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 its native European range and has been stocked for conservation management in England despite plausible non-native status. However, its impacts on English pond biota have not been broadly assessed. Freshwater invertebrates comprise a large proportion of aquatic biodiversity, encompassing many rare and endemic species, but small size and high abundance complicates their assessment. Practitioners have typically employed sweep-netting and kick-net sampling with microscopy, but specimen size/quality and experience can bias identification. DNA and eDNA metabarcoding now 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 methods were considered as standard or with potential biases removed. DNA metabarcoding reflected morphotaxonomic identification, whereas eDNA metabarcoding produced markedly different communities. Therefore, these complementary tools should be combined for comprehensive freshwater 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 invertebrate taxa to fishless ponds). Concerning pond invertebrates, the crucian carp can be considered a naturalised, non-impactful species in England, supporting continued conservation management in this region. 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

87 goldfish (*Carassius auratus*) and gibel carp (*Carassius gibelio*) (Copp, Warrington, & Wesley,
88 2008; Sayer et al., 2011), and genetic introgression through hybridisation with goldfish and
89 common carp (*Cyprinus carpio*) (Hänfling, Bolton, Harley, & Carvalho, 2005). In 2010, the
90 crucian carp was designated as a Biodiversity Action Plan (BAP) species in the county of Norfolk,
91 eastern England (Copp & Sayer, 2010). A key objective of this BAP is to increase the number of
92 viable crucian carp populations across Norfolk through pond restoration and species
93 reintroduction. Many Norfolk ponds have since been stocked with crucian carp to realise this
94 objective (Sayer et al., *in review*), but continued stocking is controversial in light of genetic
95 evidence that indicates the crucian carp is not native to the UK (Jeffries et al., 2017).
96 Nonetheless, there is support for UK conservation efforts to continue to protect the genetic
97 integrity of the crucian carp at the European level, and provide a natural stronghold for the
98 species (Harper et al., 2019; Jeffries et al., 2017; Stefanoudis et al., 2017) in the face of
99 persistent declines throughout its native range of Northwest and Central Europe (Copp et al.,
100 2008; Sayer et al., 2011).

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

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

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

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

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

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

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

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

250 The pooled libraries were quantified by qPCR using the NEBNext® Library Quant Kit for
251 Illumina® (New England Biolabs® Inc., MA, USA). An Agilent 2200 TapeStation and High
252 Sensitivity D1000 ScreenTape (Agilent Technologies, CA, USA) were used to verify removal of
253 secondary products from libraries by bead purification and that a fragment of the expected size
254 (531 bp) remained. A total of 52 bulk tissue subsamples sequenced in triplicate (n = 156), 12
255 extraction blanks, and 18 PCR controls were included alongside samples from other projects (N
256 = 188) in the DNA library. A total of 90 eDNA samples, 18 full process blanks, and 22 PCR
257 controls were included alongside samples from other projects (N = 140) in the eDNA library.
258 The DNA and eDNA libraries were sequenced on an Illumina® MiSeq using 2 x 300 bp V3
259 chemistry with 10% and 20% PhiX Sequencing Control respectively (Illumina, Inc, CA, USA).

