Development of environmental DNA surveillance 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. 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 and relative abundance estimation, specifically whether DNA copy number reflected catch-per-unit-effort (CPUE) estimate. We examined biotic and abiotic influences on eDNA detection and quantification, and compared qPCR to standard PCR. Notably, eDNA occurrence and detection probabilities in relation to biotic and abiotic factors were estimated using a hierarchical occupancy model.
3. eDNA analysis achieved 90% detection for crucian carp (N = 10), failing in only one pond where presence was known. We observed an overall positive trend between DNA copy number and CPUE estimate, but this was not significant. Macrophyte cover decreased the probability of eDNA occurrence at ponds, whereas CPUE and conductivity had positive and negative influences on eDNA detection probability in qPCR replicates respectively. Conductivity also had a negative effect on DNA copy number, but copy number increased with temperature and percentage of macrophyte cover. PCR was comparable to qPCR for species detection and may provide semi-quantitative information.
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 and sources of imperfect detection which future investigations can build upon.

50 ## 1. Introduction

51

52 The crucian carp (*Carassius carassius*) (Figure 1) is a cryptic, benthic fish species popular with
53 anglers (Copp, Warrington & Wesley, 2008b; Sayer et al., 2011). As one of few fish associated
54 with small ponds, this species may have an important ecological role but its relationship with
55 other lentic biodiversity is understudied (Copp & Sayer, 2010; Stefanoudis et al., 2017).
56 Although listed as 'Least Concern' on the International Union for Conservation of Nature
57 (IUCN) Red List of Threatened Species, the species has declined throughout its native range of
58 Northwest and Central Europe (Copp et al., 2008b; Sayer et al., 2011), with local extinctions
59 across the UK (Copp & Sayer, 2010). The county of Norfolk in eastern England was believed to
60 hold abundant and widely distributed crucian carp populations, but research indicates heavy
61 (~75%) declines in this region (Sayer et al., 2011). Declines of the crucian carp throughout its
62 range are due to habitat loss (Copp et al., 2008b; Sayer et al., 2011), species displacement by the
63 invasive gibel carp (*Carassius gibelio*) (Copp et al., 2008b; Tarkan et al., 2009; Sayer et al.,
64 2011), and genetic introgression through hybridisation (Hänfling et al., 2005). Indeed, Sayer et
65 al. (2011) observed only 50% of crucian carp ponds were not inhabited by goldfish (*Carassius*
66 *auratus*), common carp (*Cyprinus carpio*), or their hybrids with crucian carp.

67 Prior to the 1970s, crucian carp were thought to have been introduced to the UK
68 alongside common carp and were classed as non-native (Maitland, 1972). Wheeler (1977)
69 deemed the species native to southeast England based on archaeological evidence and a historic
70 distribution that mirrored native cyprinids. Conservation organisations (e.g. English Nature,
71 Environment Agency) later recognised the crucian carp as native and threatened (Smith & Moss,
72 1994; Environment Agency, 2003), but recent genetic evidence supports anthropogenic

73 introduction of the crucian carp to the UK during the 15th century (Jeffries et al., 2017).
74 Nonetheless, many introduced species in the UK are now naturalised, and several provide
75 ecological and economical benefits (Manchester & Bullock, 2000). Evidence suggests the
76 crucian carp is characteristic of species-rich ponds (Copp et al., 2008b; Sayer et al., 2011;
77 Stefanoudis et al., 2017), and English populations contain a substantial proportion of the overall
78 genetic diversity for the species across Europe. These populations may buffer species
79 displacement by gibel carp (Jeffries et al., 2017), but are threatened by hybridisation with
80 goldfish and possible displacement (Hänfling et al., 2005; Tarkan et al., 2009) as well as
81 anthropogenic activity (Copp, Černý & Kováč, 2008a).

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

91 Primarily, crucian carp are surveyed using fyke netting or electrofishing but these
92 methods can be costly and time-consuming. Environmental DNA (eDNA) analysis offers a
93 potentially rapid and cost-effective approach to fish monitoring (Jerde et al., 2011; Sigsgaard et
94 al., 2015; Wilcox et al., 2016; Hänfling et al., 2016; Hinlo et al., 2017a). Species are identified
95 using intracellular or extracellular DNA deposited in the environment by individuals via

96 secretions, excretions, gametes, blood, or decomposition (Lawson Handley, 2015). eDNA has
97 been applied worldwide to survey for invasive freshwater fish (Jerde et al., 2011; Keskin, 2014;
98 Robson et al., 2016; Hinlo et al., 2017a), and is now used routinely to monitor Asian carp
99 (*Hypophthalmichthys* spp.) invasion in the Great Lakes, USA (Farrington et al., 2015). A
100 quantitative PCR (qPCR) assay targeting crucian carp was also published in the context of early
101 warning invasion monitoring for fish species that may arrive in Canada (Roy et al., 2017), but
102 was only tested on tissue-derived DNA. Of equal importance to invasion monitoring, eDNA
103 analysis has enhanced surveys for threatened and endangered freshwater fish (Sigsgaard et al.,
104 2015; Schmelzle & Kinziger, 2016; Piggott, 2016; Bylemans et al., 2017).

105 eDNA analysis has been conducted with conventional PCR (PCR) (Ficetola et al., 2008;
106 Jerde et al., 2011), but qPCR and droplet digital PCR (ddPCR) are suggested to perform better,
107 suffer less from inhibition, and enable abundance or biomass estimation (Nathan et al., 2014).
108 However, these estimates can be inconsistent across habitats and target organisms. In flowing
109 water, Hinlo et al. (2017a) found no relationship between DNA copy number and conventional
110 density estimates of common carp, yet Takahara et al. (2012) observed a positive association
111 between common carp biomass and eDNA concentration in ponds. Environmental variables play
112 a substantial role in abundance/biomass estimation by influencing the ecology of eDNA (Barnes
113 et al., 2014). Variables examined have included temperature, pH, salinity, conductivity, anoxia,
114 sediment type, and UV light (Takahara et al., 2012; Barnes et al., 2014; Pilliod et al., 2014;
115 Keskin, 2014; Strickler, Fremier & Goldberg, 2015; Robson et al., 2016; Buxton et al., 2017b;
116 Buxton, Groombridge & Griffiths, 2017a; Weltz et al., 2017; Stoeckle et al., 2017; Goldberg,
117 Strickler & Fremier, 2018). However, these variables are not always measured and only a
118 handful of studies have assessed their effects in ponds (Takahara et al., 2012; Buxton et al.,

2017a, b; Goldberg et al., 2018).

In this study, we developed a species-specific qPCR assay for the threatened crucian carp. We evaluated presence-absence detection with eDNA compared to fyke netting, and whether our assay could estimate abundance by comparing catch-per-unit-effort (CPUE) estimates obtained by fyke netting to DNA copy number. We investigated the influence of biotic and abiotic factors on eDNA detection and quantification, and performed a small-scale comparison of qPCR and PCR for species detection. We hypothesised that: (1) eDNA and fyke netting would provide comparable presence-absence records for crucian carp; (2) DNA copy number would positively correlate with CPUE estimate; (3) eDNA detection and quantification would be influenced by crucian carp density, temperature, pH, conductivity, surface dissolved oxygen, macrophyte cover and tree shading; and (4) qPCR would possess greater detection sensitivity than PCR. We provide an eDNA framework for crucian carp monitoring which holds promise for routine survey.

2. Methods

2.1 Study sites

We studied 10 ponds with confirmed crucian carp presence at different densities and 10 fishless ponds in Norfolk (Figure 2). This region is low-lying (<100 m above sea level) and mainly agricultural. All ponds were selected to be small (<40 m in max. dimension), shallow (<2 m),

142 macrophyte-dominated, and open-canopy. Ponds were largely surrounded by arable fields,
143 excluding one located in woodland. No specific permits were required for sampling but relevant
144 landowner permissions were obtained.

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147 ## 2.2. Conventional survey

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149 Crucian carp presence-absence was confirmed at each pond by fyke netting between 2010 and
150 2016. Bar two ponds surveyed in 2013 and 2015, all crucian carp ponds were last surveyed in
151 2016. Where possible, double-ended fyke nets were set perpendicular to the bank or to beds of
152 aquatic vegetation and exposed overnight (for c. 16 h), with the number of fyke nets set being
153 proportional to pond size. This provided CPUE estimates of relative densities, which are the
154 number of fish captured per fyke net per 16 h exposure. Environmental data were collected
155 between May and August from 2010 to 2017. Conductivity, pH, surface dissolved oxygen and
156 water temperature were measured with a HACH HQ30d meter (Hach Company, CO, USA), and
157 alkalinity measured by sulphuric-acid titration using a HACH AL-DT kit (Hach Company, CO,
158 USA). Percentage of macrophyte cover and shading (trees and bushes) of ponds were estimated
159 visually.

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162 ## 2.3 eDNA sampling, capture and extraction

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164 Five 2 L surface water samples were collected from the shoreline of each pond using sterile
165 Gosselin™ HDPE plastic bottles (Fisher Scientific UK Ltd, UK) and disposable gloves. Samples
166 were taken at equidistant points around the pond perimeter where access permitted. All ponds
167 without crucian carp were sampled on 22nd August 2016. Water samples were transported on ice
168 in sterile coolboxes to the Centre for Ecology and Hydrology (CEH), Wallingford, stored at
169 4 °C, and vacuum-filtered within 24 hours of collection. Coolboxes were sterilised using 10%
170 v/v chlorine-based commercial bleach solution and 70% v/v ethanol solution before ponds
171 containing crucian carp were sampled on 25th August 2016. Samples were handled in the same
172 way. For each pond, a full process blank (1 L molecular grade water) was taken into the field and
173 stored in coolboxes with samples. Blanks were filtered and extracted alongside samples to
174 identify contamination.

175 Where possible, the full 2 L of each sample was vacuum-filtered through sterile 0.45 µm
176 cellulose nitrate membrane filters with pads (47 mm diameter; Whatman, GE Healthcare, UK)
177 using Nalgene filtration units. One hour was allowed for each sample to filter but if filters
178 clogged during this time, a second filter was used. After 2 L had been filtered or one hour had
179 passed, filters were removed from pads using sterile tweezers and placed in sterile 47 mm petri
180 dishes (Fisher Scientific UK Ltd, UK), which were sealed with parafilm (Sigma-Aldrich®, UK)
181 and stored at -20 °C. The total volume of water filtered and number of filters used per sample
182 were recorded for downstream analysis (Table S1). After each round of filtration (samples and
183 blanks from two ponds), all equipment was sterilised in 10% v/v chlorine-based commercial

184 bleach solution for 10 minutes, immersed in 5% v/v MicroSol detergent (Anachem, UK) and
185 rinsed with purified water.

