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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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 their small size and high abundance complicates their assessment. Practitioners have employed sweep-netting and kick-sampling with microscopy (morphotaxonomy), 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 morphotaxonomic identification, DNA metabarcoding, and eDNA metabarcoding. Five 2-L water samples and 3-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. 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). Crucian carp presence contributes to landscape-scale invertebrate diversity, 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 (see definition in Biggs, von Fumetti, & Kelly-Quinn, 2016) in particular provide critical habitat for biodiversity in a fragmented landscape (Céréghino, Biggs, Oertli, & Declerck, 2008), supporting many rare and specialist species not found in other waterbodies (Biggs et al., 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 purposes, 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 the 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 and cover via foraging activity of fish, or management practices (e.g. commercial farming, artificial feeding, pond depth and bank alteration, removal of aquatic vegetation and sediment) 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 a 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 fish species 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 goldfish (*Carassius auratus*) and gibel carp (*Carassius gibelio*) (Copp, Warrington, & Wesley, 2008; Sayer et al., 2011), and genetic introgression through hybridisation with goldfish, gibel carp, and common carp (*Cyprinus carpio*) (Hänfling, Bolton, Harley, & Carvalho, 2005). In 2010, the crucian carp was designated as a Biodiversity Action Plan (BAP) species in the county of Norfolk in eastern England (Copp & Sayer, 2010). A key objective of this BAP is to increase the number of viable crucian carp populations across Norfolk through pond restoration and species reintroduction. Many Norfolk ponds have since been restored and stocked with crucian carp to realise this objective (Sayer et al., 2020), but maintaining populations and continued stocking requires justification in light of genetic evidence that indicates the crucian carp is not native to the UK (Jeffries et al., 2017). Nonetheless, there is support for UK conservation efforts to continue to protect the genetic integrity of the crucian carp at the European level, and provide a natural stronghold for the species (Harper et al., 2019; Jeffries et al., 2017; Stefanoudis et al., 2017) in the face of persistent declines throughout its native range of Northwest and Central Europe (Copp et al., 2008; Sayer et al., 2011; Sayer et al., 2020).

The impact of stocking crucian carp on lentic biodiversity has been largely ignored, and little is known about its interactions with other pond species. Existing research suggests crucian carp are characteristic of ponds rich in invertebrates with extensive macrophyte cover (Copp et al., 2008; Sayer et al., 2011), and play an important ecological role (along with other pond-associated fishes) by increasing landscape-scale diversity across pond networks (Stefanoudis et al., 2017). Yet to our knowledge, only one study has assessed biodiversity (specifically macrophytes, zooplankton, and water beetles) in ponds with and without pond-associated fishes in the UK (Stefanoudis et al., 2017), and no studies have specifically focused on crucian carp. Consequently, there is a need to survey and compare fishless ponds to ponds containing crucian carp to assess the impact of maintaining existing crucian carp populations and conservation-based stocking of this species on invertebrate diversity more broadly.

Assessments of invertebrate diversity in pond networks have relied upon morphotaxonomic identification using sweep-netting together with microscopy, but this approach requires extensive resources and taxonomic expertise for accurate species-level

identification (Briers & Biggs, 2003; Haase, Pauls, Schindehütte, & Sundermann, 2010; Hill et al., 2018). 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; Leese et al., 2020), streams (Macher et al., 2018), and lakes (Klymus et al., 2017) is also gaining traction, but there are currently few published studies that have used metabarcoding for small lake or pond invertebrates (Beentjes, Speksnijder, Schilthuizen, Hoogeveen, & van der Hoorn, 2019).

In this study, 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 provided 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 meiofauna 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: 1) DNA metabarcoding and morphotaxonomic identification would produce congruent invertebrate communities (e.g. Carew et al., 2018; Emilson et al., 2017; Nichols et al. 2020), whereas eDNA metabarcoding would reveal taxa not identified by these tools (e.g. Macher et al., 2018; Mächler et al., 2019); 2) DNA and eDNA metabarcoding would enable species resolution data for problematic taxa that cannot be morphotaxonomically identified to species-level using standard keys (e.g.

Blackman et al., 2017; Elbrecht et al., 2017a; Lobo et al., 2017; Nichols et al., 2020); and 3) alpha diversity would be lower in ponds with crucian carp due to direct predation or altered habitat quality (e.g. Lemmens et al., 2013; Maceda-Veiga, López, & Green, 2017; Schilling et al., 2009a; Wood et al., 2001), but beta diversity would be enhanced due to community heterogeneity induced by crucian carp presence in the pond network (e.g. Stefanoudis et al., 2017). The third 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). Crucian carp were either present in ponds as well-established populations, or individuals that were restocked between 2010 and 2017 as part of the species' BAP (Copp & Sayer, 2010) because ponds contained populations between 1970-1980 according to local anglers (Sayer et al., 2011; Sayer et al., 2020). The fish status of ponds was confirmed by prior fyke-net surveys (Sayer et al., 2011; Sayer et al., 2020). For ponds containing crucian carp, the last fyke-net survey undertaken was 2016 (*n* = 7), 2015 (*n* = 1), and 2013 (*n* = 1). Roach (*Rutilus rutilus*) and rudd (*Scardinius erythrophthalmus*) were the only other fishes that occurred in three and one of the Norfolk ponds respectively, but at much lower density (see Table S1). All study ponds were selected to be similar in terms of morphometry and habitat structure, being <1 ha in area, <5 m in depth, dominated by submerged and floating-leaved macrophytes, 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. As invertebrate diversity often peaks in autumn (Hill, Sayer, & Wood, 2016) but large

and more easily identifiable odonate larvae are typically present in summer prior to emergence (Brooks & Cham, 2014), invertebrate specimens were collected for morphotaxonomic identification and DNA metabarcoding alongside water samples for eDNA metabarcoding in late August 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 the 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 Information). Samples were deposited in a 1.2 L sterile Whirl-Pak® stand-up bag (Cole-Palmer, London) and stored at -20 ^{o}C until laboratory processing and sorting. Samples were thawed and passed through sieves of 8 mm, 2 mm, and 0.25 mm in succession to remove large items of vegetation and detrital matter. For each sample, invertebrate specimens were sorted into three body size categories on laminated millimetre graph paper: small (<2.5 x 5 mm), medium (2.5 x 5 mm up to 5 x 10 mm), and large (>10 mm and up to 10 x 20 mm) (Elbrecht, Peinert, & Leese, 2017b). Where possible, size-sorted specimens were identified under a light microscope to species-level using Freshwater Biological Association publications (Bass, 1998; Brooks & Cham, 2014; Edington & Hildrew, 1995; Elliott, 2009; Elliott & Humpesch, 2010; Elliott & Dobson, 2015; Friday, 1988; Gledhill, Sutcliffe, & Williams, 1993; Macan, 1960; Savage, 1989; Wallace, Wallace, & Philipson, 1990). Specimens that could not be identified to species-level were identified to genus or family-level, except Hydrachnidea, Tabanini, Tanytarsini, Oligochaeata, and Collembola which were recorded as such. Terrestrial taxa and empty cases/shells were discarded. The laminated paper was sterilised with 50% v/v chlorine-based commercial bleach solution (Elliot Hygiene Ltd, UK) and 80% v/v ethanol solution between measuring specimens

203 from different ponds to minimise cross-contamination risk. Specimens were preserved in sterile
204 15 mL falcon tubes (SARSTED, Germany) containing 100% ethanol according to size category
205 and pond sampled, and stored at -20 ^{o}C until DNA extraction.
206
207 **2.3 DNA samples**
208
209 We followed the DNA extraction procedure from Blackman *et al.* (2017). Size categories from
210 each pond were dried overnight on FisherBrand cellulose filter paper (Fisher Scientific UK Ltd,
211 UK) in sterile glass funnels and conical flasks to remove excess ethanol. Size categories were
212 lysed (3 × 30 sec) using a TissueLyser II (Qiagen®, Germany) with DigiSol (50 mM Tris, 20 M
213 EDTA, 120 mM NaCl and 1% SDS). The adapter sets held 1.5 g of dried tissue and corresponding
214 volume of DigiSol. If the dry tissue weight of any size category exceeded 1.5 g, we processed
215 the size category in batches until all tissue had been lysed. The lysates from all batches were
216 pooled to recreate size categories. The size categories were incubated overnight at 55 °C with
217 SDS and Proteinase K (Bioline®, UK), following which 200 µL of lysate from each size category
218 was used for extraction with the DNeasy Blood & Tissue Kit (Qiagen®, Germany) according to
219 the manufacturer's protocol. Specimens collected from two ponds were categorised as medium
220 or large only, whereas specimens collected from other study ponds were categorised as small,
221 medium, and large (Table S2). Consequently, 16 bulk tissue samples were each represented by
222 three DNA extracts and two bulk tissue samples each represented by two DNA extracts that
223 were stored at -20 °C until metabarcoding.
224
225 **2.4 eDNA samples**
226
227 We repurposed eDNA samples from Norfolk ponds that were collected by Harper et al. (2019)
228 to validate a quantitative PCR (qPCR) assay for crucian carp. Additional samples were taken
229 from East Yorkshire ponds for the present study. Details of water sample collection, filtration,
230 and DNA extraction are provided in the Supporting Information. Briefly, five 2 L surface water
231 samples were collected at equidistant points around the pond perimeter where access