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

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

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

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

319 respectively. These values are broadly equivalent to correlation coefficients of r = 0.3 and 0.5
320 which represent moderate and strong effects accordingly (Macher et al., 2018; Nakagawa &
321 Cuthill, 2007). Jaccard dissimilarity was used as a measure of beta diversity for all analyses.
322 We tested whether the species and family-level invertebrate communities produced by
323 the combined standard or unbiased methods were influenced by pond properties in
324 conjunction with crucian carp presence-absence. Redundancy Analysis (RDA) was selected for
325 constrained ordination as it analyses variation in biological communities in response to
326 explanatory variables (Legendre & Legendre, 2012). Principal Coordinate Analysis (PCoA) was
327 performed using the *pcoa* function in ape v5.0 (Paradis & Schliep, 2018) on the turnover,
328 nestedness-resultant, and total beta diversity matrices. The Lingoes correction was employed
329 to account for negative eigenvalues (Legendre, 2014). The resultant PCoA eigenvectors
330 (principle coordinates) for each distance matrix were used as the response variable in variance
331 partitioning. Our variables were grouped as biotic (crucian carp presence-absence) or abiotic
332 (pond conductivity, area, depth, percentages of perimeter with emergent vegetation, emergent
333 macrophyte cover, submerged macrophyte cover, and shading). Abiotic variables were log10
334 transformed to eliminate their physical units (Legendre & Birks, 2012). Significant abiotic
335 variables influencing each component of beta diversity were identified using the *ordiR2step*
336 function in vegan v2.4-6 to perform separate RDA analyses under a forward selection
337 procedure. Where applicable, the relative contributions of the biotic and abiotic variables on
338 turnover, nestedness-resultant, and total beta diversity for the species and family-level
339 invertebrate communities were assessed by variance partitioning (Borcard, Legendre, &
340 Drapeau, 1992) using the *varpart* function from vegan v2.4-6. For each beta diversity
341 component, RDA was performed using biotic and significant abiotic variables, and variance
342 partitioning used to divide the total percentage of variation explained into unique and shared
343 contributions for biotic and abiotic predictor groups. The *anova* function in vegan v2.4-6 was
344 used to examine the statistical significance of the full model and the unique contributions of
345 each predictor group. We report the adjusted R^2-fractions as they are widely recommended
346 and unbiased (Peres-Neto, Legendre, Dray, & Borcard, 2006).
347

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 ±

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

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

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

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

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

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

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

522 excluded.

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

538 Marker choice and primer design can substantially influence amplification success and
539 taxonomic assignment. Although the *COI* region offers species resolution, has extensive
540 database representation, and is used as standard in DNA barcoding (Elbrecht, Hebert, &
541 Steinke, 2018), it lacks conserved primer-binding sites as a protein-coding gene (Clarke et al.,
542 2017). This introduces high risk of primer mismatch and subsequent bias during metabarcoding
543 (Clarke et al., 2017; Elbrecht et al., 2018), where preferential DNA amplification for some taxa
544 may prevent recovery of all taxa present (Elbrecht et al., 2016; Lobo et al., 2017). Consequently,
545 most metabarcoding primers designed to target the *COI* region in metazoans (Elbrecht & Leese,
546 2017; Leray et al., 2013; Meusnier et al., 2008; Zeale, Butlin, Barker, Lees, & Jones, 2011) are
547 degenerate to allow primers to bind at highly variable sites. However, highly degenerate
548 primers may bind non-target regions (Elbrecht et al., 2018) and experience biased amplification
549 toward non-metazoans, e.g. bacteria, algae, fungi (Brandon-Mong et al., 2015; Macher et al.,
550 2018). This bias can occur even when primers are designed for macroinvertebrates (Elbrecht &

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

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

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

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

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

623 The effects of environmental variables on alpha and beta diversity of pond invertebrates
624 must also be considered (Hassall et al., 2011; Hill, Heino, Thornhill, Ryves, & Wood, 2017). Our
625 study ponds were selected to have similar physical and chemical properties, which may explain
626 the minimal contribution of abiotic factors to variance in invertebrate community structure.
627 Although pond area was retained by model selection, it did not significantly influence
628 community structure and explained less variance than crucian carp presence-absence. Likewise,
629 other studies have shown a weak or no effect of pond area on invertebrate species richness and
630 community composition (Gee et al., 1997; Gledhill et al., 2008; Oertli et al., 2002; Wood et al.,
631 2001). Critically, our environmental data were collected over a 7-year period, whereas
632 contemporary data may have better explained variance in community structure. We omitted
633 variables that experience high temporal variation from our analyses, but these may be linked to
634 the Coleoptera and Mollusca alpha diversity reductions and different invertebrate communities
635 observed in crucian carp ponds, such as water temperature, pH, nutrient concentration, and
636 surface dissolved oxygen (Bendell & McNicol, 1995; Hassall et al., 2011; Menetrey, Sager, Oertli,
637 & 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).

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. L.R.H. wrote the manuscript, which all authors revised.

