Assessing the impact of the threatened crucian carp (*Carassius carassius*) on pond invertebrate diversity - a comparison of conventional and molecular tools

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Running title: Impact assessment using molecular tools

Abstract

Fishes stocked for recreation and angling can damage freshwater habitats and negatively impact biodiversity. The pond-associated crucian carp (*Carassius carassius*) is rare across Europe and stocked for conservation management in England, but impacts on pond biota are understudied. Freshwater invertebrates contribute substantially to aquatic biodiversity, encompassing many rare and endemic species, but small size and high abundance complicates their assessment. Practitioners have employed sweep-netting and kick-net sampling with microscopy, but specimen size/quality and experience can bias identification. DNA and eDNA metabarcoding offer alternative means of invertebrate assessment. We compared invertebrate diversity in ponds (N = 18) with and without crucian carp using sweep-netting and microscopy, DNA metabarcoding, and eDNA metabarcoding. Five 2-L water samples and 4-minute sweep-net samples were collected at each pond. Inventories produced by morphotaxonomic identification of netted samples, DNA metabarcoding of bulk tissue samples, and eDNA metabarcoding of water samples were compared. Alpha diversity was greatest with DNA or eDNA metabarcoding, depending on whether standard or unbiased methods were considered. DNA metabarcoding reflected morphotaxonomic identification, whereas eDNA metabarcoding produced markedly different communities. Therefore, these complementary tools should be combined for comprehensive invertebrate assessment. Crucian carp presence minimally reduced alpha diversity in ponds, but positively influenced beta diversity through taxon turnover (i.e. ponds with crucian carp contained different invertebrates to fishless ponds). The crucian carp appears to be non-impactful, supporting continued conservation management in England. Our results show that molecular tools can enhance freshwater invertebrate assessment and facilitate development of more accurate and ecologically effective pond management strategies.

Key words: community DNA, environmental DNA (eDNA), invertebrates, metabarcoding, morphotaxonomic identification, monitoring

1. Introduction

Freshwater ecosystems comprise <1% of the Earth's surface, but represent major biodiversity hotspots and provide vital ecosystem services (Dudgeon et al., 2006). Ponds in particular provide critical habitat for biodiversity in a fragmented landscape (Céréghino, Biggs, Oertli, & Declerck, 2008), supporting many rare, unique or specialist species not found in other waterbodies (Biggs, von Fumetti, & Kelly-Quinn, 2016; Wood, Greenwood, & Agnew, 2003). These highly diverse and species-rich ecosystems contribute considerably to regional-scale diversity due to high habitat heterogeneity and associated species turnover (Davies et al., 2008; Williams et al., 2003). Aquatic invertebrates are a crucial and abundant component of this diversity, and occupy the vast range of microhabitats made available in ponds by their broad-ranging physicochemical properties and habitat complexity (Davies et al., 2008; Williams et al., 2003; Wood et al., 2003).

In Europe, lakes and ponds are commonly stocked with fish for angling and recreation, despite often having negative effects on invertebrates and amphibians (Gledhill, James, & Davies, 2008; Wood, Greenwood, Barker, & Gunn, 2001). Fish can alter community structure (Schilling, Loftin, & Huryn, 2009a, 2009b; Wood et al., 2001), reduce diversity (Lemmens et al., 2013; Wood et al., 2001), and reduce abundance and biomass (Marklund, Sandsten, Hansson, & Blindow, 2002; Schilling et al., 2009a) of invertebrates. These effects may manifest through direct predation by fish, altered water quality and loss of macrophyte diversity via foraging activity of fish, or management practices associated with angling activity (Lemmens et al., 2013; Maceda-Veiga, López, & Green, 2017; Schilling et al., 2009a; Wood et al., 2001). However, the impact of fish stocking can be negligible or even beneficial to invertebrate diversity, particularly at regional-scale, provided that fish species are carefully selected and managed (Gee, Smith, Lee, & Griffiths, 1997; Hassall, Hollinshead, & Hull, 2011; Lemmens et al., 2013; Stefanoudis et al., 2017).

The crucian carp (*Carassius carassius*) is one of few fishes strongly associated with small ponds in Europe, but has suffered heavy declines and local extinctions in the last century (Copp & Sayer, 2010; Sayer et al., 2011) due to habitat loss, species displacement by the invasive

86 goldfish (*Carassius auratus*) and gibel carp (*Carassius gibelio*) (Copp, Warrington, & Wesley,
87 2008; Sayer et al., 2011), and genetic introgression through hybridisation with goldfish and
88 common carp (*Cyprinus carpio*) (Hänfling, Bolton, Harley, & Carvalho, 2005). In 2010, the
89 crucian carp was designated as a Biodiversity Action Plan (BAP) species in the county of Norfolk,
90 eastern England (Copp & Sayer, 2010). A key objective of this BAP is to increase the number of
91 viable crucian carp populations across Norfolk through pond restoration and species
92 reintroduction. Many Norfolk ponds have since been stocked with crucian carp to realise this
93 objective (Sayer et al., *in review*), but continued stocking is controversial in light of genetic
94 evidence that indicates the crucian carp is not native to the UK (Jeffries et al., 2017).
95 Nonetheless, there is support for UK conservation efforts to continue to protect the genetic
96 integrity of the crucian carp at the European level, and provide a natural stronghold for the
97 species (Harper et al., 2019; Jeffries et al., 2017; Stefanoudis et al., 2017) in the face of
98 persistent declines throughout its native range of Northwest and Central Europe (Copp et al.,
99 2008; Sayer et al., 2011).

100 The impact of stocking crucian carp on lentic biodiversity has been largely ignored, and
101 little is known about interactions between this benthic fish and other pond species. Existing
102 research suggests crucian carp are characteristic of ponds rich in invertebrates with extensive
103 macrophyte cover (Copp et al., 2008; Sayer et al., 2011), and play an important ecological role
104 (along with other pond-associated fishes) by increasing landscape-scale diversity across pond
105 networks (Stefanoudis et al., 2017). Yet to our knowledge, only one study has assessed
106 biodiversity (specifically macrophytes, zooplankton, and water beetles) in ponds with and
107 without pond-associated fishes in the UK (Stefanoudis et al., 2017), and no studies have
108 specifically focused on crucian carp. Consequently, there is a need to survey and compare
109 fishless ponds to ponds stocked with crucian carp to assess the impact of this species on
110 invertebrate diversity more broadly. However, obtaining species resolution data for
111 invertebrates is complicated by the level of taxonomic expertise needed for accurate
112 morphotaxonomic identification as well as the cost and time required to survey pond networks
113 (Briers & Biggs, 2003; Haase, Pauls, Schindehütte, & Sundermann, 2010; Hill et al., 2018).

114 Metabarcoding offers a rapid, high-resolution, cost-effective approach to biodiversity

115 assessment, where entire communities can be identified using High-Throughput Sequencing
116 (HTS) in conjunction with community DNA from bulk tissue samples (DNA metabarcoding), or
117 environmental DNA (eDNA) from environmental samples (eDNA metabarcoding), such as soil or
118 water (Deiner et al., 2017; Taberlet, Coissac, Pompanon, Brochmann, & Willerslev, 2012). DNA
119 metabarcoding of aquatic invertebrate samples has proven relatively successful, with
120 applications in biomonitoring (Andújar et al., 2017; Elbrecht, Vamos, Meissner, Aroviita, &
121 Leese, 2017a; Emilson et al., 2017; Lobo, Shokralla, Costa, Hajibabaei, & Costa, 2017). Use of
122 eDNA metabarcoding for invertebrate assessment in freshwater rivers (Blackman et al., 2017;
123 Carew, Kellar, Pettigrove, & Hoffmann, 2018; Deiner, Fronhofer, Mächler, Walser, & Altermatt,
124 2016; Klymus, Marshall, & Stepien, 2017), streams (Macher et al., 2018), and lakes (Klymus et
125 al., 2017) is also gaining traction, but there are currently no published studies that have used
126 metabarcoding for pond invertebrates.

127 We assessed invertebrate diversity in UK ponds with and without crucian carp using
128 metabarcoding in conjunction with morphotaxonomic identification. The entire taxon
129 inventories generated by each monitoring tool (standard methods) were compared to evaluate
130 which tool provides the most holistic assessment of invertebrate diversity. We also compared
131 the taxon inventories produced by each method when potential biases were removed, i.e. taxa
132 with no reference sequences and microinvertebrates that would not be captured by a 1 mm
133 mesh net (unbiased methods). The effect of crucian carp presence on invertebrate diversity was
134 then determined using the taxon inventories from the standard or unbiased methods
135 individually and combined. We hypothesised that DNA metabarcoding and morphotaxonomic
136 identification would produce congruent invertebrate communities, whereas eDNA
137 metabarcoding would reveal taxa not identified by these tools. DNA and eDNA metabarcoding
138 were expected to enable species resolution data for problematic taxa that cannot be
139 morphotaxonomically identified to species-level using standard keys. We hypothesised that
140 alpha diversity would be lower in ponds with crucian carp, but beta diversity would be
141 enhanced due to heterogeneity induced by crucian carp presence in the pond network. This
142 pattern was expected regardless of the monitoring tool used. We provide recommendations for
143 the application of molecular tools to freshwater invertebrate assessment as well as impact

assessment and conservation management of the non-native crucian carp alongside pond biodiversity.

2. Methods

2.1 Study sites

We surveyed nine ponds containing crucian carp across Norfolk (*n* = 6) and East Yorkshire (*n* = 3), and nine fishless ponds in Norfolk (Fig. S1). The fish status of ponds was determined from prior fyke-net surveys (Sayer et al., 2011; Sayer et al., *in review*). Roach (*Rutilus rutilus*) and rudd (*Scardinius erythrophthalmus*) were the only other fishes that occurred in three of the Norfolk ponds, but at lower density. All study ponds were selected to be similar in terms of morphometry and habitat structure, being <1 ha in area, <5 m in depth, macrophyte-dominated, with a largely open-canopy and thus minimal shading (≤20%) of the water surface. Ponds were mainly surrounded by arable fields, excluding one pond located in woodland. Samples for morphotaxonomic identification and metabarcoding were collected in autumn 2016, a time when invertebrate diversity often peaks (Hill, Sayer, & Wood, 2016). Data on physical (area, depth, estimated percentages of perimeter with emergent vegetation, emergent macrophyte cover, submerged macrophyte cover, and shading) and chemical (conductivity measured with a HACH HQ30d meter) properties of ponds were collected between May and August from 2010 to 2017.