186 All filters were transported on ice in a sterile coolbox to the University of Hull and stored
187 at -20 °C until DNA extraction one week later. DNA was isolated from filters using the
188 PowerWater® DNA Isolation Kit (MO BIO Laboratories, CA, USA) following the
189 manufacturer's protocol in a dedicated eDNA facility at University of Hull, devoted to pre-PCR
190 processes with separate rooms for filtration, DNA extraction and PCR preparation of
191 environmental samples. Duplicate filters from the same sample were co-extracted by placing
192 both filters in a single tube for bead milling. Eluted DNA (100 μL) concentration was quantified
193 on a Qubit™ 3.0 fluorometer using a Qubit™ dsDNA HS Assay Kit (Invitrogen, UK). DNA
194 extracts were stored at -20 °C until further analysis.
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196

197 **2.4 Assay design, specificity and sensitivity**

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199 We designed a novel assay to target a 118 bp amplicon within the mitochondrial cytochrome *b*
200 (*cytb*) gene, specific to crucian carp. Crucian carp sequences from Jeffries *et al*. (2016) were
201 aligned using MAFFT in AliView (Larsson, 2014) to sequences downloaded from the NCBI
202 nucleotide (nt) database for 24 closely related species of European freshwater fish, and a
203 consensus sequence for each species identified (Figure S1). Sequences were visually compared
204 to maximise nucleotide mismatches between crucian carp and non-target species, particularly
205 goldfish and common carp, and minimise theoretical risk of non-specific amplification.
206 Mismatches in primer regions were maximised over the probe region to increase specificity

207 (Wilcox et al., 2013). Species-specific primers CruCarp_CytB_984F (5'-
208 AGTTGCAGATATGGCTATCTTAA-3') and CruCarp_CytB_1101R (5'-
209 TGGAAAGAGGACAAGGAATAAT-3'), and corresponding probe CruCarp_CytB_1008Probe
210 (FAM 5'-ATGGATTGGAGGCATACCAGTAGAACACC-3' BHQ1) were selected on this
211 basis.

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

220 Primers were first validated *in vitro* using PCR and tissue DNA (standardised to 1 ng/µL)
221 from fin clips of crucian carp and four closely related non-target species: goldfish, common carp,
222 tench (*Tinca tinca*), and sunbleak (*Leucaspius delineatus*). An annealing temperature gradient
223 (Supporting Information: Appendix 1) was used to ensure optimal amplification of crucian carp
224 and no non-target amplification (Figure S2). The primers were also tested on eDNA samples
225 from ponds recently stocked with crucian carp to confirm potential for eDNA amplification
226 (Figure S3). Molecular grade water (Fisher Scientific UK Ltd, UK) was used as the no template
227 control (NTC) in all tests.

228 Primer and probe concentrations, standard curve preparation, and cycling conditions for
229 qPCR were then optimised (Supporting Information: Appendix 1). All subsequent qPCR

230 analyses were performed using the conditions detailed in section 2.5. Specificity tests were
231 repeated using qPCR on 10 non-target species related to crucian carp (Table S2, Figure S4) with
232 tissue DNA from fin clips (standardised to 1 ng/µL). The positive control and NTC were crucian
233 carp DNA and molecular grade water (Fisher Scientific UK Ltd, UK) respectively. The limits of
234 detection (LOD, the concentration at which no crucian carp DNA will amplify) and
235 quantification (LOQ, the concentration at which all technical replicates consistently amplify
236 crucian carp DNA) (Agersnap et al., 2017) were established using a 10-fold dilution series of
237 crucian carp DNA (1 to 1 x 10^{-8} ng/µL) and qPCR standards (10^6 to 1 copy/µL) (Figure S5). Five
238 technical replicates were performed for standards, controls, and samples in tests of assay
239 specificity and sensitivity.
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241
242 2.5 Detection and quantification of crucian carp eDNA
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244 All qPCR reactions were prepared in a UV and bleach sterilised laminar flow hood in our
245 dedicated eDNA facility. Reactions were performed in a total volume of 20 µL, consisting of 2
246 µL of template DNA, 1 µL of each primer (Forward 900 nM, Reverse 600 nM), 1 µL of probe
247 (125 nM) (Integrated DNA Technologies, Belgium), 10 µL of TaqMan® Environmental Master
248 Mix 2.0 (Life Technologies, CA, USA) and 5 µL molecular grade water (Fisher Scientific UK
249 Ltd, UK). Once eDNA samples and three NTCs were added to each 96-well plate, the plate was
250 sealed and transported to a separate laboratory on a different floor for addition of the standard
251 curve and three positive controls (crucian carp DNA, 0.01 ng/µL) in a UV and bleach sterilised
252 laminar flow hood.

253 Our standard curve was a synthesised 500 bp gBlocks® Gene Fragment (Integrated DNA
254 Technologies, Belgium) based on GenBank accessions (KT630374 - KT630380) for crucian carp
255 from Norfolk (Jeffries et al., 2016). Copy number for the gBlocks® fragment was estimated by
256 multiplying Avogadro's number by the number of moles. We performed a 10-fold serial dilution
257 of the gBlocks® fragment to generate a 6-point standard curve that ranged from 10^6 to 10
258 copies/μL. eDNA samples were compared to these known concentrations for quantification
259 (Hinlo et al., 2017a). Each standard was replicated five times on each qPCR plate. Similarly, five
260 technical replicates were performed for every sample and full process blank from each pond.

261 After addition of standards and positive controls, plates were again sealed and transported
262 to a separate laboratory on a different floor where qPCR was conducted on a StepOnePlus™
263 Real-Time PCR system (Life Technologies, CA, USA). Thermocycling conditions consisted of
264 incubation for 5 min at 50 °C, a 10 min denaturation step at 95 °C, followed by 60 cycles of
265 denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. We used 60 cycles for
266 consistency with optimisation tests, but cycling could be reduced to 45 cycles for subsequent
267 applications (see Supporting Information: Appendix 1).

268 Amplifications were considered positive detections if the exponential phase occurred
269 within 45 reaction cycles as the mean C_q value was 40.07 for the LOD (1 copy/μL). A pond was
270 considered positive for crucian carp if two or more of the five technical replicates from a sample
271 returned positive, or more than one sample returned any positive technical replicates (Goldberg
272 et al., 2016). False negatives were obtained for one pond therefore all samples were tested for
273 inhibition by spiking duplicate qPCR reactions with a known concentration of crucian carp
274 template (1000 copies/μL) (Jane et al., 2015).

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277 ## 2.6 DNA sequencing

278

279 Non-target DNA extracts and full-process blanks that amplified with qPCR were Sanger
280 sequenced alongside a representative eDNA sample from each positive pond (N = 9) to confirm
281 sequence identity. Purification and sequencing was performed by Macrogen Europe
282 (Amsterdam, The Netherlands) in triplicate in the forward direction. Sequences were edited
283 using CodonCode Aligner (CodonCode Corporation, MA, USA) with default settings. Sequences
284 were then manually aligned in AliView (Larsson, 2014) and poor quality sequences discarded
285 (Figure S6). Primers were removed from remaining sequences, and sequences identified against
286 the full NCBI nucleotide (nt) database using the NCBI BLASTn 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 five
292 technical replicates performed were discarded to minimise bias induced by pipetting error. All
293 technical replicates for eDNA samples were retained, and those which failed to amplify were
294 classed as 0 C_q (Goldberg et al., 2016). The C_q values for each set of technical replicates were
295 averaged and quantified to provide a single DNA copy number for each sample. Samples with no
296 positive amplifications were assigned a DNA copy number of zero. DNA copy numbers of
297 samples were then averaged to generate a single DNA copy number for each pond.

298 All subsequent data analyses were performed in the statistical programming environment

299 R v.3.4.2 (R Core Team, 2017). Agreement between fyke netting and qPCR for crucian carp
300 detection was assessed using Cohen's kappa coefficient (Cohen, 1960). Following this, Pearson's
301 Chi-squared Test for Independence was used to test for difference in the frequency of crucian
302 carp positive and negative ponds between methods. Prior to testing for a relationship between
303 CPUE estimate and average DNA copy number for each pond, we tested normality of the data
304 set using the Shapiro-Wilk test and visually inspected the underlying distribution using
305 histograms. All data points were included as some with the appearance of outliers may be due to
306 environmental fluctuations influencing DNA quality. A Spearman rank correlation coefficient
307 was calculated to measure strength of association as the interval data were not normally
308 distributed. Effects of water volume filtered, number of filters used, and water sample content on
309 DNA copy number of samples were also tested (see Supporting Information: Appendices 1, 2).
310 The R package 'eDNAoccupancy' v0.2.0 (Dorazio & Erickson, 2017) was used to fit a
311 Bayesian, multi-scale occupancy model to estimate crucian carp eDNA occurrence and detection
312 probabilities. Existing eDNA literature was used to identify biotic and abiotic factors reported to
313 affect eDNA detection, persistence and degradation, and construct hypotheses regarding their
314 effects on probability of eDNA occurrence in ponds (ψ), eDNA detection probability in water
315 samples (θ), and eDNA detection probability in qPCR replicates (p). Only macrophyte cover was
316 included as a covariate at site level. Vegetated ponds are more likely to contain crucian carp by
317 offering individuals refuge from predation as well as foraging and spawning opportunities (Sayer
318 et al., 2011), and have reduced UV exposure thereby preserving eDNA (Barnes et al., 2014).
319 Such ponds are susceptible to terrestrialisation which can create anoxic conditions that impede
320 crucian carp reproduction and recruitment (Sayer et al., 2011), although these conditions may
321 slow eDNA degradation and enable detection over longer periods (Barnes et al., 2014; Pilliod et

322 al., 2014; Weltz et al., 2017). At sample level, biotic and abiotic factors were included as
323 covariates. More individuals (reflected by CPUE) should increase eDNA concentration and
324 improve detection in water samples. Temperature can increase physical, metabolic, or
325 behavioural activity of organisms resulting in more eDNA release, breakdown, and degradation
326 (Takahara et al., 2012; Pilliod et al., 2014; Strickler et al., 2015; Robson et al., 2016;
327 Lacoursière-Roussel, Rosabal & Bernatchez, 2016; Buxton et al., 2017b; Bylemans et al., 2017).
328 Links established between eDNA and pH support greater detectability, concentration, and
329 persistence of eDNA in more alkaline waters (Barnes et al., 2014; Strickler et al., 2015;
330 Goldberg et al., 2018). Conductivity relates to Total Dissolved Solids (TDS) and sediment type,
331 which can impair eDNA detection due to release of inhibitory substances and their capacity to
332 bind DNA (Buxton et al., 2017a; Stoeckle et al., 2017). At qPCR replicate level, covariates again
333 included CPUE as higher eDNA concentration should improve amplification success and
334 consistency, whereas conductivity may indicate inhibitory substances that cause amplification
335 failure.

336 Prior to modeling, all environmental variables were assessed for collinearity using
337 Spearman's correlation coefficient and Variance Inflation Factors (VIFs) calculated using the R
338 package 'car' v2.1-6 (Fox & Weisberg, 2011). Variables were considered collinear and removed
339 if $r > 0.3$ and $VIF > 3$ (Zuur et al., 2009), following which candidate variables were centred and
340 scaled to have a mean of 0. We constructed 64 models which included macrophyte cover at site
341 level, and different covariate combinations at sample and qPCR replicate levels. Models were
342 ranked (Table S3) according to posterior predictive loss criterion (PPLC) under squared-error
343 loss and the widely applicable information criterion (WAIC). The model with the best support
344 was selected for comparison to models without covariates at site and the entire sampling

345 hierarchy.