232 permitted. For each pond, a full process blank (1 L molecular grade water) was taken into the
233 field, transported, filtered, and extracted alongside samples to identify contamination. Filters
234 were stored at -20 °C until DNA extraction with the PowerWater® DNA Isolation Kit (MO BIO
235 Laboratories, CA, USA) following the manufacturer's protocol. eDNA extracts were stored at -20
236 °C until metabarcoding.
237
238 **2.5 Metabarcoding workflow**
239
240 Our metabarcoding workflow is fully described in the Supporting Information. In short, a
241 comprehensive list of UK invertebrate species living in or associated with freshwater habitats
242 (expected fauna) was used to create custom, curated reference databases for UK aquatic
243 invertebrates, excluding Diptera (Appendix 1). Public GenBank records for Diptera were missing
244 record features (e.g. 'gene' or 'CDS') and/or names were not in the format required for custom
245 reference database construction using the selected bioinformatic tools. Assignments to Diptera
246 were therefore made using the entire NCBI nucleotide database (see below). For the remaining
247 taxonomic groups, 1483 species were represented in our custom reference database (78.97% of
248 the expected fauna), although representation varied strongly across groups (Fig. S2). Species
249 without database representation are listed in Table S3 (see also Figs. S2, S3). Published
250 metazoan primers mICOIintF (Leray et al., 2013) and jgHCO2198 (Geller, Meyer, Parker, &
251 Hawk, 2013), which amplify a 313 bp fragment of the mitochondrial cytochrome c oxidase
252 subunit I (*COI*) gene, were validated *in silico* using ecoPCR software (Ficetola et al., 2010)
253 against the reference databases. Parameters set allowed a 250-350 bp fragment and maximum
254 of three mismatches between each primer and each reference sequence (Appendix 2). Primers
255 were validated *in vitro* against 38 invertebrate species, representing 38 families and 10 major
256 groups, and compared to published macroinvertebrate metabarcoding primers (Elbrecht &
257 Leese, 2017; Vamos, Elbrecht, & Leese, 2017) (Figs. S4, S5, S6).
258 PCR conditions were optimised (Figs. S7, S8) before two independent libraries were
259 constructed for DNA and eDNA metabarcoding using a two-step PCR protocol (Appendix 1).
260 During the first PCR (performed in triplicate), the target region was amplified using

261 oligonucleotides comprising the locus primers, sequencing primers, and pre-adapters. DNA
262 from the exotic, terrestrial two-spotted assassin bug (*Platymeris biguttatus*) was used for PCR
263 positive controls (tissue DNA *n* = 9, eDNA *n* = 11) as this species is not found in the UK, whilst
264 sterile molecular grade water (Fisher Scientific UK Ltd, UK) substituted template DNA for PCR
265 negative controls (tissue DNA *n* = 9, eDNA *n* = 11). PCR products were individually purified using
266 a magnetic bead clean-up (VWR International Ltd, UK), following a double size selection
267 protocol from Bronner et al. (2009). The second PCR bound Multiplex Identification (MID) tags
268 to the purified products. PCR products were pooled according to PCR run and the pooled PCR
269 product purified using a magnetic bead clean-up, following a double size selection protocol
270 from Bronner et al. (2009). Each pooled purified PCR product was quantified on a Qubit™ 3.0
271 fluorometer using a Qubit™ dsDNA HS Assay Kit (Invitrogen, UK) and normalised according to
272 concentration and sample number to produce a pooled volume of 20 µL.

273 The pooled libraries were quantified by qPCR using the NEBNext® Library Quant Kit for
274 Illumina® (New England Biolabs® Inc., MA, USA). An Agilent 2200 TapeStation and High
275 Sensitivity D1000 ScreenTape (Agilent Technologies, CA, USA) were used to verify removal of
276 secondary products from libraries by bead purification and that a fragment of the expected size
277 (531 bp) remained. A total of 52 bulk tissue subsamples sequenced in triplicate (*n* = 156), 12
278 extraction blanks, and 18 PCR controls were included alongside samples from other projects (*N*
279 = 188) in the DNA library. A total of 90 eDNA samples, 18 full process blanks, and 22 PCR
280 controls were included alongside samples from other projects (*N* = 140) in the eDNA library.
281 The DNA and eDNA libraries were sequenced on an Illumina® MiSeq using 2 x 300 bp V3
282 chemistry with 10% and 20% PhiX Sequencing Control respectively (Illumina, Inc, CA, USA).

283 Raw sequences were converted to taxonomic assignments using metaBEAT v0.97.11, a
284 custom pipeline for reproducible analysis of metabarcoding data (https://github.com/HullUni-
285 bioinformatics/metaBEAT). After quality filtering, trimming, merging, chimera detection, and
286 clustering, non-redundant query sequences were compared against our reference databases
287 using BLAST (Zhang, Schwartz, Wagner, & Miller, 2000). Putative taxonomic identity was
288 assigned using a lowest common ancestor (LCA) approach based on the top 10% BLAST matches
289 for any query matching with at least 90% identity to a reference sequence across more than

290 80% of its length. Unassigned sequences were subjected to a separate BLAST against the
291 complete NCBI nucleotide (nt) database at 90% identity to determine the source via LCA as
292 described above. Bioinformatic settings (Appendix 1) were chosen based on comprehensive
293 exploration of the parameter space and comparison of metaBEAT taxonomic assignments to
294 morphotaxonomic inventories. Reference databases in GenBank or fasta format and the
295 bioinformatic analysis have been deposited in a dedicated GitHub repository, which has been
296 permanently archived for reproducibility (https://doi.org/10.5281/zenodo.3993125).
297
298 **2.6 Data analysis**
299
300 All analyses were performed in R v.3.6.3 (R Core Team, 2017), with data and R scripts deposited
301 in the GitHub repository. Manipulation of the DNA and eDNA metabarcoding datasets for
302 analysis is described in the Supporting Information. For each method, two presence-absence
303 datasets (species-level and family-level) were generated using the *decostand* function in vegan
304 v2.5-6 (Oksanen et al., 2018) for downstream analyses. This is because potential amplification
305 bias during PCR can prevent reliable abundance or biomass estimation from sequence reads
306 produced by DNA or eDNA metabarcoding (Elbrecht et al., 2017b). The entire species and
307 family inventories produced by each method (hereafter standard methods) were compared.
308 We also compared inventories from each method after correcting for known biases: taxa
309 without reference sequences (i.e. those which cannot be detected with metabarcoding) were
310 removed from the morphotaxonomic dataset, and meiofauna (i.e. taxa that would escape a 1
311 mm mesh net) were removed from the metabarcoding datasets (hereafter unbiased methods).
312 The effect of crucian carp on invertebrate diversity was then assessed at species and family-
313 level according to standard methods (main text) and unbiased methods (Supporting
314 Information) individually and combined. Finally, the impact of crucian carp alongside
315 environmental variables on invertebrate diversity was investigated using the combined
316 standard method (main text) and unbiased method (Supporting Information) datasets. For
317 species-level analyses, only species records were used, whereas family-level analyses included
318 species, genus and family records grouped at the taxonomic rank of family.

We define alpha diversity as taxon richness (species or families) within individual ponds, and beta diversity as the difference between communities present at each pond whilst accounting for taxon identity (Baselga & Orme, 2012). Jaccard dissimilarity was used as a measure of beta diversity for all analyses. For each standard or unbiased dataset, the following analyses were performed. Alpha diversity (response variable) was obtained using the *specnumber* function in vegan v2.5-6 and modelled against sampling method (explanatory variable), then modelled against 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 v1.5.1 (Baselga & Orme, 2012) to estimate total beta diversity, partitioned into nestedness-resultant and turnover components, across sampling methods and ponds using 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 v2.5-6. 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 v2.5-6. 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 $R^2 > 0.25$ respectively. These values are broadly equivalent to correlation coefficients of $r = 0.3$ and 0.5 which represent moderate and strong effects accordingly (Macher et al., 2018; Nakagawa & Cuthill, 2007).