2.2 Sweep-netting and morphotaxonomic identification

Sweep-netting was performed in accordance with the UK National Pond Survey methodology (Biggs, Fox, & Nicolet, 1998), using a 1 mm mesh long-handled net (0.3 m square bag), to generate a conventional taxonomic inventory of lentic macroinvertebrates. Sampling time at each pond comprised a 3 min sweep-net sample and 1 min hand search (see Supporting

173 Information). Samples were deposited in a 1.2 L sterile Whirl-Pak® stand-up bag (Cole-Palmer,
174 London) and stored at -20 ^{o}C until laboratory processing and sorting. Samples were thawed and
175 passed through sieves of 8 mm, 2 mm, and 0.25 mm in succession to remove large items of
176 vegetation and detrital matter. For each sample, invertebrate specimens were sorted into three
177 body size categories on laminated millimetre graph paper: small (<2.5 x 5 mm), medium (2.5 x 5
178 mm up to 5 x 10 mm), and large (>10 mm and up to 10 x 20 mm) (Elbrecht, Peinert, & Leese,
179 2017b). Where possible, size-sorted specimens were identified under a light microscope to
180 species-level using Freshwater Biological Association publications (Bass, 1998; Brooks & Cham,
181 2014; Edington & Hildrew, 1995; Elliott, 2009; Elliott & Humpesch, 2010; Elliott & Dobson,
182 2015; Friday, 1988; Gledhill, Sutcliffe, & Williams, 1993; Macan, 1960; Savage, 1989; Wallace,
183 Wallace, & Philipson, 1990). Terrestrial taxa and empty cases/shells were discarded. The
184 laminated paper was sterilised with 50% v/v chlorine-based commercial bleach solution (Elliot
185 Hygiene Ltd, UK) and 80% v/v ethanol solution between measuring specimens from different
186 ponds to minimise cross-contamination risk. Specimens were preserved in sterile 15 mL falcon
187 tubes (SARSTED, Germany) containing 100% ethanol according to size category and pond
188 sampled, and stored at -20 ^{o}C until DNA extraction.
189
190 **2.3 DNA samples**
191
192 We followed the DNA extraction procedure from Blackman *et al.* (2017). Size categories from
193 each pond were dried overnight on FisherBrand cellulose filter paper (Fisher Scientific UK Ltd,
194 UK) in sterile glass funnels and conical flasks to remove excess ethanol. Size categories were
195 lysed (3 × 30 sec) using a TissueLyser II (Qiagen®, Germany) with DigiSol (50 mM Tris, 20 M
196 EDTA, 120 mM NaCl and 1% SDS). The adapter sets held 1.5 g of dried tissue and corresponding
197 volume of DigiSol. If the dry tissue weight of any size category exceeded 1.5 g, we processed
198 the size category in batches until all tissue had been lysed. The lysates from all batches were
199 pooled to recreate size categories. The size categories were incubated overnight at 55 °C with
200 SDS and Proteinase K (Bioline®, UK), following which 200 µL of lysate from each size category
201 was used for extraction with the DNeasy Blood & Tissue Kit (Qiagen®, Germany) according to

202 the manufacturer's protocol. Consequently, 16 bulk tissue samples were each represented by
203 three DNA extracts and two bulk tissue samples each represented by two DNA extracts that
204 were stored at -20 °C until metabarcoding.
205
206 **2.4 eDNA samples**
207
208 We repurposed eDNA samples from Norfolk ponds that were collected by Harper et al. (2019)
209 to validate a quantitative PCR (qPCR) assay for crucian carp. Additional samples were taken
210 from East Yorkshire ponds for the present study. Details of water sample collection, filtration,
211 and DNA extraction are provided in the Supporting Information. Briefly, five 2 L surface water
212 samples were collected at equidistant points around the pond perimeter where access
213 permitted. For each pond, a full process blank (1 L molecular grade water) was taken into the
214 field, transported, filtered, and extracted alongside samples to identify contamination. Filters
215 were stored at -20 °C until DNA extraction with the PowerWater® DNA Isolation Kit (MO BIO
216 Laboratories, CA, USA) following the manufacturer's protocol. eDNA extracts were stored at -20
217 °C until metabarcoding.
218
219 **2.5 Metabarcoding workflow**
220
221 Our metabarcoding workflow is fully described in the Supporting Information. In short, a
222 comprehensive list of UK invertebrate species living in or associated with freshwater habitats
223 was used to create custom, curated reference databases for UK aquatic invertebrates,
224 excluding Diptera (Appendix 1). Species without database representation are listed in Table S1
225 (see also Figs. S2, S3). Published metazoan primers mlCOIintF (Leray et al., 2013) and
226 jgHCO2198 (Geller, Meyer, Parker, & Hawk, 2013), which amplify a 313 bp fragment of the
227 mitochondrial cytochrome c oxidase subunit I (*COI*) gene, were validated *in silico* using ecoPCR
228 software (Ficetola et al., 2010) against the reference databases. Parameters set allowed a 250-
229 350 bp fragment and maximum of three mismatches between each primer and each reference
230 sequence (Appendix 2). Primers were validated *in vitro* against 38 invertebrate species,

representing 38 families and 10 major groups, and compared to published macroinvertebrate metabarcoding primers (Elbrecht & Leese, 2017; Vamos, Elbrecht, & Leese, 2017) (Figs. S4, S5, S6).

PCR conditions were optimised (Figs. S7, S8) before two independent libraries were constructed for DNA and eDNA metabarcoding using a two-step PCR protocol (Appendix 1). During the first PCR (performed in triplicate), the target region was amplified using oligonucleotides comprising the locus primers, sequencing primers, and pre-adapters. DNA from the exotic, terrestrial two-spotted assassin bug (*Platymeris biguttatus*) was used for PCR positive controls (tissue DNA n = 9, eDNA n = 11) as this species is not found in the UK, whilst sterile molecular grade water (Fisher Scientific UK Ltd, UK) substituted template DNA for PCR negative controls (tissue DNA n = 9, eDNA n = 11). PCR products were individually purified using a magnetic bead clean-up (VWR International Ltd, UK), following a double size selection protocol from Bronner *et al*. (2009). The second PCR bound Multiplex Identification (MID) tags to the purified products. PCR products were pooled according to PCR run and the pooled PCR product purified using a magnetic bead clean-up, following a double size selection protocol from Bronner *et al*. (2009). Each purified PCR product was quantified on a Qubit™ 3.0 fluorometer using a Qubit™ dsDNA HS Assay Kit (Invitrogen, UK) and normalised according to concentration and sample number to produce a pooled volume of 20 µL.

The pooled libraries were quantified by qPCR using the NEBNext® Library Quant Kit for Illumina® (New England Biolabs® Inc., MA, USA). An Agilent 2200 TapeStation and High Sensitivity D1000 ScreenTape (Agilent Technologies, CA, USA) were used to verify removal of secondary products from libraries by bead purification and that a fragment of the expected size (531 bp) remained. A total of 52 bulk tissue subsamples sequenced in triplicate (n = 156), 12 extraction blanks, and 18 PCR controls were included alongside samples from other projects (N = 188) in the DNA library. A total of 90 eDNA samples, 18 full process blanks, and 22 PCR controls were included alongside samples from other projects (N = 140) in the eDNA library. The DNA and eDNA libraries were sequenced on an Illumina® MiSeq using 2 x 300 bp V3 chemistry with 10% and 20% PhiX Sequencing Control respectively (Illumina, Inc, CA, USA).

Raw sequences were converted to taxonomic assignments using metaBEAT v0.97.11, a

260 custom pipeline for reproducible analysis of metabarcoding data (https://github.com/HullUni-
261 bioinformatics/metaBEAT). After quality filtering, trimming, merging, chimera detection, and
262 clustering, non-redundant query sequences were compared against our reference databases
263 using BLAST (Zhang, Schwartz, Wagner, & Miller, 2000). Putative taxonomic identity was
264 assigned using a lowest common ancestor (LCA) approach based on the top 10% BLAST matches
265 for any query matching with at least 90% identity to a reference sequence across more than
266 80% of its length. Unassigned sequences were subjected to a separate BLAST against the
267 complete NCBI nucleotide (nt) database at 90% identity to determine the source via LCA as
268 described above. Bioinformatic settings (Appendix 1) were chosen based on comprehensive
269 exploration of the parameter space and comparison of metaBEAT taxonomic assignments to
270 morphotaxonomic inventories. Reference databases in GenBank format and the bioinformatic
271 analysis have been deposited in a dedicated GitHub repository, which has been permanently
272 archived for reproducibility (https://doi.org/10.5281/zenodo.3733127).
273
274 **2.6 Data analysis**
275
276 All analyses were performed in R v.3.4.3 (R Core Team, 2017), with data and R scripts deposited
277 in the GitHub repository. Manipulation of the DNA and eDNA metabarcoding datasets for
278 analysis is described in the Supporting Information. For each method, two presence-absence
279 datasets (species-level and family-level) were generated using the *decostand* function in vegan
280 v2.4-6 (Oksanen et al., 2018) for downstream analyses. This is because potential amplification
281 bias during PCR can prevent reliable abundance or biomass estimation from sequence reads
282 produced by DNA or eDNA metabarcoding (Elbrecht et al., 2017b). The entire species and
283 family inventories produced by each method (hereafter standard methods) were compared.
284 We also compared inventories from each method after removing potential biases: taxa without
285 reference sequences were removed from the morphotaxonomic dataset, and
286 microinvertebrates (i.e. taxa that would escape a 1 mm mesh net) were removed from the
287 metabarcoding datasets (hereafter unbiased methods). The effect of crucian carp on
288 invertebrate diversity was then assessed at species and family-level according to standard

289 methods (main text) and unbiased methods (Supporting Information) individually and
290 combined. Finally, the impact of crucian carp alongside environmental variables on invertebrate
291 diversity was investigated using the combined standard method (main text) and unbiased
292 method (Supporting Information) datasets.