346 We examined the influence of abiotic factors on eDNA quantification using a generalised
347 linear mixed effects model (GLMM) within the R package 'glmmTMB' v0.2.0 (Brooks et al.,
348 2017). Collinearity was assessed as above, leaving temperature, pH, conductivity, and percentage
349 of tree shading as explanatory variables. Pond was modelled as a random effect to account for
350 spatial autocorrelation in our data set and the influence of other properties inherent to each pond,
351 whereas all other explanatory variables were fixed effects. A Poisson distribution was specified
352 as the nature of the response variable (DNA copy number) was integer count data. Validation
353 checks were performed to ensure all model assumptions were met and absence of overdispersion
354 (Zuur et al., 2009). Model fit was assessed visually and with the Hosmer and Lemeshow
355 Goodness of Fit Test (Hosmer & Lemeshow, 2000) using the R package 'ResourceSelection'
356 v0.3-0 (Lele et al., 2014). Model predictions were obtained using the predict() function and
357 upper and lower 95% CIs were calculated from the standard error of the predictions. All values
358 were bound in a new data frame and model results plotted for evaluation using the R package
359 'ggplot2' v2.2.1 (Wickham, 2009).

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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 truncoulata* (plant, 8 mismatches). Our probe sequence could not be included *in silico* but would likely increase specificity. All crucian carp DNA amplified by PCR, with non-target amplification removed above 58 °C. 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 material, where sequences were either identified as crucian carp or poor quality (Table S4). Our assay was highly sensitive with a LOD of 1 copy/µL and LOQ of 10 copies/µL. The lowest concentration of crucian carp tissue DNA that amplified was 0.0001 ng/µL.

384 ## 3.2 qPCR analysis

385

386 The qPCR assay had average amplification efficiency of 93.614% (range 79.607-102.489%) and
387 average R^2 value of 0.998 (range 0.995-0.999) for the standard curve. No amplification occurred
388 in NTCs but the POFA4 full process blank amplified (<10 copies/µL). This was the only
389 contaminated blank as the POHI blank filtered alongside POFA4 and POHI samples, and blanks
390 downstream of these samples did not amplify. Partial inhibition occurred in a single sample from
391 four different ponds: PYES2 (no crucian carp), RAIL, POHI, and GUES1 (crucian carp present).
392 However, amplification of other samples enabled ponds to meet established detection criteria,
393 thus problematic samples were not treated for inhibition or qPCRs repeated.

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396 ## 3.3 Presence-absence detection

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398 eDNA surveillance detected crucian carp in 90% of ponds (N = 10) with confirmed presence.
399 Sanger sequencing of representative samples confirmed species identity as crucian carp (Table
400 S5). eDNA failed entirely in one pond (CHIP) that contained a sizeable crucian carp population
401 (CPUE = 60.50), but samples from CHIP were not inhibited. Crucian carp DNA was not
402 detected at any sites where the species was absent. Cohen's kappa coefficient ($\kappa = 0.9$) indicated
403 strong agreement between fyke netting and eDNA analysis, further supported by no significant
404 difference in frequency of crucian carp positive and negative ponds by each monitoring tool (χ^2 =
405 0.1003, df = 1, $P = 0.752$).

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407

3.4 Relative abundance estimation

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410 We identified a weak, positive trend between CPUE estimate and DNA copy number (Figure 3),
411 but this was not significant (r_S = 0.334, df = 8, *P* = 0.345). The association was unchanged when
412 ponds not surveyed by fyke netting in 2016 were removed, or DNA copy number was set as 10
413 copies/μL for amplifications below the LOQ.

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3.5 Factors influencing eDNA detection and quantification

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418 The occupancy model with the best support included macrophyte cover as a covariate of eDNA
419 occurrence probability at sites (ψ), and CPUE and conductivity as covariates of eDNA detection
420 probability in qPCR replicates (p). The model did not include any covariates of eDNA detection
421 probability in water samples (θ). The probability of eDNA occurrence in a pond (Figure 4a)
422 ranged between 0.34 to 0.73 (Table 1) and was negatively influenced by macrophyte cover
423 (parameter estimate = -0.294). Estimates of eDNA detection probability in a qPCR replicate
424 ranged between 0.15 to 1.00 (Table 1), where crucian carp CPUE and conductivity played
425 positive (parameter estimate = 1.409) and negative (parameter estimate = -1.923) roles in eDNA
426 availability respectively (Figures 4b, c). The GLMM identified temperature (0.711 ± 0.284, χ^2_1 =
427 5.223, *P* = 0.022), conductivity (-0.006 ± 0.002, χ^2_1 = 6.017, *P* = 0.014), and macrophyte cover
428 (0.035 ± 0.015, χ^2_1 = 4.167, *P* = 0.041) as significant predictors of DNA copy number, where
429 DNA copy number was greater at higher temperatures (Figure 5a) but decreased as conductivity

430 and macrophyte cover increased (Figures. 5b, c).

431

432

433 3.6 PCR versus qPCR

434

435 Crucian carp eDNA was amplified by PCR in all samples that amplified using qPCR (Table 2).
436 PCR also provided semi-quantitative estimates of eDNA concentration when PCR products for
437 eDNA samples were run on gels alongside qPCR standards (Figure 6).

438

439

440

441 4. Discussion

442

443 We developed a novel species-specific qPCR assay to enable large-scale distribution monitoring
444 of the threatened crucian carp using eDNA. Crucian carp were detected at almost all sites with
445 confirmed presence and no false positives were generated. Our eDNA approach may have
446 limited suitability for abundance estimation as DNA copy number did not correlate with crucian
447 carp density estimated from netting. However, several biotic and abiotic factors that influence
448 eDNA detection and quantification were identified. Finally, PCR provided semi-quantitative
449 estimates of eDNA concentration and may be a viable alternative to qPCR where funding or
450 laboratory facilities are limited. We discuss areas for improvement in our workflow and provide
451 recommendations for future study.

452

453

454 ## 4.1 Using eDNA for crucian carp conservation

455

456 eDNA analysis is often compared to conventional monitoring tools to assess performance,
457 reliability, reproducibility, and prospective applications in conservation programmes. We found
458 strong agreement between eDNA and fyke netting for crucian carp detection, where eDNA
459 detected crucian carp in 90% of ponds with presence confirmed by netting. This high detection
460 and low false negative rate supports applicability of eDNA to crucian carp presence-absence
461 monitoring, particularly at large spatial scales where fyke netting is too costly and time-
462 consuming. Abundance estimation is less straightforward as DNA copy number did not directly
463 correspond to crucian carp density. This inconsistency is more likely to stem from eDNA than
464 fyke netting due to effects exerted by external factors (section 4.2) and sample processing
465 (section 4.3) on eDNA quality. However, fyke netting also has detection biases that may
466 influence performance comparisons with eDNA. Fyke net surveys are restricted spatially and
467 temporally (to pre- and post-spawning, as well as spring and autumn when temperatures are low
468 to reduce fish stress in nets), and may fail to capture species that do not have homogenous
469 distribution in their environment (Turner et al., 2012). Netting can be biased towards a particular
470 sex and size class, and catchability dependent on time of year (Ruane, Davenport & Igoe, 2012)
471 and even time of day (Hardie, Barmuta & White, 2006). Therefore, effectiveness of standard
472 methods must also be evaluated and eDNA compared to multiple tools before deemed capable or
473 incapable of estimating abundance.

474

475

476 ## 4.2 Factors influencing eDNA detection and quantification

477

478 Effects of biotic and abiotic factors on eDNA may vary across target species and ecosystems
479 (Barnes et al., 2014). We found that macrophyte cover negatively influenced eDNA occurrence
480 in ponds, but positively influenced DNA copy number. Crucian carp prefer ponds with stands of
481 aquatic vegetation; however, target DNA may experience interference from plant DNA during
482 qPCR or qPCR reactions may be inhibited by substances in plants, impairing eDNA detection
483 (Jane et al., 2015; Stoeckle et al., 2017). Whilst aquatic vegetation may impede eDNA detection,
484 it may facilitate eDNA preservation and accumulation through reduced UV exposure or induced
485 anoxia (Barnes et al., 2014; Pilliod et al., 2014; Weltz et al., 2017).

486 eDNA detection probability in qPCR replicates increased at higher crucian carp densities,
487 but decreased as conductivity increased. DNA copy number and conductivity were also
488 negatively correlated. Density is frequently reported to improve detection probability of aquatic
489 species due to more eDNA deposition in the environment (Schmelzle & Kinziger, 2016; Buxton
490 et al., 2017b; Stoeckle et al., 2017). Conductivity has been suggested to influence eDNA
491 detection and quantification, but studies that directly measured this variable found no discernable
492 effect (Takahara et al., 2012; Keskin, 2014; Goldberg et al., 2018). Conductivity (also measured
493 as TDS) relates to sediment type which influences eDNA detection probability, the rate at which
494 sediment binds eDNA, and release of inhibitory substances (Buxton et al., 2017a; Stoeckle et al.,
495 2017). Notably, the only false negative pond in our study was also the most conductive (760
496 µs/cm). Therefore, conductivity may lead to incorrect inferences about species presence and
497 impact conservation management decisions.

498 Our results indicate that samples may have been affected by inhibitory substances despite
499 tests performed to identify inhibition. We spiked qPCR reactions with a known amount of
500 synthetic target DNA. However, an artificial Internal Positive Control gene assay may identify
501 inhibition more effectively (Goldberg et al., 2016). Dilution of eDNA samples (and inhibitory
502 substances present) can release inhibition, but also reduce detection probability (Piggott, 2016)
503 and induce false negatives (Buxton et al., 2017a). We used TaqMan® Environmental Master Mix
504 2.0 (Life Technologies, CA, USA) in qPCR reactions to counter inhibition (Jane et al., 2015), but
505 it may be advisable to use DNA extraction kits that perform inhibitor removal (Sellers et al.,
506 2018) or include Bovine-serum albumin (BSA) in qPCR reactions (Jane et al., 2015).
507 Alternatively, ddPCR may handle inhibitors better than qPCR and provide more accurate
508 abundance or biomass estimates (Nathan et al., 2014).

509 In addition to effects of macrophyte cover and conductivity, water temperature positively
510 influenced DNA copy number. Although warmer temperature coincided with breeding activity
511 and heightened DNA release in other fish and amphibian species (Buxton et al., 2017b;
512 Bylemans et al., 2017), water sample collection in late August was outwith the reported
513 spawning period for crucian carp (Aho & Holopainen, 2000). The association observed here may
514 instead reflect increased DNA shedding rates caused by higher metabolic activity in response to
515 warm temperature, as reported for other fish species (Takahara et al., 2012; Robson et al., 2016;
516 Lacoursière-Roussel et al., 2016).

517 Crucially, environmental data were not collected in 2016 for every pond in our study. Our
518 results indicate direction of effects of biotic and abiotic factors on eDNA detection and
519 quantification, but contemporary data (particularly temperature) are needed for comprehensive
520 interpretation of these relationships. However, it is clear that eDNA practitioners must account

521 for these effects as well as sample inhibition. The uncertainty around the estimated effects of
522 covariates at each level of our hierarchical occupancy model and GLMM also imply that greater
523 sample volume, sample number, and/or qPCR replication are required to improve the ability and
524 precision of our assay to detect crucian carp eDNA and reduce the potential for false negatives
525 (Schultz & Lance, 2015; Goldberg et al., 2018).

