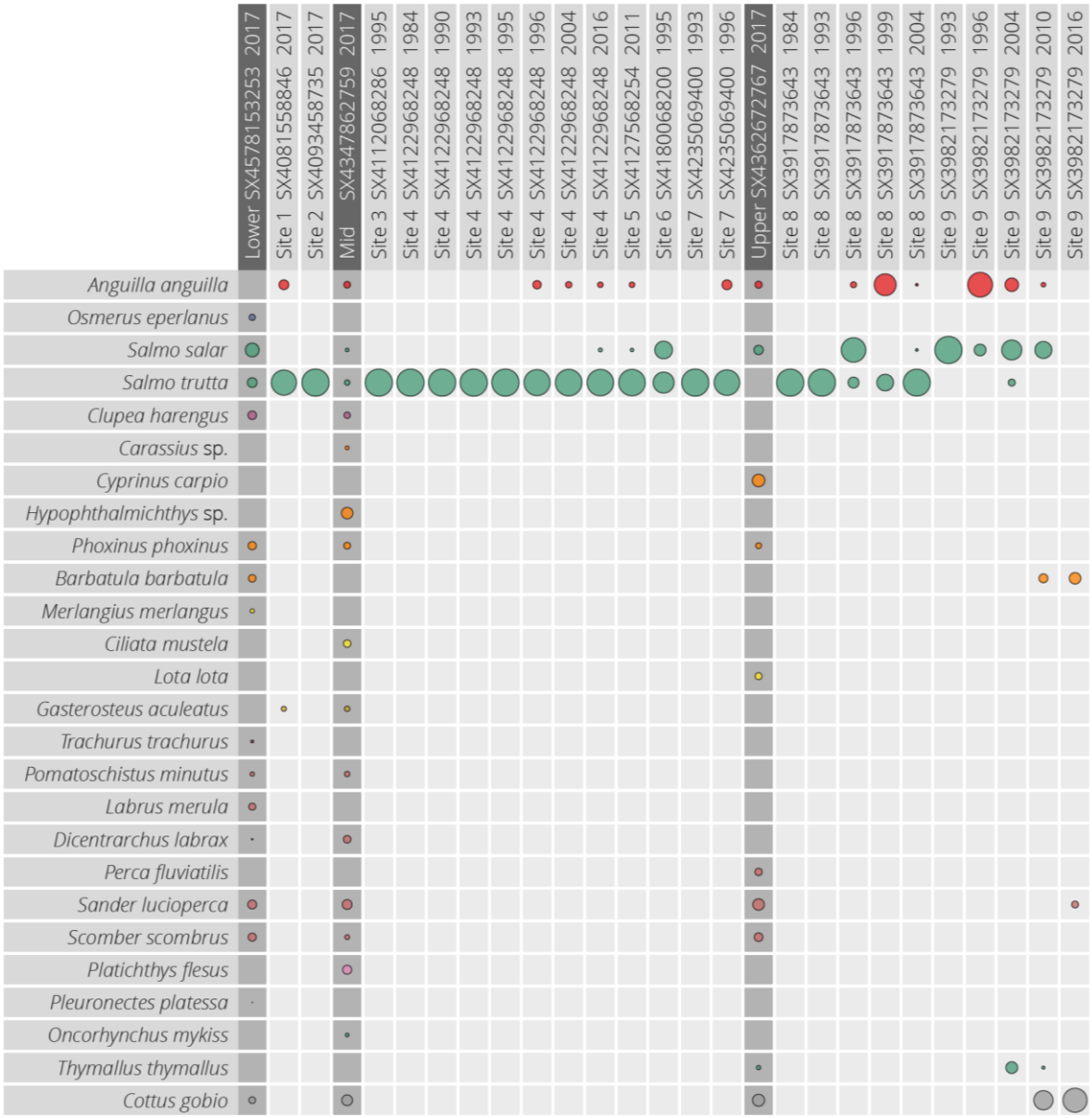

Figure 4. The proportion of fish (sequences for eDNA and individuals for traditional surveys) detected across 3 eDNA surveys and 24 traditional surveys across the closest 9 sites to the eDNA samples.

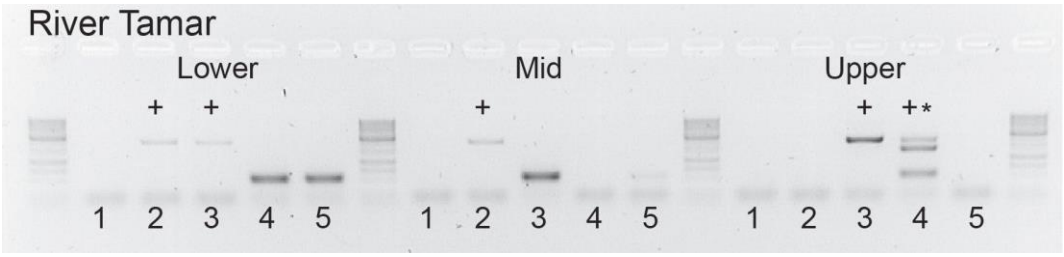

Figure 5. Gel electrophoresis image of the lamprey PCR assay split among the 3 sections of the Tamar (Lower, Mid, Upper). There were two different sized products from the assay: The lamprey specific band (indicated by an asterisk *), and a larger product denoted by a plus (+).