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966

967 **Table 1.** Summary of analyses (GLM) statistically comparing alpha diversity at species-level and
968 family-level between ponds with and without crucian carp using independent and combined
969 standard methods.
970

	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

971
972

973 **Table 2.** Relative contribution of taxon turnover and nestedness-resultant to total beta diversity
974 (Jaccard dissimilarity) using independent and combined standard methods. A value of 1
975 corresponds to all sites containing different species.
976

	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%)

977

978 **Table 3.** Summary of analyses (PERMANOVA) statistically examining variation in community
979 composition between ponds with and without crucian carp at species-level and family-level
980 using independent and combined standard methods.
981

	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

982

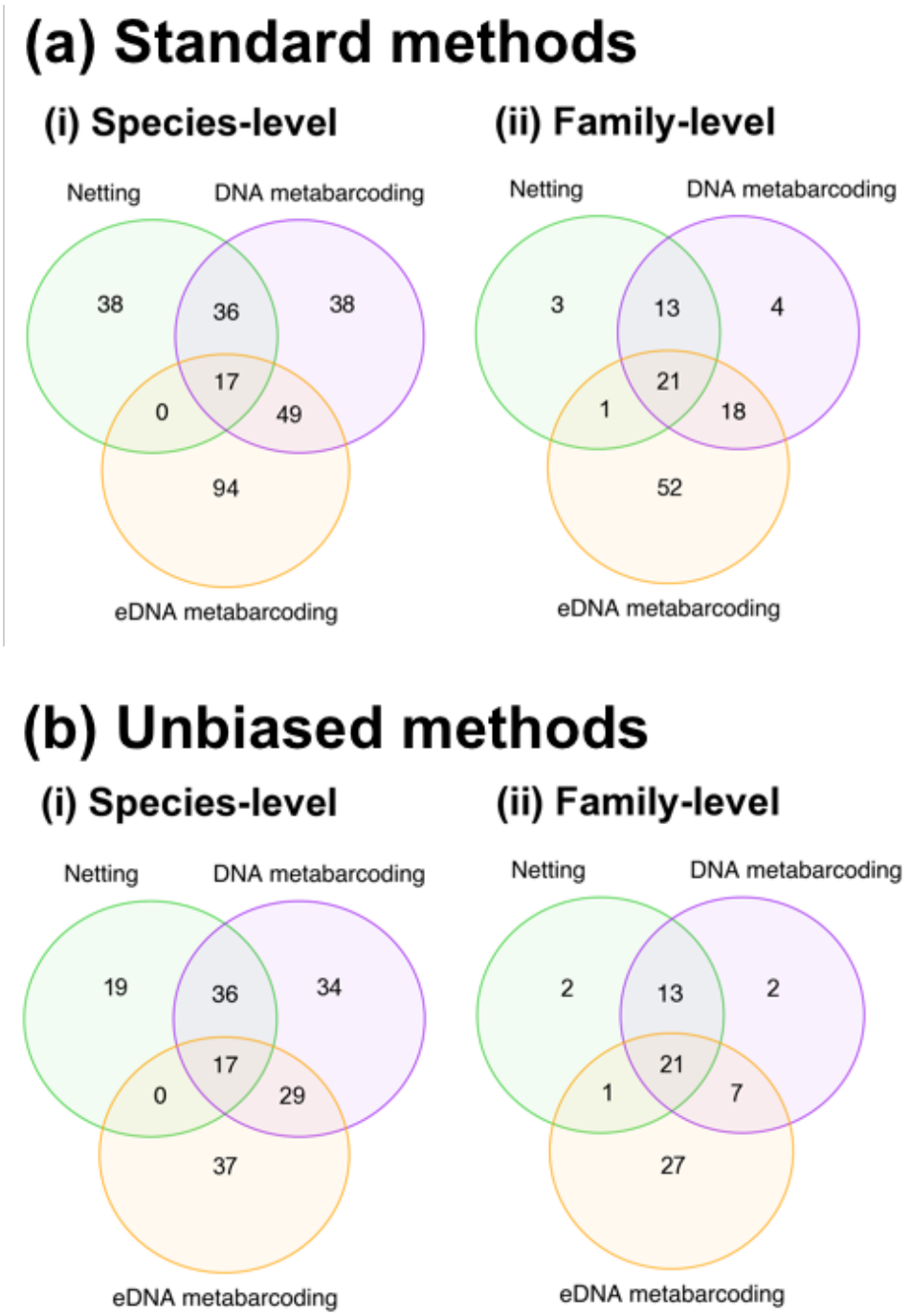

Figure 1. Venn diagram which summarises taxon detection by standard **(a)** and unbiased **(b)** methods of invertebrate assessment. The number of invertebrate species **(i)** and families **(ii)** detected across the 18 study ponds by sweep-netting and microscopy (green circle), DNA metabarcoding (purple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

