Environmental DNA as a non-invasive sampling tool to detect the spawning distribution of European anadromous shads (*Alosa* spp.)

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Abstract

1. Populations of the European shads *Alosa alosa* and *Alosa fallax* (*Alosa* spp.) are protected under legislation due to their vulnerability to human disturbances. In particular, river impoundments block their upstream migration, preventing access to spawning areas. Knowledge on the spatial extent of their spawning is important for informing conservation and river management plans.

2. Determining the spatial extent of *Alosa* spp. spawning is challenging. They enter rivers over a two to three-month period and the species potentially migrate different distances upstream. Capture and handling can be problematic, spawning events generally occur at night, and kick sampling for eggs is limited to shallow water. Assessing their spatial extent of spawning could, however, incorporate non-invasive sampling tools, such as environmental DNA (eDNA).

3. An eDNA assay for *Alosa* spp. was successfully developed, based on the Cytochrome *c* Oxidase Subunit I gene segment and quantitative polymerase chain reaction (qPCR). Application in spring 2017 to the River Teme (River Severn catchment, Western England) revealed high sensitivity in both laboratory and field trials. Field data indicated *Alosa* spp. spawning between May and June, with migrants mainly restricted to areas downstream of the final impoundment.

4. eDNA can thus be utilised as a non-invasive sampling tool to determine the freshwater distribution of these fishes in Europe, enhancing their conservation at local and regional scales.

38 Keywords: detection, environmental DNA, impoundment, migratory fish, monitoring, qPCR

1. Introduction

European shads *Alosa alosa* and *Alosa fallax* are cryptic, anadromous fishes whose distributions overlap (Alexandrino et al., 2006). In general, their populations have declined throughout their geographical range (Aprahamian, Aprahamian, Bagliniére, Sabatié, & Alexandrino, 2003), with both species listed in the Bern Convention (Appendix V) and Habitats Directive of the European Union (Annexes II and V) (Aprahamian, Lester, & Aprahamian, 1999; Aprahamian, et al., 2003). Where they spawn in close proximity, the fishes tend to produce reproductively viable hybrids (Jolly et al., 2012).

The spawning behaviour of these *Alosa* spp. involves migration into freshwater in spring (timing dependent on location, but usually April to July; Kottelat & Freyhof, 2007). Of the two species, *A. alosa* tends to migrate the furthest upstream to spawn and so when unimpeded the two fishes can segregate in their spawning areas. However, the construction of weirs on many European rivers now largely prevents this segregation, resulting in high genetic introgression (Jolly et al., 2012), with *A. alosa* largely absent from many of its former rivers (Aprahamian et al., 1999).

The conservation of *Alosa* spp. in European rivers requires spatial and temporal information on their spawning distributions and how these relate to river impoundments. Assessments of their spawning distributions can, however, be difficult to complete using capture methods due to, for example, the general sensitivity of the fishes to handling and anaesthesia (Breine et al., 2017). Egg sampling can provide positive indications of spawning activity (Caswell & Aprahamian, 2001; JNCC, 2015), but can be labour intensive when applied across large spatial areas. It is also limited to areas of relatively shallow waters, with spawning of *Alosa* spp. in some European rivers occurring in the deeper, lower reaches, including estuarine

areas (Magath & Thiel, 2013; Briene et al., 2017). Detection of spawning events can be completed, but these tend to occur at night. An alternative is environmental DNA (eDNA), a non-invasive sampling tool that has increasingly been shown to provide a reliable method for detecting rare and endangered aquatic species (Pilliod, Goldberg, Arkle, & Waits, 2013). Although there remains some uncertainties in the application and interpretation of eDNA data (e.g. Roussel, Paillisson, Treguier & Petit, 2015), evidence increasingly suggests it can provide greater probabilities of detection of aquatic species when compared to the use of traditional sampling techniques (Jerde et al., 2011; Dejean et al., 2012), especially when 'best practice' methodologies are used (Wilcox et al., 2018)

