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Article

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1 **Development and application of environmental DNA surveillance**

2 **for the threatened crucian carp (*Carassius carassius*)**

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Abstract

1. The crucian carp (*Carassius carassius*) is one of few fish species associated with small ponds in the UK. These populations contain genetic diversity not found in Europe and are important to conservation efforts for the species, which has declined across its range in Europe. Detection and monitoring of extant crucian carp populations are crucial for conservation success. Environmental DNA (eDNA) analysis could be very useful in this respect as a rapid, cost-efficient monitoring tool.

2. We developed a species-specific quantitative PCR (qPCR) assay for eDNA surveillance of crucian carp to enable non-invasive, large-scale distribution monitoring. We compared fyke netting and eDNA at ponds with (N = 10) and without (N = 10) crucian carp for presence-absence detection. We examined biotic (crucian carp density represented by catch-per-unit-effort estimate - CPUE) and abiotic influences on eDNA detection probability using a hierarchical occupancy model, and eDNA quantification using a mixed-effects model.

3. eDNA analysis achieved 90% detection for crucian carp (N = 10), failing in only one pond where presence was known. CPUE estimate and conductivity had positive and negative influences on eDNA detection probability in qPCR replicates respectively. Similarly, conductivity had a negative effect on DNA copy number, whereas copy number increased with CPUE estimate.

4. Our results demonstrate that eDNA could enable detection of crucian carp populations in ponds and benefit ongoing conservation efforts, but imperfect species detection in relation to biotic and abiotic factors and eDNA workflow requires further investigation. Nonetheless, we have established an eDNA framework for crucian carp as well as sources of imperfect detection which future investigations can build upon.

1. Introduction

The crucian carp (*Carassius carassius*) (Figure 1) is an elusive, benthic fish species popular with anglers (Copp, Warrington & Wesley, 2008b; Sayer et al., 2011). As one of few fish associated with small ponds, this species may have an important ecological role but its relationship with other lentic biodiversity is understudied (Copp & Sayer, 2010; Stefanoudis et al., 2017). Although listed as 'Least Concern' on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species, the species has declined throughout its native range of Northwest and Central Europe (Copp et al., 2008b; Sayer et al., 2011), with local extinctions across the UK (Copp & Sayer, 2010). The county of Norfolk in eastern England was believed to hold abundant and widely distributed crucian carp populations, but research indicates heavy (~75%) declines in this region (Sayer et al., 2011). Declines of the crucian carp throughout its range are due to habitat loss (Copp et al., 2008b; Sayer et al., 2011), species displacement by the invasive gibel carp (*Carassius gibelio*) (Copp et al., 2008b; Tarkan et al., 2009; Sayer et al., 2011), and genetic introgression through hybridisation (Hänfling et al., 2005). Indeed, Sayer et al. (2011) observed only 50% of crucian carp ponds to be uninhabited by goldfish (*Carassius auratus*), common carp (*Cyprinus carpio*), or their hybrids with crucian carp.

Prior to the 1970s, crucian carp were thought to have been introduced to the UK alongside common carp and were classed as non-native (Maitland, 1972). Wheeler (1977) deemed the species native to southeast England based on archaeological evidence and a historic distribution that mirrored native cyprinids. Conservation organisations (e.g. English Nature, Environment Agency) later recognised the crucian carp as native and threatened (Smith & Moss, 1994; Environment Agency, 2003), but recent genetic evidence supports anthropogenic introduction of the crucian carp to the UK during the 15th century (Jeffries et

al., 2017). Nonetheless, many introduced species in the UK are now naturalised, and several provide ecological and economical benefits (Manchester & Bullock, 2000). Evidence suggests that the crucian carp is characteristic of small, plant-dominated, high-quality ponds (Copp et al., 2008b; Sayer et al., 2011; Stefanoudis et al., 2017), and English populations contain a substantial proportion of the overall genetic diversity for the species across Europe. English crucian carp populations may buffer species displacement by gibel carp at the European level (Jeffries et al., 2017), but are threatened by hybridisation with goldfish and possible displacement (Hänfling et al., 2005; Tarkan et al., 2009) as well as anthropogenic activity (Copp, Černý & Kováč, 2008a).

In 2010, the crucian carp was designated as a Biodiversity Action Plan (BAP) species in the county of Norfolk (Copp & Sayer, 2010; Sayer et al., 2011). To meet the BAP aims, local conservation efforts have included species reintroduction, pond restoration, and eradication of goldfish (Sayer et al., 2011). However, current distribution records are unreliable as individuals are frequently misidentified as the feral brown variety of goldfish due to high physical similarity (Copp et al., 2008a; Tarkan et al., 2009), and many pond populations are mixtures of true crucian carp and crucian carp x goldfish hybrids (Hänfling et al., 2005). Consequently, distribution maps have been called into question and further monitoring is needed to ensure long-term success of established and reintroduced crucian carp populations (Copp et al., 2008a; Tarkan et al., 2009).

Primarily, crucian carp are surveyed using fyke netting or electrofishing, but these methods can be costly and time-consuming. Environmental DNA (eDNA) analysis offers a potentially rapid and cost-effective approach to fish monitoring (Jerde et al., 2011; Sigsgaard et al., 2015; Wilcox et al., 2016; Hänfling et al., 2016; Hinlo et al., 2017a). Species are identified using DNA deposited in the environment by individuals via secretions, excretions, gametes, blood, or decomposition (Lawson Handley, 2015). eDNA has been applied

100   worldwide to survey for invasive freshwater fish (Jerde et al., 2011; Keskin, 2014; Robson et

101   al., 2016; Hinlo et al., 2017a), and is now used routinely to monitor Asian carp

102   (*Hypophthalmichthys* spp.) invasion in the Great Lakes, USA (Farrington et al., 2015). A

103   quantitative PCR (qPCR) assay targeting crucian carp was also published in the context of

104   early warning invasion monitoring for fish species that may arrive in Canada (Roy et al.,

105   2017), but was only tested on tissue-derived DNA. Of equal importance to invasion

106   monitoring, eDNA analysis has enhanced surveys for threatened and endangered freshwater

107   fish (Sigsgaard et al., 2015; Schmelzle & Kinziger, 2016; Piggott, 2016; Bylemans et al.,

108   2017).