We tested whether the species and family-level invertebrate communities produced by the combined standard or unbiased methods were influenced by pond properties in conjunction with crucian carp presence-absence. Redundancy Analysis (RDA) was selected for constrained ordination as it analyses variation in biological communities in response to explanatory variables (Legendre & Legendre, 2012). Principal Coordinate Analysis (PCoA) was performed using the *pcoa* function in ape v5.3 (Paradis & Schliep, 2018) on the turnover, nestedness-resultant, and total beta diversity matrices. The Lingoes correction was employed to account for negative eigenvalues (Legendre, 2014). The resultant PCoA eigenvectors (principle coordinates) for each distance matrix were used as the response variable in variance partitioning. Our variables were grouped as biotic (crucian carp presence-absence) or abiotic (pond conductivity, area, depth, percentages of perimeter with emergent vegetation, emergent macrophyte cover, submerged macrophyte cover, and shading). Abiotic variables were log10 transformed to eliminate their physical units (Legendre & Birks, 2012). Significant abiotic variables influencing each component of beta diversity were identified using the *ordiR2step* function in vegan v2.5-6 to perform separate RDA analyses under a forward selection procedure. Where applicable, the relative contributions of the biotic and abiotic variables on turnover, nestedness-resultant, and total beta diversity for the species and family-level invertebrate communities were assessed by variance partitioning (Borcard, Legendre, & Drapeau, 1992) using the *varpart* function in vegan v2.5-6. For each beta diversity component, RDA was performed using biotic and significant abiotic variables, and variance partitioning used to divide the total percentage of variation explained into unique and shared contributions for biotic and abiotic predictor groups. The *anova* function in vegan v2.5-6 was used to examine the statistical significance of the full model and the unique contributions of each predictor group. We report the adjusted R^2-fractions as they are widely recommended and unbiased (Peres-Neto, Legendre, Dray, & Borcard, 2006).

375 # 3. Results

376

377 ## 3.1 Comparison of methods for freshwater invertebrate assessment

378

379 Summaries of taxon detection by standard and unbiased methods are reported in the
380 Supporting Information (Appendix 2; Tables S4, S5, S6, S7; Figs. S9, S10). Briefly, using
381 microscopy, we identified 2,281 specimens belonging to 38 families, of which 1,404 specimens
382 belonged to 91 species. With DNA metabarcoding, 2,906,869 sequence reads were assigned to
383 55 families, of which 2,448,078 sequence reads were assigned to 131 species. With eDNA
384 metabarcoding, 813,376 sequence reads were assigned to 90 families, of which 346,163
385 sequence reads were assigned to 145 species. Only 19 species (Figs. 1ai, bi) and 22 families
386 (Figs. 1aii, bii) were shared by all three methods (standard or unbiased). Standard methods
387 detected more distinct taxa than unbiased methods, especially morphotaxonomic identification
388 and eDNA metabarcoding. eDNA metabarcoding detected the most unique species and
389 families, whereas DNA metabarcoding and morphotaxonomic identification were more
390 comparable (Fig. 1). The number of taxa that morphotaxonomic identification shared with DNA
391 and eDNA metabarcoding was unchanged using standard or unbiased methods. Detection of
392 one family was exclusive to morphotaxonomic identification and eDNA metabarcoding,
393 whereas detection of 36 species and 13 families was exclusive to morphotaxonomic
394 identification and DNA metabarcoding (Fig. 1). Morphotaxonomic identification detected 19
395 species without reference sequences, whereas DNA and eDNA metabarcoding detected 4 and
396 41 meiofaunal species respectively (Fig. 1a). The metabarcoding approaches either shared 44
397 species and 16 families (standard methods, Fig. 1a) or 33 species and 8 families (unbiased
398 methods, Fig. 1b).

399 Standard sampling method influenced alpha diversity of ponds at species (GLM χ^2_2 =
400 26.058, $P < 0.001$) and family-level (GLM χ^2_2 = 48.238, $P < 0.001$). Significant differences ($P <$
401 0.05) between the alpha diversity means for morphotaxonomic identification and DNA
402 metabarcoding, morphotaxonomic identification and eDNA metabarcoding, and DNA and eDNA
403 metabarcoding (family-level only) were observed at species and family-level (Table 1).

Conversely, unbiased sampling method influenced alpha diversity of ponds at species-level (GLM χ^2_2 = 14.614, P < 0.001), but not family-level (GLM χ^2_2 = 5.214, P = 0.074). Significant differences between the species-level alpha diversity means of morphotaxonomic identification and DNA metabarcoding, and DNA and eDNA metabarcoding were observed (Table 1). With standard methods, species and family-level alpha diversity was highest using eDNA metabarcoding, followed by DNA metabarcoding then morphotaxonomic identification (Figs. 2ai, aii). With unbiased methods, DNA metabarcoding detected more species than morphotaxonomic identification and eDNA metabarcoding, but alpha diversity was comparable at family-level (Figs. 2bi, bii). For beta diversity, MVDISP did not significantly differ between standard or unbiased methods for turnover, nestedness-resultant or total beta diversity at either taxonomic rank (Table S8). Both standard and unbiased sampling method had moderate and strong positive effects on turnover and total beta diversity at species (Figs. 3ai, aiii, ci, ciii) and family-level (Figs. 3aii, aiv, cii, civ) respectively, but not nestedness-resultant (Table S9).

3.2 Impact of crucian carp stocking on pond invertebrates

Here, we report the impact of crucian carp stocking assessed using standard methods as these provide the most comprehensive evaluation of invertebrate diversity. Crucian carp impact assessment using unbiased methods is reported in the Supporting Information (Appendix 2; Tables S11, S12, S13, S14; Figs. S13, S14, S15, S16). Independently and combined, standard methods showed alpha diversity of invertebrates to be marginally lower in ponds containing crucian carp at species (Figs. 4ai-iv) and family-level (Figs. 4bi-iv), but this difference was not statistically significant (P > 0.05, Table 2). Detailed examination of alpha diversity within the major invertebrate groups identified by all three methods combined revealed that Coleoptera and Mollusca diversity was reduced (Coleoptera borderline significant and Mollusca highly significant) in ponds with crucian carp at species-level (GLM χ^2_{21} = 28.033, P = 0.139; Coleoptera -0.481 ± 0.254, Z = -1.897, P = 0.058; Mollusca -0.925 ± 0.278, Z = -3.324, P < 0.001), but not family-level (GLM χ^2_{22} = 18.366, P = 0.684; Coleoptera -0.544 ± -0.296, Z = -1.834, P = 0.067; Mollusca -0.442 ± -0.302, Z = -1.462, P = 0.144). However, differences in alpha diversity

433 between ponds with or without crucian carp were not significant for other invertebrate groups
434 at either taxonomic rank (Fig. S11). Alpha diversity relationships held true for the unbiased
435 methods (Table S11; Fig. S13, S14).

436 Total beta diversity of ponds was consistently high at species and family-level for
437 independent and combined methods (standard or unbiased). Variation in invertebrate
438 community composition was driven by turnover rather than nestedness-resultant (Tables 3,
439 S12). MVDISP differed between ponds for species-level total beta diversity with eDNA
440 metabarcoding, and species and family-level total beta diversity with all methods combined.
441 Fishless ponds had significantly lower dispersion than ponds with crucian carp (Table S10).
442 MVDISP patterns were not consistent across standard and unbiased methods (Table S13). At
443 species-level, morphotaxonomic identification, eDNA metabarcoding, and all methods
444 combined revealed a weak or moderate positive influence of crucian carp presence on turnover
445 (Table 4; Figs. 5ai, iii, iv) and total beta diversity (Table 4; Figs. 5ci, iii, iv) between ponds, but
446 not nestedness-resultant (Table 4; Figs. 5bi, iii, iv). At family-level, eDNA metabarcoding and all
447 methods combined revealed a weak positive influence of crucian carp presence on total beta
448 diversity (Table 4; Fig. S12ciii) between ponds, but not turnover (Table 4; Fig. S12aiii) or
449 nestedness-resultant (Table 4; Fig. S12biii). Crucian carp presence did not influence any beta
450 diversity component produced by DNA metabarcoding (Table 4; Figs. 5aii, bii, cii). Similar beta
451 diversity patterns were obtained using unbiased eDNA metabarcoding and combined methods
452 (Table S14; Figs. S15, S16).

453 Additional analyses undertaken on the combined method datasets supported an effect
454 of crucian carp presence and excluded the influence of abiotic variables on invertebrate
455 diversity. At species-level, only pond area was identified by forward selection as a significant
456 abiotic variable for turnover and total beta diversity. Variance partitioning analysis was
457 undertaken for turnover and total beta diversity using crucian carp presence-absence and pond
458 area. Using the adjusted R^2 values, biotic and abiotic variables explained 7.04% and 4.75% of
459 the total variation in species turnover and total beta diversity respectively (Fig. 6). Crucian carp
460 presence-absence significantly contributed to turnover (Fig. 6a; adjusted R^2 = 5.55%, F_1 = 2.031,
461 P = 0.001) and total beta diversity (Fig. 6b; adjusted R^2 = 4.05%, F_1 = 1.730, P = 0.001), whereas

pond area explained less variance and did not influence turnover (Fig. 6a; adjusted R^2 = 2.94%, F_1 = 0.340, P = 0.852) or total beta diversity (Fig. 6b; adjusted R^2 = 1.93%, F_1 = 1.118, P = 0.218). RDA of nestedness-resultant without abiotic data indicated no influence of crucian carp presence-absence (F_1 = 0.340, P = 0.850). At family-level, forward selection did not identify any significant abiotic variables for turnover, nestedness-resultant, or total beta diversity. Therefore, variance partitioning was not undertaken for any component of beta diversity. RDA of each beta diversity component minus abiotic data showed that crucian carp presence-absence influenced total beta diversity (F_1 = 1.474, P = 0.034), but not turnover (F_1 = 1.399, P = 0.106) or nestedness-resultant (F_1 = 1.970, P = 0.141). Variance partitioning results from the unbiased methods are presented in Appendix 2.