The aim of this study was to thus develop and test an eDNA sampling tool for the detection of *Alosa* spp. in rivers during their spawning migrations. A quantitative PCR (qPCR) was developed to detect *Alosa* spp; and its utility was tested using laboratory and field trials. The field trials were completed on the River Teme, a major tributary of the River Severn, western England, where current data suggest *Alosa* spawning is restricted to the area below the final impoundment (Powick Weir) close to the Severn confluence (Pinder, Andreou, Hardouin, Sana, Gillingham & Gutmann Roberts, 2016). The field trials determined the duration of *Alosa* spawning period and the spatial extent of their distribution. The spatial distribution of the fish was assessed to enable subsequent assessment of how the partial removal of this final impoundment will subsequently affect the spatial distribution of spawning *Alosa* spp. in the river (Environment Agency, 2018).

2. Methods

2.1 eDNA filtering and extraction

Samples were collected across four sites of the River Teme in 2017 (Table 1). The primary focus was on Site 1, located downstream of the final weir impoundment where *Alosa* spp. have been historically been observed to spawn, enabling the duration of the spawning season to be determined. To assess their spatial distribution, three additional sites were used, all upstream of the weir at Site 1, at distances to 48 km upstream. Initial samples were collected in March (as controls) and then between late May and early July (Table 1). All water samples were collected in 1 L sterile plastic bottles.

Water samples were collected by two methods. Firstly, they were collected by samplers standing in the riparian zone. Sampling bottles were attached to an extendible pole (1.8 to 3.7 m). Equipment was cleaned after collecting each sample (10 % microsol detergent; Anachem, UK). Ten water samples were collected per site, comprising of paired samples (at 1.8 and 3.7 m) from five sampling points (10 m intervals). Two negative controls were taken; after 5 samples (1.8 m) and 10 samples (3.7 m). These were the same type of bottles, but filled with sterile water and treated in the same manner as the sample collection bottles. The sampling equipment was changed and sterilised between sampling points. Secondly, samples were collected from bridges, with 10 samples and 2 negative controls initially collected from each bridge from across the river's wetted width. This reduced to 5 and 1 negative control following initial analyses. During sampling, each bottle had been pre-weighted (700 g) and placed individually in a plastic sample bag. In the field, each bottle was lowered into the river on a rope to collect the sample.

2.3 *eDNA qPCR assay development*

The primer and probe specific for *Alosa* spp. Cytochrome *c* Oxidase Subunit I gene segment (COI gene) was designed by Applied Biosystems (assay ID: APMFW3H). Probe and

primers sequences were designed using European *Alosa* spp. (*A. alosa, A. fallax* and hybrids) sequences in the National Centre for Biotechnology Information nucleotide database (NCBI - https://www.ncbi.nlm.nih.gov/). Specificity to European *Alosa* spp. was determined in an *in-silico* test using target and off target species commonly found in British freshwaters (Table S1). The TaqMan® Gene Expression Master Mix UDG was used for this assay (Applied Biosystems). Extracted DNA from scales of *Alosa* spp. collected from the River Severn catchment was used as a template for assay validation and standard curves for qPCR.

The *Alosa* spp. specific COI gene assay was tested for cross-reactivity with pure fish DNA present in the freshwater areas of the River Severn catchment (10 ng for each of the following fish species: roach *Rutilus rutilus*, minnow *Phoxinus phoxinus*, common bream *Abramis brama*, chub *Squalius cephalus,* perch *Perca fluviatilis*, dace *Leuciscus leuciscus*, bleak *Alburnus alburnus*, grayling *Thymallus thymallus*, brown trout, *Salmo trutta*, Atlantic salmon *Salmo salar*, gudgeon *Gobio gobio*, eel *Anguilla Anguilla*, sea lamprey *Petromyzon marinus,* brook lamprey *Lampetra planeri*, carp *Cyprinus carpio* and European barbel *Barbus barbus*). Note that as the eDNA water samples were being collected from freshwater areas only then cross-reactivity was not tested for other fishes of the Clupeidae family that occur in marine and estuarine waters (e.g. *Clupea harengus*). The assay was also not tested on North American *Alosa* spp. (e.g. *Alosa sapidissima*; *Alosa pseudoharengus*). To determine the sensitivity of the assay, a calibration curve was generated using genomic DNA extracted from *Alosa* spp. scales. A ten-fold serial dilution of *Alosa* spp. genomic DNA was prepared to give a template concentration from 10 ng/µl to 1 fg/µl. The detection limit was defined as the lowest genomic *Alosa* DNA concentration detected at least 95 % of the times by the qPCR assay. qPCR was run for each eDNA sample in triplicate in 20 µl, under manufacture's instruction, with 2 µl of DNA template (undiluted). The qPCR run method

used warm-up conditions of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles between 95 °C for 15 s and 60 °C for 1 min. All negative controls were performed in triplicate.