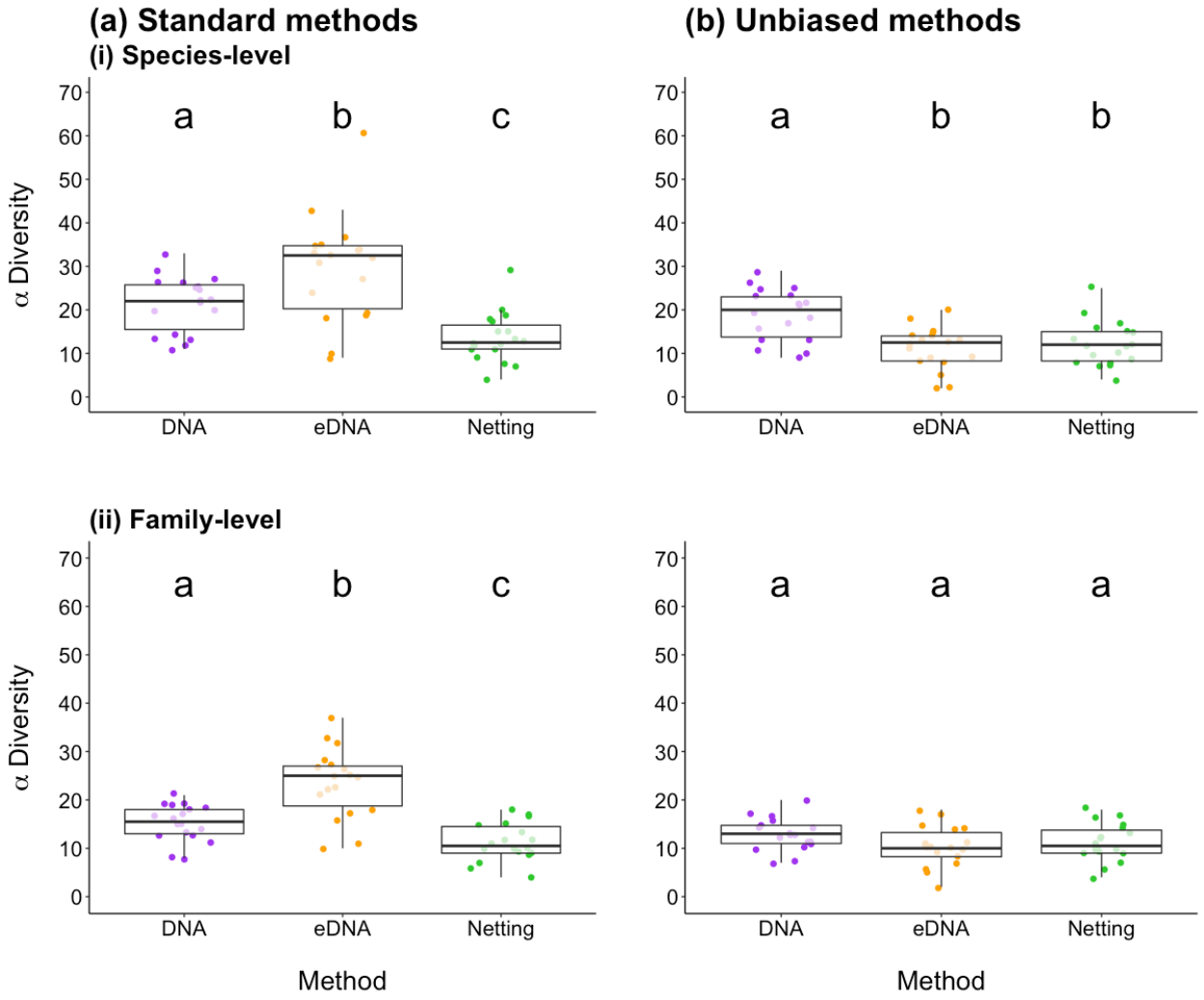

Figure 2. Mean alpha diversity of invertebrates in ponds across Norfolk and East Yorkshire. Alpha diversity is displayed according to standard **(a)** and unbiased **(b)** methods of invertebrate assessment. Species **(i)** and family **(ii)** richness generated by netting and microscopy (green points),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