293 We define alpha diversity as taxon richness within individual ponds, and beta diversity
294 as the difference between communities present at each pond whilst accounting for taxon
295 identity (Baselga & Orme, 2012). For each standard or unbiased dataset, the following analyses
296 were performed. Alpha diversity (response variable) was obtained using the *specnumber*
297 function in vegan v2.4-6 and modelled against sampling method (explanatory variable), then
298 crucian carp presence-absence (explanatory variable). Using the combined method datasets,
299 alpha diversity of the major invertebrate groups, i.e. orders, classes, or phyla (Dobson, Pawley,
300 Fletcher, & Powell, 2012), in each pond (response variable), was modelled against crucian carp
301 presence-absence (explanatory variable). All alpha diversity comparisons were made using
302 negative binomial Generalized Linear Models (GLMs), and Pairwise Tukey's HSD tests were used
303 to assess significance of differences. We used betapart v1.5.0 (Baselga & Orme, 2012) to
304 estimate total beta diversity, partitioned by nestedness-resultant and turnover, across sampling
305 methods and ponds with the *beta.multi* function. These three beta diversity components were
306 estimated for inventories produced by each sampling method, and ponds with or without
307 crucian carp using the *beta.pair* function (pairwise beta diversity scores). For each beta diversity
308 component, we compared community heterogeneity in each group (sampling method or
309 crucian carp presence-absence) by calculating homogeneity of multivariate dispersions
310 (MVDISP) using the *betadisper* function from vegan v2.4-6. Variation in MVDISP was statistically
311 tested using an analysis of variance (ANOVA), and pairwise Tukey's HSD tests used to determine
312 if there were significant differences between the groups. The effect of sampling method and
313 crucian carp on each beta diversity component was visualised using Non-metric
314 Multidimensional Scaling (NMDS) ordination plots with the *metaMDS* function, and tested
315 statistically using permutational multivariate analysis of variance (PERMANOVA) with the
316 *adonis* function in vegan v2.4-6. Pre-defined cut-off values were used for effect size, where
317 PERMANOVA results were interpreted as moderate and strong effects if $R^2 > 0.09$ and $R^2 > 0.25$

318 respectively. These values are broadly equivalent to correlation coefficients of r = 0.3 and 0.5
319 which represent moderate and strong effects accordingly (Macher et al., 2018; Nakagawa &
320 Cuthill, 2007). Jaccard dissimilarity was used as a measure of beta diversity for all analyses.
321 We tested whether the species and family-level invertebrate communities produced by
322 the combined standard or unbiased methods were influenced by pond properties in
323 conjunction with crucian carp presence-absence. Redundancy Analysis (RDA) was selected for
324 constrained ordination as it analyses variation in biological communities in response to
325 explanatory variables (Legendre & Legendre, 2012). Principal Coordinate Analysis (PCoA) was
326 performed using the *pcoa* function in ape v5.0 (Paradis & Schliep, 2018) on the turnover,
327 nestedness-resultant, and total beta diversity matrices. The Lingoes correction was employed
328 to account for negative eigenvalues (Legendre, 2014). The resultant PCoA eigenvectors
329 (principle coordinates) for each distance matrix were used as the response variable in variance
330 partitioning. Our variables were grouped as biotic (crucian carp presence-absence) or abiotic
331 (pond conductivity, area, depth, percentages of perimeter with emergent vegetation, emergent
332 macrophyte cover, submerged macrophyte cover, and shading). Abiotic variables were log10
333 transformed to eliminate their physical units (Legendre & Birks, 2012). Significant abiotic
334 variables influencing each component of beta diversity were identified using the *ordiR2step*
335 function in vegan v2.4-6 to perform separate RDA analyses under a forward selection
336 procedure. Where applicable, the relative contributions of the biotic and abiotic variables on
337 turnover, nestedness-resultant, and total beta diversity for the species and family-level
338 invertebrate communities were assessed by variance partitioning (Borcard, Legendre, &
339 Drapeau, 1992) using the *varpart* function from vegan v2.4-6. For each beta diversity
340 component, RDA was performed using biotic and significant abiotic variables, and variance
341 partitioning used to divide the total percentage of variation explained into unique and shared
342 contributions for biotic and abiotic predictor groups. The *anova* function in vegan v2.4-6 was
343 used to examine the statistical significance of the full model and the unique contributions of
344 each predictor group. We report the adjusted R^2-fractions as they are widely recommended
345 and unbiased (Peres-Neto, Legendre, Dray, & Borcard, 2006).
346

3. Results

3.1 Comparison of methods for freshwater invertebrate assessment

Summaries of taxon detection by standard and unbiased methods are reported in the Supporting Information (Appendix 2; Tables S2, S3, S4, S5; Figs. S9, S10). Briefly, 1,404 specimens belonging to 91 species and 2,281 specimens belonging 38 families were identified by microscopy. With DNA metabarcoding, 2,454,206 and 2,907,021 sequence reads were assigned to 140 species and 56 families. With eDNA metabarcoding, 389,766 and 831,073 sequence reads were assigned to 160 species and 92 families respectively. Only 17 species (Figs. 1ai, bi) and 21 families (Figs. 1aii, bii) were shared by all three methods (standard or unbiased). eDNA metabarcoding detected the most unique species and families, whereas DNA metabarcoding and morphotaxonomic identification were more comparable (Fig. 1). The number of taxa that morphotaxonomic identification shared with DNA and eDNA metabarcoding was unchanged using standard or unbiased methods. Morphotaxonomic identification shared one family with eDNA metabarcoding, but 36 species and 13 families with DNA metabarcoding (Fig. 1). Morphotaxonomic identification detected 19 species without reference sequences, whereas DNA and eDNA metabarcoding detected 4 and 57 microinvertebrate species respectively (Fig. 1a). The metabarcoding approaches either shared 49 species and 18 families (standard methods, Fig. 1a) or 29 species and 7 families (unbiased methods, Fig. 1b).

Standard sampling method influenced alpha diversity of ponds at species (GLM χ^2_2 = 36.999, $P < 0.001$) and family-level (GLM χ^2_2 = 54.800, $P < 0.001$). Significant differences between the alpha diversity means for morphotaxonomic identification and DNA metabarcoding (species-level -0.452 ± 0.131, Z = -3.446, P = 0.002; family-level -0.295 ± 0.109, Z = -2.719, P = 0.018), morphotaxonomic identification and eDNA metabarcoding (species-level -0.779 ± 0.128, Z = -6.069, $P < 0.001$; family-level -0.729 ± 0.102, Z = -7.122, $P < 0.001$), and DNA and eDNA metabarcoding (species-level 0.327 ± 0.123, Z = 2.671, P = 0.021; family-level 0.434 ±

376 0.096, Z = 4.519, P < 0.001) were observed. Conversely, unbiased sampling method influenced
377 alpha diversity of ponds at species-level (GLM χ^2_2 = 20.767, P < 0.001), but not family-level
378 (GLM χ^2_2 = 3.832, P = 0.147). Significant differences between the species-level alpha diversity
379 means of morphotaxonomic identification and DNA metabarcoding (species-level -0.443 ±
380 0.126, Z = -3.506, P = 0.001; family-level -0.134 ± 0.106, Z = -1.267, P = 0.414),
381 morphotaxonomic identification and eDNA metabarcoding (species-level 0.086 ± 0.134, Z =
382 0.639, P = 0.798; family-level 0.072 ± 0.110, Z = 0.650, P = 0.792), and DNA and eDNA
383 metabarcoding (species-level -0.529 ± 0.128, Z = -4.132, P < 0.001; family-level -0.206 ± 0.108, Z
384 = -1.915, P = 0.134) were observed. With standard methods, species and family-level alpha
385 diversity was highest using eDNA metabarcoding, followed by DNA metabarcoding then
386 morphotaxonomic identification (Figs. 2ai, aii). With unbiased methods, DNA metabarcoding
387 detected more species than morphotaxonomic identification and eDNA metabarcoding, but
388 alpha diversity was comparable at family-level (Figs. 2bi, bii). Regarding beta diversity, MVDISP
389 only significantly differed between unbiased methods for family-level turnover and total beta
390 diversity (Table S6). Both standard and unbiased sampling method had moderate and strong
391 positive effects on turnover and total beta diversity at species (Figs. 3ai, aiii, ci, ciii) and family-
392 level (Figs. 3aii, aiv, cii, civ) respectively, but not nestedness-resultant (Table S7).
393
394 **3.2 Impact of crucian carp stocking on pond invertebrates**
395
396 Here, we report the impact of crucian carp stocking assessed using standard methods as these
397 provide the most comprehensive evaluation of invertebrate diversity. Crucian carp impact
398 assessment using unbiased methods is reported in the Supporting Information (Appendix 2;
399 Tables S9, S10, S11, S12; Figs. S13, S14, S15, S16). Independently and combined, standard
400 methods showed alpha diversity of invertebrates to be marginally reduced in ponds containing
401 crucian carp at species (Figs. 4ai-iv) and family-level (Figs. 4bi-iv), but not significantly so (P >
402 0.05, Table 1). Detailed examination of alpha diversity within the major invertebrate groups
403 identified by all three methods combined revealed that Coleoptera and Mollusca diversity was
404 significantly reduced in ponds with crucian carp at species-level (GLM χ^2_{24} = 30.029, P = 0.184;

405 Coleoptera -0.517 ± 0.255, Z = -2.025, P = 0.043; Mollusca -0.815 ± 0.268, Z = -3.046, P = 0.002),
406 but not family-level (GLM χ^2_{24} = 1.503, P = 0.220; Coleoptera -0.511 ± -0.298, Z = -1.713, P =
407 0.087; Mollusca -0.442 ± -0.302, Z = -1.462, P = 0.144). However, differences in alpha diversity
408 between ponds with or without crucian carp were not significant for other invertebrate groups
409 at either taxonomic rank (Fig. S11). Alpha diversity relationships held true for the unbiased
410 methods (Table S9; Fig. S13, S14).