526

527

528
4.3 Optimisation of eDNA workflow

529

530 Some non-target DNA extracts used to validate assay specificity were contaminated with crucian
531 carp DNA. Field cross-contamination can occur if reference tissue material is collected from
532 multiple species without sterilising equipment, or eDNA is present on the material collected
533 (Rodgers, 2017). Collection and storage of reference tissue material is an important consideration
534 for eDNA practitioners, particularly those using highly sensitive assays (LOD = 1 copy/μL)
535 (Wilcox et al., 2013, 2016). Dedicated, sterilised equipment should be used when collecting new
536 reference material from different species. From existing reference collections, only non-target
537 samples that were collected on separate and chronologically distinct occasions from target
538 samples should be used (Rodgers, 2017).

539 Cross-contamination can also arise during water sampling, filtration, DNA extraction and
540 qPCR preparation. Low-level contamination was found in one full process blank but detections
541 from this pond were not omitted as it contained crucian carp and contamination was not observed
542 downstream. All equipment in our study was sterilised by immersion in 10% chlorine-based
543 commercial bleach solution for 10 mins, followed by 5% MicroSol detergent (Anachem, UK),

544 and rinsed with purified water. However, sterilisation with 50% chlorine-based commercial
545 bleach solution (Goldberg et al., 2016) or single-use, sterile materials (Wilcox et al., 2016) may
546 further minimise contamination risk.

547 Many of our eDNA samples were low concentration (<100 copies/µL) which can cause
548 inconsistent qPCR amplification (Goldberg et al., 2016), thus we discuss approaches to
549 maximise eDNA concentration and improve detection probability. The probability of eDNA
550 detection depends heavily on the number of samples and volume of water collected, time of
551 sampling, and sample concentration (Schultz & Lance, 2015; Goldberg et al., 2018). We sampled
552 5 x 2 L water samples from each pond in autumn 2016, but timing and/or sampling effort may
553 have been inappropriate. A seasonal effect on common carp eDNA detection was observed,
554 where spring sampling generated higher eDNA concentration and detection rate due to greater
555 common carp activity (Turner et al., 2014) and density (Hinlo et al., 2017a). As water sampling
556 did not coincide with fyke netting (spring 2016) in our study, eDNA concentration may not
557 reflect CPUE estimates. Water samples in spring may contain more crucian carp eDNA due to
558 higher activity of individuals, or autumn fyke netting may produce lower CPUE estimates.
559 Parallel seasonal sampling, where water sampling is performed in conjunction with fyke netting
560 throughout the year, may better align eDNA concentration with CPUE estimates and enable
561 eDNA-based abundance estimates for crucian carp.

562 Representative sampling is crucial in eDNA surveys. Individuals of a species can be
563 unevenly distributed in the environment, which impacts eDNA detection, distribution, and
564 concentration (Takahara et al., 2012; Eichmiller, Bajer & Sorensen, 2014; Schmelzle &
565 Kinziger, 2016; Goldberg et al., 2018). In lentic ecosystems, eDNA has a patchy horizontal and
566 sometimes vertical distribution, resulting in fine spatial variation (Eichmiller et al., 2014).

567 Studies on common carp revealed eDNA was more concentrated near the shoreline of lentic
568 water bodies (Takahara et al., 2012; Eichmiller et al., 2014), due to aggregations of individuals
569 (Eichmiller et al., 2014). We collected surface water (5 x 2 L) from the shoreline and sampled at
570 equidistant points around the pond perimeter where possible; however, more samples and greater
571 water volumes may be required to improve detection probability (Schultz & Lance, 2015;
572 Goldberg et al., 2018). Fine spatial sampling and occupancy modelling are needed to determine
573 the sample number required to achieve high detection probability and eliminate false negatives
574 (Goldberg et al., 2018). However, the number of samples required will inevitably vary across
575 habitats due to inherently variable physical properties (Schmelzle & Kinziger, 2016).

576 Method of eDNA capture can dictate success of this monitoring tool. Studies of eDNA in
577 ponds (Ficetola et al., 2008; Biggs et al., 2015) have used an ethanol precipitation approach, but
578 this is restricted to small volumes. Filtration allows more water to be processed and minimises
579 capture of non-target DNA, with macro-organism eDNA effectively captured by pore sizes of 1 -
580 10 μm (Turner et al., 2014). We used a small pore size of 0.45 μm to capture most eDNA particle
581 sizes, although filter clogging prevented the entire sample being processed and may have
582 affected eDNA concentration downstream. Pre-filtering can reduce clogging, but is labour-
583 intensive and increases cost (Takahara et al., 2012). Larger pore sizes have been used in
584 temperate and tropical lentic environments (Takahara et al., 2012; Robson et al., 2016; Goldberg
585 et al., 2018), though independent investigation is needed to determine which pore size maximises
586 target DNA concentration.

587 Comparisons of eDNA yield across filter types and DNA extraction protocols have
588 shown that cellulose nitrate filters stored at -20 °C (this study) often provide best eDNA yield
589 (Piggott, 2016; Spens et al., 2016; Hinlo et al., 2017b). However, different filter types may be

590 optimal for different species, which has consequences for detectability (Spens et al., 2016) and
591 relationships between eDNA concentration and abundance/biomass (Lacoursière-Roussel et al.,
592 2016). Extraction method used, regardless of filter type, will ultimately influence DNA quality
593 and yield. We used the PowerWater® DNA Isolation Kit (MO BIO Laboratories, CA, USA), but
594 the DNeasy Blood and Tissue kit (Qiagen®, Hilden, Germany) has demonstrated greater yield
595 (Hinlo et al., 2017b). We also combined filters from the same sample for DNA extraction at the
596 bead milling stage, but independent lysis may recover more DNA (Hinlo et al., 2017b). A
597 comparison of DNA extraction protocols is necessary to assess which maximises crucian carp
598 eDNA concentration. A new modular extraction method shows promise for eDNA but has yet to
599 be evaluated for targeted qPCR (Sellars et al., 2018).

600 Finally, detection sensitivity can be enhanced by increasing the number of qPCR
601 technical replicates (Schultz & Lance, 2015; Piggott, 2016). We performed five technical
602 replicates for each of our samples, but other studies have used as many as twelve and only one
603 may amplify (Biggs et al., 2015). More replication may have enabled amplification from CHIP
604 samples, but qPCR cost would inevitably increase. Further replication may also be unnecessary
605 if steps are taken to improve initial concentration of samples instead (Schultz & Lance, 2015).

606

607

608 **4.4 PCR or qPCR?**

609

610 Our study is not the first to compare eDNA detection using different means of DNA
611 amplification (Nathan et al., 2014; Farrington et al., 2015; Piggott, 2016; De Ventura et al.,
612 2017). Like Nathan et al. (2014), we found PCR had comparable sensitivity to qPCR and band

strength of PCR products may indicate eDNA concentration; however, we also translated band strength to approximate DNA copy number. PCR may require more replication to achieve set detection probabilities (Piggott, 2016), but lower sensitivity could make this approach more robust to false positives from cross-contamination than qPCR (De Ventura et al., 2017). Large-scale comparisons of PCR and qPCR across study systems and species are needed to truly assess performance of each approach. Nonetheless, our findings support PCR as a cost-efficient, semi-quantitative alternative to qPCR for conservation programmes wishing to utilise eDNA (Nathan et al., 2014; De Ventura et al., 2017).

4.5 Concluding remarks

A primary objective of the Norfolk crucian carp BAP was to obtain a basic understanding of species distribution and population status across Norfolk (Copp & Sayer, 2010). eDNA surveillance for crucian carp will provide a useful, cost-effective alternative to established survey methods where the aim is determining presence-absence. Our assay may detect hybrids where crucian carp were the maternal parent due to use of a mitochondrial marker; however, these detections are also beneficial to the crucian carp conservation effort by identifying ponds where true crucian carp may still exist and contamination with goldfish, common carp and their hybrids has occurred. Alternatively, our assay could be used as an early warning tool in countries where the crucian carp is considered invasive. The areas we have highlighted require further investigation before eDNA can be used routinely. Nevertheless, eDNA survey could enable large-scale distribution monitoring for crucian carp through rapid screening of existing and new

ponds. Fyke netting could then be used to investigate age, sex and size structure of populations, and remove hybrids.

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651 ## References
652

653 Agersnap, S., Larsen, W.B., Knudsen, S.W., Strand, D., Thomsen, P.F., Hesselsøe, M., …
654 Møller, P.R. (2017). Monitoring of noble, signal and narrow-clawed crayfish using
655 environmental DNA from freshwater samples. *PLoS ONE*, *12*, e0179261.
656 https://dx.doi.org/10.1371/journal.pone.0179261
657 Aho, J., & Holopainen, I.J. (2000). Batch spawning of crucian carp (*Carassius carassius* (L.)) in
658 mono- and multispecies communities. *Annales Zoologici Fennici*, *37*, 101–111.
659 Barnes, M.A., Turner, C.R., Jerde, C.L., Renshaw, M.A., Lindsay Chadderton, W., & Lodge,
660 D.M. (2014). Environmental Conditions Influence eDNA Persistence in Aquatic Systems.
661 *Environmental Science & Technology*, *48*, 1819−1827.
662 https://dx.doi.org/10.1021/es404734p
663 Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C., Dejean, T., Griffiths, R.A., … Dunn, F.
664 (2015). Using eDNA to develop a national citizen science-based monitoring programme for
665 the great crested newt (*Triturus cristatus*). *Biological Conservation*, *183*, 19–28.
666 https://dx.doi.org/10.1016/j.biocon.2014.11.029
667 Brooks, M.E., Kristensen, K., van Benthem, K.J., Magnusson, A., Berg, C.W., Nielsen A., …
668 Bolker, B.M. (2017). Modeling zero-inflated count data with glmmTMB. *bioRxiv*, 132753.
669 https://dx.doi.org/10.1101/132753
670 Buxton, A.S., Groombridge, J.J., & Griffiths, R.A. (2017a). Is the detection of aquatic
671 environmental DNA influenced by substrate type? *PLoS ONE*, *12*, e0183371. |
672 https://doi.org/10.1371/journal.pone.0183371
673 Buxton, A.S., Groombridge, J.J., Zakaria, N.B., & Griffiths, R.A. (2017b). Seasonal variation in
674 environmental DNA in relation to population size and environmental factors. *Scientific*
675 *Reports*, *7*, 46294. https://doi.org/10.1038/srep46294
676 Bylemans, J., Furlan, E.M., Hardy, C.M., McGuffie, P., Lintermans, M., & Gleeson, D.M.
677 (2017). An environmental DNA-based method for monitoring spawning activity: a case
678 study, using the endangered Macquarie perch (*Macquaria australasica*). *Methods in*
679 *Ecology and Evolution*, *8*, 646–655. https://doi.org/10.1111/2041-210X.12709
680 Cohen, J. (1960). A Coefficient of Agreement for Nominal Scales. *Educational and*
681 *Psychological Measurement*, *20*, 37–46.
682 Copp, G.H., Černý, J., & Kováč, V. (2008a). Growth and morphology of an endangered native
683 freshwater fish, crucian carp *Carassius carassius*, in an English ornamental pond. *Aquatic*
684 *Conservation: Marine and Freshwater Ecosystems*, *18*, 32–43.
685 https://doi.org/10.1002/aqc.820
686 Copp, G.H., Warrington, S., & Wesley, K.J. (2008b). Management of an ornamental pond as a
687 conservation site for a threatened native fish species, crucian carp *Carassius carassius*.
688 *Hydrobiologia*, *597*, 149-155. https://doi.org/10.1007/s10750-007-9220-0
689 Copp, G.H., & Sayer, C.D. (2010). Norfolk Biodiversity Action Plan–Local Species Action Plan
690 for Crucian Carp (*Carassius carassius*). Norfolk Biodiversity Partnership Reference: LS/ 3.
691 Centre for Environment, Fisheries & Aquaculture Science, Lowestoft, Suffolk.
692 De Ventura, L., Kopp, K., Seppälä, K., & Jokela, J. (2017). Tracing the quagga mussel invasion
693 along the Rhine river system using eDNA markers: early detection and surveillance of
694 invasive zebra and quagga mussels. *Management of Biological Invasions*, *8*, 101–112.
695 https://doi.org/10.3391/mbi.2017.8.1.10