Fish assemblage

A total of 26 fish taxa were detected across the 15 samples (excluding non-metazoan and contaminant taxa) (Table 4), of which 23 could be identified to species level. These 26 taxa belong to 10 orders (Anguilliformes, Clupeiformes, Cypriniformes, Gadiformes, Gasterosteiformes, Osmeriformes, Perciformes, Pleuronectiformes, Salmoniformes, and Scorpaeniformes), 17 families (Anguillidae, Carangidae, Clupeidae, Cottidae, Cyprinidae, Gadidae, Gasterosteidae, Gobiidae, Labridae, Lotidae, Moronidae, Nemacheilidae, Osmeridae, Percidae, Pleuronectidae, Salmonidae, and Scombridae), and 25 genera. The relative proportion of the fish sequences found in each of the samples is shown in Figure 4, and Table 4.

The average species richness was 3.6 and ranged from 0 (Upper 3 (TAMAR)) to 11 (Lower 4 (TAMAR)), and the diversity is summarised in Table 5. The diversity and the proportion of sequence reads predominantly comprised Atlantic salmon (*Salmo salar*), which was the most commonly detected species (detected in 5 samples) and accounted for 17.8% of the total sequence reads.

The low number of detected species and complete lack of detected fish species in Upper 3, is likely to due to the lower amount of DNA captured from these filters. Previously only the DNA captured on the filter was extracted, whereas now DNA is simultaneously extracted from the preservation buffer. Increasing the DNA yield has a direct effect on the detectability of the fish in the sample. At low DNA concentrations, the stochastic effect of PCR is greater. Some interesting detections in these samples include bighead/ silver carp in the middle of Tamar, burbot in the upper Tamar, and zander in the lower, mid and upper Tamar. All three of these unexpected detections are a perfect match to those species on our database and make up a decent proportion of the sequence reads for those samples.

Table 4. Proportion of the sequencing output allocated to the different species among the River Tamar samples. Care should be taken in interpreting the numbers in terms of relative species abundance, but a high sequence proportion can be interpreted as lending greater confidence to a detection. Darker shades of blue correspond to higher proportion of sequence output per site. No data is presented for 'Upper 3' because no fish sequences were returned from the analyses.

	Lower 1	Lower 2	Lower 3	Lower 4	Lower 5	Mid 1	Mid 2	Mid 3	Mid 4	Mid 5	Upper 1	Upper 2	Upper 3	Upper 4	Upper 5
Atlantic herring				11.70	15.71			9.74	5.31						
Atlantic mackerel				6.35	16.06				8.67					32.23	
Atlantic salmon		100.00		5.51					5.04					10.30	17.00
Bass				2.71		29.45									
Bighead carp / Silver carp						70.55									
Brown trout				18.14	19.34			9.97							
Brown wrasse					14.24										
Burbot														20.44	
Carp															43.38
Crucian carp / Goldfish								5.23							
Eel species							55.34		3.44		26.61				
European bullhead					12.76		44.66	32.94							39.62
Five-bearded rockling									6.99	100.00					
Flounder								21.09	9.12						
Grayling												100.00			
Horse mackerel				5.01											
Minnow				21.24	9.23				18.16					13.90	
Perch														23.13	
Plaice				2.36											
Rainbow trout								4.78							
Sand goby				10.14					10.94						
Smelt	100.00														
Stone loach				7.31	12.65										
Three-spined stickleback									11.22						
Whiting				9.53											
Zander			100.00					16.25	21.10		73.39				

Table 5. Frequency of occurrence of all detected families obtained from 15 samples. Numbers correspond to the number of taxa belonging to those families in those samples.
L = Lower, M = Mid, U = Upper.