3. Results

3.1 eDNA assay validation

Using a ten-fold serial solution of *Alosa* spp. genomic DNA, the limit of detection of the assay was 1 pg/µl, with a mean C_t-value of 37 (± 0.02 SD). The C_t-values with standard genomic DNA dilutions in the late cycle (> 37), which corresponded to 0.1 pg/µl, were unreliable as the probability of detection was < 95%. No amplification was detected in all negative controls. The qPCR was also found to be highly specific to *Alosa* spp., with no cross-species amplification detected.

3.2 Comparing eDNA sampling methods

Both water sampling methods resulted in positive detections of *Alosa* DNA (Table 1). Sampling from the riparian zone resulted in significantly higher C_t values and eDNA concentration than from bridges (non-parametric Wilcoxon rank test: Z = -2.59; and Z = -3.39, respectively, $P < 0.05$). However, bridge sampling was more time efficient in the field as equipment was pre-prepared and pre-sterilised in the laboratory, and thus was the preferred method.

3.3 eDNA detection of Alosa *spp.*

Water samples collected from the River Teme in March were negative but were all positive at the end of May; peak DNA concentrations occurred in mid-June and final detections were in early July (Table 1; Fig. 1). Spatially, *Alosa* spp. DNA was most frequently detected in

169 Site 1 (Table 1). No positive samples were recorded from Sites 2 and 3, but at Site 4, *Alosa*
170 DNA was detected in two water samples in early June (Table 1).
171
172 **4. Discussion**
173
174 An eDNA method to detect the presence of *Alosa* spp. in rivers was successfully developed
175 and tested. This assay had a discrete level of resolution (detection limit: 1 pg/µl) and high
176 specificity for *Alosa* spp.. Temporally, positive samples were recorded between May and
177 early July at Site 1, with peak DNA concentrations in mid-June. Only two positive samples
178 were recorded further upstream. These initial data thus suggest the primary spawning area in
179 this river was in Site 1, downstream of the final weir, with a much smaller number of
180 individuals by-passing this weir and moving further upstream. The spawning activity in
181 Section 1 was validated by the presence of *Alosa* eggs that were regularly sampled in the
182 section between mid-May and mid-June (unpublished data).
183
184 The detection rates of eDNA can be relatively high in river water samples (Pilliod et al.,
185 2013), although information on the spatial resolution of these detections often remains
186 uncertain (Goldberg, Strickler & Pilliod, 2015). For example, macro-invertebrate DNA can
187 be detected from source populations up to 10 km upstream (Deiner & Altermatt, 2014). For
188 fish, distances tend to be closer to 1 km upstream (Balasingham, Walter, & Heath, 2017).
189 However, the absence of a consistent relationship between eDNA concentration and
190 downstream distances (Laramie, Pilliod, & Goldberg, 2015) suggest that consistent DNA
191 accumulations do not occur. This is due to DNA settlement on the riverbed and subsequent
192 re-suspension and degradation (Shogren et al., 2017; Wilcox et al., 2016). The positive
193 detections of *Alosa* at Site 1 were all from samples collected approximately 0.5 km
194 downstream of the final impoundment. Consequently, it was assumed to all be from fish

present downstream of this weir. It was less clear where the *Alosa* spp. detected at Site 4 were located, and further investigation will represent an important step to understand this result. Moreover, the general lack of species-specific marker to discriminate between these *Alosa* species (Faria, Weiss, & Alexandrino, 2012) meant it could not be determined whether this DNA originated from *A. alosa*, *A. fallax* or a hybrid form. Whilst potentially important, as *A. alosa* tend to migrate greater distances than *A. fallax* (Kottelat and Freyhof, 2007), the River Teme is a relatively small catchment. Correspondingly, the distances from the Severn estuary to Site 4 of the study were within the migration range of both European *Alosa* spp. (Aprahamian et al., 2003). In general, this aspect of the results highlight the need to complete further work on how the spatial extent of *Alosa* spawning in non-impounded rivers is related to spatial variability in the genetic composition of populations.