696 Dorazio, R.M., & Erickson, R.A. (2017). eDNAoccupancy: An R package for multi-scale
697 occupancy modeling of environmental DNA data. *Molecular Ecology Resources*, *18*, 368-
698 380. https://doi.org/10.1111/1755-0998.12735
699 Eichmiller, J.J., Bajer, P.G., & Sorensen, P.W. (2014). The relationship between the distribution
700 of common carp and their environmental DNA in a small lake. *PLoS ONE*, *9*, e112611.
701 https://doi.org/10.1371/journal.pone.0112611
702 Environment Agency (2003). Crucian carp field guide. National Coarse Fish Centre,
703 Environment Agency, Bristol.
704 Farrington, H.L., Edwards, C.E., Guan, X., Carr, M.R., Baerwaldt, K., & Lance R.F. (2015).
705 Mitochondrial genome sequencing and development of genetic markers for the detection of
706 DNA of invasive bighead and silver carp (*Hypophthalmichthys nobilis* and *H. molitrix*) in
707 environmental water samples from the United States. *PLoS ONE*, *10*, e0117803.
708 https://doi.org/10.1371/journal.pone.0117803
709 Ficetola, G.F., Coissac, E., Zundel, S., Riaz, T., Shehzad, W., Bessière, J., … Pompanon, F.
710 (2010). An In silico approach for the evaluation of DNA barcodes. *BMC Genomics* **11**, 434.
711 https://doi.org/10.1186/1471-2164-11-434
712 Ficetola, G.F., Miaud, C., Pompanon, F., & Taberlet, P. (2008). Species detection using
713 environmental DNA from water samples. *Biology Letters* **4**, 423–425.
714 https://doi.org/10.1098/rsbl.2008.0118
715 Fox, J., & Weisberg S. (2011). *An R Companion to Applied Regression, Second Edition*. Sage,
716 Thousand Oaks, CA.
717 Goldberg, C.S., Strickler, K.M., & Fremier, A.K. (2018). Degradation and dispersion limit
718 environmental DNA detection of rare amphibians in wetlands: Increasing efficacy of
719 sampling designs. *Science of The Total Environment*, *633*, 695–703.
720 https://doi.org/10.1016/j.scitotenv.2018.02.295
721 Goldberg, C.S., Turner, C.R., Deiner, K., Klymus, K.E., Thomsen, P.F., Murphy,
722 M.A., …Taberlet, P. (2016). Critical considerations for the application of environmental
723 DNA methods to detect aquatic species. *Methods in Ecology and Evolution*, *7*, 1299–1307.
724 https://doi.org/10.1111/2041-210X.12595
725 Hänfling, B., Bolton, P., Harley, M., & Carvalho, G.R. (2005). A molecular approach to detect
726 hybridisation between crucian carp (*Carassius carassius*) and non-indigenous carp species
727 (*Carassius* spp. and *Cyprinus carpio*). *Freshwater Biology*, *50*, 403–417.
728 https://doi.org/10.1111/j.1365-2427.2004.01330.x
729 Hänfling, B., Lawson Handley, L., Read, D.S., Hahn, C., Li, J., Nichols, P., … Winfield, I.J.
730 (2016). Environmental DNA metabarcoding of lake fish communities reflects long-term
731 data from established survey methods. *Molecular Ecology*, *25*, 3101–3119.
732 https://doi.org/10.1111/mec.13660
733 Hardie, S.A., Barmuta, L.A., & White, R.W.G. (2006). Comparison of Day and Night Fyke
734 Netting, Electrofishing and Snorkelling for Monitoring a Population of the Threatened
735 Golden Galaxias (*Galaxias auratus*). *Hydrobiologia*, *560*, 145–158. https://10.1007/s10750-
736 005-9509-9
737 Hinlo, R., Furlan, E., Suitor, L., & Gleeson, D. (2017a). Environmental DNA monitoring and
738 management of invasive fish: comparison of eDNA and fyke netting. *Management of*
739 *Biological Invasions*, *8*, 89–100. https://doi.org/10.3391/mbi.2017.8.1.09

740 Hinlo, R., Gleeson, D., Lintermans, M., & Furlan, E. (2017b). Methods to maximise recovery of
741 environmental DNA from water samples. *PLoS ONE*, *12*, e0179251.
742 https://doi.org/10.1371/ journal.pone.0179251
743 Hosmer D.W. & Lemeshow S. (2000) Multiple Logistic Regression. In: *Applied Logistic*
744 *Regression*. pp. 31–46. John Wiley & Sons, Inc.
745 Jane, S.F., Wilcox, T.M., McKelvey, K.S., Young, M.K., Schwartz, M.K., Lowe, W.H., …
746 Whiteley, A.R. (2015). Distance, flow and PCR inhibition: eDNA dynamics in two
747 headwater streams. *Molecular Ecology Resources*, *15*, 216–227.
748 https://doi.org/10.1111/1755-0998.12285
749 Jeffries, D.L., Copp, G.H., Lawson Handley, L., Olsén, K.H., Sayer, C.D., & Hänfling, B.
750 (2016). Comparing RADseq and microsatellites to infer complex phylogeographic patterns,
751 an empirical perspective in the Crucian carp, *Carassius carassius*, L. *Molecular Ecology*,
752 *25*, 2997-3018. https://doi.org/10.1111/mec.13660
753 Jeffries, D.L., Copp, G.H., Maes, G.E., Lawson Handley, L., Sayer, C.D., & Hänfling, B. (2017).
754 Genetic evidence challenges the native status of a threatened freshwater fish (*Carassius*
755 *carassius*) in England. *Ecology and Evolution*, *7*, 2871-2882.
756 https://doi.org/10.1002/ece3.2831
757 Jerde, C.L., Mahon, A.R., Chadderton, W.L., & Lodge, D.M. (2011). “Sight-unseen” detection
758 of rare aquatic species using environmental DNA. *Conservation Letters*, *4*, 150–157.
759 https://doi.org/10.1111/j.1755-263X.2010.00158.x
760 Keskin, E. (2014). Detection of invasive freshwater fish species using environmental DNA
761 survey. *Biochemical Systematics and Ecology*, *56*, 68–74.
762 https://dx.doi.org/10.1016/j.bse.2014.05.003
763 Lacoursière-Roussel, A., Rosabal, M., & Bernatchez, L. (2016). Estimating fish abundance and
764 biomass from eDNA concentrations: variability among capture methods and environmental
765 conditions. *Molecular Ecology Resources*, *16*, 1401-1414. https://doi.org/10.1111/1755-
766 0998.12522
767 Larsson, A. (2014). AliView: a fast and lightweight alignment viewer and editor for large
768 datasets. *Bioinformatics*, *30*, 3276–3278. https://doi.org/10.1093/bioinformatics/btu531
769 Lawson Handley, L. (2015). How will the ‘molecular revolution’ contribute to biological
770 recording? *Biological Journal of the Linnean Society*, *115*, 750–766.
771 Lele, S.R., Keim, J.L., & Solymos, P. (2016). ResourceSelection: Resource Selection
772 (Probability) Functions for Use-Availability Data. R package version 0.3-2.
773 Maitland, P.S. (1972). A key to the freshwater fishes of the British Isles with notes on their
774 distribution and ecology. Scientific Publication no. 27, Freshwater Biological Association,
775 Ambleside, UK.
776 Manchester, S.J., & Bullock, J.M. (2000). The impacts of non-native species on UK biodiversity
777 and the effectiveness of control. *Journal of Applied Ecology*, *37*, 845–864.
778 Nathan, L.M., Simmons, M., Wegleitner, B.J., Jerde, C.L., & Mahon, A.R. (2014). Quantifying
779 environmental DNA signals for aquatic invasive species across multiple detection
780 platforms. *Environmental Science & Technology*, *48*, 12800–12806.
781 https://dx.doi.org/10.1021/es5034052
782 Piggott, M.P. (2016). Evaluating the effects of laboratory protocols on eDNA detection
783 probability for an endangered freshwater fish. *Ecology and Evolution*, *6*, 2739–2750.
784 https://doi.org/10.1002/ece3.2083

785 Pilliod, D.S., Goldberg, C.S., Arkle, R.S., & Waits, L.P. (2014). Factors influencing detection of
786 eDNA from a stream-dwelling amphibian. *Molecular Ecology Resources* **14**, 109–116.
787 https://doi.org/10.1111/1755-0998.12159
788 Robson, H.L.A., Noble, T.H., Saunders, R.J., Robson, S.K.A., Burrows, D.W., & Jerry, D.R.
789 (2016). Fine tuning for the tropics: application of eDNA technology for invasive fish
790 detection in tropical freshwater ecosystems. *Molecular Ecology Resources*, *16*, 922-932.
791 https://doi.org/10.1111/1755-0998.12505
792 Rodgers, T. (2017). Proper fin-clip sample collection for molecular analyses in the age of eDNA.
793 *Journal of Fish Biology*, *91*, 1265–1267. https://doi.org/10.1111/jfb.13485
794 Roy, M., Belliveau, V., Mandrak, N.E., & Gagné, N. (2018). Development of environmental
795 DNA (eDNA) methods for detecting high-risk freshwater fishes in live trade in Canada.
796 *Biological Invasions*, *20*, 299-314. https://doi.org/10.1111/jfb.13485
797 Ruane, N.M., Davenport, J., & Igoe, F. (2012). NON-DESTRUCTIVE TECHNIQUES FOR
798 THE MONITORING OF ARCTIC CHAR SALVELINUS ALPINUS (L.) IN IRISH
799 LOUGHS I. FYKE NETTING. *Biology and Environment. Proceedings of the Royal Irish*
800 *Academy*, *112B*, 301–304.
801 Sayer, C.D., Copp, G.H., Emson, D., Godard, M.J., Zięba, G., & Wesley, K.J. (2011). Towards
802 the conservation of crucian carp *Carassius carassius*: understanding the extent and causes
803 of decline within part of its native English range. *Journal of Fish Biology*, *79*, 1608–1624.
804 https://doi.org/10.1111/j.1095-8649.2011.03059.x
805 Schmelzle, M.C., & Kinziger, A.P. (2016). Using occupancy modelling to compare
806 environmental DNA to traditional field methods for regional-scale monitoring of an
807 endangered aquatic species. *Molecular Ecology Resources*, *16*, 895-908. https://doi.org/
808 10.1111/1755-0998.12501
809 Schultz, M.T., & Lance, R.F. (2015). Modeling the Sensitivity of Field Surveys for Detection of
810 Environmental DNA (eDNA). *PLoS ONE*, *10*, e0141503.
811 https://doi.org/10.1371/journal.pone.0141503
812 Sellers, G.S., Di Muri, C., Hänfling, B., Gómez, A. (2018) Mu-DNA: a modular universal DNA
813 extraction method adaptable for a wide range of sample types.
814 http://dx.doi.org/10.17504/protocols.io.nbedaje
815 Sigsgaard, E.E., Carl, H., Møller, P.R., & Thomsen, P.F. (2015). Monitoring the near-extinct
816 European weather loach in Denmark based on environmental DNA from water samples.
817 *Biological Conservation*, *183*, 46–52. http://dx.doi.org/10.1016/j.biocon.2014.11.023
818 Smith, P, & Moss, B. (1994). The role of fish in the management of freshwater Sites of Special
819 Scientific Interest. English Nature Research Report no. 111.
820 Spens, J., Evans, A.R., Halfmaerten, D., Knudsen, S.W., Sengupta, M.E., Mak, S.S.T., …
821 Hellström, M. (2016). Comparison of capture and storage methods for aqueous macrobial
822 eDNA using an optimized extraction protocol: advantage of enclosed filter. *Methods in*
823 *Ecology and Evolution*, *8*, 635–645. https://doi.org/10.1111/2041-210X.12683
824 Stefanoudis P.V., Sayer C.D., Greaves H.M., Davidson T.A., Robson H., Almeida D., & Smith,
825 E. (2017) Consequences of fish for cladoceran, water beetle and macrophyte communities
826 in a farmland pond landscape: implications for conservation. *Fundamental and Applied*
827 Limnology, *190*, 141–156. https://doi.org/10.1127/fal/2017/1004