4. Discussion

4.1 Comparison of methods for freshwater invertebrate assessment

DNA (unbiased methods) or eDNA (standard methods) metabarcoding generated the highest species and family-level alpha diversity. Differences in alpha diversity were more pronounced between standard methods, but unique species were detected with unbiased methods nonetheless. Morphotaxonomic identification and DNA metabarcoding produced more similar communities, and performed best for Coleoptera, Hemiptera, Hirudinea, Megaloptera, and Odonata. eDNA metabarcoding generated a markedly different community, and detected aquatic taxa overlooked in pond net samples (Crustacea, Lepidoptera [i.e. Crambidae species with aquatic larvae such as *Cataclysta lemnata*], Trichoptera) alongside singleton terrestrial taxa (Arachnida, Hymenoptera, Psocoptera, Thysanoptera). Both metabarcoding approaches performed well for Annelida, Collembola, Diptera and Ephemeroptera, particularly at species-level.

Critically, we used a 1 mm mesh net which is designed to sample macroinvertebrates, whereas a 500 µm mesh net would also sample the meiofauna detected by metabarcoding.

491 Meiofauna may inflate metabarcoding diversity estimates compared to morphotaxonomic
492 identification, but may also prevent PCR amplification of macroinvertebrates (Beentjes et al.,
493 2019; Deiner et al., 2016; Macher et al., 2018). To mitigate bias, we performed all analyses
494 excluding meiofauna not captured by netting and excluding morphotaxonomically identified
495 taxa without reference sequences that metabarcoding would not recover. Alpha diversity
496 results from the standard and unbiased method comparisons were variable, but beta diversity
497 results were more consistent. Both method comparisons echo other studies where
498 metabarcoding captured greater diversity than morphotaxonomic approaches and resolved
499 problematic groups (e.g. Diptera) for species-level morphotaxonomic identification (Andújar et
500 al., 2017; Carew et al., 2018; Clarke, Beard, Swadling, & Deagle, 2017; Elbrecht et al., 2017a;
501 Emilson et al., 2017; Klymus et al., 2017; Lobo et al., 2017). Standard and unbiased methods
502 broadly revealed the same ecological relationships, although standard methods may yield more
503 insights. Regardless, all three methods will be most powerful when used in combination for
504 ecological investigation.

505 Each method has inherent biases that influence performance for freshwater
506 invertebrate assessment. The UK National Pond Survey methodology recommends that pond
507 net samples are placed into buckets/zip-lock bags, followed by sorting and identification in the
508 laboratory as soon as possible. Samples are typically preserved in ethanol or refrigerated to
509 prolong processing time (Biggs et al., 1998), but this standard methodology does not lend itself
510 to molecular applications. After collection, samples were transported on ice then frozen at -20
511 °C to prevent predation within samples, minimise organismal decay and subsequent DNA
512 degradation, and allow samples to be processed as required in the laboratory. Using this
513 strategy, small or inconspicuous dead specimens would not have been recovered during sorting
514 (Biggs et al., 1998) and therefore excluded from morphotaxonomic and DNA metabarcoding
515 inventories. These losses are surplus to the estimated 29% of specimens already overlooked
516 during sorting due to smaller body size (Haase et al., 2010). Sorting may also damage or
517 completely destroy some recovered specimens, influencing morphotaxonomic identification
518 (Lobo et al., 2017; Zizka, Leese, Peinert, & Geiger, 2018).

Human error is another source of discrepancy between morphotaxonomic identification and metabarcoding (Carew, Pettigrove, Metzeling, & Hoffmann, 2013; Elbrecht et al., 2017a; Haase et al., 2010). Some taxa were identified to genus or family-level and omitted from our species-level inventories, whereas other taxa may have been falsely identified and inventoried despite caution exercised to only assign taxa to species-level where there was high confidence in identification. Losses incurred by the preservation and sorting treatments for morphotaxonomic identification cannot be mitigated, and human error can only be reduced with taxonomic expertise. Conversely, taxon recovery by DNA metabarcoding can be improved with protocols that preserve and extract DNA from intact, unsorted samples (Elbrecht et al., 2017a; Nichols et al., 2020). For example, ethanol used to preserve specimens (Zizka et al., 2018), or replacement of ethanol with DNA extraction buffer followed by incubation then specimen removal (Carew, Coleman, & Hoffmann, 2018), offer alternative starting materials to bulk tissue for DNA metabarcoding. However, these alternative DNA sources tend to produce false negatives for schlerotised groups, such as Coleoptera, Trichoptera, and Mollusca (Carew et al., 2018; Zizka et al., 2018). More recently, Nichols et al. (2020) demonstrated that unsorted samples, including the sample matrix, recovered the same taxa as sorted samples, thus sorting may be unnecessary.

Despite successful application of DNA metabarcoding to bulk tissue samples for biomonitoring (Andújar et al., 2017; Carew et al., 2018; Elbrecht et al., 2017a; Emilson et al., 2017; Macher et al., 2018), recurring issues have been encountered. Arguably, the most pressing issue is size bias, where DNA from large and/or high biomass taxa can outcompete DNA of smaller and/or low biomass taxa during PCR amplification and sequencing (Elbrecht et al., 2017b). Although we used the size-sorting approach of Elbrecht *et al*. (2017b) and sequenced body size categories independently with data pooling downstream, DNA metabarcoding failed to detect several taxa which have reference sequences and were reliably identified by microscopy, including multiple coleopterans (*Agabus sturmii*, *Hydroporus erythrocephalus*, *Rhantus frontalis*, *Haliplus confinis*, *Haliplus ruficollis*), a small mollusc (*Gyraulus crista*), a medium-sized hirudinean (*Erpobdella lineata*), and two large anisopterans (*Aeshna mixta*, *Anax imperator*). Primer bias may be partly responsible for these nondetections

548 (see below), but non-delivery of desired results by size-sorting is problematic as it is a time-
549 consuming, labour-intensive process that potentially increases cross-contamination risk
550 between samples (Elbrecht et al., 2017b), and still requires samples to be sorted from
551 vegetation and substrate (Elbrecht et al., 2017a). Size-sorting may therefore be a drain on
552 resources and time allocated to DNA metabarcoding projects, but other sources of false
553 negatives must be excluded.

554 In contrast, eDNA metabarcoding preferentially amplifies DNA from small, floating
555 organisms (Beentjes et al., 2019; Deiner et al., 2016; Leese et al., 2020; Macher et al., 2018),
556 and these organisms are often retained on filter membranes used for eDNA capture (*pers.*
557 *obs.*). Consequently, their DNA is abundant in samples and overwhelms DNA from other taxa
558 during sequencing. Pre-filtering with or only using large pore size filter membranes may reduce
559 this bias (Macher et al., 2018). eDNA metabarcoding may also detect different taxa due to
560 species variability in eDNA production and shedding rates. The species infrequently detected by
561 eDNA metabarcoding were those that possess thicker exoskeletons composed of chitin and
562 occasionally calcium carbonate, e.g. Coleoptera, Hemiptera. Exoskeletons may restrict the
563 release of DNA into the water column (Tréguier et al., 2014) as opposed to organisms that are
564 filter-feeders or produce slime (e.g. Mollusca), ectoparasites (i.e. Acari) that feed on blood or
565 skin of other species, or use external versus internal fertilisation (Mächler et al., 2019; Wacker
566 et al., 2019). Furthermore, species may differ in their habitat preferences and utilisation,
567 potentially resulting in highly localised distributions (Klymus et al., 2017; Mächler et al., 2019).
568 Therefore, more samples or greater volumes may be required to improve detection of pond
569 biota (Harper et al., 2019).

