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SHORT COMMUNICATION

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

Caterina Maria Antognazza | **J. Robert Britton** | **Caitlin Potter** | **Elizabeth Franklin** | **Emilie A. Hardouin** | **Catherine Gutmann Roberts** | **Miran Aprahamian** | **Demetra Andreou**

Department of Life and Environmental Sciences, Bournemouth University, Poole, UK

Correspondence
Caterina Maria Antognazza, Bournemouth University, Fern Barrow, Poole, BH12 5BB, UK.
Email: cm.antognazza@gmail.com

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Abstract

1. Populations of the European shads *Alosa alosa* (Linnaeus, 1758) and *Alosa fallax* Lacépède, 1800 (*Alosa* spp.) are protected under legislation because of 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 2-3-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 used as a non-invasive sampling tool to determine the freshwater distribution of these fishes in Europe, enhancing their conservation at local and regional scales.

KEYWORDS

detection, environmental DNA, impoundment, migratory fish, monitoring, qPCR

1 | INTRODUCTION

European shads *Alosa alosa* (Linnaeus, 1758) and *Alosa fallax* Lacépède, 1800 are cryptic, anadromous fishes, the distributions of which overlap (Alexandrino et al., 2006). In general, their populations have declined throughout their geographical range (Aprahamian, Aprahamian, Baglinière, Sabatié, & Alexandrino, 2003), with both species listed in the Bern Convention (Appendix V) and in the Habitats Directive of the European Union (Annexes II and V) (Aprahamian et al., 2003; Aprahamian, Lester, & Aprahamian, 1999; Council of the European

Communities, 1992). 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 fresh water in spring (with the timing dependent on location, but usually in April–July; Kottelat & Freyhof, 2007). Of the two species, *A. alosa* tends to migrate the furthest upstream to spawn, so when unimpeded the two fishes can segregate 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, owing to 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 this can be labour intensive when applied across large spatial areas. It is also limited to areas of relatively shallow waters, with the spawning of *Alosa* spp. in some European rivers occurring in the deeper, lower reaches, including estuarine areas (Breine et al., 2017; Magath & Thiel, 2013). The detection of spawning events can be completed, but these tend to occur at night. An alternative is to use 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 that it can provide greater probabilities of detection of aquatic species when compared with the use of traditional sampling techniques (Dejean et al., 2012; Jerde, Mahon, Chadderton, & Lodge, 2011), especially when 'best practice' methods are used (Wilcox et al., 2018).

The aim of this study was to 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 carried out on the River Teme, a major tributary of the River Severn, western England, where current data suggest that *Alosa* spawning is restricted to the area below the final impoundment (Powick Weir), close to the Severn confluence (Pinder et al., 2016). The field trials determined the duration of the *Alosa* spawning period and the spatial extent of their distribution. The spatial distribution of the fish was assessed to enable an assessment of how the partial removal of this 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 at four sites on 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 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 of up to 48 km upstream. Initial samples were collected in March (as controls), and then again 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; first, by samplers standing in the riparian zone. Sampling bottles were attached to an extendible pole (1.8–3.7 m). Equipment was cleaned after collecting each sample (with 10% microsol detergent; Anachem, Leicester, UK). Ten water samples were collected per site, comprising paired samples (at 1.8 and 3.7 m) from five sampling points (at 10-m intervals). Two negative controls were taken: after five samples (1.8 m) and after 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 sterilized between sampling points. The second sampling method collected the samples from bridges, with water from 10 samples and two negative controls initially collected from each bridge across the wetted width of the river. This was reduced to five and one 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.

TABLE 1 Description of sampling sites. Site, 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	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

2.2 | eDNA qPCR assay development

The primer and probe specific for the *Alosa* spp. cytochrome *c* oxidase subunit I gene segment (*COI*) was designed by Applied Biosystems (assay ID: APMFW3H; Applied Biosystems, Foster City, CA). Probe and primer 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 with an *in silico* test using target and off-target species commonly found in British fresh waters (Table S1). The TaqMan® Gene Expression Master Mix UDG was used for this assay (Applied Biosystems). DNA extracted 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* species-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: *Abramis brama* (Linnaeus, 1758) (common bream), *Alburnus alburnus* (Linnaeus, 1758) (bleak), *Anguilla anguilla* (Linnaeus, 1758) (eel), *Barbus barbus* (Linnaeus, 1758) (European barbel), *Cyprinus carpio* Linnaeus, 1758 (carp), *Gobio gobio* (Linnaeus, 1758) (gudgeon), *Lampetra planeri* (Bloch, 1784) (brook lamprey), *Leuciscus leuciscus* (Linnaeus, 1758) (dace), *Perca fluviatilis* Linnaeus, 1758 (perch), *Petromyzon marinus* Linnaeus, 1758 (sea lamprey), *Phoxinus phoxinus* (Linnaeus, 1758) (minnow), *Rutilus rutilus* (Linnaeus, 1758) (roach), *Salmo salar* Linnaeus, 1758 (Atlantic salmon), *Salmo trutta* Linnaeus, 1758 (brown trout), *Squalius cephalus* (Linnaeus, 1758) (chub), and *Thymallus thymallus* (Linnaeus, 1758) (grayling). Note that because the eDNA water samples were being collected from freshwater areas only, cross-reactivity was not tested for other fishes of the Clupeidae family that occur in marine and estuarine waters (e.g. *Clupea harengus* Linnaeus, 1758). The assay was also not tested on North American *Alosa* spp. (e.g. *Alosa sapidissima* (A. Wilson, 1811) and *Alosa pseudoharengus* (A. Wilson, 1811)). To determine the sensitivity of the assay, a calibration curve was generated using genomic DNA extracted from the scales of *Alosa* spp. A 10-fold serial dilution of *Alosa* spp. genomic DNA was prepared to give a template concentration from 10 ng μL^{-1} to 1 fg μL^{-1}. The detection limit was defined as the lowest genomic *Alosa* DNA concentration detected at least 95% of the time by the qPCR assay. qPCR was run for each eDNA sample in triplicate in 20 µL, under the manufacturer's instructions, with 2 µL of DNA template (undiluted). The qPCR 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 10-fold serial solution of *Alosa* spp. genomic DNA, the limit of detection of the assay was 1 pg μL^{-1}, with a mean cycle threshold (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^{-1}, 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 concentrations than from bridges (non-parametric Wilcoxon rank test: $Z = -2.59$ and $Z = -3.39$, respectively; $P < 0.05$). Bridge sampling was more time efficient in the field, however, as equipment was pre-prepared and pre-sterilized 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 found to be positive at the end of May. Peak DNA concentrations occurred in mid-June, and final detections were recorded in early July (Figure 1; Table 1;). Spatially, *Alosa* spp. DNA was most frequently detected at site 1 (Table 1). No positive samples were recorded from sites 2 and 3, but *Alosa* DNA was detected in two water samples in early June at site 4 (Table 1).