Further investigations and more stringent analyses could enable further examination of the eDNA field results, especially in areas upstream of Site 1. This is because both site-specific and environmental conditions can influence eDNA detection (Stoeckle et al., 2017), potentially leading to the detection of false positive recordings. In addition, factors such as humic acid, non-target eDNA and other particles, are responsible for PCR interference that can lead to false negative data (Goldberg et al., 2016), which decreases the potential level of resolution of the assay. Moreover, sampling for *Alosa* eggs at each site and completing spawning observations would provide complementary data and assist validation of the eDNA results. Indeed, complementary sampling by egg collection (by kick sampling or drift nets) or, where river conditions do not permit this, then spawning observations, is recommended wherever the eDNA assay is applied. This would also enable the cost-effectiveness of the eDNA assay versus traditional sampling techniques to be determined. In addition, effectiveness of the assay to detect migrating *Alosa* spp. in the lower reaches of rivers, including estuaries, requires testing. However, it is argued that the most appropriate

application of the assay is the determination of the upstream limits of *Alosa* spp. migration and detecting their presence/ absence in rivers where anecdotal evidence suggests fish are present but this has not been confirmed by traditional sampling methods.

In summary, an eDNA assay for European *Alosa* fishes was successfully developed that, when applied to the River Teme, revealed the temporal and spatial extent of their 2017 spawning migration. Spatially, spawning *Alosa* spp. were primarily restricted to the area below the final impoundment, although the results suggested small numbers of *Alosa* spp. can occasionally pass this barrier and move up to 48 km upstream. The planned modification of this impoundment should thus open up more of the catchment to migrating *Alosa* spp. than is the case at present (Environment Agency, 2018). Subsequent refinement and testing of the assay will specifically enable this to be tested and, in general, will improve the power of this assay to assess the temporal and spatial patterns of migrating *Alosa* spp. in European rivers.

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Table 1: Description of sampling site, site ID, GPS coordinates, date of sampling, number of water samples collected and the number of samples with eDNA detection of *Alosa* spp. DNA are indicated.

Location	Site ID	Sampling method	GPS Coordinates	Date	Water Samples	eDNA detection of *Alosa spp.*
Powick	1	Bridge	52.170497, -2.242295	30/05/17	8	8
				12/06/17	10	4
				19/06/17	10	6
				02/07/17	10	2
				18/07/17	5	0
				08/08/17	5	0
		Riparian zone	52.169564, -2.240533	23/03/17	10	0
				30/05/17	10	9
Bransford	2	Bridge	52.176929, -2.288100	30/05/17	10	0
Knightwick	3	Bridge	52.201276, -2.392410	30/05/17	10	0
Tenbury Wells	4	Bridge	52.313900, -2.594711	05/06/17	10	2
				18/07/17	5	0
				08/08/17	5	0

Figure captions

Figure 1: Mean cycle threshold (C_t, black squares) and eDNA concentration (ng/µl) for *Alosa* spp. (grey circles) date in the River Teme below Powick Weir. Errors around means are 95% confidence limits.