570 Marker choice and primer design can substantially influence amplification success and
571 taxonomic assignment. Although the *COI* region offers species resolution, has extensive
572 database representation, and is used as standard in DNA barcoding (Elbrecht, Hebert, &
573 Steinke, 2018), it lacks conserved primer-binding sites as a protein-coding gene (Clarke et al.,
574 2017; Leese et al., 2020). This introduces high risk of primer mismatch and subsequent bias
575 during metabarcoding (Clarke et al., 2017; Elbrecht et al., 2016; Elbrecht et al., 2018; Lobo et
576 al., 2017). Most *COI* metabarcoding primers (Elbrecht & Leese, 2017; Leray et al., 2013;

Meusnier et al., 2008; Zeale, Butlin, Barker, Lees, & Jones, 2011) are degenerate to enable binding, but degenerate primers may bind non-target regions (Elbrecht et al., 2018) and amplify non-metazoans, e.g. bacteria, algae, fungi (Brandon-Mong et al., 2015; Leese et al., 2020; Macher et al., 2018). Degenerate primers can experience primer slippage and produce variable length sequences across species, which has implications for bioinformatic processing and taxonomic assignment (Elbrecht et al., 2018). Using different (e.g. 16S ribosomal RNA) or multiple markers may counter these issues, but different markers often lack reference database representation, hindering taxonomic resolution, and use of multiple markers will increase PCR and sequencing costs (Clarke et al., 2017; Deiner et al., 2016; Elbrecht et al., 2016; Elbrecht et al., 2017a).

We obtained an excessive number (~60%) of unassigned reads from eDNA metabarcoding, despite using a relaxed BLAST identity (90%) against custom and public reference databases. This suggests that sequences were of poor quality, could not be assigned to reference sequences, or lacked reference database representation (Macher et al., 2018). Public reference database records may be few, mislabelled, or have limited geographic coverage (Curry, Gibson, Shokralla, Hajibabaei, & Baird, 2018; Elbrecht et al., 2017a; Klymus et al., 2017; Weigand et al., 2019). We morphotaxonomically identified 19 species (Hemiptera, Mollusca, Odonata) without reference sequences whose DNA may have been sequenced, but would not have been taxonomically assigned to species-level. Future studies must develop more specific primers that have binding and flanking regions of high nucleotide diversity (Elbrecht et al., 2018; Leese et al., 2020) and/or target particular invertebrate orders or families (Klymus et al., 2017) as well as procure reference sequences for different markers (Curry et al., 2018; Elbrecht et al., 2016; Weigand et al., 2019). These are essential steps to improve the reliability and accuracy of molecular monitoring in freshwater ecosystems.

4.2 Impact of crucian carp stocking on pond invertebrates

Our findings indicate that the crucian carp can be an important driver of invertebrate community heterogeneity in ponds. Crucian carp had a negligible influence on alpha diversity

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

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

Critically, only two of the aforementioned studies accounted for the identity of fish

species assemblages present (Stefanoudis et al., 2017; Wood et al., 2001) and just one was based on standardised fish surveys (Stefanoudis et al., 2017), whereas other studies only considered the presence of a particular species (Schilling et al., 2009a) or fish presence-absence generally (Bendell & McNicol, 1995; Gee et al., 1997; Hassall et al., 2011; Schilling et al., 2009b). The contrasting results produced by these studies and our own indicate that the impact of fish on pond biodiversity will likely depend on the species present, population density, and management strategy. Wetland fishes vary in dietary preference and consume different proportions of invertebrate taxa, thus different fishes will suppress numbers of and confer benefits to different invertebrate taxa (Batzer, Pusateri, & Vetter, 2000). Invasive fish species may be more detrimental than non-invasive species, for example, the mosquitofish (*Gambusia affinis*) reduced zooplankton abundance and macroinvertebrate density by 90% and 50% respectively after introduction in a wetland ecosystem experiment (Preston et al., 2017). Additionally, invertebrate species richness and abundance was found to decrease as duration of stocking increased (Schilling et al., 2009a). Therefore, local- and regional-scale diversity may benefit most from pond mosaics that maintain fish-free and fish-containing (at low-moderate densities) ponds to prevent increases in fish biomass and predation pressure over time (Lemmens et al., 2013).

The effects of environmental variables on alpha and beta diversity of pond invertebrates must also be considered (Hassall et al., 2011; Hill, Heino, Thornhill, Ryves, & Wood, 2017). Our study ponds were selected to have similar physical and chemical properties, which may explain the minimal contribution of abiotic factors to variance in invertebrate community structure. Although pond area was retained by model selection, it did not significantly influence community structure and explained less variance than crucian carp presence-absence. Likewise, other studies have shown a weak or no effect of pond area on invertebrate species richness and community composition (Gee et al., 1997; Gledhill et al., 2008; Oertli et al., 2002; Wood et al., 2001). Critically, our environmental data were collected over a 7-year period, whereas contemporary data may have better explained variance in community structure. We omitted variables that experience high temporal variation from our analyses, but these may be linked to the Coleoptera and Mollusca alpha diversity reductions and different invertebrate communities

observed in crucian carp ponds, such as water temperature, pH, nutrient concentration, and surface dissolved oxygen (Bendell & McNicol, 1995; Hassall et al., 2011; Menetrey, Sager, Oertli, & 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 and likely being non-native (Jeffries et al., 2017). Foraging activities of introduced common carp are known to reduce invertebrate density and macrophyte cover, with knock-on effects for amphibian and waterfowl species richness and abundance (Chan, 2011; Haas et al., 2007; Maceda-Veiga et al., 2017). However, all crucian carp ponds studied here 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. Additionally, no impact of crucian carp on amphibian presence, oviposition, larval behaviour, or recruitment success has been found (Chan, 2011; Harper et al., 2019; Jarvis, 2012). Despite its probable non-native status, cumulative evidence suggests that the crucian carp does not have invasive potential. Instead, the crucian carp is naturalised and faces the same threats as native UK pond biodiversity, such as the great crested newt (*Triturus cristatus*), including terrestrialisation, dessication and infilling of ponds as well as predation by other fishes, e.g. pike (*Esox lucius*) and three-spined stickleback (*Gasterosteus aculeatus*) (Sayer et al., 2020). Additionally, the crucian carp is threatened by hybridisation with goldfish, common carp, and gibel carp across Europe (Hänfling et al., 2005). Gibel carp are though to be absent from UK waters and given the severe threat that this species poses to crucian carp persistence in ponds, England can be regarded as an important centre for crucian carp conservation (Jeffries et al., 2017; Sayer et al., 2020). Moreover, pond restoration as part of crucian carp conservation efforts in Norfolk has substantially benefitted macrophyte, invertebrate, amphibian, and farmland bird diversity (Sayer et al., 2013; Lewis-Phillips et al., 2019).

Our study demonstrates that crucian carp presence enhances invertebrate diversity

across pond networks, and that current conservation management of established and stocked populations in England is appropriate. 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, although stocking should only occur in artificially created ponds or in ponds where fish were previously known to occur (Sayer et al., 2020). The crucian carp is likely to be an important species in this respect, but more research is required to verify that crucian carp presence increases community heterogeneity without contributing to the decline of endangered invertebrates, albeit this is unlikely. Furthermore, the impact of crucian carp on all pond biodiversity, especially amphibians, must be studied more broadly with respect to crucian carp population density, seasonality, and the aforementioned environmental variables to determine whether maintaining populations and 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.3993125).

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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1056

1057 **Table 1.** Summary of analyses (Pairwise Tukey's HSD Test) statistically comparing alpha diversity
1058 at species-level and family-level between standard and unbiased methods.
1059

	Species-level			**Family-level**		
	Pairwise Tukey's HSD Test			**Pairwise Tukey's HSD Test**		
	Estimate ± SE	***Z***	***P***	**Estimate ± SE**	***Z***	***P***
Standard methods						
Sweep-netting and microscopy :DNA metabarcoding	-0.399 ± 0.129	-3.087	**0.006**	-0.284 ± 0.108	-2.630	**0.023**
Sweep-netting and microscopy : eDNA metabarcoding	-0.645 ± 0.127	-5.087	**<0.001**	-0.686 ± 0.102	-6.712	**<0.001**
DNA metabarcoding : eDNA metabarcoding	0.246 ± 0.121	2.030	0.105	0.402 ± 0.096	4.183	**<0.001**
Unbiased methods						
Sweep-netting and microscopy : DNA metabarcoding	-0.463 ± 0.128	-3.632	**<0.001**	-0.134 ± 0.106	-1.267	0.414
Sweep-netting and microscopy : eDNA metabarcoding	0.128 ± 0.132	-0.973	0.594	0.072 ± 0.110	0.650	0.792
DNA metabarcoding : eDNA metabarcoding	-0.335 ± 0.125	-2.671	**0.021**	-0.206 ± 0.108	-1.915	0.134

1060

1061 **Table 2.** Summary of analyses (GLM) statistically comparing alpha diversity at species-level and
1062 family-level between ponds with and without crucian carp using independent and combined
1063 standard methods.
1064