Family	L1	L2	L3	L4	L5	M1	M2	M3	M4	M5	U1	U2	U3	U4	U5	Sum
Anguillidae	0	0	0	0	0	0	**1**	0	**1**	0	**1**	0	0	0	0	**3**
Carangidae	0	0	0	**1**	0	0	0	0	0	0	0	0	0	0	0	**1**
Clupeidae	0	0	0	**1**	**1**	0	0	**1**	**1**	0	0	0	0	0	0	**4**
Cottidae	0	0	0	0	**1**	0	**1**	**1**	0	0	0	0	0	0	**1**	**4**
Cyprinidae	0	0	0	**1**	**1**	**1**	0	**1**	**1**	0	0	0	0	**1**	**1**	**7**
Gadidae	0	0	0	**1**	0	0	0	0	0	0	0	0	0	0	0	**1**
Gasterosteidae	0	0	0	0	0	0	0	0	**1**	0	0	0	0	0	0	**1**
Gobiidae	0	0	0	**1**	0	0	0	0	**1**	0	0	0	0	0	0	**2**
Labridae	0	0	0	0	**1**	0	0	0	0	0	0	0	0	0	0	**1**
Lotidae	0	0	0	0	0	0	0	0	**1**	**1**	0	0	0	**1**	0	**3**
Moronidae	0	0	0	**1**	0	**1**	0	0	0	0	0	0	0	0	0	**2**
Nemacheilidae	0	0	0	**1**	**1**	0	0	0	0	0	0	0	0	0	0	**2**
Osmeridae	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**
Percidae	0	0	**1**	0	0	0	0	**1**	**1**	0	**1**	0	0	**1**	0	**5**
Pleuronectidae	0	0	0	**1**	0	0	0	**1**	**1**	0	0	0	0	0	0	**3**
Salmonidae	0	**1**	0	**2**	**1**	0	0	**2**	**1**	0	0	**1**	0	**1**	**1**	**8**
Scombridae	0	0	0	**1**	**1**	0	0	0	**1**	0	0	0	0	**1**	0	**4**

River Wye

Migratory fish

Among the migratory species found were Atlantic salmon, trout and eel (Figure 6). Atlantic salmon was detected in 40% of the samples (4 of the 10 replicates) with 2 incidences in the Brockweir (Mid) and 2 in the Bigweir (Upper). No evidence of Atlantic salmon was detected from the estuary samples. Brown trout were detected only at 8 Wye Bigsweir (Upper). Eel were detected in 60% of the samples (6 of 10) with one incidence in the last estuary sample (Lower) and in both Brockweir (Mid) and all three Bigsweir samples (Upper). The lamprey assay was positive in two samples, one in the Mid Wye (Brockweir) and one in the Upper Wye (Bigweir) (Figure 7).
No Environment Agency data for a similar location exists for comparison. An APEM electrofishing survey conducted in 2012-2013 detected Atlantic salmon, trout, eel, and sea lamprey.

Fish assemblage

A total of 24 fish taxa were detected across the 10 samples (excluding non-metazoan and contaminant taxa) (Table 6), of which 21 could be identified to species level. These 24 taxa belong to 10 orders (Anguilliformes, Atheriniformes, Clupeiformes, Cypriniformes, Esociformes, Gasterosteiformes, Perciformes, Pleuronectiformes, Salmoniformes, and Scorpaeniformes), 11 families (Anguillidae, Atherinidae, Clupeidae, Cottidae, Cyprinidae, Esocidae, Gasterosteidae, Nemacheilidae, Percidae, Pleuronectidae, and Salmonidae), and 23 genera. The relative proportion of the fish sequences found in each of the samples is shown in Figure 6, and Table 6.

The average species richness was 8.7 and ranged from 1 (4 Wye Estuary) to 20 (7 Wye - Brockweir), and the diversity is summarised in Table 7. The diversity and the proportion of sequence reads predominantly comprised Minnow (*Phoxinus phoxinus*), which accounted for 24.4% of the total sequence reads. The most commonly detected species were Eel (*Anguilla* sp.) and Roach (*Rutilus rutilus*), which were each detected 6 times.

The sequence output and the subsequent detectable diversity for the Wye estuary samples was very low, for example, the average species richness for the estuary samples was 2, while the weir sample average species richness was 15. The reasons for this are twofold:

1) **Contaminant DNA:** There was a huge amount of cow and sheep DNA in the samples, which can come from either laboratory additives (e.g. bovine serum albumin used in PCR) or cow and sheep near to the sampling site. The amount of contaminant sequence reads ranged from 67% (1 Wye Estuary) to 80% (4 Wye Estuary) with an average of 75%, whereas the weir samples had much less contaminant DNA with an average of 17%, ranging from 2% (8 Wye - Bigsweir) to 25% (6 Wye - Brockweir).

2) **Low volume:** Only 200 ml of water was sampled with each filter compared with ~1000 ml everywhere else. The amount of water sampled and filtered is correlated with the detection probability of fish and has a more pronounced effect in larger water bodies. Moreover, the dropout rate is higher with rarer species.

The lack of consistency among the five estuarine samples is surprising given that these samples were taken from the same large pot of mixed water collected around the mouth of the Wye. We don't think the results for these samples are representative of the fish fauna and hypothesise that the inconsistency among the samples might be a result of the small volume of water sampled or the longer than usual time taken to filter and preserve the water after sampling it.