109   eDNA analysis has been conducted with conventional PCR (PCR) (Ficetola et al.,

110   2008; Jerde et al., 2011), but qPCR and droplet digital PCR (ddPCR) are suggested to

111   perform better, suffer less from inhibition, and enable abundance or biomass estimation

112   (Nathan et al., 2014). However, these estimates can be inconsistent across habitats and target

113   organisms. In flowing water, Hinlo et al. (2017a) found no relationship between DNA copy

114   number and conventional density estimates of common carp, yet Takahara et al. (2012)

115   observed a positive association between common carp biomass and eDNA concentration in

116   ponds. Environmental variables play a substantial role in abundance/biomass estimation by

117   influencing the ecology of eDNA (Barnes et al., 2014). Variables examined have included

118   temperature, pH, salinity, conductivity, anoxia, sediment type, and UV light (Takahara et al.,

119   2012; Barnes et al., 2014; Pilliod et al., 2014; Keskin, 2014; Strickler, Fremier & Goldberg,

120   2015; Robson et al., 2016; Buxton et al., 2017b; Buxton, Groombridge & Griffiths, 2017a;

121   Weltz et al., 2017; Stoeckle et al., 2017; Goldberg, Strickler & Fremier, 2018). However,

122   these variables are not always measured and only a handful of studies have assessed their

123   effects in ponds (Takahara et al., 2012; Buxton et al., 2017a, b; Goldberg et al., 2018).

124   In this study, we developed a species-specific qPCR assay for the threatened crucian

125 carp. We evaluated presence-absence detection with eDNA compared to fyke netting, and

126 investigated the influence of biotic and abiotic factors on eDNA detection and quantification.

127 We hypothesised that: (1) eDNA and fyke netting would provide comparable presence-

128 absence records for crucian carp, and (2) eDNA detection and quantification would be

129 influenced by crucian carp density, temperature, pH, conductivity, surface dissolved oxygen,

130 macrophyte cover, and tree shading. We provide an eDNA framework for crucian carp

131 monitoring which holds promise for routine survey.

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135 **2. Methods**

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137 **2.1 Study sites**

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139 We studied 10 ponds with confirmed crucian carp presence at different densities and 10

140 fishless ponds in Norfolk (Figure 2). This region is low-lying (<100 m above sea level) and

141 mainly agricultural. All study ponds were selected to be small (<40 m in max. dimension),

142 shallow (<2 m), macrophyte-dominated, with a largely open-canopy and thus minimal

143 shading of the water surface. Ponds were largely surrounded by arable fields, excluding one

144 located in woodland. No specific permits were required for sampling but relevant landowner

145 permissions were obtained.

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148 **2.2. Conventional survey**

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150 Crucian carp presence-absence was confirmed at each pond by fyke netting between 2010

151 and 2016. Bar two ponds surveyed in 2013 and 2015, all crucian carp ponds were last

152 surveyed in 2016. Where possible, double-ended fyke nets were set perpendicular to the bank

153 or to beds of aquatic vegetation and exposed overnight (for c. 16 h), with the number of fyke

154 nets set being proportional to pond size. This provided CPUE estimates of relative densities,

155 which are the number of fish captured per fyke net per 16 h exposure. Environmental data

156 were collected between May and August from 2010 to 2017. Conductivity, pH, surface

157 dissolved oxygen, and water temperature were measured with a HACH HQ30d meter (Hach

158 Company, CO, USA), and alkalinity was determined by sulphuric-acid titration using a

159 HACH AL-DT kit (Hach Company, CO, USA). Percentages of macrophyte cover and

160 shading of ponds by trees and scrub were estimated visually.

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163 **2.3 eDNA sampling, capture and extraction**

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165 Five 2 L surface water samples were collected from the shoreline of each pond using sterile

166 Gosselin™ HDPE plastic bottles (Fisher Scientific UK Ltd, UK) and disposable gloves.

167 Samples were taken at equidistant points around the pond perimeter where access permitted.

168 All ponds without crucian carp were sampled on 22nd August 2016. Water samples were

169 transported on ice in sterile coolboxes to the Centre for Ecology and Hydrology (CEH),

170 Wallingford, stored at 4 °C, and vacuum-filtered within 24 hours of collection. Coolboxes

171 were sterilised using 10% v/v chlorine-based commercial bleach (Elliott Hygiene Ltd, UK)

172 solution and 70% v/v ethanol solution before ponds containing crucian carp were sampled on

173 25th August 2016. Samples were handled in the same way as those from fishless ponds. For

174 each pond, a full process blank (1 L molecular grade water) was taken into the field and

175 stored in coolboxes with samples. Blanks were filtered and extracted alongside pond samples

176 to identify contamination.

177 Where possible, the full 2 L of each sample was vacuum-filtered through sterile 0.45

178 µm cellulose nitrate membrane filters with pads (47 mm diameter; Whatman, GE Healthcare,

179 UK) using Nalgene filtration units. One hour was allowed for each sample to filter but if

180 filters clogged during this time, a second filter was used. After 2 L had been filtered or one

181 hour had passed, filters were removed from pads using sterile tweezers and placed in sterile

182 47 mm petri dishes (Fisher Scientific UK Ltd, UK), sealed with parafilm (Sigma-Aldrich®,

183 UK), and stored at -20 °C. The total volume of water filtered and the number of filters used

184 per sample were recorded for downstream analysis (Table S1). After each round of filtration

185 (samples and blanks from two ponds), all equipment was sterilised in 10% v/v chlorine-based

186 commercial bleach (Elliott Hygiene Ltd, UK) solution for 10 minutes, immersed in 5% v/v

187 MicroSol detergent (Anachem, UK), and rinsed with purified water.

188 All filters were transported on ice in a sterile coolbox to the University of Hull and

189 stored at -20 °C until DNA extraction one week later. DNA was isolated from filters using

190 the PowerWater® DNA Isolation Kit (MO BIO Laboratories, CA, USA) following the