	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	0.060 ± 0.141	0.429	0.668	0.184	0.668	1	-0.007 ± 0.122	-0.061	0.952	0.004	0.952
eDNA metabarcoding	1	-0.072 ± 0.194	-0.375	0.708	0.141	0.708	1	-0.173 ± 0.140	-1.234	0.217	1.524	0.217
Combined methods	1	-0.062 ± 0.122	-0.506	0.613	0.256	0.613	1	-0.128 ± 0.095	-1.349	0.177	1.822	0.177

1065
1066

1067 **Table 3.** Relative contribution of taxon turnover and nestedness-resultant to total beta diversity
1068 (Jaccard dissimilarity) using independent and combined standard methods. A value of 1
1069 corresponds to all sites containing different species.
1070

	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	0.938 (98.53%)	0.014 (1.47%)	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%)	0.871 (95.29%)	0.043 (4.71%)	0.914 (100%)
Combined methods	0.927 (98.41%)	0.015 (1.59%)	0.942 (100%)	0.871 (96.46%)	0.032 (3.54%)	0.903 (100%)

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1072 **Table 4.** Summary of analyses (PERMANOVA) statistically examining variation in community
1073 composition between ponds with and without crucian carp at species-level and family-level
1074 using independent and combined standard methods.
1075

	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	**0.018**	1	1.198	0.070	0.358
Nestedness-resultant	1	-3.454	-0.275	0.949	1	-0.103	-0.007	0.620
Total beta diversity	1	1.369	0.079	**0.033**	1	1.136	0.066	0.306
DNA metabarcoding								
Turnover	1	1.305	0.075	0.137	1	1.668	0.094	0.098
Nestedness-resultant	1	-0.578	-0.038	0.664	1	-0.116	-0.007	0.717
Total beta diversity	1	1.163	0.068	0.176	1	1.277	0.074	0.197
eDNA metabarcoding								
Turnover	1	2.374	0.129	**0.001**	1	1.551	0.088	0.097
Nestedness-resultant	1	-2.515	-0.187	0.967	1	0.586	0.035	0.560
Total beta diversity	1	1.790	0.101	**0.004**	1	1.502	0.086	**0.041**
Combined methods								
Turnover	1	1.999	0.111	**0.001**	1	1.452	0.083	0.136
Nestedness-resultant	1	-1.663	-0.116	0.966	1	3.103	0.162	0.128
Total beta diversity	1	1.717	0.097	**0.001**	1	1.474	0.084	**0.027**

1076

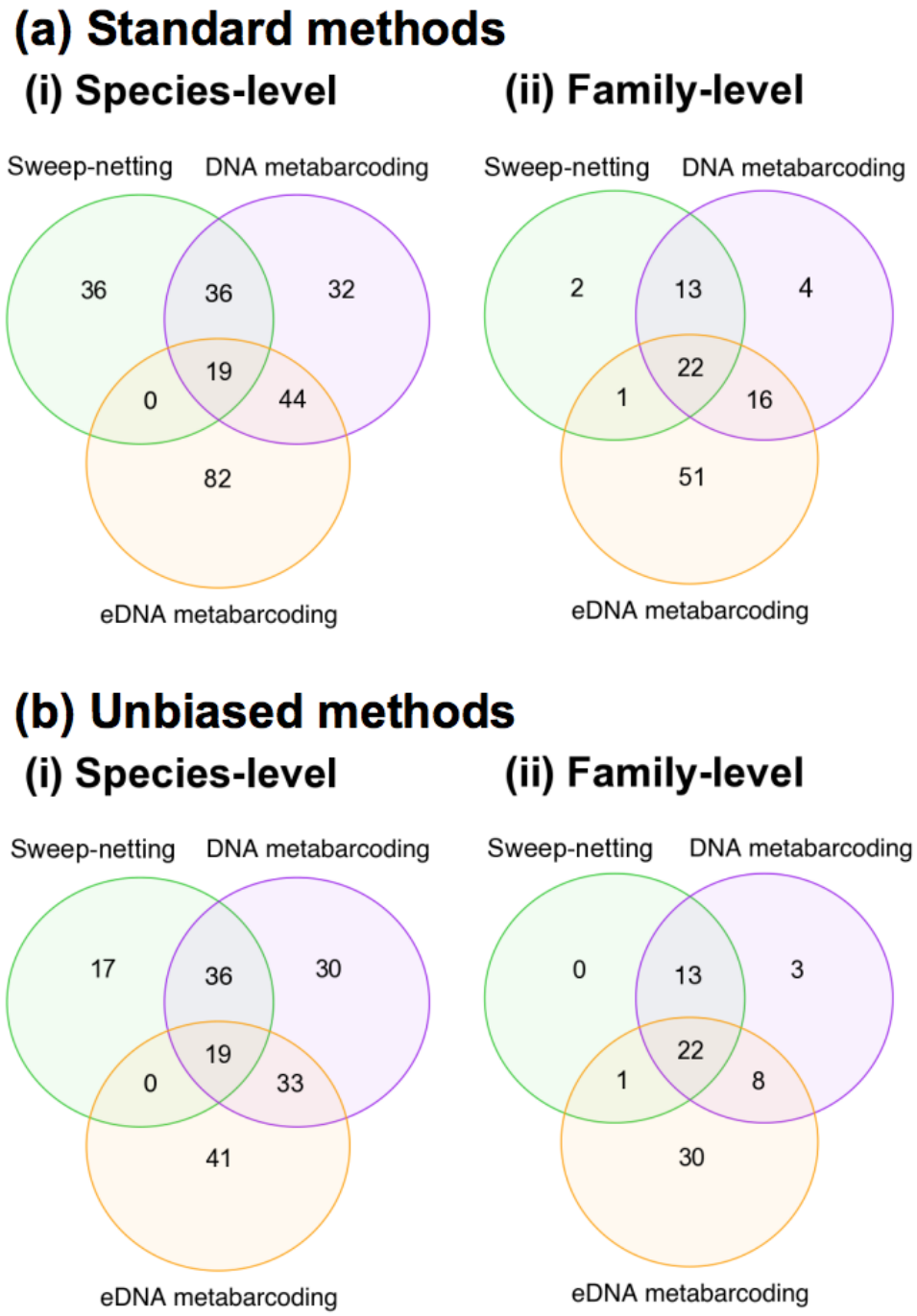

Figure 1. Venn diagrams summarising taxon detection by standard **(a)** and unbiased **(b)** methods of invertebrate assessment. The number of invertebrate species **(i)** and families **(ii)** detected across the 18 study ponds by sweep-netting and microscopy (green circle), DNA metabarcoding (purple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