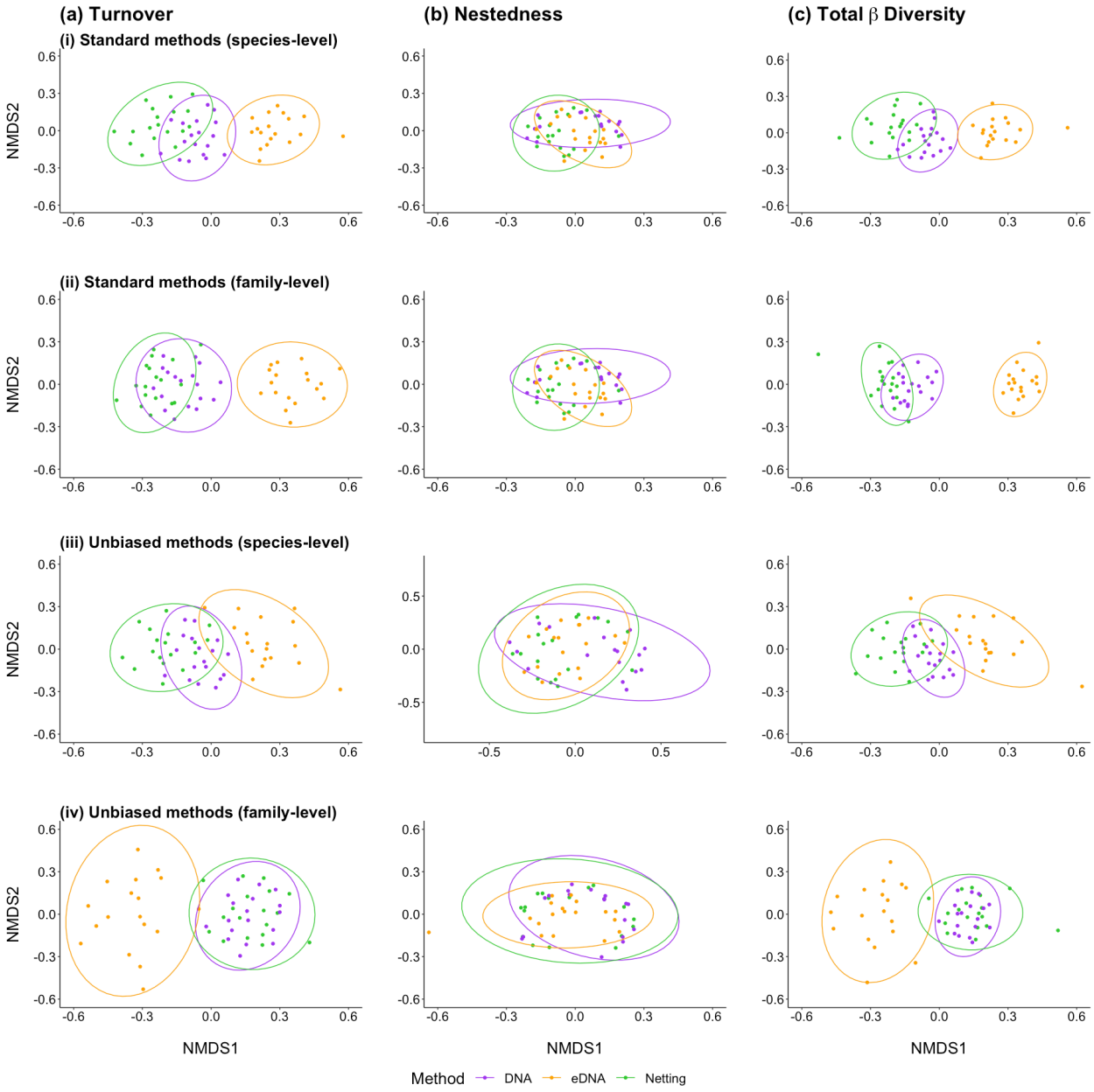

Figure 3. Non-metric Multidimensional Scaling (NMDS) plots of invertebrate communities (Jaccard dissimilarity) produced by sweep-netting and microscopy (green points/ellipse), DNA metabarcoding (purple points/ellipse), and eDNA metabarcoding (orange points/ellipse) for the 18 study ponds. The turnover **(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.