4 | DISCUSSION

An eDNA method to detect the presence of *Alosa* spp. in rivers was successfully developed and tested. This assay had a discrete level of resolution (detection limit: 1 pg μL^{-1}) and high specificity for *Alosa* spp. Temporally, positive samples were recorded between May and early July at site 1, with peak DNA concentrations in mid-June. Only two positive samples were recorded further upstream. These initial data thus suggest that the primary spawning area in this river was at site 1, downstream of the final weir, with a much smaller number of individuals by-passing this weir and moving further upstream. The spawning activity in site 1 was validated by the presence of *Alosa* eggs

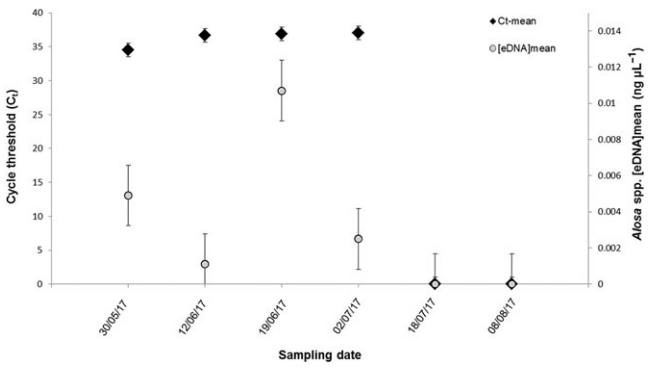

FIGURE 1 Mean cycle threshold (C_t, black squares) and eDNA concentration (ng μL^{-1}) for *Alosa* spp. (grey circles) in the River Teme below Powick Weir. Errors around the means represent the 95% confidence limits

that were regularly sampled in this section between mid-May and mid-June (unpublished data).

The detection rates of eDNA can be relatively high in river water samples (Pilliod et al., 2013), although information on the spatial resolution of these detections often remains uncertain (Goldberg, Strickler, & Pilliod, 2015). For example, macroinvertebrate DNA can be detected from source populations up to 10 km upstream (Deiner & Altermatt, 2014). For fish, distances tend to be closer to 1 km upstream (Balasingham, Walter, & Heath, 2017). However, the absence of a consistent relationship between eDNA concentration and downstream distances (Laramie, Pilliod, & Goldberg, 2015) suggest that consistent DNA accumulations do not occur. This is because of DNA settlement on the river bed and subsequent re-suspension and degradation (Shogren et al., 2017; Wilcox et al., 2016). The positive detections of *Alosa* at site 1 were all from samples collected approximately 0.5 km downstream of the final impoundment. Consequently, it was assumed that the DNA was all 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 understanding this result. Moreover, the general lack of species-specific markers to discriminate between these *Alosa* species (Faria, Weiss, & Alexandrino, 2012) meant that it was not possible to determine whether this DNA originated from *A. alosa*, *A. fallax*, or a hybrid form. Although potentially important, as *A. alosa* tend to migrate greater distances than *A. fallax* (Kottelat & 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, Aprahamian, et al., 2003). In general, this aspect of the results highlights 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 the 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 would assist with the validation of the eDNA results. Indeed, complementary sampling by egg collection (by kick-sampling or drift nets) or, where the river conditions do not permit this, 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, the 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, as well as detecting the presence or absence of *Alosa* spp. in rivers where anecdotal evidence suggests that fish are present, but where this has not been confirmed by traditional sampling methods.

In this study of the River Teme, the results suggested that small numbers of *Alosa* spp. can occasionally pass the final barrier and move as far as 48 km upstream. The planned modification of this impoundment should thus open up more of the catchment to migrating *Alosa* spp. than is presently the case (Environment Agency, 2018). Subsequent refinement and testing of the assay will 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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ORCID

Caterina Maria Antognazza https://orcid.org/0000-0001-5344-8245

J. Robert Britton https://orcid.org/0000-0003-1853-3086

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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