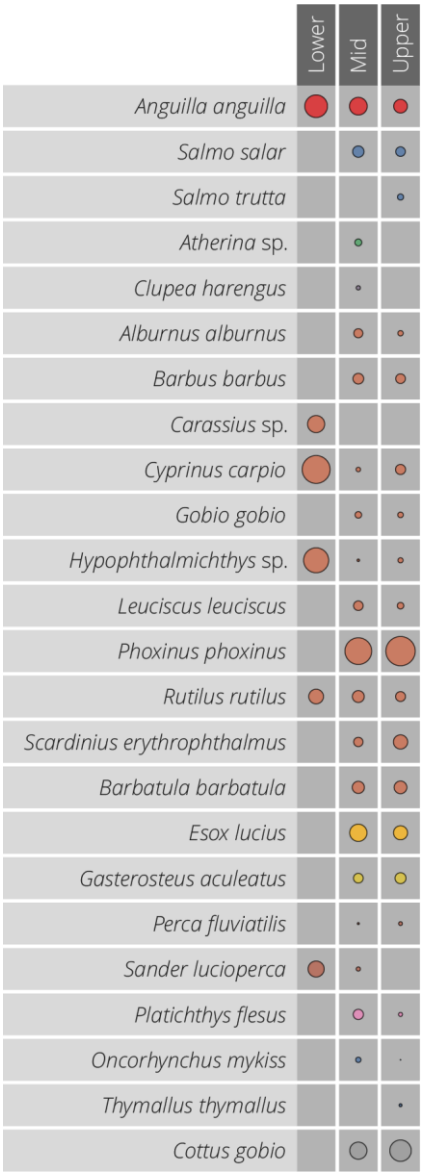

Figure 6. Proportion of the sequencing output allocated to the different species among the River Wye samples.

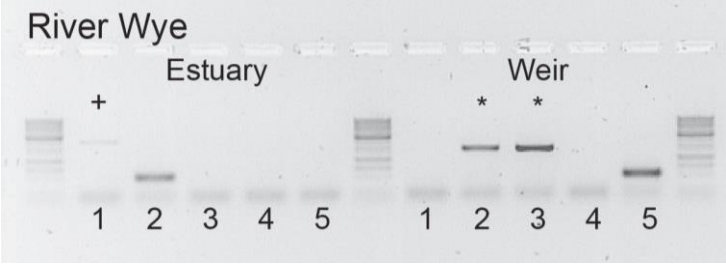

Figure 7. Gel electrophoresis image of the Lamprey PCR assay split among the 3 sections of the Wye (Lower, Mid, Upper). There were two different sized products from the assay: The lamprey specific band (indicated by an asterisk *), and a larger product denoted by a plus (+).

Table 6. Proportion of the sequencing output allocated to the different species among the River Wye samples. Care should be taken in interpreting the numbers in terms of relative species abundance, but a high sequence proportion can be interpreted as lending greater confidence to a detection. Darker shades of blue correspond to higher proportion of sequence output per site.

	1 Wye – Estuary	2 Wye – Estuary	3 Wye – Estuary	4 Wye – Estuary	5 Wye – Estuary	6 Wye – Brockweir	7 Wye – Brockweir	8 Wye – Bigsweir	9 Wye – Bigsweir	10 Wye – Bigsweir
Atlantic herring							0.84			
Atlantic salmon						5.79	3.72	2.40		5.37
Barbel						3.21	4.38	1.56	2.02	5.14
Big-scale sand smelt						4.30				
Bighead carp / Silver carp	42.17		61.72		25.62		0.30		3.56	
Bleak						4.35	1.87	0.64		1.50
Brown trout								3.28		
Carp		68.99	37.25				0.92	0.29	15.21	
Chub						3.31	2.69	4.91	3.79	11.08
Crucian carp / Goldfish		31.01	1.02							
Dace						4.10	2.55	1.77		1.71
Eel					74.38	8.71	13.13	6.72	4.90	7.53
European bullhead						5.52	13.70	19.29	10.09	18.89
Flounder							5.62	1.32		
Grayling								0.30		0.35
Gudgeon							2.13	1.68	1.51	
Minnow						32.58	23.75	30.06	36.90	32.65
Northern pike						11.36	10.64	8.04	13.82	2.60
Perch							0.22	1.16		
Rainbow trout						1.09	0.75	0.26		
Roach				100.00		6.58	4.03	2.44	4.36	3.51
Stone loach						4.28	5.69	9.37	3.83	3.52
Three-spined stickleback						4.84	2.27	4.49		6.15
Zander	57.83						0.80			

Table 7. Frequency of occurrence of all detected families obtained from 10 samples. Numbers correspond to the number of taxa belonging to those families in those samples.