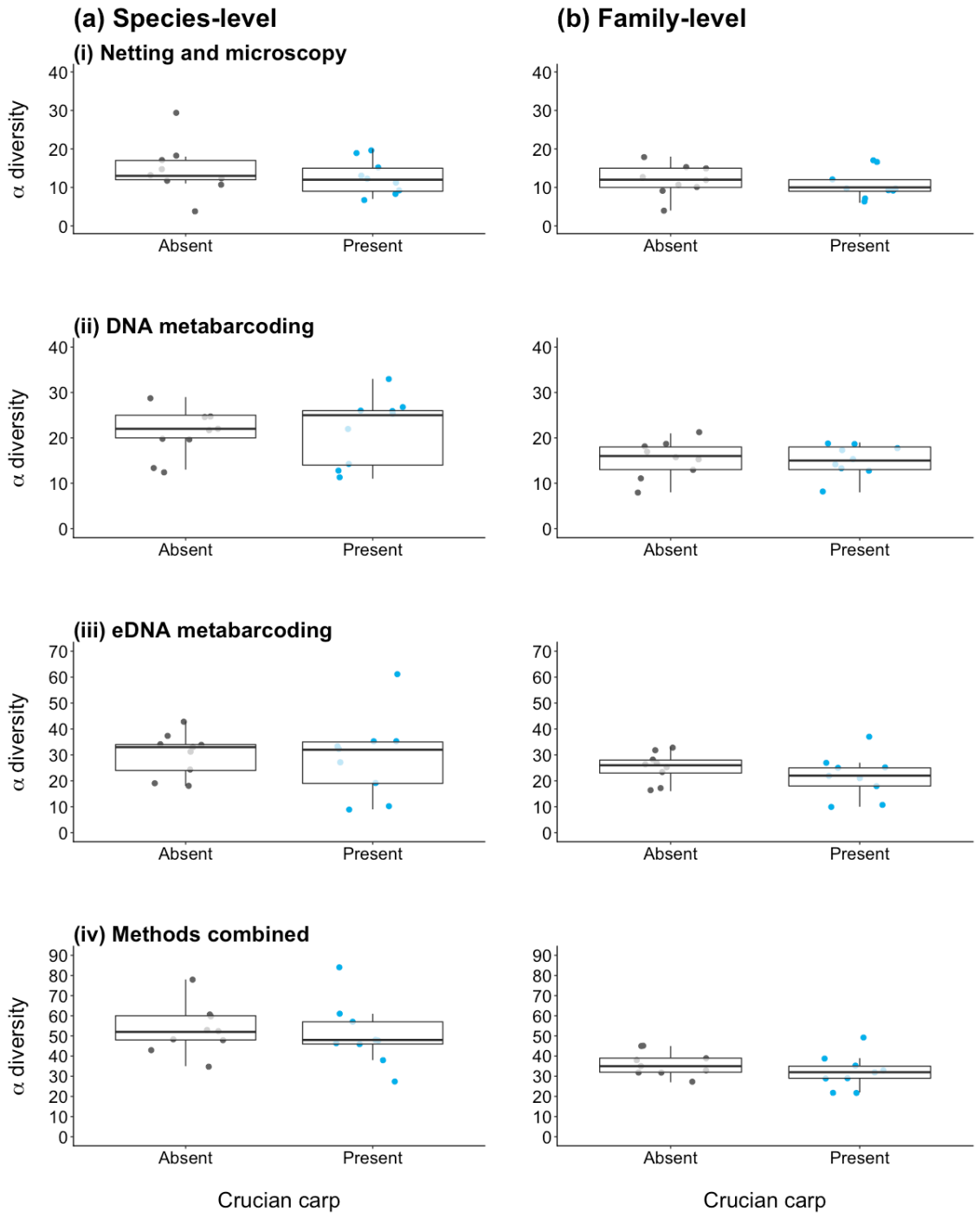

Figure 4. Mean alpha diversity of invertebrates in ponds with crucian carp (blue points) and without fish (grey points) across Norfolk and East Yorkshire. Alpha diversity at species-level **(a)** and family-level **(b)** is shown according to standard methods of invertebrate assessment: sweep-netting 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.