191 manufacturer's protocol in a dedicated eDNA facility at the University of Hull, devoted to

192 pre-PCR processes with separate rooms for filtration, DNA extraction, and PCR preparation

193 of environmental samples. Duplicate filters from the same sample were co-extracted by

194 placing both filters in a single tube for bead milling. Eluted DNA (100 µL) concentration was

195 quantified on a Qubit™ 3.0 fluorometer using a Qubit™ dsDNA HS Assay Kit (Invitrogen,

196 UK). DNA extracts were stored at -20 °C until further analysis.

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2.4 Assay design, specificity and sensitivity

We designed a novel qPCR assay to target a 118 bp amplicon (73 bp excluding primers) within the mitochondrial cytochrome *b* (*cytb*) gene, specific to crucian carp. Crucian carp sequences from Jeffries et al. (2016) were aligned using MAFFT in AliView (Larsson, 2014) to sequences downloaded from the NCBI nucleotide (nt) database for 23 closely related species of European freshwater fish (Table S2), and a consensus sequence for each species was identified (Figure 3). Sequences were visually compared to maximise nucleotide mismatches between crucian carp and non-target species, particularly goldfish and common carp, and minimise theoretical risk of non-specific amplification. Mismatches in primer regions were maximised over the probe region to increase specificity (Wilcox et al., 2013). Species-specific primers CruCarp_CytB_984F (5'-AGTTGCAGATATGGCTATCTTAA-3') and CruCarp_CytB_1101R (5'-TGGAAAGAGGACAAGGAATAAT-3'), and corresponding probe CruCarp_CytB_1008Probe (FAM 5'-ATGGATTGGAGGCATACCAGTAGAACACC-3' BHQ1) were selected on this basis.

Primers without probe were tested *in silico* using ecoPCR (Ficetola et al., 2010) against a custom, phylogenetically curated reference database that was constructed for eDNA metabarcoding of lake fish communities in Windermere, Lake District National Park, England, which contains 67 freshwater fish species confirmed or potentially present in the UK (Hänfling et al., 2016). Parameters set allowed a 50-150 bp fragment and maximum of three mismatches between each primer and each sequence in the reference database. Specificity of primers (without probe) was also tested against the full NCBI nucleotide (nt) database using Primer-BLAST (Ye et al., 2012) with default settings.

The primers were tested with PCR, following which primer and probe concentrations, standard curve preparation, and cycling conditions for qPCR were optimised (Supporting

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224 Information: Appendix 1). All subsequent qPCR analyses were performed using the

225 conditions detailed in section 2.5. Primers and probe were validated *in vitro* using tissue

226 DNA (standardised to 1 ng/µL) from fin clips of 10 non-target species (1 UK individual per

227 species) related to crucian carp (Table S3, Figures S1-3). The positive control and No

228 Template Control (NTC) were crucian carp DNA and molecular grade water (Fisher

229 Scientific UK Ltd, UK) respectively. The limits of detection (LOD, the lowest concentration

230 where at least one technical replicate amplified crucian carp DNA) and quantification (LOQ,

231 the concentration at which all technical replicates consistently amplified crucian carp DNA)

232 (Agersnap et al., 2017) were established using the qPCR standards (10^6 to 1 copy/µL) (Figure

233 S4). Five technical replicates were performed for standards, controls, and samples in tests of

234 assay specificity and sensitivity.

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2.5 Detection and quantification of crucian carp eDNA

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239 All qPCR reactions were prepared in a UV and bleach (Elliott Hygiene Ltd, UK) sterilised

240 laminar flow hood in the dedicated eDNA facility at the University of Hull. Reactions were

241 performed in a total volume of 20 µL, consisting of 2 µL of template DNA, 1 µL of each

242 primer (Forward 900 nM, Reverse 600 nM), 1 µL of probe (125 nM) (Integrated DNA

243 Technologies, Belgium), 10 µL of TaqMan® Environmental Master Mix 2.0 (Life

244 Technologies, CA, USA), and 5 µL molecular grade water (Fisher Scientific UK Ltd, UK).

245 Once eDNA samples and three NTCs were added to each 96-well plate, the plate was sealed

246 and transported to a separate laboratory on a different floor for addition of the standard curve

247 and three positive controls (crucian carp DNA, 0.01 ng/µL) in a UV and bleach sterilised

248 laminar flow hood.

249 Our standard curve was a synthesised 500 bp gBlocks® Gene Fragment (Integrated

250 DNA Technologies, Belgium) based on GenBank accessions (KT630374 - KT630380) for

251 crucian carp from Norfolk (Jeffries et al., 2016). Copy number for the gBlocks® fragment

252 was estimated by multiplying Avogadro's number by the number of moles. We performed a

253 10-fold serial dilution of the gBlocks® fragment to generate a 6-point standard curve that

254 ranged from 10^6 to 10 copies/µL. eDNA samples were compared to these known

255 concentrations for quantification (Hinlo et al., 2017a). Each standard was replicated five

256 times on each qPCR plate. Similarly, five technical replicates were performed for every

257 sample and full process blank from each pond.

258 After addition of standards and positive controls, plates were again sealed and

259 transported to a separate laboratory on a different floor where qPCR was conducted on a

260 StepOnePlus™ Real-Time PCR system (Life Technologies, CA, USA). Thermocycling

261 conditions consisted of incubation for 5 min at 50 °C, a 10 min denaturation step at 95 °C,

262 followed by 60 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. We

263 used 60 cycles for consistency with optimisation tests, but cycling could be reduced to 45

264 cycles for subsequent applications (see Supporting Information: Appendix 1). A small-scale

265 comparison of eDNA detection and concentration using PCR and qPCR was also conducted

266 (Supporting Information: Appendix 1).

267 Amplifications were considered positive detections if the exponential phase occurred

268 within 45 reaction cycles as the mean C_q value was 40.07 for the LOD (1 copy/µL). A pond

269 was considered positive for crucian carp if two or more of the five technical replicates from a

270 sample returned positive, or more than one sample returned any positive technical replicates

271 (Goldberg et al., 2016). False negatives were obtained for one pond, therefore all samples

272 were tested for inhibition by spiking duplicate qPCR reactions with a known concentration of

273 synthetic crucian carp template (1000 copies/µL) (Jane et al., 2015).

