

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

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12 **Abstract**

13

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

19

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

26

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

33

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

37

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

39       **1. Introduction**

40

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

48

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

56

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

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

74

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

86

87

88

## 89 **2. Methods**

90

91        *2.1 eDNA filtering and extraction*

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

99

100        Water samples were collected by two methods. Firstly, they were collected by samplers  
101        standing in the riparian zone. Sampling bottles were attached to an extendible pole (1.8 to  
102        3.7 m). Equipment was cleaned after collecting each sample (10 % microsolv detergent;  
103        Anachem, UK). Ten water samples were collected per site, comprising of paired samples (at  
104        1.8 and 3.7 m) from five sampling points (10 m intervals). Two negative controls were  
105        taken; after 5 samples (1.8 m) and 10 samples (3.7 m). These were the same type of bottles,  
106        but filled with sterile water and treated in the same manner as the sample collection bottles.  
107        The sampling equipment was changed and sterilised between sampling points. Secondly,  
108        samples were collected from bridges, with 10 samples and 2 negative controls initially  
109        collected from each bridge from across the river's wetted width. This reduced to 5 and 1  
110        negative control following initial analyses. During sampling, each bottle had been pre-  
111        weighted (700 g) and placed individually in a plastic sample bag. In the field, each bottle  
112        was lowered into the river on a rope to collect the sample.

113

114        *2.3 eDNA qPCR assay development*

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

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

125

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

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

146

### 147 **3. Results**

148

#### 149 *3.1 eDNA assay validation*

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

156

#### 157 *3.2 Comparing eDNA sampling methods*

158 Both water sampling methods resulted in positive detections of *Alosa* DNA (Table 1).  
159 Sampling from the riparian zone resulted in significantly higher C<sub>t</sub> values and eDNA  
160 concentration than from bridges (non-parametric Wilcoxon rank test: Z = -2.59; and Z = -  
161 3.39, respectively, P < 0.05). However, bridge sampling was more time efficient in the field  
162 as equipment was pre-prepared and pre-sterilised in the laboratory, and thus was the  
163 preferred method.

164

#### 165 *3.3 eDNA detection of Alosa spp.*

166 Water samples collected from the River Teme in March were negative but were all positive  
167 at the end of May; peak DNA concentrations occurred in mid-June and final detections were  
168 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/ $\mu$ l) 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

195 present downstream of this weir. It was less clear where the *Alosa* spp. detected at Site 4  
196 were located, and further investigation will represent an important step to understand this  
197 result. Moreover, the general lack of species-specific marker to discriminate between these  
198 *Alosa* species (Faria, Weiss, & Alexandrino, 2012) meant it could not be determined  
199 whether this DNA originated from *A. alosa*, *A. fallax* or a hybrid form. Whilst potentially  
200 important, as *A. alosa* tend to migrate greater distances than *A. fallax* (Kottelat and Freyhof,  
201 2007), the River Teme is a relatively small catchment. Correspondingly, the distances from  
202 the Severn estuary to Site 4 of the study were within the migration range of both European  
203 *Alosa* spp. (Aprahamian et al., 2003). In general, this aspect of the results highlight the need  
204 to complete further work on how the spatial extent of *Alosa* spawning in non-impounded  
205 rivers is related to spatial variability in the genetic composition of populations.

206

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

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

224

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

235

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237

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242

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244

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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 <i>Alosa</i> 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	4	Bridge	52.313900, -2.594711	05/06/17	10	2
Wells				18/07/17	5	0
				08/08/17	5	0

## Figure captions

Figure 1: Mean cycle threshold ( $C_t$ , black squares) and eDNA concentration ( $\text{ng}/\mu\text{l}$ ) for *Alosa* spp. (grey circles) data in the River Teme below Powick Weir. Errors around means are 95% confidence limits.



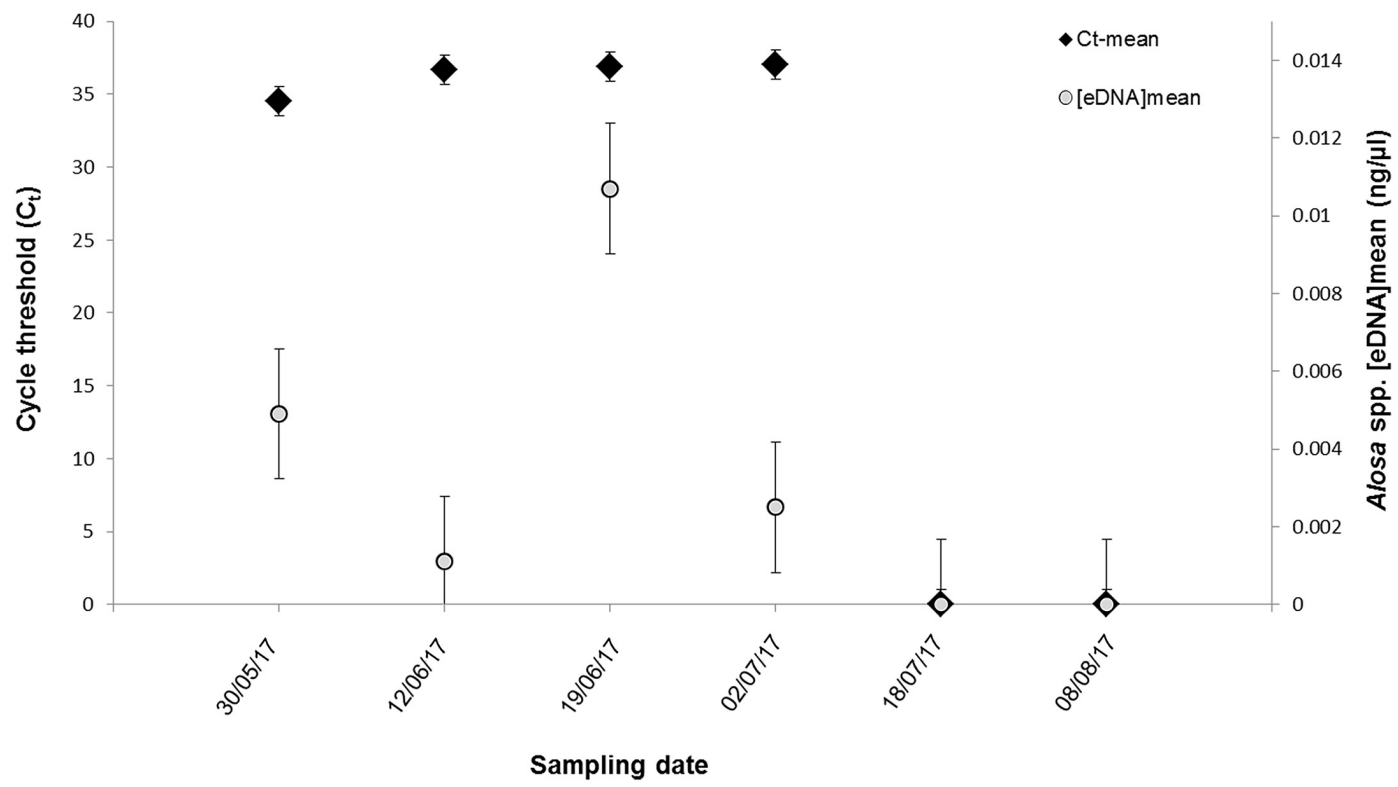


Figure 1.

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

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Off target species

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*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*

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*Squalius cephalus*

*Thymallus thymallus*

*Tinca tinca*

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