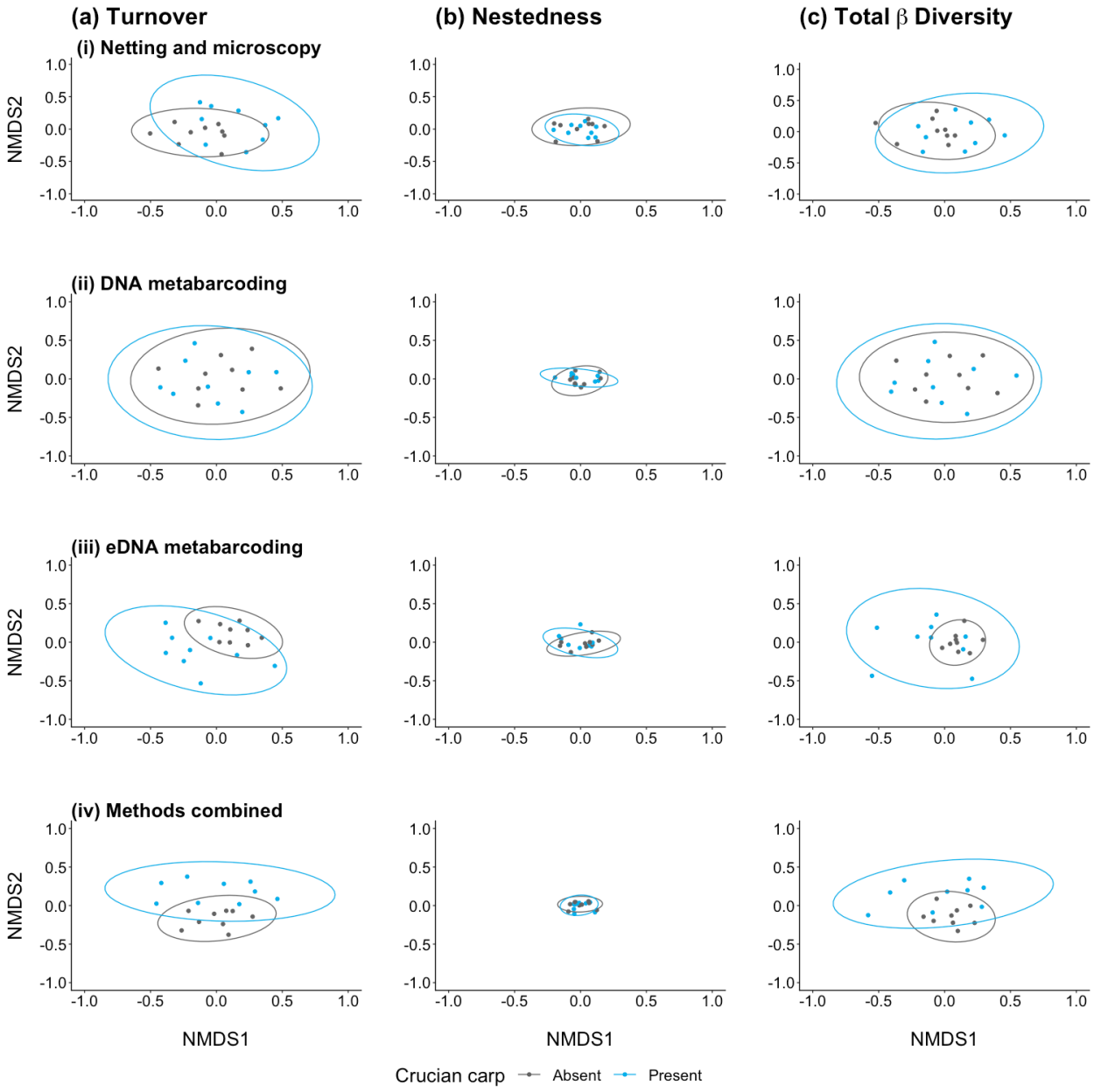

Figure 5. Non-metric Multidimensional Scaling (NMDS) plots of species-level invertebrate communities (Jaccard dissimilarity) from ponds with crucian carp (blue points/ellipse) and without fish (grey points/ellipse) across Norfolk and East Yorkshire. The turnover **(a)** and nestedness-resultant **(b)** 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)**.