Family	1	2	3	4	5	6	7	8	9	10	Sum
Anguillidae	0	0	0	0	**1**	**1**	**1**	**1**	**1**	**1**	**6**
Atherinidae	0	0	0	0	0	**1**	0	0	0	0	**1**
Clupeidae	0	0	0	0	0	0	**1**	0	0	0	**1**
Cottidae	0	0	0	0	0	**1**	**1**	**1**	**1**	**1**	**5**
Cyprinidae	**1**	**2**	**3**	**1**	**1**	**6**	**9**	**8**	**7**	**6**	**10**
Esocidae	0	0	0	0	0	**1**	**1**	**1**	**1**	**1**	**5**
Gasterosteidae	0	0	0	0	0	**1**	**1**	**1**	0	**1**	**4**
Nemacheilidae	0	0	0	0	0	**1**	**1**	**1**	**1**	**1**	**5**
Percidae	**1**	0	0	0	0	0	**2**	**1**	0	0	**3**
Pleuronectidae	0	0	0	0	0	0	**1**	**1**	0	0	**2**
Salmonidae	0	0	0	0	0	**2**	**2**	**4**	0	**2**	**4**

River Frome

Migratory fish

Sampling locations are shown in Figure 8. Among the migratory species found were Atlantic salmon, trout, and eel (Figure 9), all three species were detected in the lower, mid and upper Frome. Atlantic salmon and brown trout were detected in all 15 replicate samples with seemingly even numbers of sequence reads throughout the river system (Figure 9). Eel were detected in 80% of the samples (12 of 15) with 5 incidences in the lower Frome, 4 in mid Frome and 3 in the upper Frome. The lamprey assay was positive in two samples, one in the lower Frome and one in the mid Frome (Figure 10).

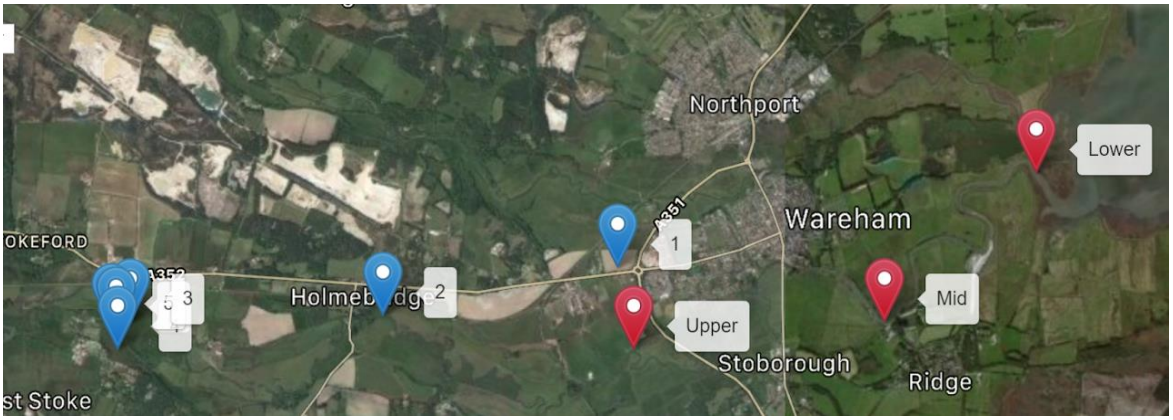

Figure 8. Sampling locations for the eDNA samples (red) and the six sites from which 16 different fish surveys were conducted by the Environment Agency (blue).

The eDNA results were compared to Environment Agency data from a similar area dating from 2002 to 2018 at 6 different sites in and around the eDNA sampling points (Figure 8). Unfortunately no Environment Agency data was available closer to the lower part of the Frome. Across 16 surveys, over 12 years, 2625 individual fish were counted and these included 506 salmon, 159 brown trout, and 466 eels. According to the Environment Agency data for these 6 sites, 3 individual brook lamprey (*Lampetra planeri*) were detected in 2011 in Mill Stream at East Stoke (SY8725186665). Salmon were detected in 15 surveys, trout were detected in 14 surveys and eel were detected in 12 surveys.

Fish assemblage

A total of 28 fish taxa were detected across the 15 samples (excluding non-metazoan and contaminant taxa) (Table 8), of which 24 could be identified to species level. These 28 taxa belong to 10 orders (Anguilliformes, Clupeiformes, Cypriniformes, Esociformes, Gasterosteiformes, Mugiliformes, Perciformes, Pleuronectiformes, Salmoniformes, and Scorpaeniformes), 14 families (Anguillidae, Clupeidae, Cottidae, Cyprinidae, Esocidae, Gasterosteidae, Gobiidae, Labridae, Moronidae, Mugilidae, Nemacheilidae, Percidae, Pleuronectidae, and Salmonidae), and 27 genera. The relative proportion of the fish sequences found in each of the samples is shown in Figure 9 and Table 8.

The average species richness was 13 and ranged from 8 (River Frome upper site 1) to 17

(River Frome Estuary 1, River Frome Estuary 2, River Frome Estuary 5, and River Frome middle site 2), and the diversity is summarised in Table 9. The diversity and the proportion of sequence reads predominantly comprised European bullhead (*Cottus gobio*), which was the most commonly detected species (detected in all 15 samples) and accounted for 42% of the total sequence reads. Other commonly detected species included minnow (*Phoxinus phoxinus*), brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*), which were detected in all 15 samples, and three-spined stickleback (*Gasterosteus aculeatus*) and stone loach (*Barbatula barbatula*), which were each found in 14 of the samples.