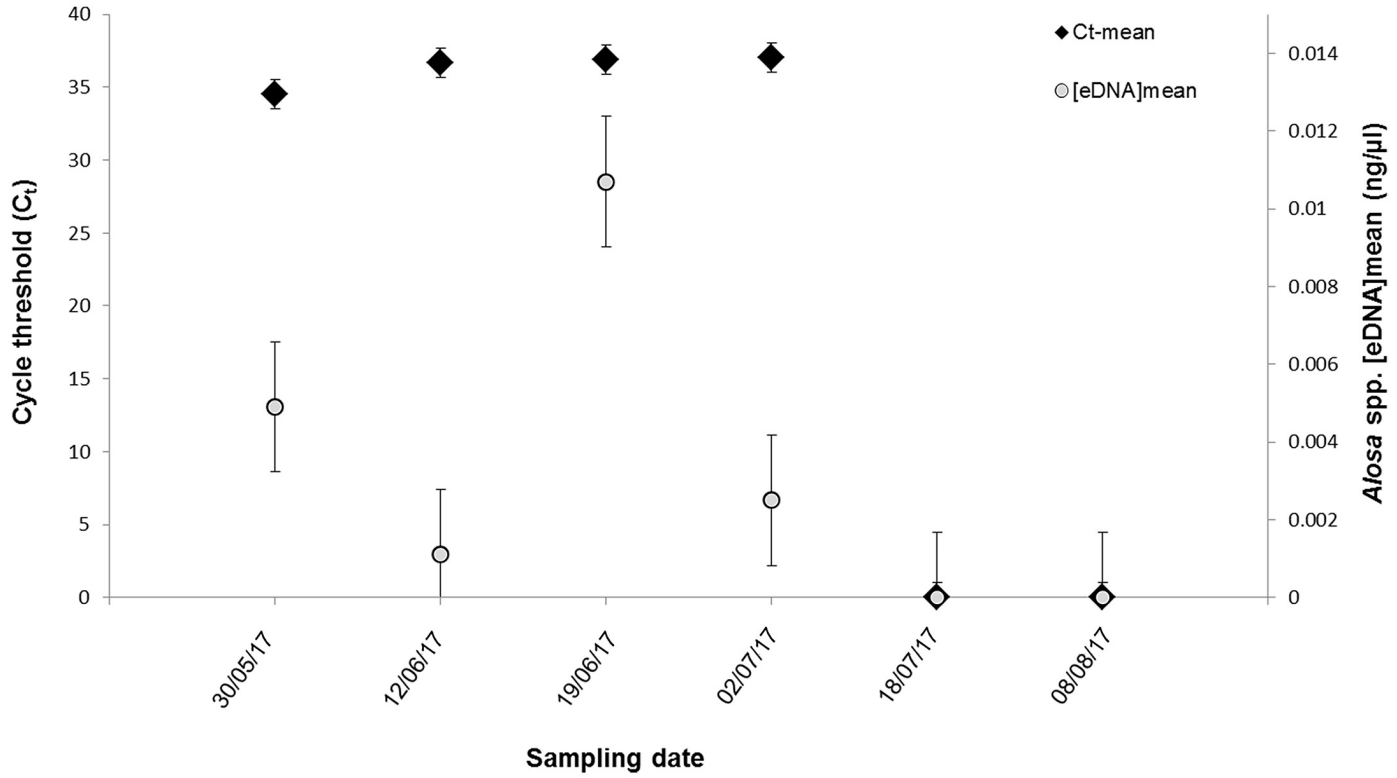
Ct-mean
[eDNA]mean
Cycle threshold (Ct)
Alosa spp. [eDNA]mean (ng/µl)
Sampling date
30/05/17
12/06/17
19/06/17
02/07/17
18/07/17
08/08/17
40
35
30
25
20
15
10
5
0
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0

Figure 1.

Table S1: List of the off target species commonly found in British freshwaters used to design probe and primers specific to European *Alosa* spp.

Off target species
Abramis brama
Alburnus alburnus
Anguilla anguilla
Barbatula barbatula
Barbus barbus
Blicca bjoerkna
Carassius carassius
Cottus gobio
Cyprinus carpio
Esox lucius
Gasterosteus aculeatus
Gobio gobio
Lampetra fluviatilis
Lampetra planeri
Leuciscus idus
Leuciscus leuciscus
Oncorhynchus mykiss
Osmerus eperlanus
Perca fluviatilis
Petromyzon marinus
Phoxinus phoxinus
Platichthys flesus
Pseudorasbora parva
Rhodeus sericeus
Rutilus rutilus
Salmo salar
Salmo trutta
Sander lucioperca
Scardinius erythrophthalmus
Silurus glanis
Squalius cephalus

Thymallus thymallus

Tinca tinca