411 Total beta diversity of ponds was consistently high at species and family-level for
412 independent and combined methods (standard or unbiased). Variation in invertebrate
413 community composition was driven by turnover rather than nestedness-resultant (Tables 2,
414 S10). MVDISP differed between ponds for species-level nestedness-resultant with
415 morphotaxonomic identification, species-level total beta diversity with eDNA metabarcoding,
416 and species and family-level total beta diversity with all methods combined. Bar nestedness-
417 resultant with morphotaxonomic identification, fishless ponds had significantly lower
418 dispersion than ponds with crucian carp (Table S8). MVDISP patterns were not consistent across
419 standard and unbiased methods (Table S11). At species-level, morphotaxonomic identification,
420 eDNA metabarcoding, and all methods combined revealed a weak or moderate positive
421 influence of crucian carp presence on turnover (Table 3; Figs. 5ai, iii, iv) and total beta diversity
422 (Table 3; Figs. 5ci, iii, iv) between ponds, but not nestedness-resultant (Table 3; Figs. 5bi, iii, iv).
423 At family-level, only eDNA metabarcoding revealed a weak or moderate positive influence of
424 crucian carp presence on turnover (Table 3; Fig. S12aiii) and total beta diversity (Table 3; Fig.
425 S12ciii) between ponds, but not nestedness-resultant (Table 3; Fig. S12biii). Crucian carp
426 presence did not influence any beta diversity component with DNA metabarcoding (Table 3;
427 Figs. 5aii, bii, cii). Less insights on beta diversity were obtained using unbiased methods (Table
428 S12; Figs. S15, S16) than standard methods.

429 Additional analyses undertaken on the combined method dataset supported an effect of
430 crucian carp presence and excluded the influence of abiotic variables on invertebrate diversity.
431 At species-level, only pond area was identified as a significant abiotic variable for turnover and
432 total beta diversity by forward selection. Variance partitioning analysis was undertaken for
433 turnover and total beta diversity using crucian carp presence-absence and pond area. Using the

434 adjusted R^2 values, biotic and abiotic variables explained 6.30% and 4.33% of the total variation
435 in species turnover and total beta diversity respectively (Fig. 6). Crucian carp presence-absence
436 significantly contributed to turnover (Fig. 6a; adjusted R^2 = 5.15%, F_1 = 1.947, P = 0.001) and
437 total beta diversity (Fig. 6b; adjusted R^2 = 3.79%, F_1 = 1.679, P = 0.001), whereas pond area
438 explained less variance and did not influence turnover (Fig. 6a; adjusted R^2 = 2.57%, F_1 = 1.197,
439 P = 0.170) or total beta diversity (Fig. 6b; adjusted R^2 = 1.72%, F_1 = 1.090, P = 0.276). RDA of
440 nestedness-resultant without abiotic data indicated no influence of crucian carp presence-
441 absence (F_1 = 0.364, P = 0.844). At family-level, forward selection did not identify any significant
442 abiotic variables for turnover, nestedness-resultant, or total beta diversity. Therefore, variance
443 partitioning was not undertaken for any component of beta diversity. RDA of each beta
444 diversity component minus abiotic data revealed crucian carp presence-absence influenced
445 total beta diversity (F_1 = 1.430, P = 0.047), but not turnover (F_1 = 1.410, P = 0.111) or
446 nestedness-resultant (F_1 = 1.793, P = 0.178). Variance partitioning results using the unbiased
447 methods are presented in Appendix 2.
448
449
450 ## 4. Discussion
451
452 ### 4.1 Comparison of methods for freshwater invertebrate assessment
453
454 DNA (unbiased methods) or eDNA (standard methods) metabarcoding generated the highest
455 species and family-level alpha diversity. Differences in alpha diversity were more pronounced
456 between standard methods, but unique species were detected nonetheless. Morphotaxonomic
457 identification and DNA metabarcoding produced more similar communities, and performed
458 best for Coleoptera, Hemiptera, Hirudinea, Megaloptera, and Odonata. eDNA metabarcoding
459 generated a markedly different community, and detected aquatic taxa overlooked in pond net
460 samples (Arachnida, Crustacea, Lepidoptera, Psocoptera, Trichoptera) alongside singleton
461 terrestrial taxa (Hymenoptera, Thysanoptera). Both metabarcoding approaches performed well
462 for Annelida, Collembola, Diptera, and Ephemeroptera, particularly at species-level.

463 Critically, we used a 1 mm mesh net which is designed to sample macroinvertebrates,
464 whereas a 500 µm mesh net would also sample the microinvertebrates detected by
465 metabarcoding. Microinvertebrates may inflate metabarcoding diversity estimates against
466 morphotaxonomic identification, but may also prevent PCR amplification of
467 macroinvertebrates. To mitigate bias, we performed all analyses excluding microinvertebrates
468 not captured by netting and excluding morphotaxonomically identified taxa without reference
469 sequences that metabarcoding would not recover. Alpha diversity results from the standard
470 and unbiased method comparisons were variable, but beta diversity results were more
471 consistent. Both method comparisons echo other studies where metabarcoding captured
472 greater diversity than morphotaxonomic approaches and resolved problematic groups (e.g.
473 Diptera) for species-level morphotaxonomic identification (Andújar et al., 2017; Carew et al.,
474 2018; Clarke, Beard, Swadling, & Deagle, 2017; Elbrecht et al., 2017a; Emilson et al., 2017;
475 Klymus et al., 2017; Lobo et al., 2017). Standard and unbiased methods broadly revealed the
476 same ecological relationships, although standard methods may yield more insights. All three
477 methods will be most powerful when used in combination for ecological investigation.
478 Each method has inherent biases that influence performance for freshwater
479 invertebrate assessment. The UK National Pond Survey methodology recommends that pond
480 net samples are placed into buckets/zip-lock bags, followed by sorting and identification in the
481 laboratory as soon as possible. Samples are typically preserved in ethanol or refrigerated to
482 prolong processing time (Biggs et al., 1998), but this standard methodology does not lend itself
483 to molecular applications. After collection, samples were transported on ice then frozen at -20
484 °C to prevent predation within samples, minimise organismal decay and subsequent DNA
485 degradation, and allow samples to be processed as required in the laboratory. Using this
486 strategy, small or inconspicuous dead specimens would not have been recovered during sorting
487 (Biggs et al., 1998) and therefore excluded from morphotaxonomic and DNA metabarcoding
488 inventories. These losses are surplus to the 29% of specimens already overlooked during sorting
489 due to smaller body size (Haase et al., 2010). Sorting may also damage or completely destroy
490 some recovered specimens, influencing morphotaxonomic identification (Lobo et al., 2017;
491 Zizka, Leese, Peinert, & Geiger, 2018).

492 Human error is another source of discrepancy between morphotaxonomic identification
493 and metabarcoding. Some taxa were identified to genus or family-level and omitted from our
494 species-level inventories, whereas other taxa may have been falsely identified and inventoried
495 (Carew, Pettigrove, Metzeling, & Hoffmann, 2013; Elbrecht et al., 2017a; Haase et al., 2010).
496 Losses incurred by the preservation and sorting treatments for morphotaxonomic identification
497 cannot be mitigated, and human error can only be reduced with taxonomic expertise.
498 Conversely, taxon recovery by DNA metabarcoding can be improved with protocols that
499 preserve and extract DNA from intact, unsorted samples (Elbrecht et al., 2017a). For example,
500 ethanol used to preserve specimens (Zizka et al., 2018), or replacement of ethanol with DNA
501 extraction buffer followed by incubation then specimen removal (Carew, Coleman, &
502 Hoffmann, 2018), offer alternative starting materials to bulk tissue for DNA metabarcoding.
503 However, these alternative DNA sources tend to produce false negatives for schlerotised
504 groups, such as Coleoptera and Trichoptera (Carew et al., 2018; Zizka et al., 2018).
505 Despite successful application of DNA metabarcoding to bulk tissue samples for
506 biomonitoring (Andújar et al., 2017; Carew et al., 2018; Elbrecht et al., 2017a; Emilson et al.,
507 2017; Macher et al., 2018), recurring issues have been encountered. Arguably, the most
508 pressing issue is size bias, where DNA from large and/or high biomass taxa can outcompete
509 DNA of smaller and/or low biomass taxa during PCR amplification and sequencing (Elbrecht et
510 al., 2017b). Although we used the size-sorting approach of Elbrecht *et al*. (2017b) and
511 sequenced body size categories independently with data pooling downstream, DNA
512 metabarcoding failed to detect some taxa that have reference sequences and were reliably
513 identified by microscopy, including several Coleopterans (*Agabus sturmii*, *Hydroporus*
514 *erythrocephalu*s, *Rhantus frontalis*, *Haliplus confinis*, *Haliplus ruficollis*), a small Mollusc
515 (*Gyraulus crista*), a medium Hirudinean (*Erpobdella lineata*), and two large Anisopterans
516 (*Aeshna mixta*, *Anax imperator*). Non-delivery of desired results is problematic as size-sorting is
517 time-consuming, labour-intensive, potentially increases cross-contamination risk between
518 samples (Elbrecht et al., 2017b), and still requires samples to be sorted from vegetation and
519 substrate (Elbrecht et al., 2017a). Size-sorting may therefore be a drain on resources and time
520 allocated to DNA metabarcoding projects, but other sources of false negatives must be

521 excluded.

522 In contrast, eDNA metabarcoding preferentially amplifies DNA from small, floating
523 organisms (Deiner et al., 2016; Macher et al., 2018), and these organisms are often retained on
524 filter membranes used for eDNA capture (*pers. obs.*). Consequently, their DNA is abundant in
525 samples and overwhelms DNA from other taxa during sequencing. Pre-filtering with or only
526 using large pore size filter membranes may reduce this bias (Macher et al., 2018). eDNA
527 metabarcoding may also detect different taxa due to species variability in eDNA production and
528 shedding rates. The species infrequently detected by eDNA metabarcoding were those that
529 possess thicker exoskeletons composed of chitin and occasionally calcium carbonate, e.g.
530 Coleoptera, Hemiptera. Exoskeletons may restrict the release of DNA into the water column
531 (Tréguier et al., 2014) as opposed to organisms that are filter-feeders or produce slime (e.g.
532 Crustacea, Mollusca), ectoparasites (i.e. Acari) that feed on blood or skin of other species, or
533 use external versus internal fertilisation (Wacker et al., 2019). Furthermore, species may differ
534 in their habitat preferences and utilisation, potentially resulting in highly localised distributions
535 (Klymus et al., 2017). Therefore, more samples or greater volumes may be required to improve
536 detection of pond biota (Harper et al., 2019).