Sunbleak and Zander, which are non-native species were detected in the River Frome. Sunbleak was detected in the Frome estuary (sample 2) and Zander was detected in both the Frome estuary (sample 3) and Upper Frome (samples 1 and 2). The sequence reads for these taxa were a perfect match to previously sequenced individuals from these species.

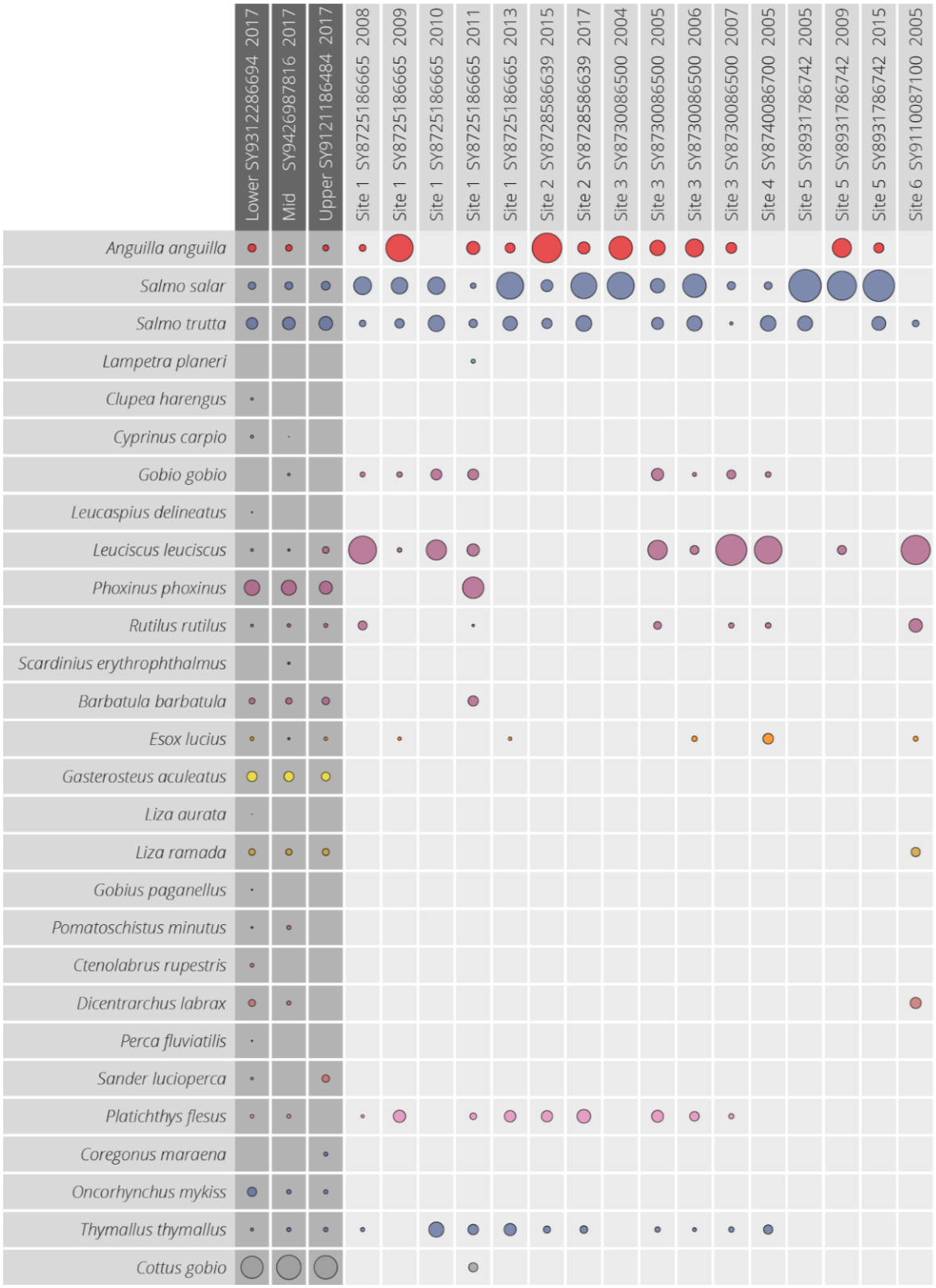

Figure 9. Proportion of the sequencing output allocated to the different species among the River Frome samples.

Figure 10. Gel electrophoresis image of the Lamprey PCR assay split among the 3 sections of the Frome (Lower, Mid, Upper). There were two different sized products from the assay: The lamprey specific band (indicated by an asterisk *), and a larger product denoted by a plus (+).