276 **2.6 DNA sequencing**

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278 Non-target DNA extracts and full-process blanks that amplified with qPCR were Sanger

279 sequenced alongside a representative eDNA sample from each positive pond (N = 9) to

280 confirm sequence identity. Purification and sequencing was performed by Macrogen Europe

281 (Amsterdam, The Netherlands) in triplicate in the forward direction. Sequences were edited

282 using CodonCode Aligner (CodonCode Corporation, MA, USA) with default settings.

283 Sequences were then manually aligned in AliView (Larsson, 2014) and poor quality

284 sequences were discarded (Figure S5). Primers were removed from remaining sequences, and

285 sequences identified against the full NCBI nucleotide (nt) database using the NCBI BLASTn

286 tool.

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289 **2.7 Data analysis**

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291 Technical replicates for each qPCR standard that differed by >0.5 C_q from the average of the

292 five technical replicates performed were discarded to minimise bias induced by pipetting

293 error. All technical replicates for eDNA samples were retained, and those which failed to

294 amplify were classed as 0 copies/µL (Goldberg et al., 2016). The C_q values for each set of

295 technical replicates were averaged and quantified to provide a single DNA copy number for

296 each sample. Samples with no positive amplifications were assigned a DNA copy number of

297 zero. DNA copy numbers of samples were then averaged to generate a single DNA copy

298 number for each pond.

299   All subsequent data analyses were performed in the statistical programming

300   environment R v.3.4.2 (R Core Team, 2017). Effects of water volume filtered, number of

301   filters used, and water sample content on DNA copy number of samples were tested and

302   reported in Supporting Information (see Appendices 1, 2; Figures S6, S7). Results and

303   discussion of the PCR-qPCR comparison are also reported in Supporting Information

304   (Appendices 2-3; Table S4; Figure S8). The R package 'eDNAoccupancy' v0.2.0 (Dorazio &

305   Erickson, 2017) was used to fit a Bayesian, multi-scale occupancy model to estimate eDNA

306   detection probability at sites where crucian carp were confirmed as present by fyke netting.

307   Existing eDNA literature was used to identify biotic and abiotic factors reported to affect

308   eDNA detection, persistence and degradation, and construct hypotheses regarding their

309   effects on eDNA detection probability in water samples (θ), and eDNA detection probability

310   in qPCR replicates (p). No covariates were included at the site level (ψ) as ponds were

311   occupied by crucian carp and eDNA should have been present. At the sample level, more

312   individuals (reflected by CPUE) should increase eDNA concentration and improve detection.

313   Temperature can increase physical, metabolic, or behavioural activity of organisms resulting

314   in more eDNA release, breakdown, and degradation (Takahara et al., 2012; Pilliod et al.,

315   2014; Strickler et al., 2015; Robson et al., 2016; Lacoursière-Roussel, Rosabal & Bernatchez,

316   2016; Buxton et al., 2017b; Bylemans et al., 2017). Links established between eDNA and pH

317   support greater detectability, concentration, and persistence of eDNA in more alkaline waters

318   (Barnes et al., 2014; Strickler et al., 2015; Goldberg et al., 2018). Conductivity relates to

319   Total Dissolved Solids (TDS) and sediment type, which can impair eDNA detection due to

320   release of inhibitory substances and their capacity to bind DNA (Buxton et al., 2017a;

321   Stoeckle et al., 2017). Vegetated ponds reduce UV exposure thereby preserving eDNA

322   (Barnes et al., 2014), and are susceptible to terrestrialisation which can create anoxic

323   conditions that may slow eDNA degradation (Barnes et al., 2014; Pilliod et al., 2014; Weltz

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324 et al., 2017). At the qPCR replicate level, covariates again included CPUE as higher eDNA

325 concentration should improve amplification success and consistency, whereas conductivity

326 may indicate inhibitory substances that cause amplification failure.

327   Prior to modeling, all environmental variables were assessed for collinearity using

328 Spearman's correlation coefficient and Variance Inflation Factors (VIFs) calculated using the

329 R package 'car' v2.1-6 (Fox & Weisberg, 2011). Variables were considered collinear and

330 removed if r $>$0.3 and VIF $>$3 (Zuur et al., 2009), following which candidate variables (CPUE,

331 conductivity, pH, and percentage of macrophyte cover) were centred and scaled to have a

332 mean of 0 and standard deviation of 1. We constructed 64 models which assumed a constant

333 probability of eDNA occurrence at the site level, and different covariate combinations at the

334 sample and qPCR replicate levels. Models were ranked (Table S5) according to posterior

335 predictive loss criterion (PPLC) under squared-error loss and the widely applicable

336 information criterion (WAIC). The model with the best support was selected for comparison

337 to the null model without covariates at the entire sampling hierarchy.

338   We examined the influence of biotic and abiotic factors on eDNA quantification using

339 a generalised linear mixed effects model (GLMM) within the R package 'glmmTMB' v0.2.0

340 (Brooks et al., 2017). Collinearity was assessed as above, leaving CPUE, pH, conductivity,

341 and percentage of macrophyte cover as explanatory variables. Pond was modeled as a random

342 effect to account for spatial autocorrelation in our data set and the influence of other

343 properties inherent to each pond, whereas all other explanatory variables were fixed effects.

344 A Poisson distribution was specified as the nature of the response variable (DNA copy

345 number) was integer count data. Validation checks were performed to ensure all model

346 assumptions were met and absence of overdispersion (Zuur et al., 2009). Model fit was

347 assessed visually and with the Hosmer and Lemeshow Goodness of Fit Test (Hosmer &

348 Lemeshow, 2000) using the R package 'ResourceSelection' v0.3-0 (Lele et al., 2014). Model

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predictions were obtained using the predict() function and upper and lower 95% CIs were calculated from the standard error of the predictions. All values were bound in a new data frame and model results plotted for evaluation using the R package 'ggplot2' v2.2.1 (Wickham, 2009). All R scripts and corresponding data have been deposited in a dedicated GitHub repository (https://github.com/lrharper1/crucian_carp_eDNA_surveillance), which has been permanently archived (https://doi.org/10.5281/zenodo.1421602).