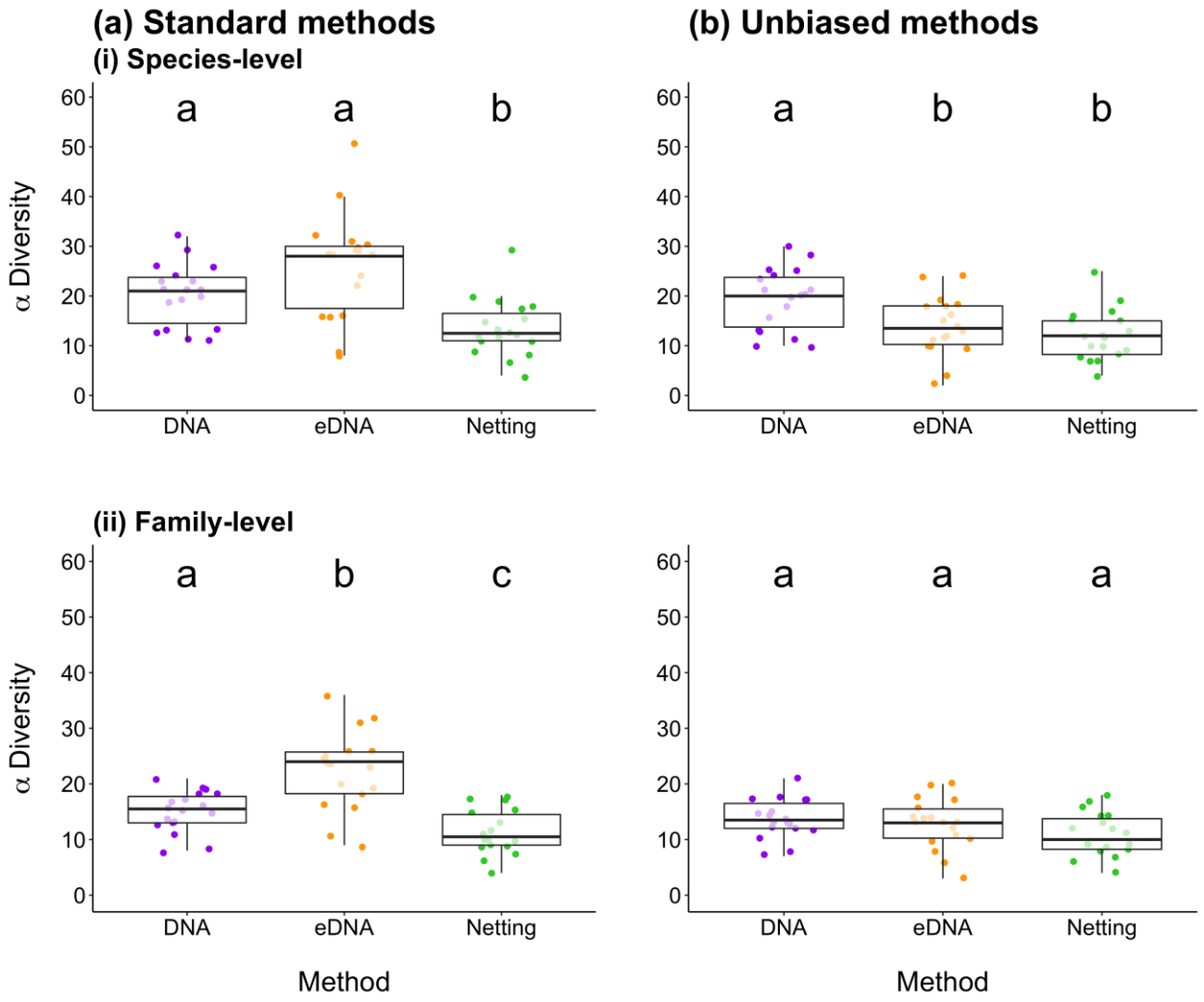

Figure 2. Mean alpha diversity of invertebrates in ponds across Norfolk and East Yorkshire. Alpha diversity is displayed according to standard **(a)** and unbiased **(b)** methods of invertebrate assessment. Species **(i)** and family **(ii)** richness generated by sweep-netting and microscopy (green 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.

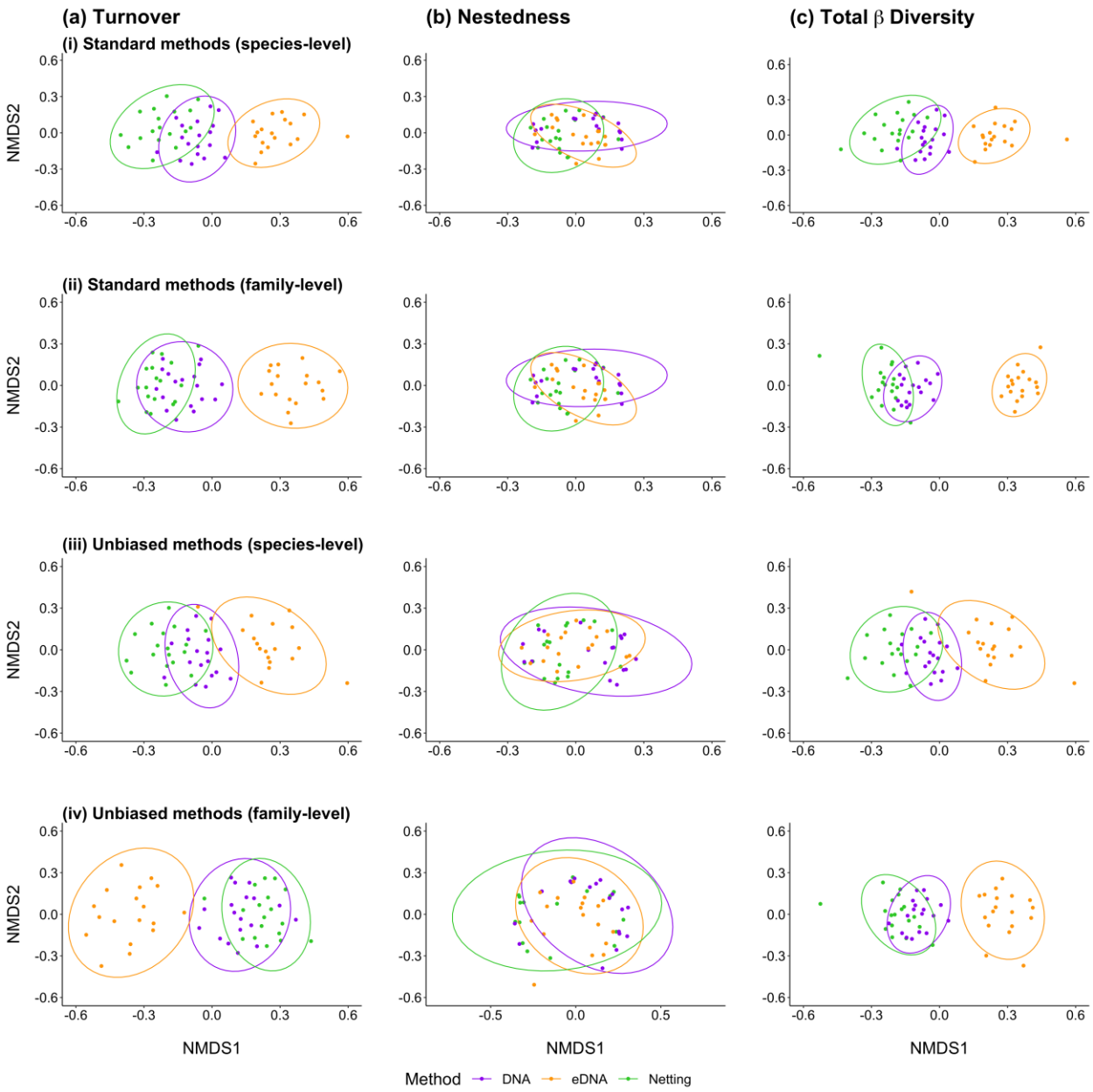

Figure 3. Non-metric Multidimensional Scaling (NMDS) plots of invertebrate communities (Jaccard dissimilarity) produced by sweep-netting and microscopy (green points/ellipse), DNA metabarcoding (purple points/ellipse), and eDNA metabarcoding (orange points/ellipse) for the 18 study ponds. The turnover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

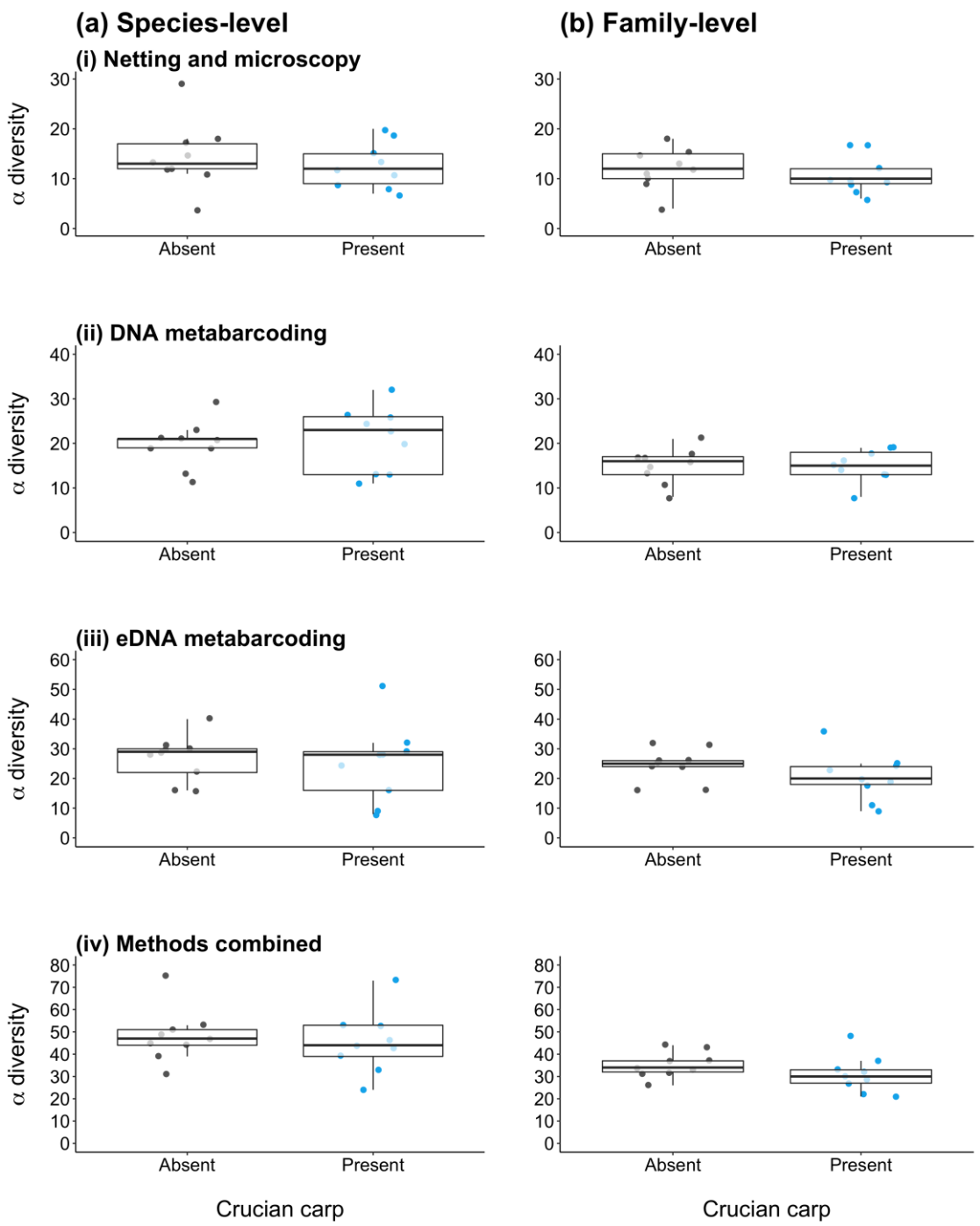

Figure 4. Mean alpha diversity of invertebrates in ponds with crucian carp (blue points) and without fish (grey points) across Norfolk and East Yorkshire. Alpha diversity at species-level **(a)** and family-level **(b)** is shown according to standard methods of invertebrate assessment: sweep-netting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