537 Marker choice and primer design can substantially influence amplification success and
538 taxonomic assignment. Although the *COI* region offers species resolution, has extensive
539 database representation, and is used as standard in DNA barcoding (Elbrecht, Hebert, &
540 Steinke, 2018), it lacks conserved primer-binding sites as a protein-coding gene (Clarke et al.,
541 2017). This introduces high risk of primer mismatch and subsequent bias during metabarcoding
542 (Clarke et al., 2017; Elbrecht et al., 2018), where preferential DNA amplification for some taxa
543 may prevent recovery of all taxa present (Elbrecht et al., 2016; Lobo et al., 2017). Consequently,
544 most metabarcoding primers designed to target the *COI* region in metazoans (Elbrecht & Leese,
545 2017; Leray et al., 2013; Meusnier et al., 2008; Zeale, Butlin, Barker, Lees, & Jones, 2011) are
546 degenerate to allow primers to bind at highly variable sites. However, highly degenerate
547 primers may bind non-target regions (Elbrecht et al., 2018) and experience biased amplification
548 toward non-metazoans, e.g. bacteria, algae, fungi (Brandon-Mong et al., 2015; Macher et al.,
549 2018). This bias can occur even when primers are designed for macroinvertebrates (Elbrecht &

550 Leese, 2017a; Elbrecht et al., 2017b), and is more pronounced in sequences obtained from
551 eDNA samples (Macher et al., 2018). Invertebrate metabarcoding may benefit from the use of
552 different (e.g. 16S ribosomal RNA) or multiple markers (Deiner et al., 2016; Elbrecht et al.,
553 2016). Unfortunately, alternative markers often provide less taxonomic resolution due to lack
554 of reference database representation (Clarke et al., 2017; Elbrecht et al., 2016). Use of multiple
555 markers will also increase PCR and sequencing costs, preventing metabarcoding uptake by
556 freshwater monitoring schemes, but these are expected to subside (Elbrecht et al., 2017a).
557 We obtained an excessive number (~60%) of unassigned reads from eDNA
558 metabarcoding, despite using a relaxed BLAST identity (90%) against custom and public
559 reference databases. This suggests that sequences were of poor quality, could not be assigned
560 to reference sequences, or lacked reference database representation (Macher et al., 2018).
561 Elbrecht *et al*. (2018) demonstrated that degenerate primers like mlCOIintF can experience
562 primer slippage and produce variable length sequences when they bind to low diversity DNA
563 regions. This differs across species with implications for bioinformatic processing and
564 taxonomic assignment. Public reference database records may be few, mislabelled, or have
565 limited geographic coverage (Curry, Gibson, Shokralla, Hajibabaei, & Baird, 2018; Elbrecht et al.,
566 2017a; Klymus et al., 2017). We morphotaxonomically identified 19 species (Hemiptera,
567 Mollusca, Odonata) without reference sequences whose DNA may have been sequenced, but
568 would not have been taxonomically assigned to species-level. Future studies must develop
569 more specific primers that have binding and flanking regions of high nucleotide diversity
570 (Elbrecht et al., 2018) and/or target particular invertebrate orders or families (Klymus et al.,
571 2017) as well as procure reference sequences for different markers (Curry et al., 2018; Elbrecht
572 et al., 2016). These are essential steps to improve the reliability and accuracy of molecular
573 monitoring in freshwater ecosystems.
574
575 **4.2 Impact of crucian carp stocking on pond invertebrates**
576
577 Crucian carp had a negligible influence on alpha diversity and a positive influence on beta
578 diversity of pond invertebrates. Alpha diversity in ponds with crucian carp was marginally

579 reduced compared to alpha diversity in fishless ponds, but this difference was not significant
580 across methods used at either taxonomic rank. Within the major invertebrate groups identified
581 by all methods combined, species-level alpha diversity of Coleoptera and Mollusca was
582 significantly reduced in ponds containing crucian carp as opposed to fishless ponds. This may be
583 because Coleopterans are large, active swimmers that are visible and easy to hunt, and
584 Molluscs are slow-moving prey. Across ponds, total beta diversity of invertebrate communities
585 was driven by turnover (taxon replacement) rather than nestedness-resultant (taxon subsets)
586 (Baselga & Orme, 2012). Our results revealed crucian carp to positively influence turnover and
587 total beta diversity between ponds. Therefore, taxa in fishless ponds were replaced by different
588 taxa in ponds with crucian carp, resulting in dissimilar community composition. Removal of top
589 predators and dominant species, such as Coleopterans, by crucian carp may allow other prey
590 species to colonise but further research is needed.

591 Our results both echo and contradict Stefanoudis *et al*. (2017), where the presence of
592 fish (including crucian carp) in ponds altered macrophyte and cladoceran community
593 composition, but not water beetle composition. Hassall *et al*. (2011) also found that fish
594 presence in ponds had a positive effect on species richness of most invertebrate taxa, but a
595 negative effect on Coleoptera. Gee *et al*. (1997) observed no influence of fish stocking on
596 macrophyte and macroinvertebrate species richness, although Odonata richness was lower and
597 Trichoptera richness higher in stocked ponds. Conversely, other research found that
598 managed/stocked ponds, some of which contained crucian carp, had lower invertebrate
599 diversity than unmanaged sites, which were characterised by Trichoptera, Coleoptera and
600 Zygoptera larvae (Wood et al., 2001). Similarly, large, active and free-swimming taxa
601 (Notonectidae, Corixidae, Gyrinidae, Dytiscidae, Aeshnidae, Libellulidae and Chaoboridae) were
602 strongly associated with fish absence as well as more diverse and abundant in fishless lakes
603 (Bendell & McNicol, 1995; Schilling et al., 2009a, 2009b).

604 Critically, only two of the aforementioned studies accounted for the identity of fish
605 species assemblages present (Stefanoudis et al., 2017; Wood et al., 2001) and just one was
606 based on fish surveys (Stefanoudis et al., 2017), whereas other studies only considered the
607 presence of a particular species (Schilling et al., 2009a) or fish presence-absence generally

608 (Bendell & McNicol, 1995; Gee et al., 1997; Hassall et al., 2011; Schilling et al., 2009b). The
609 contrasting results produced by these studies and our own indicate that the impact of fish on
610 pond biodiversity will likely depend on the species present, population density, and
611 management strategy. Wetland fishes vary in dietary preference and consume different
612 proportions of invertebrate taxa, thus different fishes will suppress numbers of and confer
613 benefits to different invertebrate taxa (Batzer, Pusateri, & Vetter, 2000). Invasive fish species
614 may be more detrimental than non-invasive species, for example, the mosquitofish (*Gambusia*
615 *affinis*) reduced zooplankton abundance and macroinvertebrate density by 90% and 50%
616 respectively after introduction in a wetland ecosystem experiment (Preston et al., 2017).
617 Additionally, duration of stocking was found to substantially reduce invertebrate species
618 richness and abundance (Schilling et al., 2009a). Therefore, local- and regional-scale diversity
619 may benefit most from pond mosaics that maintain fish-free and stocked (at low-moderate
620 densities) ponds to prevent increases in fish biomass and predation pressure over time
621 (Lemmens et al., 2013).

622 The effects of environmental variables on alpha and beta diversity of pond invertebrates
623 must also be considered (Hassall et al., 2011; Hill, Heino, Thornhill, Ryves, & Wood, 2017). Our
624 study ponds were selected to have similar physical and chemical properties, which may explain
625 the minimal contribution of abiotic factors to variance in invertebrate community structure.
626 Although pond area was retained by model selection, it did not significantly influence
627 community structure and explained less variance than crucian carp presence-absence. Likewise,
628 other studies have shown a weak or no effect of pond area on invertebrate species richness and
629 community composition (Gee et al., 1997; Gledhill et al., 2008; Oertli et al., 2002; Wood et al.,
630 2001). Critically, our environmental data were collected over a 7-year period, whereas
631 contemporary data may have better explained variance in community structure. We omitted
632 variables that experience high temporal variation from our analyses, but these may be linked to
633 the Coleoptera and Mollusca alpha diversity reductions and different invertebrate communities
634 observed in crucian carp ponds, such as water temperature, pH, nutrient concentration, and
635 surface dissolved oxygen (Bendell & McNicol, 1995; Hassall et al., 2011; Menetrey, Sager, Oertli,
636 & Lachavanne, 2005). Investigations examining contemporary physicochemical variables

alongside crucian carp presence-absence will help disentangle the effects of stocking and habitat. The degree of connectivity between ponds may also substantially influence invertebrate community structure, both independently and in combination with crucian carp presence (Hill et al., 2017).

The crucian carp might be assumed to have negative impacts on pond biodiversity by having "carp" in its name. Common carp foraging activities are known to reduce invertebrate density and macrophyte cover, with knock-on effects for waterfowl species richness and abundance (Haas et al., 2007; Maceda-Veiga et al., 2017). However, all of our crucian carp ponds were dominated by floating and/or submerged macrophyte beds as in Stefanoudis *et al*. (2017), and we observed no evidence for negative impacts on vegetation. Our study demonstrates that crucian carp can be an important, positive driver of invertebrate community heterogeneity and subsequently beta diversity in ponds. Our findings indicate that crucian carp presence enhances invertebrate diversity across pond networks, and that current conservation management of stocked populations in England is appropriate. English waters are absent of gibel carp and subsequently hybridisation with this species that is threatening the crucian carp across Europe, thus England is an important epicentre for crucian carp conservation (Jeffries et al., 2017; Sayer et al., *in review*). Crucian carp may contribute to taxon turnover and subsequent community heterogeneity in ponds by providing colonisation opportunities for smaller invertebrates through predator (e.g. Coleopterans) removal. We conclude that management strategies to support high landscape-scale pond biodiversity should consider a patchwork of fish-containing and fishless ponds, with the crucian carp likely to be an important species in this respect. However, the impact of crucian carp on pond biodiversity, especially amphibians, must be studied more broadly with respect to crucian carp population density and the aforementioned environmental variables to determine whether stocking is invariably beneficial.