3. Results

3.1 Assay specificity and sensitivity

Only crucian carp amplified in ecoPCR, confirming primer specificity. Non-target species returned by primer-BLAST against the full NCBI nucleotide (nt) database were *Barilius bakeri* (a Cyprinid fish restricted to India, 6 mismatches), *Naumovozyma dairensis* (fungi, 8 mismatches), and *Medicago trunculata* (plant, 8 mismatches). Our probe sequence could not be included *in silico* but would likely increase specificity. Tissue extracts from common rudd (*Scardinius erythrophthalmus*) and European chub (*Squalius cephalus*) included in qPCR assay specificity tests were amplified by primers and probe, but possessed low DNA copy number (<10 copies/μL). In a later test, common carp DNA also amplified (<10 copies/μL). However, no amplification was observed for NTCs, fresh tissue extracts obtained from rudd and chub, or eDNA samples from locations where crucian carp were absent and these species were present (data not shown). DNA sequencing confirmed cross-contamination of reference

373 material, where sequences were either identified as crucian carp or of poor quality (Table 2).

374 Our assay was highly sensitive with a LOD of 1 copy/µL and LOQ of 10 copies/µL.

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377 **3.2 qPCR analysis**

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379 The qPCR assay had an average amplification efficiency of 93.61% (range 79.61-102.49%)

380 and an average R^2 value of 0.998 (range 0.995-0.999) for the standard curve. Only one plate

381 had a qPCR efficiency lower than 90% but the standard curve quantified as expected, thus

382 qPCR was not repeated. No amplification occurred in NTCs, but the full process blank for

383 one pond (POFA4) amplified (<10 copies/µL). This was the only contaminated blank as the

384 blank for pond POHI filtered alongside POFA4 and POHI samples, and blanks downstream

385 of these samples did not amplify. Partial inhibition (<1000 copies/µL) occurred in a single

386 sample from four different ponds: PYES2 (no crucian carp), RAIL, POHI, and GUES1

387 (crucian carp present). However, partially inhibited samples all possessed >0 copies/µL when

388 originally tested, and copy number did not differ substantially (higher in one instance) from

389 other samples belonging to the same pond (Table S1). Consequently, partial inhibition did not

390 influence detectability in our study, and problematic samples were not treated for inhibition

391 and qPCRs were not repeated.

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394 **3.3 Presence-absence detection**

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396 eDNA surveillance detected crucian carp in 90% of the study ponds (N = 10) with confirmed

397 presence. Sanger sequencing of representative samples confirmed species identity as crucian

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398   carp (Table 2). eDNA failed entirely in one pond (CHIP) that contained a sizeable crucian

399   carp population (CPUE = 60.50), but samples from CHIP were not inhibited. Crucian carp

400   DNA was not detected at any sites where the species was absent.

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403   **3.4 Factors influencing eDNA detection and quantification**

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405   The occupancy model with the best support included CPUE and conductivity as covariates of

406   eDNA detection probability in qPCR replicates (p). The model did not include any covariates

407   of eDNA occurrence probability at sites (ψ) or eDNA detection probability in water samples

408   (θ). Estimates of eDNA detection probability in a qPCR replicate ranged between 0.12 to

409   1.00 (Table 1), where crucian carp CPUE (parameter estimate = 1.357) and conductivity

410   (parameter estimate = -2.112) played positive and negative roles in eDNA availability

411   respectively (Figures 4a, b). The GLMM identified CPUE (0.020 ± 0.007, χ^2_1 = 5.426, P =

412   0.020) and conductivity (-0.007 ± 0.002, χ^2_1 = 8.709, P = 0.003) as significant predictors of

413   DNA copy number, where DNA copy number was greater at higher CPUE (Figure 5a) but

414   decreased as conductivity increased (Figure 5b).

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418   **4. Discussion**

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420   We developed a novel species-specific qPCR assay to enable large-scale distribution

421   monitoring of the threatened crucian carp using eDNA. Crucian carp were detected at almost

422   all sites with confirmed presence and no false positives were generated. Furthermore, biotic

423 and abiotic factors that influence eDNA detection and quantification were identified. We

424 discuss areas for improvement in our workflow and provide recommendations for future

425 study.

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427

428 **4.1 Using eDNA for crucian carp conservation**

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430 eDNA analysis is often compared to conventional monitoring tools to assess performance,

431 reliability, reproducibility, and prospective applications in conservation programmes. We

432 found strong agreement between eDNA and fyke netting for crucian carp detection, where

433 eDNA detected crucian carp in 90% of ponds with presence confirmed by netting. This high

434 detection and low false negative rate supports the applicability of eDNA analysis to crucian

435 carp presence-absence monitoring, particularly at large spatial scales where fyke netting can

436 be costly and time-consuming, and where ponds are remote with poor access. Abundance

437 estimation is less straightforward as there was uncertainty around the relationship between

438 DNA copy number and crucian carp density. This inconsistency is more likely to result from

439 eDNA than fyke netting due to effects exerted by external factors (section 4.2) and sample

440 processing (section 4.3) on eDNA quality. However, fyke netting also has detection biases

441 that may influence performance comparisons with eDNA. Fyke net surveys are restricted

442 spatially and temporally to pre- and post-spawning as well as spring and autumn when

443 temperatures are low to reduce fish stress in nets. Furthermore, fyke net surveys may fail to

444 capture species that do not have homogenous distribution in their environment, especially

445 where populations contain few individuals (Turner et al., 2012). Netting is also biased

446 towards particular fish size classes that can enter nets through standard European otter (*Lutra*

447 *lutra*) guards (75 mm in UK), and catchability is further dependent on time of year (Ruane,

448   Davenport & Igoe, 2012) and even time of day (Hardie, Barmuta & White, 2006). Therefore,

449   effectiveness of standard methods must also be evaluated and eDNA compared to multiple

450   tools before deemed capable or incapable of estimating abundance.