828 Stoeckle, B.C., Beggel, S., Cerwenka, A.F., Motivans, E., Kuehn, R., & Geist, J. (2017). A
829 systematic approach to evaluate the influence of environmental conditions on eDNA
830 detection success in aquatic ecosystems. *PLoS ONE*, *12*, e0189119. .
831 https://doi.org/10.1371/journal. pone.0189119
832 Strickler, K.M., Fremier, A.K., & Goldberg, C.S. (2015). Quantifying effects of UV-B,
833 temperature, and pH on eDNA degradation in aquatic microcosms. *Biological*
834 *Conservation*, *183*, 85–92. http://dx.doi.org/10.1016/j.biocon.2014.11.038
835 Takahara, T., Minamoto, T., Yamanaka, H., Doi, H. & Kawabata, Z. (2012). Estimation of fish
836 biomass using environmental DNA. *PLoS ONE*, *7*, e35868. https://doi.org/
837 10.1371/journal.pone.0035868
838 Tarkan, A.S., Copp, G.H., Zięba, G., Godard, M.J., & Cucherousset, J. (2009). Growth and
839 reproduction of threatened native crucian carp *Carassius carassius* in small ponds of
840 Epping Forest, south-east England. *Aquatic Conservation: Marine and Freshwater*
841 *Ecosystems*, *19*, 797–805. https://doi.org/10.1002/aqc.1028
842 Turner, C.R., Barnes, M.A., Xu, C.C.Y., Jones, S.E., Jerde, C.L., & Lodge, D.M. (2014). Particle
843 size distribution and optimal capture of aqueous macrobial eDNA. *Methods in Ecology and*
844 Evolution, *5*, 676–684. https://doi.org/10.1002/aqc.1028
845 Turner, C.R., Lodge, D.M., Xu, C., Cooper, M.J., & Lamberti, G.A. (2012). Evaluating
846 environmental DNA detection alongside standard fish sampling in Great Lakes coastal
847 wetland monitoring (seed project). Final Report, Illinois-Indiana Sea Grant.
848 Weltz, K., Lyle, J.M., Ovenden, J., Morgan, J.A.T., Moreno, D.A., & Semmens, J.M. (2017).
849 Application of environmental DNA to detect an endangered marine skate species in the
850 wild. *PLoS One*, *12*, e0178124. https://doi.org/10.1371/journal. pone.0178124
851 Wheeler, A. (1977). The Origin and Distribution of the Freshwater Fishes of the British Isles.
852 *Journal of Biogeography*, *4*, 1–24.
853 Wickham, H. (2009). ggplot2: elegant graphics for data analysis. Springer, New York, NY.
854 Wilcox, T.M., McKelvey, K.S., Young, M.K., Jane, S.F., Lowe, W.H., Whiteley, A.R.,
855 Schwartz, M.K. (2013). Robust detection of rare species using environmental DNA: the
856 importance of primer specificity. *PLoS ONE*, *8*, e59520.
857 https://doi.org/10.1371/journal.pone.0059520
858 Wilcox, T.M., McKelvey, K.S., Young, M.K., Sepulveda, A.J., Shepard, B.B., Jane, S.F., …
859 Schwartz, M.K. (2016). Understanding environmental DNA detection probabilities: A case
860 study using a stream-dwelling char *Salvelinus fontinalis*. *Biological Conservation*, *194*,
861 209–216. http://dx.doi.org/10.1016/j.biocon.2015.12.023
862 Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T.L. (2012). Primer-
863 BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC*
864 *Bioinformatics*, *13*, 1-11. https://doi.org/10.1186/1471-2105-13-134
865 Zuur, A.F., Ieno, E.N., Walker, N., Saveliev, A.A., & Smith, G.M. (2009). Mixed effects models
866 and extensions in ecology with R. *Springer, New York*. https://doi.org/10.1007/978-0-387-
867 87458-6
868

869 **Table 1**. Bayesian estimates of crucian carp eDNA occurrence probability at a pond (ψ), eDNA detection
870 probability in a water sample (θ), and eDNA detection probability in a qPCR replicate (p). Posterior median and
871 95% credible interval (CI) are given for each parameter of the occupancy model. Ponds were all located in Norfolk,
872 eastern England.

873

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	0.73	0.19 - 0.99	0.83	0.70 - 0.92	0.17	0.06 - 0.34
CHIP	Y	60.50	0.43	0.23 - 0.65	0.83	0.70 - 0.92	0.16	0.05 - 0.40
GUES1	Y	121.75	0.34	0.11 - 0.67	0.83	0.70 - 0.92	0.99	0.90 - 1.00
MYST	Y	6.17	0.68	0.22 - 0.98	0.83	0.70 - 0.92	0.93	0.85 - 0.97
OTOM	Y	14.67	0.37	0.15 - 0.65	0.83	0.70 - 0.92	0.96	0.90 - 0.99
POFA4	Y	13.67	0.43	0.23 - 0.65	0.83	0.70 - 0.92	0.89	0.81 - 0.95
POHI	Y	7.25	0.52	0.29 - 0.76	0.83	0.70 - 0.92	0.42	0.26 - 0.59
RAIL	Y	58.17	0.37	0.15 - 0.65	0.83	0.70 - 0.92	1.00	0.99 - 1.00
SKEY1	Y	31.38	0.37	0.15 - 0.65	0.83	0.70 - 0.92	1.00	1.00 - 1.00
WADD3	Y	126.00	0.58	0.28 - 0.86	0.83	0.70 - 0.92	1.00	1.00 - 1.00
LDUN2	N	0	0.46	0.26 - 0.67	0.83	0.70 - 0.92	0.86	0.74 - 0.94
LDUN3	N	0	0.49	0.28 - 0.71	0.83	0.70 - 0.92	0.15	0.05 - 0.33
PYES2	N	0	0.46	0.26 - 0.67	0.83	0.70 - 0.92	0.89	0.78 - 0.96
SABA	N	0	0.37	0.15 - 0.65	0.83	0.70 - 0.92	0.97	0.91 - 0.99
VALE	N	0	0.55	0.29 - 0.82	0.83	0.70 - 0.92	1.00	1.00 - 1.00
WADD10	N	0	0.40	0.19 - 0.65	0.83	0.70 - 0.92	0.31	0.15 - 0.50
WADD11	N	0	0.37	0.15 - 0.65	0.83	0.70 - 0.92	0.15	0.04 - 0.32
WADD17	N	0	0.46	0.26 - 0.68	0.83	0.70 - 0.92	0.95	0.88 - 0.99
WOOD	N	0	0.55	0.29 - 0.81	0.83	0.70 - 0.92	0.91	0.80 - 0.96
WRONG	N	0	0.34	0.11 - 0.67	0.83	0.70 - 0.92	0.93	0.83 - 0.98

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876 **Table 2**. Summary of eDNA amplification by PCR and qPCR for all samples from two ponds.

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Sample	PCR amplification (Y/N)	Band strength (copies/µL)	qPCR amplification (Y/N)	DNA copy number (copies/µL)
RAIL1	Y	10-100	Y	78
RAIL2	N	0	N	0
RAIL3	Y	100-1000	Y	306
RAIL4	Y	100-1000	Y	460
RAIL5	Y	10-100	Y	86
MYST1	Y	10-100	Y	11
MYST2	Y	10-100	Y	10
MYST3	Y	10-100	Y	19
MYST4	Y	10-100	Y	15
MYST5	Y	10-100	Y	12

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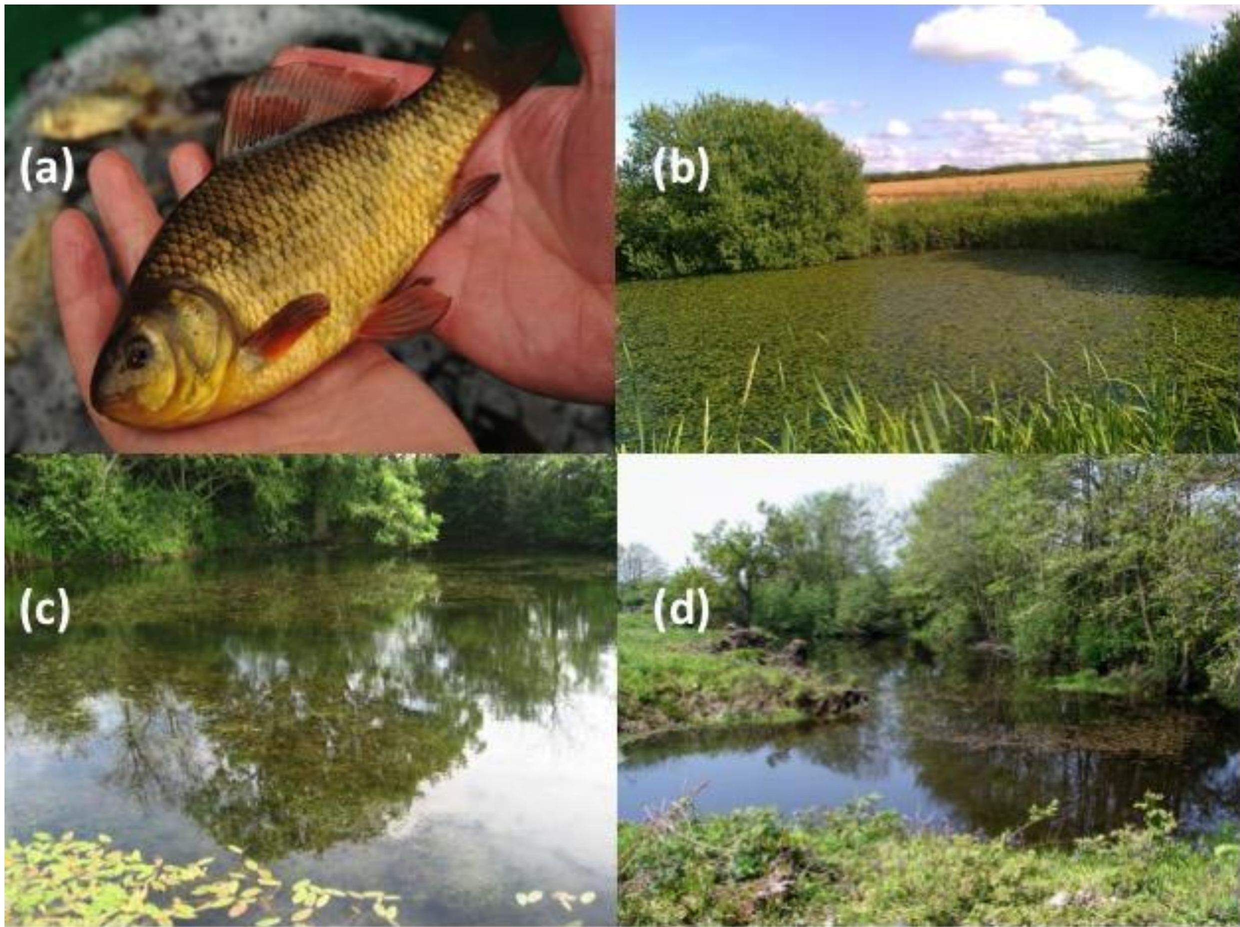

881 **Figure 1.** Crucian carp (*Carassius carassius*) individual **(a)** and examples of ponds inhabited by crucian carp **(b-d)**.