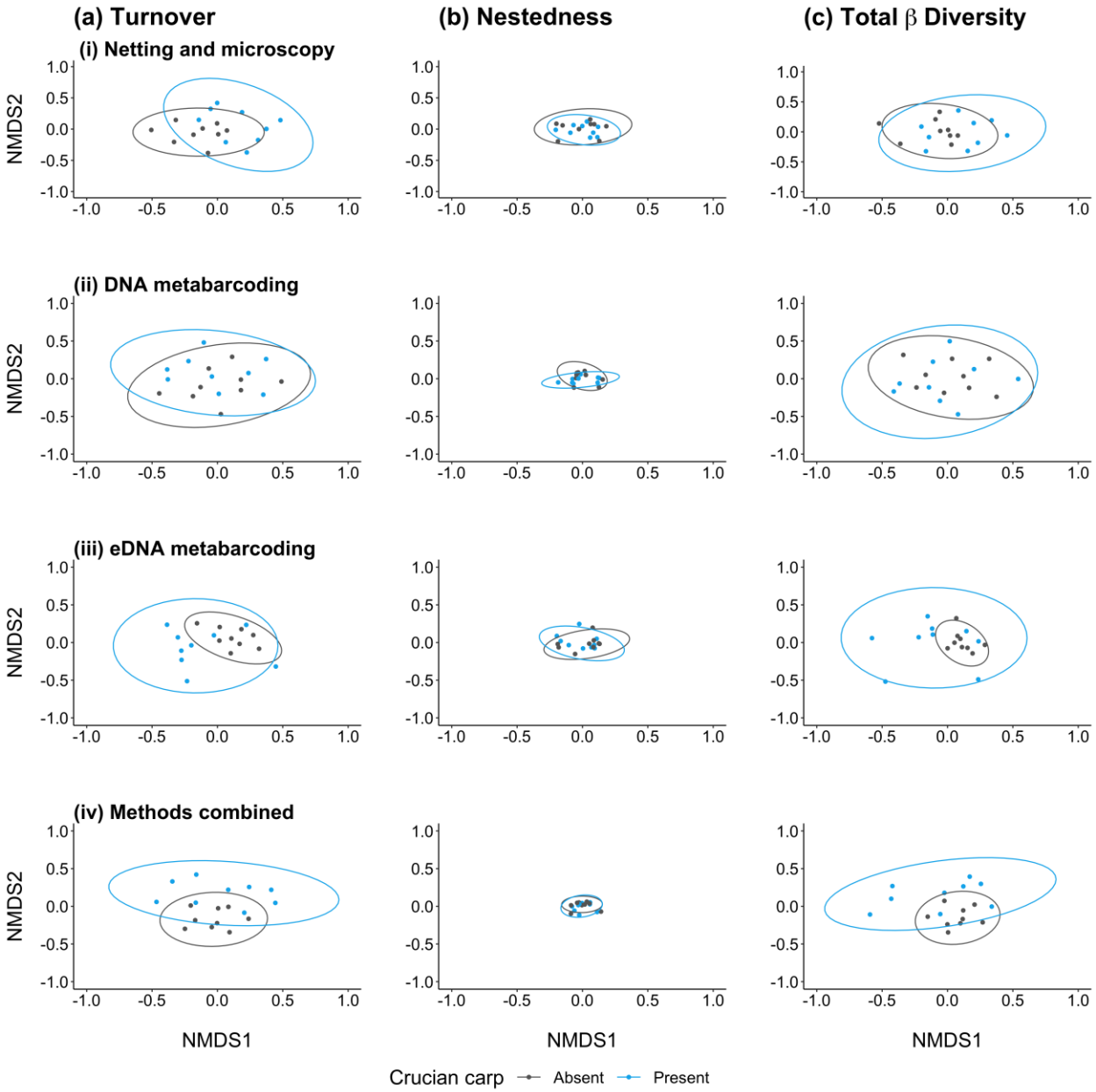

Figure 5. Non-metric Multidimensional Scaling (NMDS) plots of species-level invertebrate communities (Jaccard dissimilarity) from ponds with crucian carp (blue points/ellipse) and without fish (grey points/ellipse) across Norfolk and East Yorkshire. The turnover **(a)** and nestedness-resultant **(b)**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

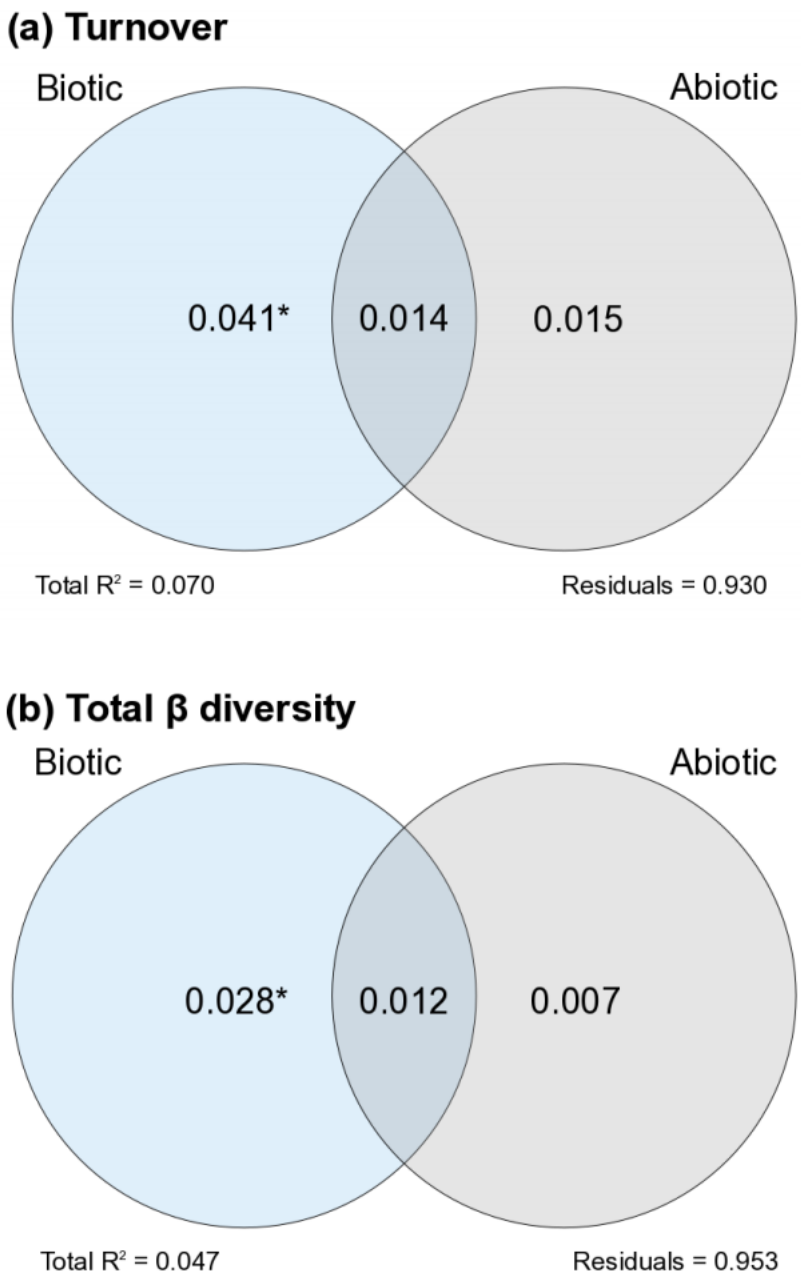

Figure 6. The relative contribution of biotic (crucian carp presence-absence) and abiotic variables (pond area) to species-level turnover **(a)** and total beta diversity **(b)** when the combined invertebrate data from all three standard sampling methods were considered. Values within circles and circle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