451

452

453   **4.2 Factors influencing eDNA detection and quantification**

454

455   Effects of biotic and abiotic factors on eDNA may vary across target species and ecosystems

456   (Barnes et al., 2014). We found crucian carp density (CPUE) positively influenced eDNA

457   detection probability and DNA copy number. Density is frequently reported to improve

458   detection probability of aquatic species due to more eDNA deposition in the environment

459   (Schmelzle & Kinziger, 2016; Buxton et al., 2017b; Stoeckle et al., 2017), but this

460   relationship is highly variable across study systems and species due to influence of external

461   factors (Strickler et al., 2015; Buxton et al., 2017a; Goldberg et al., 2018). For example,

462   increase in water temperature coincided with breeding activity and heightened DNA release

463   in other fish and amphibian species (Buxton et al., 2017b; Bylemans et al., 2017). In our

464   study, CPUE was collinear with water temperature and thus water temperature was not

465   included in our occupancy model or GLMM. We performed water sample collection in late

466   August, which is outside the reported spawning period for crucian carp (Aho & Holopainen,

467   2000). However, the association between CPUE and DNA copy number may be linked to

468   increased DNA shedding rates caused by higher metabolic activity in response to warm

469   temperature, as reported for other fish species (Takahara et al., 2012; Robson et al., 2016;

470   Lacoursière-Roussel et al., 2016).

471   In contrast to CPUE, conductivity had a negative effect on eDNA detection and

472   concentration. Conductivity has been suggested to influence eDNA detection and

19

473 quantification, but studies that directly measured this variable have found no discernable

474 effect (Takahara et al., 2012; Keskin, 2014; Goldberg et al., 2018). Conductivity (also

475 measured as TDS) relates to sediment type which influences eDNA detection probability, the

476 rate at which sediment binds eDNA, and release of inhibitory substances (Buxton et al.,

477 2017a; Stoeckle et al., 2017). Notably, the only false negative pond in our study was also the

478 most conductive (760 μs/cm) and possessed dense beds of rigid hornwort (*Ceratophyllum*

479 *demersum*) that could restrict water movement. Therefore, conductivity may lead to incorrect

480 inferences about species presence and impact conservation management decisions. Further

481 investigation into the effects of conductivity on eDNA detection and quantification is clearly

482 needed.

483   Our results indicate that samples may have been affected by inhibitory substances

484 despite tests performed to identify inhibition. We spiked qPCR reactions with a known

485 amount of synthetic target DNA; however, an artificial Internal Positive Control gene assay

486 may identify inhibition more effectively (Goldberg et al., 2016). Dilution of eDNA samples

487 (and inhibitory substances present) can release inhibition, but also reduce detection

488 probability (Piggott, 2016) and induce false negatives (Buxton et al., 2017a). We used

489 TaqMan® Environmental Master Mix 2.0 (Life Technologies, CA, USA) in qPCR reactions

490 to counter inhibition (Jane et al., 2015), but it may be advisable to use DNA extraction kits

491 that perform inhibitor removal (Sellers et al., 2018) or include Bovine-serum albumin (BSA)

492 in qPCR reactions (Jane et al., 2015). Alternatively, ddPCR may handle inhibitors better than

493 qPCR and provide more accurate abundance or biomass estimates (Nathan et al., 2014).

494   Crucially, environmental data were not collected in 2016 for every pond in our study.

495 Our results indicate direction of effects of biotic and abiotic factors on eDNA detection and

496 quantification, but contemporary data are needed for comprehensive interpretation of these

497 relationships. However, it is clear that eDNA practitioners must account for these effects as

498 well as sample inhibition. The uncertainty around the estimated effects of covariates in our

499 hierarchical occupancy model and GLMM also imply that greater sample volume, sample

500 number, and/or qPCR replication are required to improve the ability and precision of our

501 assay to detect crucian carp eDNA and reduce the potential for false negatives (Schultz &

502 Lance, 2015; Goldberg et al., 2018).

503

504

505 **4.3 Optimisation of eDNA workflow**

506

507 Some non-target DNA extracts used to validate assay specificity were contaminated with

508 crucian carp DNA. Field cross-contamination can occur if reference tissue material is

509 collected from multiple species without sterilising equipment, or eDNA is present on the

510 material collected (Rodgers, 2017). Collection and storage of reference tissue material is an

511 important consideration for eDNA practitioners, particularly those using highly sensitive

512 assays (LOD = 1 copy/μL) (Wilcox et al., 2013, 2016). Dedicated, sterilised equipment

513 should be used when collecting new reference material from different species. From existing

514 reference collections, only non-target samples that were collected on separate and

515 chronologically distinct occasions from target samples should be used (Rodgers, 2017).

516 Cross-contamination can also arise during water sampling, filtration, DNA extraction

517 and qPCR preparation. Low-level contamination was found in one full process blank but

518 detections from this pond were not omitted as it contained crucian carp and contamination

519 was not observed downstream. All equipment in our study was sterilised by immersion in 10%

520 chlorine-based commercial bleach (Elliott Hygiene Ltd, UK) solution for 10 mins, followed

521 by rinsing in 5% MicroSol detergent (Anachem, UK), and then purified water. However,

522 sterilisation with 50% chlorine-based commercial bleach solution (Goldberg et al., 2016) or

21

523   single-use, sterile materials (Wilcox et al., 2016) may further minimise contamination risk.

524   Many of our eDNA samples were low concentration (<100 copies/μL) which can

525   cause inconsistent qPCR amplification (Goldberg et al., 2016), thus we discuss approaches to

526   maximise eDNA concentration and improve detection probability. The probability of eDNA

527   detection depends heavily on the number of samples and volume of water collected, time of

528   sampling, and sample concentration (Schultz & Lance, 2015; Goldberg et al., 2018). We

529   sampled 5 x 2 L water samples from each pond in autumn 2016, but timing and/or sampling

530   effort may have been inappropriate. A seasonal effect on common carp eDNA detection was

531   observed, where spring sampling generated higher eDNA concentration and detection rates

532   due to greater common carp activity (Turner et al., 2014) and density (Hinlo et al., 2017a). As

533   water sampling did not coincide with fyke netting (spring 2016) in our study, eDNA

534   concentration may not reflect CPUE estimates. Water samples in spring may contain more

535   crucian carp eDNA due to higher activity of individuals, or autumn fyke netting may produce

536   lower CPUE estimates. Parallel seasonal sampling, where water sampling is performed in

537   conjunction with fyke netting at different times of the year, may better align eDNA

538   concentration with CPUE estimates and enable eDNA-based abundance estimates for crucian

539   carp. This is certainly a worthwhile area of research.