Table 8. Proportion of the sequencing output allocated to the different species among the River Frome samples. Care should be taken in interpreting the numbers in terms of relative species abundance, but a high sequence proportion can be interpreted as lending greater confidence to a detection. Darker shades of blue correspond to higher proportion of sequence output per site.

	River Frome Estuary 1	River Frome Estuary 2	River Frome Estuary 3	River Frome Estuary 4	River Frome Estuary 5	River Frome middle site 1	River Frome middle site 2	River Frome middle site 3	River Frome middle site 4	River Frome middle site 5	River Frome upper site 1	River Frome upper site 2	River Frome upper site 3	River Frome upper site 4	River Frome upper site 5
Atlantic herring		1.00													
Atlantic salmon	3.29	2.99	2.94	3.71	5.22	2.09	4.14	1.22	5.40	7.88	5.72	6.92	5.32	8.28	0.75
Bass	6.34	0.64		6.36	4.45	1.02	1.41		1.60						
Bream species				0.48											
Brown trout	6.89	10.41	3.18	11.22	11.91	12.15	15.32	18.87	6.02	9.72	23.18	4.32	13.63	9.07	25.02
Carp	1.84			0.51	0.66		0.28								
Dace	0.76	0.80			0.19		0.36			1.34			9.57	3.71	0.54
Eel	2.74	5.75	2.22	5.11	4.15	4.27	5.30		1.27	3.82			5.75	1.45	4.58
European bullhead	43.89	41.45	32.52	38.14	35.06	49.05	42.94	57.34	44.79	34.70	18.58	48.35	42.19	55.39	45.91
Flounder	0.76	1.68		0.73		0.75	1.38	2.52							
Golden mullet				0.24											
Goldsinny wrasse					2.70										
Grayling	0.62	0.51		0.67	0.48	0.53	1.04		1.87	0.67		2.99		1.06	0.75
Gudgeon						0.55	0.30			1.13					
Minnow	16.95	14.86	22.67	18.39	18.46	18.41	15.10	9.47	18.34	21.39	15.55	14.75	6.26	12.53	13.58
Northern pike		0.95	0.47	1.12	0.74	0.47	0.33	0.36						3.09	0.89
Perch	0.79														
Rainbow trout	3.71	3.64	12.96	3.90	6.77	0.32	0.97		1.20	4.02		1.43	1.20		2.06
Roach	0.30	0.24	1.22		0.32	0.51	0.87			2.84		2.57			1.04
Rock goby	0.98														
Rudd						0.85			0.23						
Sand goby					0.58			5.61							
Stone loach	4.24	0.90	1.15	3.94	2.50	1.64	1.58	1.20	4.19	3.20	3.13		9.72	2.77	3.69
Sunbleak		0.21													
Thick/Thin-lipped mullet	3.91	3.47	3.44	2.64	1.26	1.04	1.19		3.77	6.84		9.69	1.89		
Three-spined stickleback	2.00	10.50	15.09	2.84	4.56	6.35	7.49	3.41	11.33	2.46	21.46		4.46	2.65	1.20
Whitefish species											5.24				
Zander			2.15								7.13	8.99			

Table 9. Frequency of occurrence of all detected families obtained from 15 samples from the River Frome. Numbers correspond to the number of taxa belonging to those families in those samples. E = Estuary, M = Mid, U = Upper.

Family	E1	E2	E3	E4	E5	M1	M2	M3	M4	M5	U1	U2	U3	U4	U5	Sum
Anguillidae	**1**	**1**	**1**	**1**	**1**	**1**	**1**	0	**1**	**1**	0	0	**1**	**1**	**1**	**12**
Clupeidae	0	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**
Cottidae	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**15**
Cyprinidae	**4**	**4**	**2**	**3**	**4**	**4**	**5**	**1**	**2**	**4**	**1**	**2**	**2**	**2**	**3**	**15**
Esocidae	0	**1**	**1**	**1**	**1**	**1**	**1**	**1**	0	0	0	0	0	**1**	**1**	**9**
Gasterosteidae	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	0	**1**	**1**	**1**	**14**
Gobiidae	**1**	0	0	0	**1**	0	0	**1**	0	0	0	0	0	0	0	**3**
Labridae	0	0	0	0	**1**	0	0	0	0	0	0	0	0	0	0	**1**
Moronidae	**1**	**1**	0	**1**	**1**	**1**	**1**	0	**1**	0	0	0	0	0	0	**7**
Mugilidae	**1**	**1**	**1**	**2**	**1**	**1**	**1**	0	**1**	**1**	0	**1**	**1**	0	0	**11**
Nemacheilidae	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	0	**1**	**1**	**1**	**14**
Percidae	**1**	0	**1**	0	0	0	0	0	0	0	**1**	**1**	0	0	0	**4**
Pleuronectidae	**1**	**1**	0	**1**	0	**1**	**1**	**1**	0	0	0	0	0	0	0	**6**
Salmonidae	**4**	**4**	**3**	**4**	**4**	**4**	**4**	**2**	**4**	**4**	**3**	**4**	**3**	**3**	**4**	**15**

Table 10. Unresolved taxa.