882 Photo **(a)** by John Bailey.

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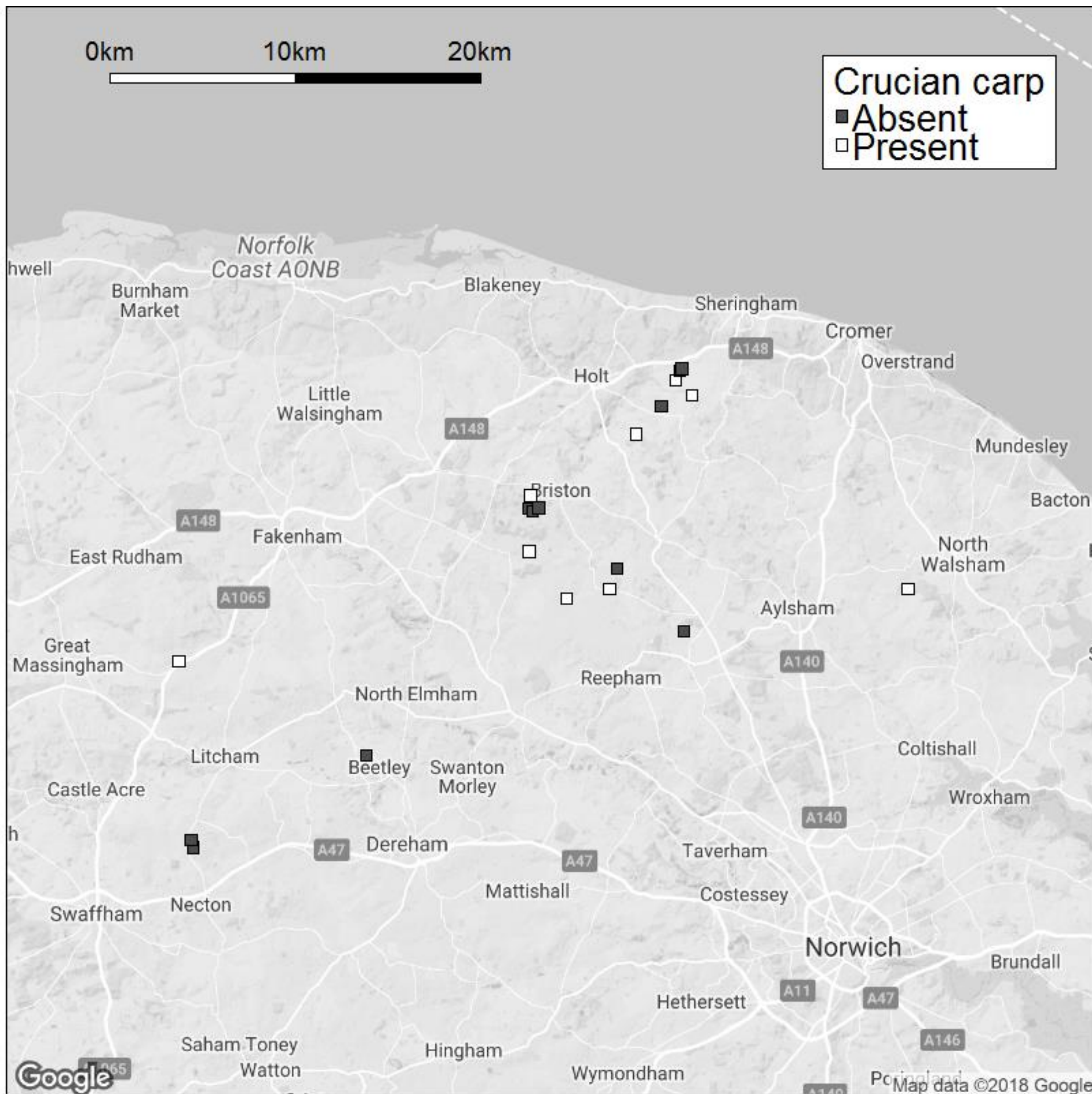

885 **Figure 2.** Google map of North Norfolk, eastern England, showing the distribution of ponds stocked with crucian

886 carp (white squares) and ponds where the species is absent (grey squares).

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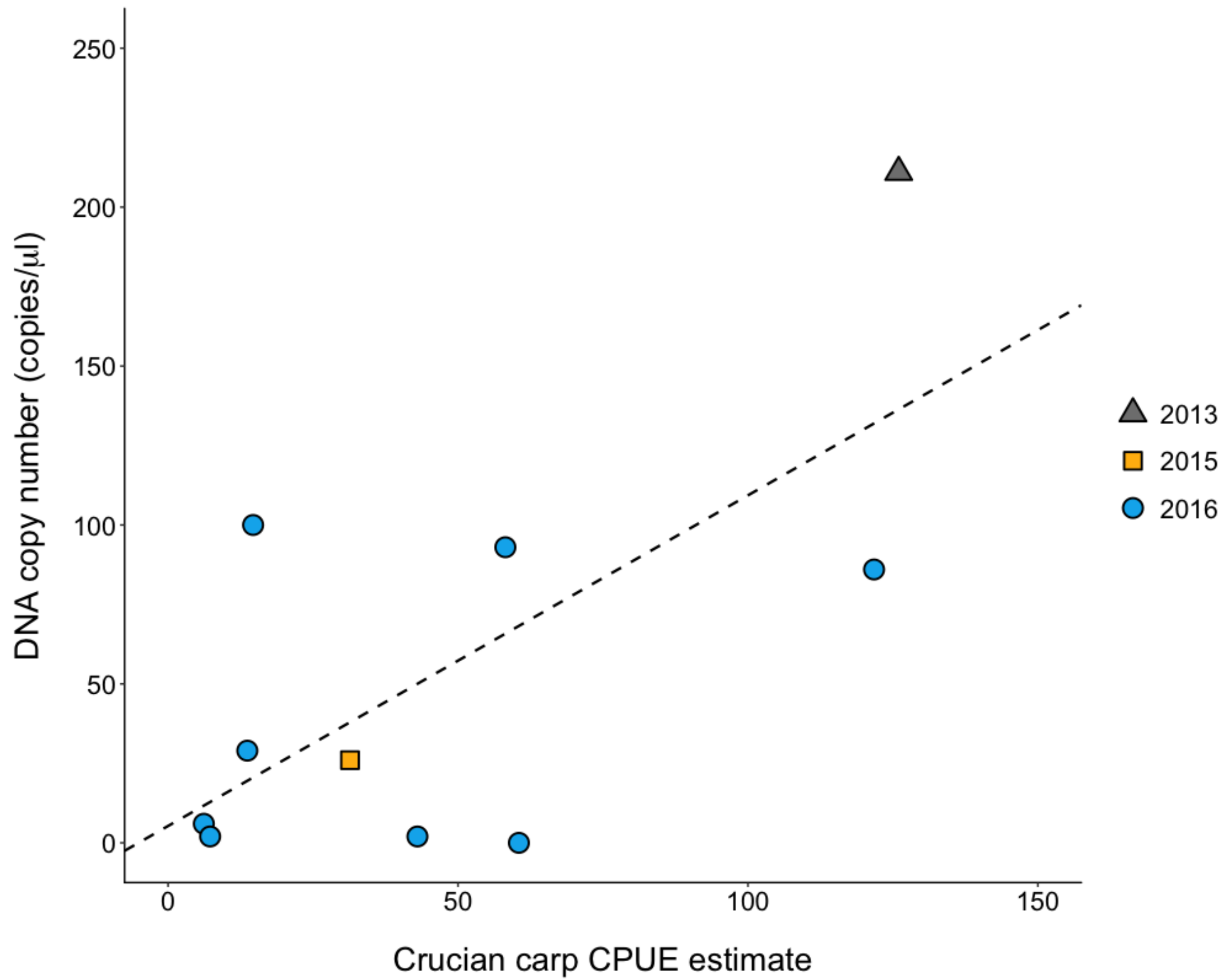

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889 **Figure 3.** Relationship between DNA copy number and CPUE estimate for crucian carp. A broad positive trend was
890 observed but the relationship was variable, where some ponds with high CPUE had low DNA copy number and vice
891 versa. Points are coloured and shaped by the last year that ponds were surveyed using fyke netting. Three ponds fell
892 below the LOQ (10 copies/µL) and one pond did not amplify during qPCR.