540   Representative sampling is crucial in eDNA surveys. Individuals of a species can be

541   unevenly distributed in the environment, which impacts eDNA detection, distribution, and

542   concentration (Takahara et al., 2012; Eichmiller, Bajer & Sorensen, 2014; Schmelzle &

543   Kinziger, 2016; Goldberg et al., 2018). In lentic ecosystems, eDNA has a patchy horizontal

544   and sometimes vertical distribution, resulting in fine spatial variation (Eichmiller et al., 2014).

545   Studies on common carp revealed eDNA was more concentrated near the shoreline of lentic

546   water bodies (Takahara et al., 2012; Eichmiller et al., 2014), due to aggregations of

547   individuals (Eichmiller et al., 2014). We collected surface water (5 x 2 L) from the shoreline

548 and sampled at equidistant points around the pond perimeter where possible; however, more

549 samples and greater water volumes may be required to improve detection probability (Schultz

550 & Lance, 2015; Goldberg et al., 2018). Fine spatial sampling and occupancy modelling are

551 needed to determine the sample number required to achieve high detection probability and

552 eliminate false negatives (Goldberg et al., 2018). However, the number of samples required

553 will inevitably vary across habitats due to inherently variable physical properties (Schmelzle

554 & Kinziger, 2016).

555   Method of eDNA capture can dictate success of this monitoring tool. Studies of

556 eDNA in ponds (Ficetola et al., 2008; Biggs et al., 2015) have used an ethanol precipitation

557 approach, but this is restricted to small volumes. Filtration allows more water to be processed

558 and minimises capture of non-target DNA, with macro-organism eDNA effectively captured

559 by pore sizes of 1 - 10 μm (Turner et al., 2014). We used a small pore size of 0.45 μm to

560 capture most eDNA particle sizes, although filter clogging prevented the entire sample being

561 processed and may have affected eDNA concentration downstream. Pre-filtering can reduce

562 clogging, but is labour-intensive and increases cost (Takahara et al., 2012). Larger pore sizes

563 have been used in temperate and tropical lentic environments (Takahara et al., 2012; Robson

564 et al., 2016; Goldberg et al., 2018), though independent investigation is needed to determine

565 which pore size maximises target DNA concentration.

566   Comparisons of eDNA yield across filter types and DNA extraction protocols have

567 shown that cellulose nitrate filters stored at -20 °C (this study) often provide best eDNA yield

568 (Piggott, 2016; Spens et al., 2016; Hinlo et al., 2017b). However, different filter types may be

569 optimal for different species, which has consequences for detectability (Spens et al., 2016)

570 and relationships between eDNA concentration and abundance/biomass (Lacoursière-Roussel

571 et al., 2016). Extraction method used, regardless of filter type, will ultimately influence DNA

572 quality and yield. We used the PowerWater® DNA Isolation Kit (MO BIO Laboratories, CA,

573 USA), but the DNeasy Blood and Tissue kit (Qiagen®, Hilden, Germany) has demonstrated

574 greater yield (Hinlo et al., 2017b). We also combined filters from the same sample for DNA

575 extraction at the bead milling stage, but independent lysis may recover more DNA (Hinlo et

576 al., 2017b). A comparison of DNA extraction protocols is necessary to assess which approach

577 maximises crucian carp eDNA concentration. A new modular extraction method shows

578 promise for eDNA but has yet to be evaluated for targeted qPCR (Sellars et al., 2018).

579 Finally, detection sensitivity can be enhanced by increasing the number of qPCR

580 technical replicates (Schultz & Lance, 2015; Piggott, 2016). We performed five technical

581 replicates for each of our samples, but other studies have used as many as twelve and only

582 one may amplify (Biggs et al., 2015). More replication may have enabled amplification from

583 the CHIP pond samples, but qPCR cost would inevitably increase. Further replication may

584 also be unnecessary if steps are taken to improve initial concentration of samples instead

585 (Schultz & Lance, 2015).

586

587

588 **4.4 Concluding remarks**

589

590 A primary objective of the Norfolk crucian carp BAP was to obtain a basic understanding of

591 species distribution and population status across Norfolk (Copp & Sayer, 2010). eDNA

592 surveillance for crucian carp will provide a useful, cost-effective alternative to established

593 survey methods where the aim is determining presence-absence. Our assay may detect

594 hybrids where crucian carp were the maternal parent due to use of a mitochondrial marker;

595 however, these detections are also beneficial to the crucian carp conservation effort through

596 the identification of ponds where true crucian carp may still exist, and where contamination

597 with goldfish, common carp and their hybrids has occurred. Alternatively, our assay could be

598   used as an early warning tool in countries where the crucian carp is considered invasive. The

599   areas we have highlighted require further investigation before eDNA can be used routinely.

600   Nevertheless, eDNA survey could enable large-scale distribution monitoring for crucian carp

601   through rapid screening of existing and new ponds. Fyke netting could then be used to

602   investigate age, sex and size structure of populations, and remove hybrids.

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606   ## Acknowledgements

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616   ## Conflict of interest

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618   The authors declare no conflict of interest.

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837

838 **Table 1**. Bayesian estimates of crucian carp eDNA occurrence probability at a pond (ψ), eDNA detection

839 probability in a water sample (θ), and eDNA detection probability in a qPCR replicate (p). Posterior median and

840 95% credible interval (CI) are given for each parameter of the occupancy model. The corresponding catch-per-

841 unit-effort estimate (CPUE) is given for each pond.