Identification	Match (ID%)	Comment
Bighead carp / Silver carp	100	This sequence is either bighead carp (*Hypophthalmichthys nobilis*) or silver carp (*H. molitrix*), which are indistinguishable based on this particular DNA barcode.
Bream species	100	This sequence is either vimba bream (*Vimba vimba*) or silver bream (*Abramis bjoerkna*), which are indistinguishable based on this particular DNA barcode. We therefore conservatively identify this sequence as a bream species.
Crucian carp / Goldfish	100	These species are so closely related that they are able to hybridise in the wild. It is thought that goldfish are actually a cultivated breed of crucian carp taken from the wild. It is more likely that the observed species is a crucian carp.
Eel species	100	This sequence is a perfect match to either the European eel (*Anguilla anguilla*) or the American eel (*Anguilla rostrata*), which are indistinguishable based on this particular DNA barcode. It is more likely that the observed species is a European eel.
Thin/thick - lipped mullet	100	This sequence is either *Liza ramado* or *Chelon labrosus*, which are thin and thick-lipped mullets, and may be incorrectly attributed on the reference database.
Whitefish species	100	This sequence is houting (*Coregonus oxyrinchus*), Pollan (*Coregonus autumnalis*) or powan (*Coregonus lavaretus*), which are indistinguishable based on this particular DNA barcode. We therefore conservatively identify this sequence as a whitefish species. This species complex is currently argued to be a single species - *Coregonus maraena*.

Discussion

Here we have shown that a modest eDNA sampling effort (10-15 samples taken in a single day by a single team) can capture the same diversity of migratory fish species as has been recorded from similar locations with decades of Pulsed Direct Current Electrofishing. We detect salmon (*Salmo salar*), brown trout (*Salmo trutta*), eel (*Anguilla anguilla*) and lamprey (Petromyzontidae) in the Tamar, the Wye and the Frome, but also smelt (*Osmerus eperlanus*) in the Tamar estuary. Our fish eDNA pipeline is quite well established and the major shortcomings of the pipeline are known to us: these include a minimum volume of water to be filtered and an improved extraction process. Variability in the consistency of the diversity detected between the Tamar samples and the Frome samples trace the evolution of our method.

When comparing these data to the 'Environment Agency Freshwater Fish Counts for all Species' we should note that the data are not completely comparable in terms of effort, specific timing, or spatial scale. However, the similarities in these data highlight the potential for eDNA in migratory fish monitoring. With the exception of the smelt found in the Tamar estuary, the exact same migratory species have been found in all three river systems.
One thing is very clear, for the same amount of time and sampling effort, much more can be routinely done with eDNA than with traditional methods. For instance, at each location, all of the eDNA sampling was done in a day and could have been done by a single person or a small team, no special equipment is required and the barrier to entry for the data collection is low. It must be stressed that the barrier to entry for the lab work and analysis is much higher. Having said that, the additional effort taken to conventionally sample the fish will afford the user with additional information on size, sex ratios, condition, and abundance. An additional benefit of the eDNA metabarcoding method is that you get a picture of the fuller fish community rather than just the target species. For Tamar and Frome (where comparable data are available), a total of 38 species were found by eDNA metabarcoding of which only 45% were found by electrofishing. Bullhead (*Cottus gobio*), an Annex II species was also found in all three river systems. Monitoring the community as a whole is especially important because this biological layer is important in determining understanding the dynamics of a whole fish assemblage.

eDNA methods are non-invasive and so circumvent the documented negative effects of electric fishing treatments on fish (e.g. reduced the growth, spinal misalignments, lower condition, and reduced survival), which is contrary to a mandate for protection and monitoring of threatened taxa. While really good presence-absence data is readily obtainable with eDNA metabarcoding, important limitations exist when compared to traditional monitoring, and so different methods will need to be employed to gain different aspects of information. As the method stands, eDNA is not strictly quantitative, although there is a growing body of literature that suggests that data generated using eDNA has strong and meaningful trends when compared to fish abundances (e.g. Pont et al. 2018 - Scientific reports 8.1: 10361; Li et al. 2019 - Journal of Applied Ecology). eDNA cannot inform you of the life history, age class distribution, condition, or measurements of the target species, for this information more thorough trapping techniques need to be employed. In addition, the data needs to be interpreted carefully, for example the presence of Whitefish in the upper River Frome is possibly because it has been used as a bait species.

We have shown that eDNA metabarcoding shows promise as an efficient, cost-effective, and sensitive means of monitoring fish communities, which could be narrowed down to investigate the migration patterns of certain fish. The key benefits of using eDNA for monitoring fish is that whole communities can be characterised simultaneously and that the sampling is easy and convenient to perform, which makes it possible to employ widely, flexibly and frequently for high temporal resolution, in response to particular impact events that may affect the species in question.