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Data accessibility

Raw sequence reads have been archived on the NCBI Sequence Read Archive (Study: SRP163672; BioProject: PRJNA494857; BioSamples: SAMN10181701 - SAMN10182084 [bulk tissue DNA] and SAMN10187732 - SAMN10188115 [eDNA]; SRA accessions: SRR7969394 - SRR796977 [bulk tissue DNA] and SRR7985814 - SRR7986197 [eDNA]). Jupyter notebooks, R scripts and corresponding data are deposited in a dedicated GitHub repository (https://github.com/HullUni-bioinformatics/Harper_et_al_2020_crucian_carp_impact_invertebrates_conventional_molecular_tools) which has been permanently archived (https://doi.org/10.5281/zenodo.3733127).

Author contributions

L.R.H, B.H, and L.L.H conceived and designed the study. C.D.S selected the study ponds in Norfolk and provided associated environmental data. L.R.H, M.B, and R.C.B built the custom reference sequence database for invertebrates. L.R.H and R.C.B sampled the Norfolk ponds for invertebrates. L.R.H and M.B collected and filtered water samples from all study ponds. D.S.R assisted with water filtration of eDNA samples and provided advice on primers, PCR protocols, and sequencing. L.R.H performed laboratory work, and analysed the data with assistance from M.J.H. L.R.H wrote the manuscript, which all authors revised.

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965

966 **Table 1.** Summary of analyses (GLM) statistically comparing alpha diversity at species-level and
967 family-level between ponds with and without crucian carp using independent and combined
968 standard methods.
969

	Species-level						Family-level					
	GLM				LRT		GLM				LRT	
	df	Estimate ± SE	*Z*	*P*	χ^2	*P*	df	Estimate ± SE	*Z*	*P*	χ^2	*P*
Sweep-netting and microscopy	1	-0.139 ± 0.186	-0.746	0.456	0.556	0.456	1	-0.098 ± 0.158	-0.620	0.535	0.385	0.535
DNA metabarcoding	1	0.047 ± 0.141	0.332	0.740	0.110	0.740	1	-0.015 ± 0.121	-0.121	0.904	0.015	0.904
eDNA metabarcoding	1	-0.045 ± 0.198	-0.227	0.820	0.052	0.820	1	0.147 ± 0.141	-1.043	0.297	1.089	0.297
Combined methods	1	-0.049 ± 0.121	-0.409	0.683	0.167	0.683	1	-0.117 ± 0.096	-1.225	0.220	1.503	0.220

970
971

972 **Table 2.** Relative contribution of taxon turnover and nestedness-resultant to total beta diversity
973 (Jaccard dissimilarity) using independent and combined standard methods. A value of 1
974 corresponds to all sites containing different species.
975

	Species-level			Family-level		
	Turnover	**Nestedness-resultant**	**Total beta diversity**	**Turnover**	**Nestedness-resultant**	**Total beta diversity**
Sweep-netting and microscopy	0.935 (98.01%)	0.019 (1.99%)	0.954 (100%)	0.867 (94.75%)	0.048 (5.25%)	0.915 (100%)
DNA metabarcoding	0.939 (98.63%)	0.013 (1.37%)	0.952 (100%)	0.885 (96.83%)	0.029 (3.17%)	0.914 (100%)
eDNA metabarcoding	0.917 (97.24%)	0.026 (2.76%)	0.943 (100%)	0.870 (95.29%)	0.043 (4.71%)	0.913 (100%)
Combined methods	0.927 (98.41%)	0.015 (1.59%)	0.942 (100%)	0.870 (96.35%)	0.033 (3.65%)	0.903 (100%)

976

977 **Table 3.** Summary of analyses (PERMANOVA) statistically examining variation in community
978 composition between ponds with and without crucian carp at species-level and family-level
979 using independent and combined standard methods.
980

	Community similarity (PERMANOVA)							
	Species-level				Family-level			
	df	*F*	R^2	*P*	df	*F*	R^2	*P*
Sweep-netting and microscopy								
Turnover	1	1.673	0.095	**0.022**	1	1.198	0.070	0.371
Nestedness-resultant	1	-3.454	-0.275	0.954	1	-0.103	-0.007	0.649
Total beta diversity	1	1.369	0.079	**0.029**	1	1.136	0.066	0.311
DNA metabarcoding								
Turnover	1	1.271	0.074	0.154	1	1.668	0.094	0.106
Nestedness-resultant	1	-1.082	-0.073	0.748	1	-0.113	-0.007	0.731
Total beta diversity	1	1.121	0.066	0.224	1	1.259	0.073	0.213
eDNA metabarcoding								
Turnover	1	2.484	0.134	**0.002**	1	1.850	0.104	**0.033**
Nestedness-resultant	1	-2.136	-0.154	0.963	1	-0.015	-0.001	0.687
Total beta diversity	1	1.841	0.103	**0.003**	1	1.521	0.087	**0.032**
Combined methods								
Turnover	1	1.924	0.107	**0.001**	1	1.494	0.085	0.106
Nestedness-resultant	1	-1.449	-0.099	0.954	1	2.621	0.141	0.162
Total beta diversity	1	1.669	0.095	**0.003**	1	1.430	0.082	0.051

981

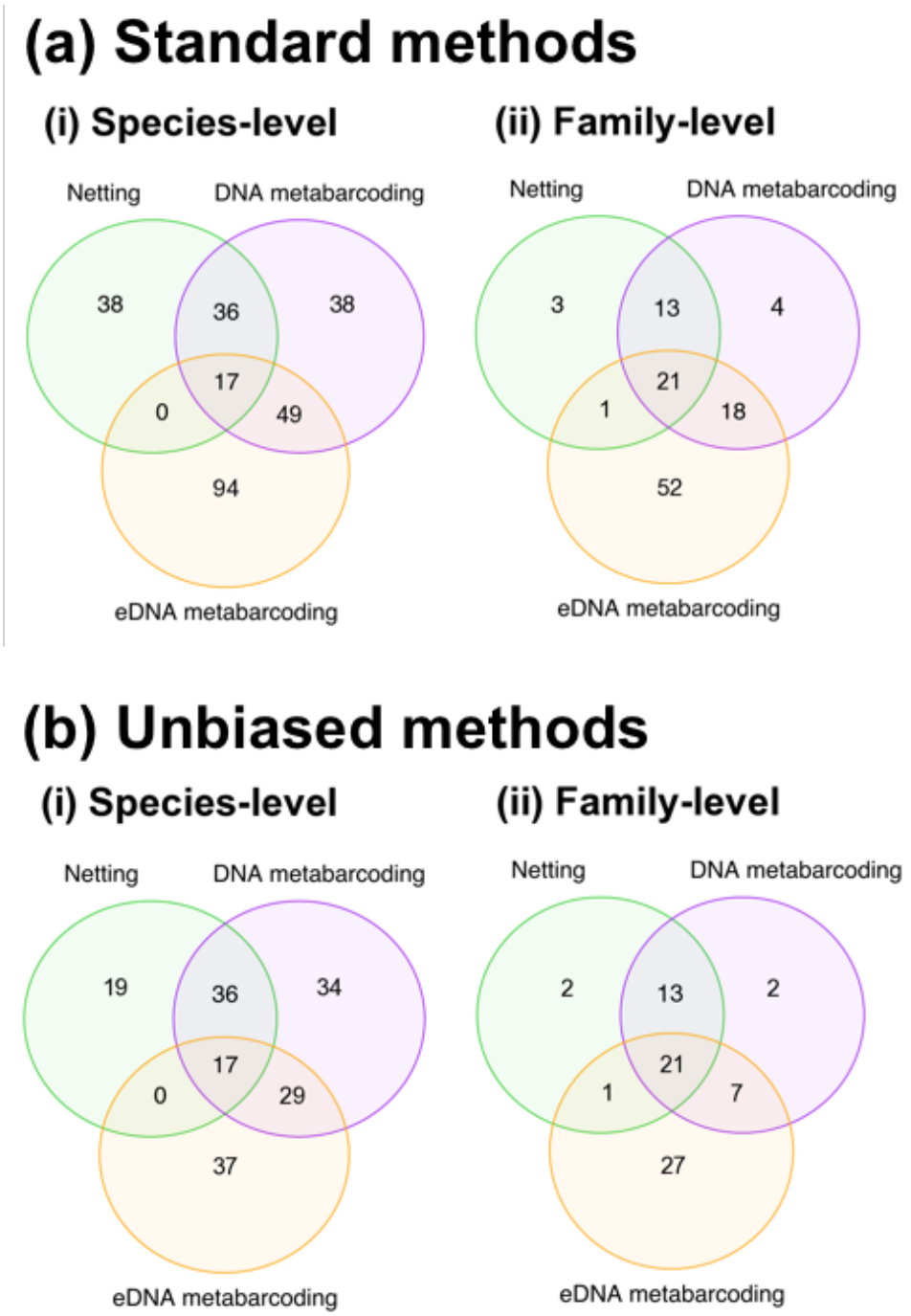
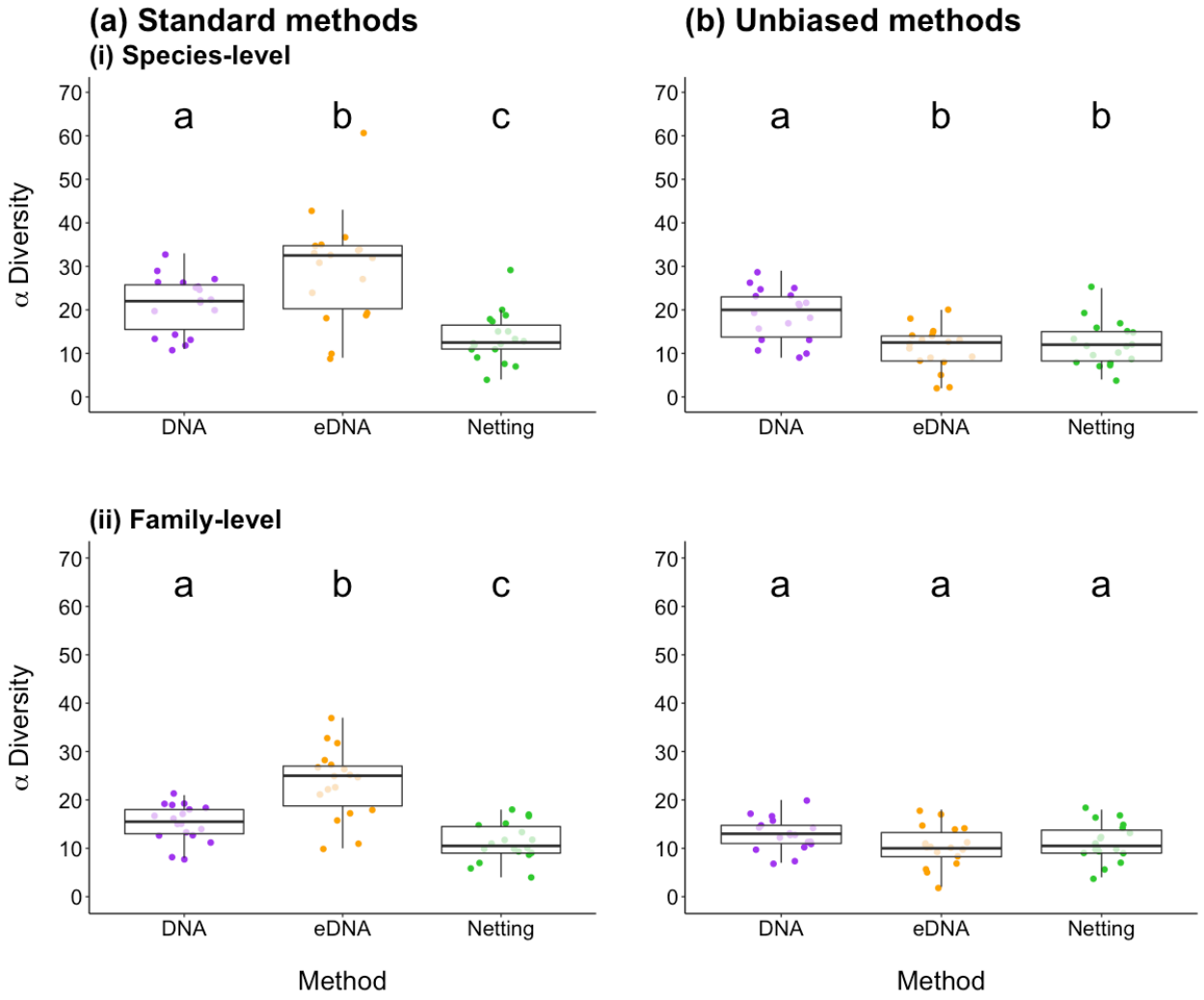