842

Pond	Crucian carp (Y/N)	CPUE estimate	ψ		θ		p	
			Posterior median	95% CI	Posterior median	95% CI	Posterior median	95% CI
CAKE	Y	43.00	0.90	0.62 – 1.00	0.83	0.70 - 0.92	0.14	0.05 - 0.33
CHIP	Y	60.50	0.90	0.62 – 1.00	0.83	0.70 - 0.92	0.12	0.03 - 0.36
GUES1	Y	121.75	0.90	0.62 – 1.00	0.83	0.70 - 0.92	0.98	0.86 - 1.00
MYST	Y	6.17	0.90	0.62 – 1.00	0.83	0.70 - 0.92	0.93	0.86 - 0.98
OTOM	Y	14.67	0.90	0.62 – 1.00	0.83	0.70 - 0.92	0.96	0.91 - 0.99
POFA4	Y	13.67	0.90	0.62 – 1.00	0.83	0.70 - 0.92	0.89	0.81 - 0.95
POHI	Y	7.25	0.90	0.62 – 1.00	0.83	0.70 - 0.92	0.44	0.28 - 0.59
RAIL	Y	58.17	0.90	0.62 – 1.00	0.83	0.70 - 0.92	1.00	0.99 - 1.00
SKEY1	Y	31.38	0.90	0.62 – 1.00	0.83	0.70 - 0.92	1.00	1.00 - 1.00
WADD3	Y	126.00	0.90	0.62 – 1.00	0.83	0.70 - 0.92	1.00	1.00 - 1.00

843

844 **Table 2.** Top NCBI BLASTn hit for Sanger sequences obtained from target DNA (tissue extracts and synthetic

845 gBlocks® Gene Fragment), non-target tissue DNA extracts, full process blanks, and representative eDNA

846 samples that amplified during qPCR. Sample descriptions marked with '!' indicate a poor quality, discarded

847 sequence.

848

Sample	Description	Query length	Coverage	E value	Identity	Accession
CrucianCarp-01	*Carassius carassius*	37	100%	3E-09	100%	KR131843.1
CrucianCarp-02	*Carassius carassius*	37	100%	3E-09	100%	KR131843.1
CrucianCarp-03	!					
Gblock-100copies-01	*Carassius carassius*	37	100%	3E-09	100%	KR131843.1
Gblock-100copies-02	!					
Gblock-100copies-03	!					
Rudd-JL-01	*Carassius carassius*	38	100%	9E-10	100%	KR131843.1
Rudd-JL-02	*Carassius carassius*	38	100%	9E-10	100%	KR131843.1
Rudd-JL-03	!					
Rudd-PS-01	!					
Rudd-PS-02	!					
Rudd-PS-03	!					
Chub-PS-01	!					
Chub-PS-02	!					
Chub-PS-03	!					
Chub-JL-01	!					
Chub-JL-02	!					
Chub-JL-03	!					
CommonCarp-01	!					
CommonCarp-02	!					
CommonCarp-03	!					
POFA4-B-01	!					
POFA4-B-02	!					

POFA4-B-03	!					
GUES1-5-01	*Carassius carassius*	37	100%	3E-09	100%	KR131843.1
GUES1-5-02	*Carassius carassius*	41	100%	1E-07	95%	KR131843.1
GUES1-5-03	*Carassius carassius*	41	100%	2E-11	100%	KR131843.1
MYST-3-01	*Carassius carassius*	46	100%	4E-14	100%	KR131843.1
MYST-3-02	!					
MYST-3-03	*Carassius carassius*	41	100%	1E-07	95%	KR131843.1
SKEY1-4-01	*Carassius carassius*	35	100%	4E-08	100%	KR131843.1
SKEY1-4-02	!					
SKEY1-4-03	*Carassius carassius*	37	100%	3E-09	100%	KR131843.1
OTOM-4-01	!					
OTOM-4-02	!					
OTOM-4-03	*Carassius carassius*	37	100%	3E-09	100%	KR131843.1
POHI-2-01	*Carassius carassius*	41	100%	2E-11	100%	KR131843.1
POHI-2-02	!					
POHI-2-03	*Carassius carassius*	37	100%	3E-09	100%	KR131843.1
RAIL-4-01	!					
RAIL-4-02	*Carassius carassius*	38	100%	9E-10	100%	KR131843.1
RAIL-4-03	*Carassius carassius*	46	100%	4E-14	100%	KR131843.1
WADD3-4-01	*Carassius carassius*	25	96%	0.034	100%	KR131843.1
WADD3-4-02	!					
WADD3-4-03	*Carassius carassius*	38	100%	9E-10	100%	KR131843.1
CAKE-1-01	!					
CAKE-1-02	!					
CAKE-1-03	!					
POFA4-5-01	!					
POFA4-5-01	!					
POFA4-5-01	!					

849

850   **Figure 1.** A crucian carp (*Carassius carassius*) individual **(a)** and examples of the study ponds **(b-d)**. Photo **(a)**

851   by John Bailey and photo **(d)** by Sacha Dench.

852

853   **Figure 2.** Map of pond locations in North Norfolk, eastern England, showing the distribution of ponds

854   containing crucian carp (black dots) and ponds where the species is absent (grey dots).

855

856   **Figure 3.** Alignment of consensus sequences for a region of the mitochondrial cytochrome *b* (*cytb*) gene in 24

857   European freshwater fishes, including the crucian carp. Species-specific primers and probe for the crucian carp

858   are given on the first line. Consensus with primer and probe sequence across species is highlighted in white

859   whereas mismatches are coloured by nucleotide base.

860

861   **Figure 4.** Estimated probability of eDNA detection in qPCR replicates. Points are estimates of posterior

862   medians with 95% credible intervals. Probability of eDNA detection in qPCR replicates increased with higher

863   catch-per-unit-effort (CPUE) estimate **(a)** but decreased as conductivity increased **(b)**.

864

865   **Figure 5.** Relationship between fixed effects and response variable (DNA copy number) in ponds, as predicted

866   by the Poisson GLMM. The 95% CIs, as calculated using the model predictions and standard error for these

867   predictions, are given for each relationship. The observed data (points) are also displayed against the predicted

868   relationships (line). DNA copy number increased with catch-per-unit-effort (CPUE) estimate **(a)**, but decreased

869   as conductivity **(b)** increased.

870