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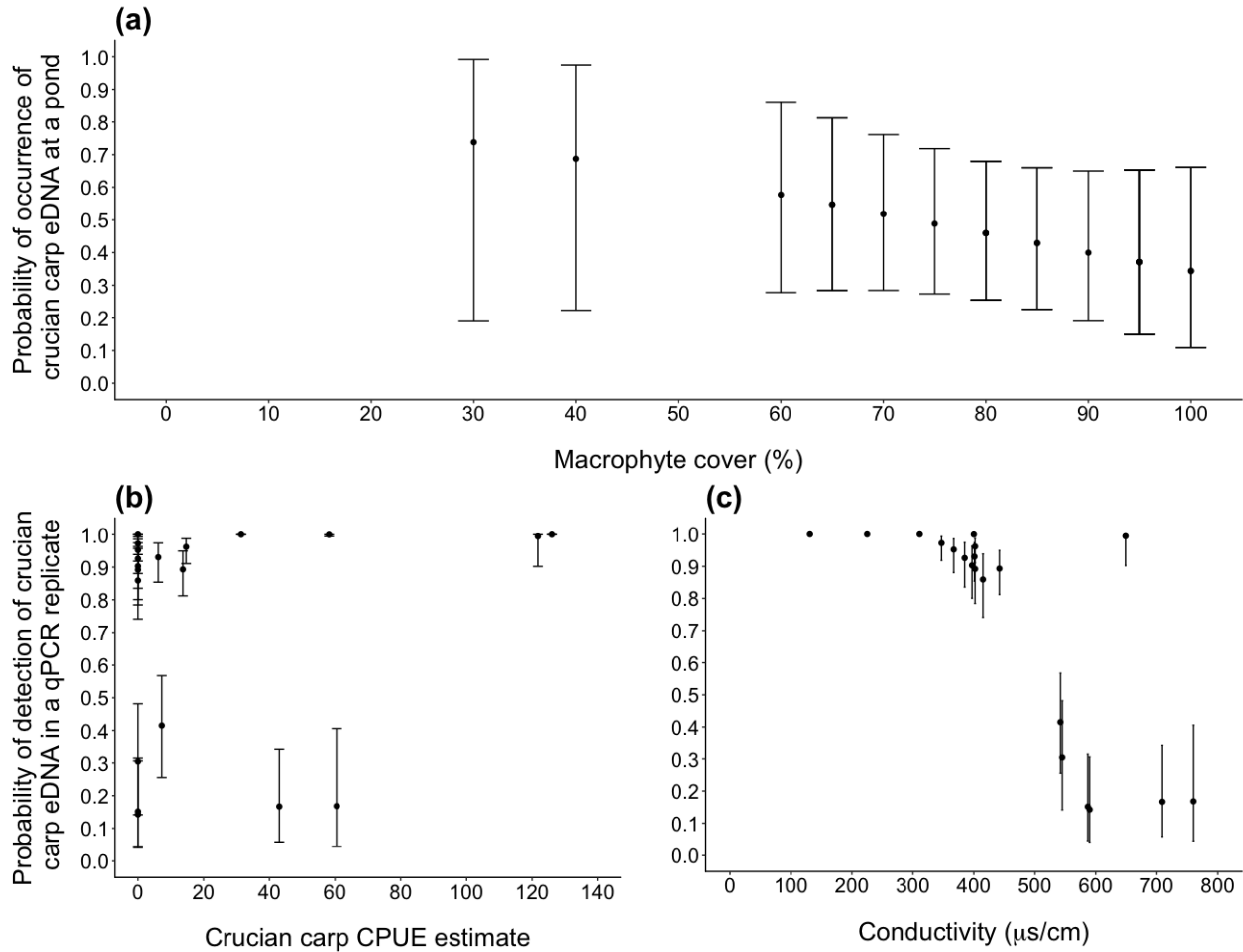

895 **Figure 4.** Estimated probabilities of eDNA occurrence in ponds, and eDNA detection in qPCR replicates. Points are
896 estimates of posterior medians with 95% credible intervals. Probability of eDNA occurrence in ponds decreased as
897 percentage of macrophyte cover increased **(a)**. Probability of eDNA detection in qPCR replicates increased with
898 higher CPUE **(b)** but decreased as conductivity increased **(c)**.

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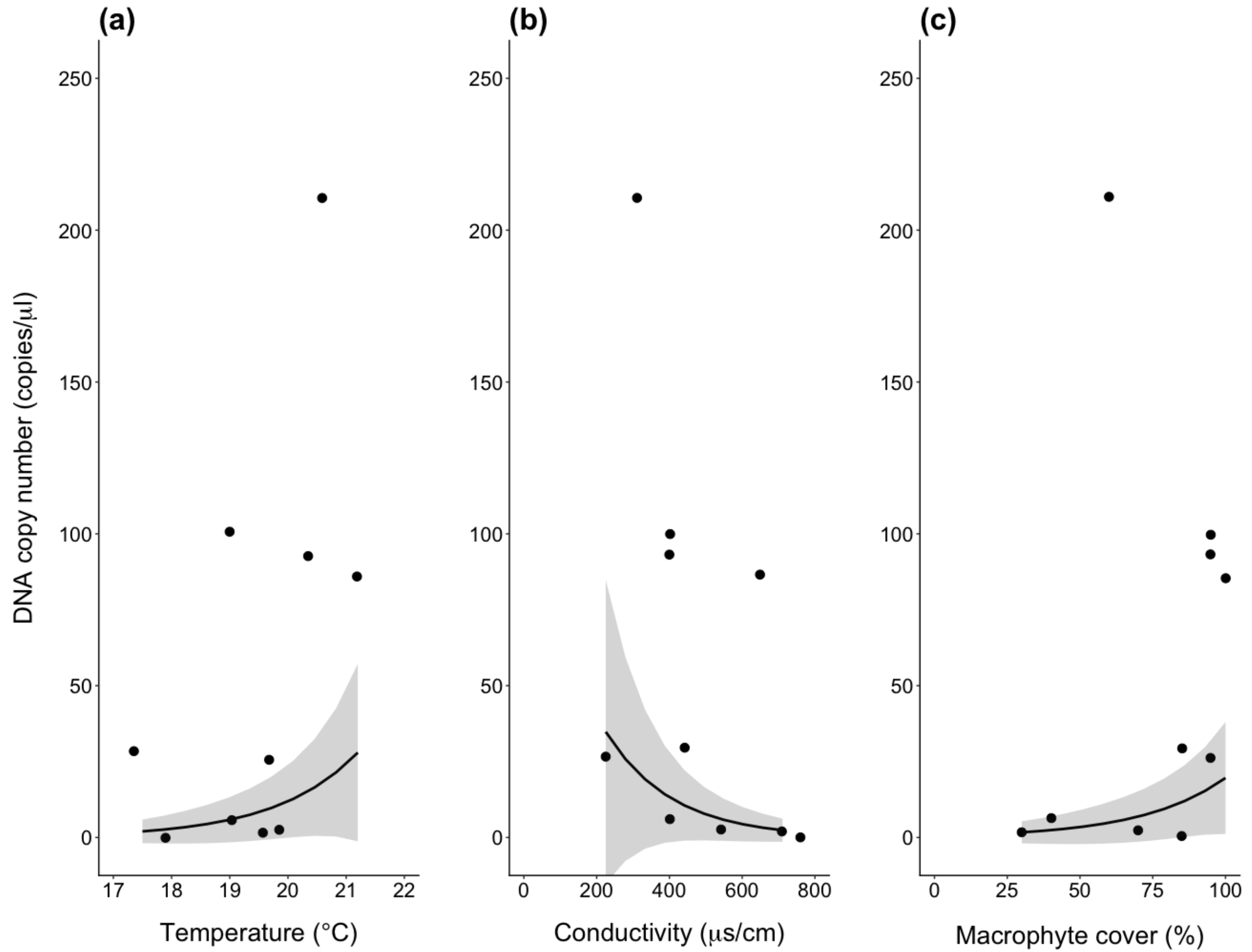

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901 **Figure 5.** Relationship between fixed effects and response variable (DNA copy number) in ponds, as predicted by
902 the Poisson GLMM. The 95% CIs, as calculated using the model predictions and standard error for these
903 predictions, are given for each relationship. The observed data (points) are also displayed against the predicted
904 relationships (line). DNA copy number increased with water temperature **(a)**, but decreased as conductivity **(b)** and
905 percentage of macrophyte cover **(c)** increased.

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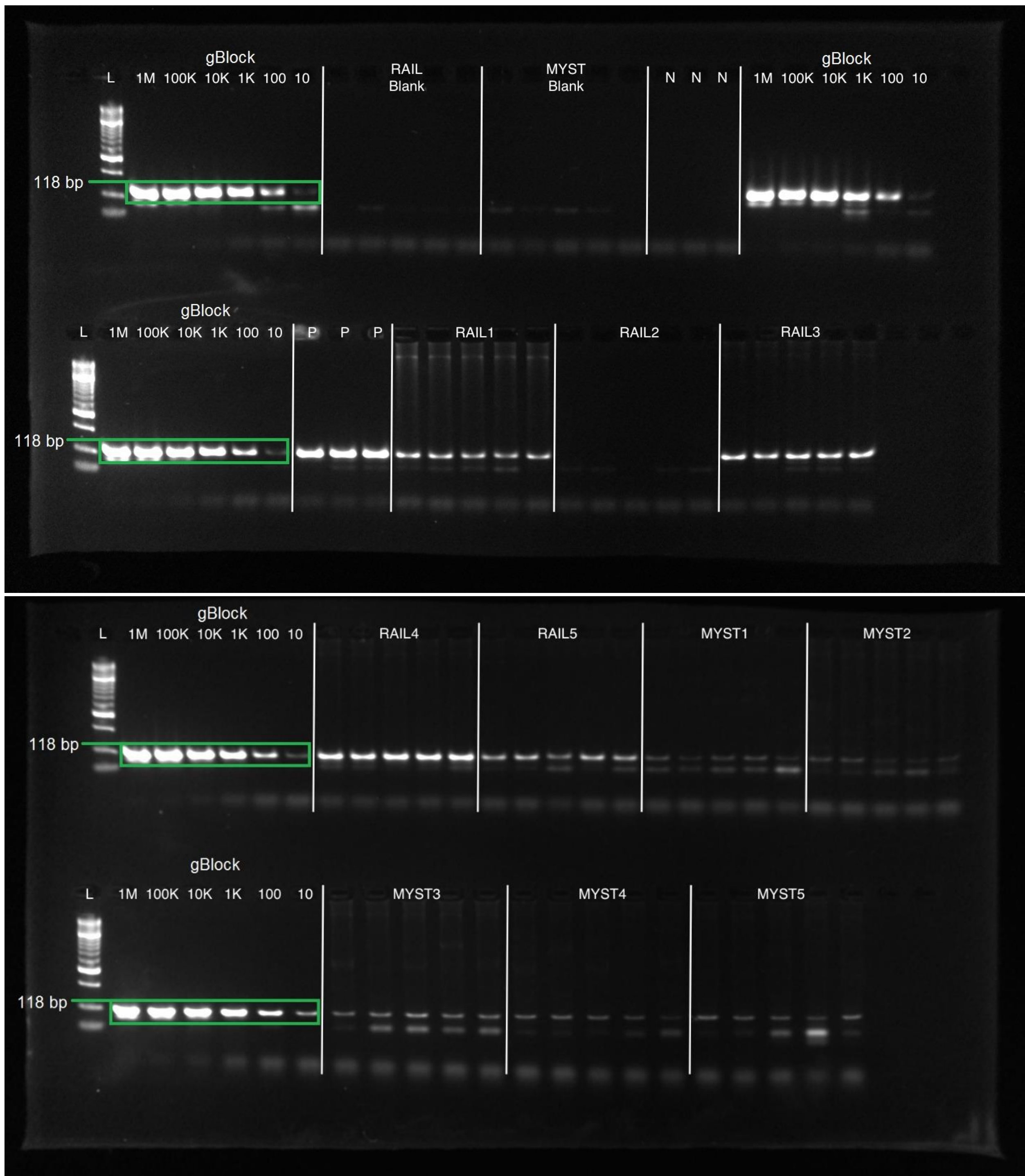

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910 **Figure 6.** PCR products of gBlocks® standards and five eDNA samples from two ponds. Products were run on 2%
911 agarose gels with Hyperladder™ 50bp (Bioline®, London, UK) molecular weight marker (L). Five replicates were
912 performed for each standard curve point and each eDNA sample. Sample ID is given for each set of replicates,
913 confined by white lines. Exemplary bands of expected size (118 bp) are highlighted in green.