Figure 1. Venn diagram which summarises taxon detection by standard **(a)** and unbiased **(b)** methods of invertebrate assessment. The number of invertebrate species **(i)** and families **(ii)** detected across the 18 study ponds by sweep-netting and microscopy (green circle), DNA metabarcoding (purple circle), and eDNA metabarcoding (orange circle) is displayed. Overlap in species or family detections between methods is shown within circle intersections.



990
991
992 **Figure 2.** Mean alpha diversity of invertebrates in ponds across Norfolk and East Yorkshire.
993 Alpha diversity is displayed according to standard **(a)** and unbiased **(b)** methods of invertebrate
994 assessment. Species **(i)** and family **(ii)** richness generated by netting and microscopy (green
995 points), DNA metabarcoding (purple points), and eDNA metabarcoding (orange points) is
996 shown. Boxes show 25th, 50th, and 75th percentiles, and whiskers show 5th and 95th
997 percentiles.
998

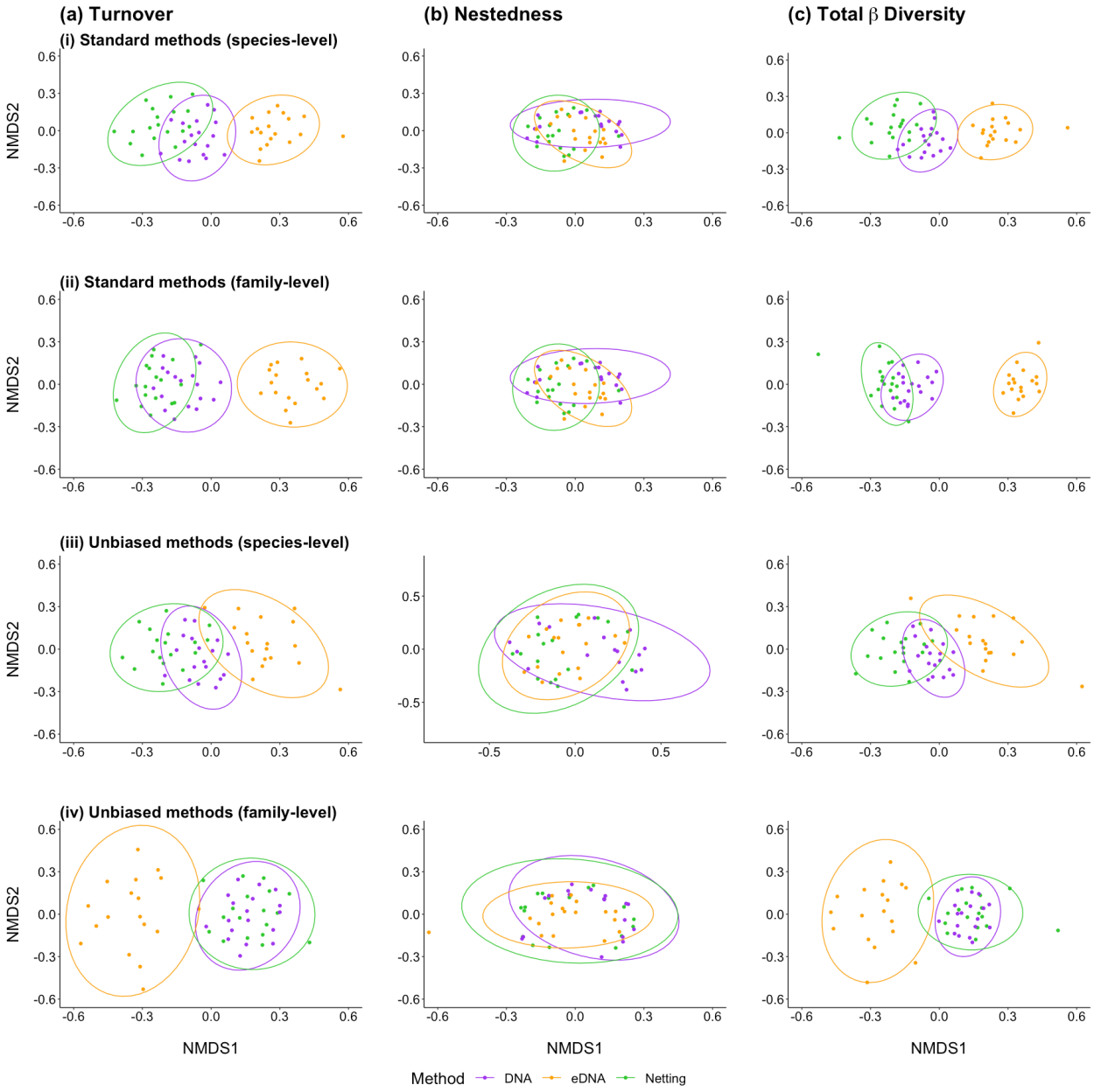


Figure 3. Non-metric Multidimensional Scaling (NMDS) plots of invertebrate communities (Jaccard dissimilarity) produced by sweep-netting and microscopy (green points/ellipse), DNA metabarcoding (purple points/ellipse), and eDNA metabarcoding (orange points/ellipse) for the 18 study ponds. The turnover **(a)** and nestedness-resultant **(b)** partitions of total beta diversity **(c)** are shown at species-level and family-level for standard **(i, ii)** and unbiased **(iii, iv)** methods.