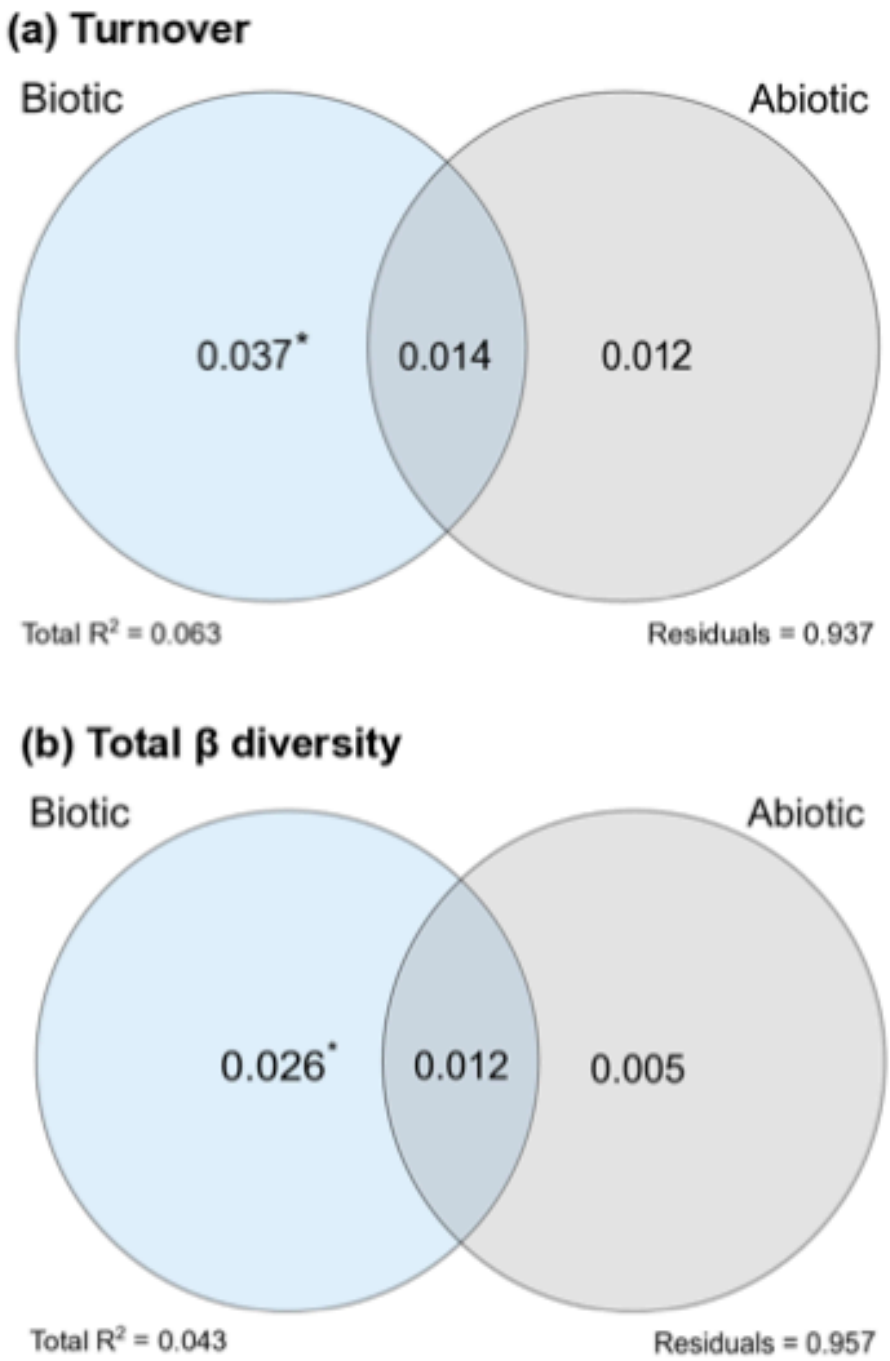

Figure 6. The relative contribution of biotic (crucian carp presence-absence) and abiotic variables (pond area) to species-level turnover **(a)** and total beta diversity **(b)** when the combined invertebrate data from all three standard sampling methods were considered. Values within circles and circle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