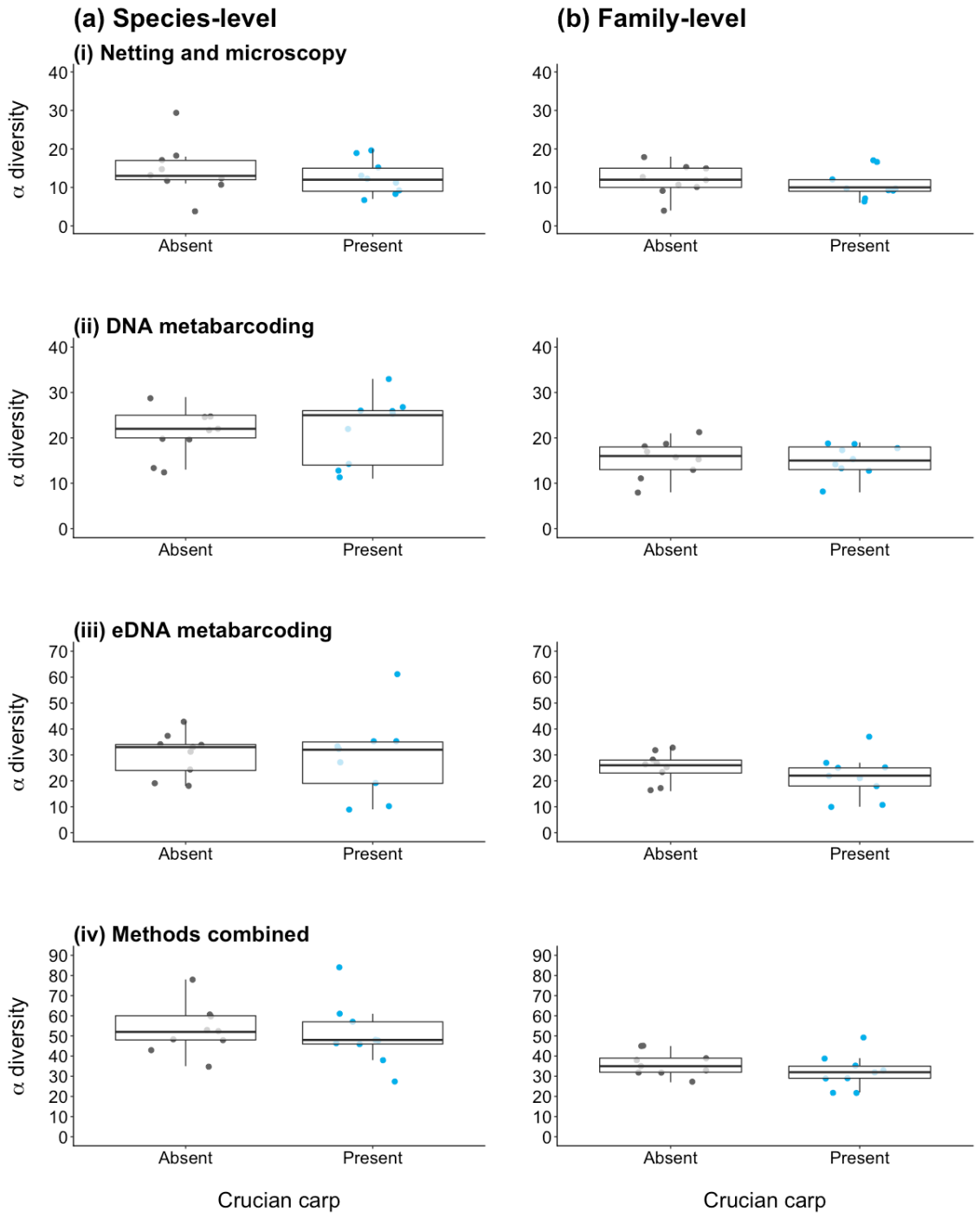


Figure 4. Mean alpha diversity of invertebrates in ponds with crucian carp (blue points) and without fish (grey points) across Norfolk and East Yorkshire. Alpha diversity at species-level **(a)** and family-level **(b)** is shown according to standard methods of invertebrate assessment: sweep-netting and microscopy **(i)**, DNA metabarcoding **(ii)**, eDNA metabarcoding **(iii)**, and all methods combined **(iv)**. Boxes show 25th, 50th, and 75th percentiles, and whiskers show 5th and 95th percentiles.

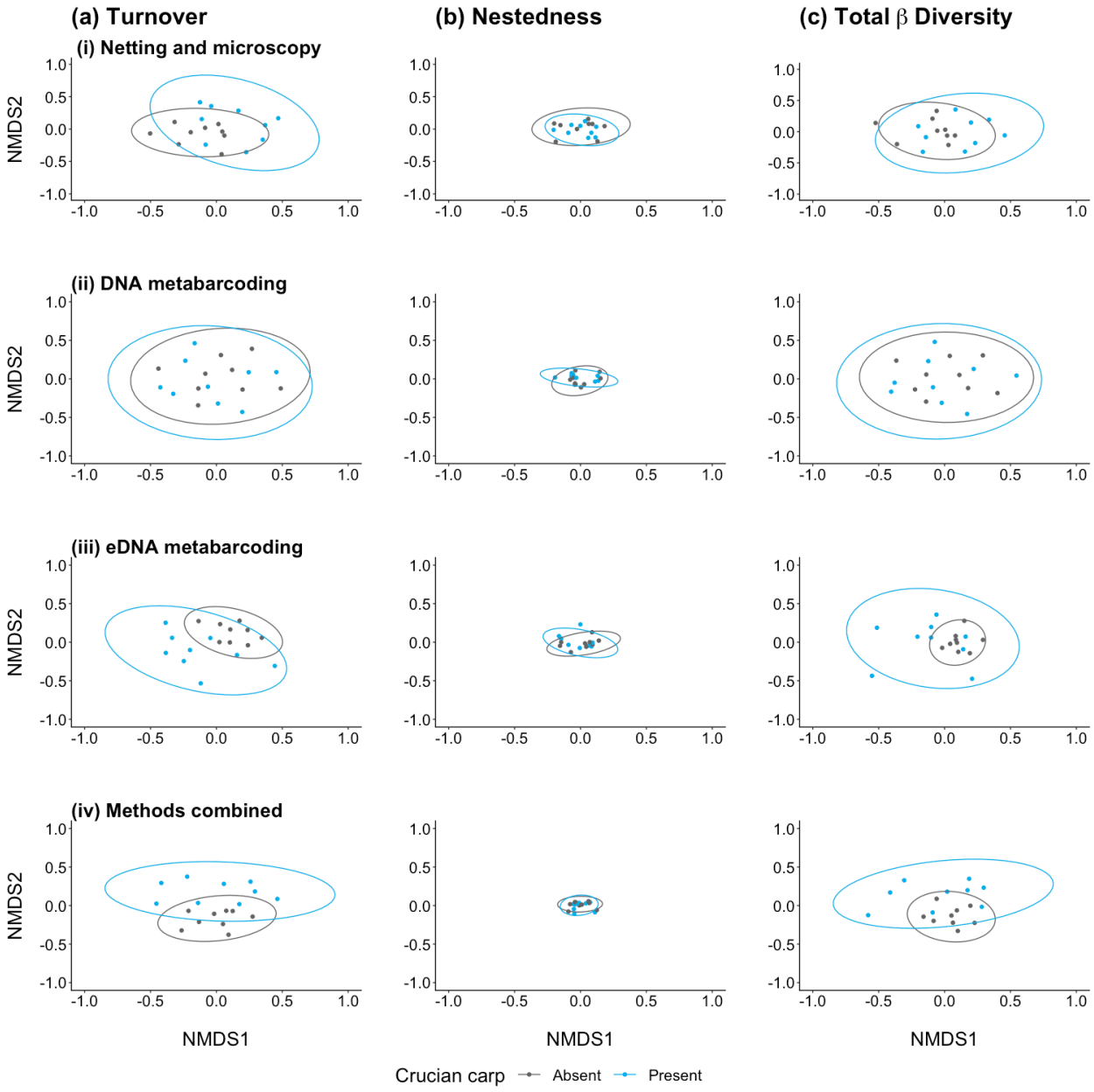


Figure 5. Non-metric Multidimensional Scaling (NMDS) plots of species-level invertebrate communities (Jaccard dissimilarity) from ponds with crucian carp (blue points/ellipse) and without fish (grey points/ellipse) across Norfolk and East Yorkshire. The turnover **(a)** and nestedness-resultant **(b)** partitions of total beta diversity **(c)** are shown according to standard methods of invertebrate assessment: netting and microscopy **(i)**, DNA metabarcoding **(ii)**, eDNA metabarcoding **(iii)**, and all methods combined **(iv)**.

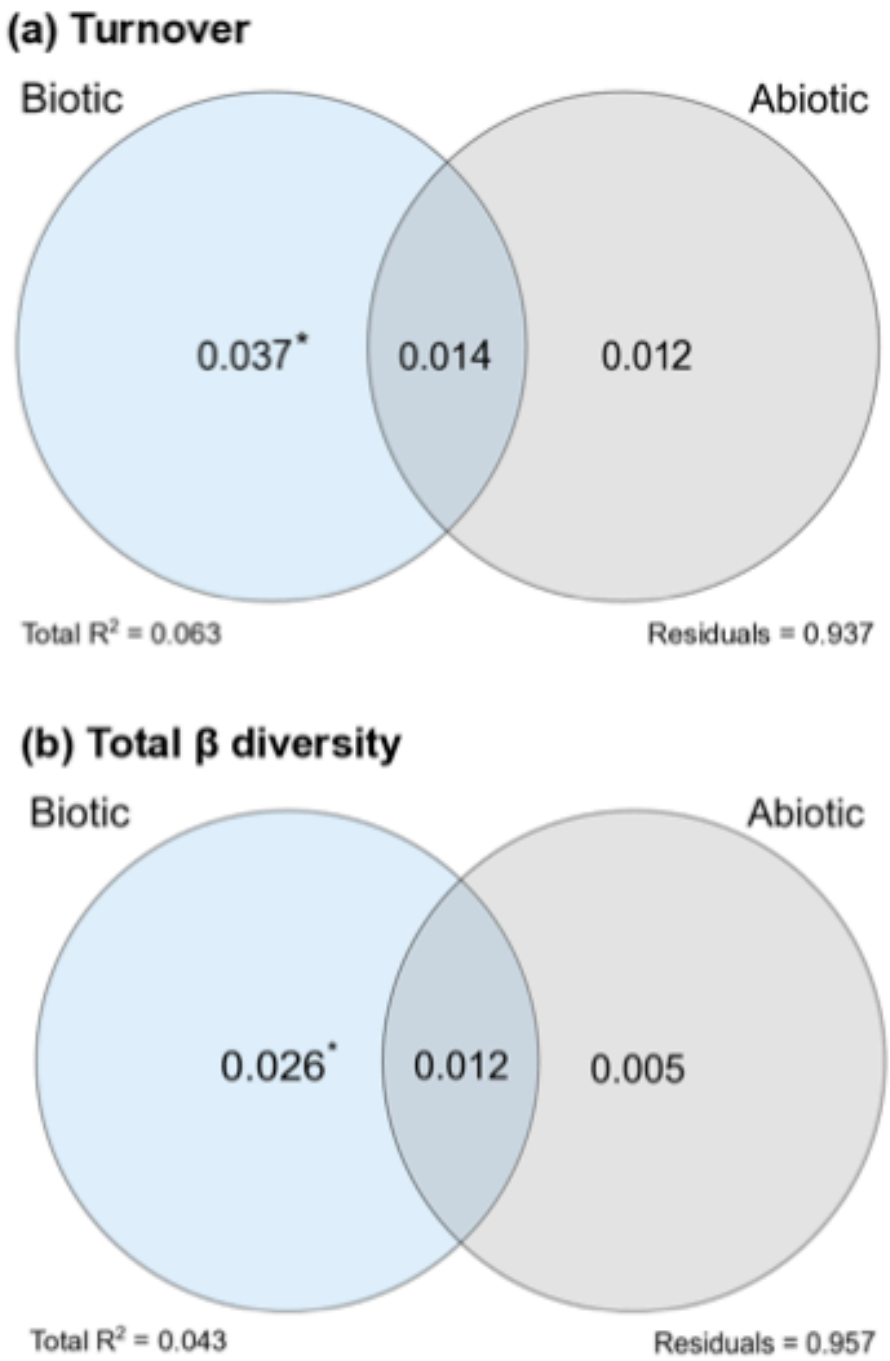


Figure 6. The relative contribution of biotic (crucian carp presence-absence) and abiotic variables (pond area) to species-level turnover **(a)** and total beta diversity **(b)** when the combined invertebrate data from all three standard sampling methods were considered. Values within circles and circle intersections represent the adjusted R^2 values. Significant variables are indicated by asterisks (* = $P < 0.05$).