Development of an environmental DNA method for monitoring fish communities: ground truthing in diverse lakes with characterised fish faunas

Running title: eDNA method for lake fish monitoring

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Abstract

1. Accurate, cost-effective monitoring of fish is required to assess the quality of lakes under the European Water Framework Directive (WFD). Recent studies have shown that environmental DNA (eDNA) metabarcoding is an effective and non-invasive method, which can provide semi-quantitative information on fish communities in large lakes.
2. This study further investigated the potential of eDNA metabarcoding as a tool for WFD status assessment by collecting and analysing water samples from eight Welsh lakes and six meres in Cheshire, England, with well described fish faunas. Water samples ($N = 252$) were assayed using two mitochondrial DNA regions (Cytb and 12S rRNA).
3. eDNA sampling indicated the presence of very similar species in the lakes compared to those expected on the basis of existing and historical information. In total, 24 species were detected with a total of 111 species occurrences in the lakes studied using eDNA. Secondly, there was a significant positive correlation between expected faunas and eDNA data in terms of confidence of species occurrence (Spearman's $r = 0.74$, $df = 109$, $p < 0.001$). Thirdly, eDNA data can estimate relative abundance with the standard five-level classification scale ("DAFOR"). Lastly, four ecological fish communities were characterised using eDNA data which agrees with the pre-defined lake types according to environmental characteristics.
4. *Synthesis and applications*. This study provides further evidence that eDNA metabarcoding could be a powerful and non-invasive monitoring tool for WFD purpose in a wide range of lake types, considerably outperforming other methods for community level analysis.

Key-words: eDNA, metabarcoding, fish monitoring, community ecology, EC Water Framework Directive, lakes, lentic systems

Introduction

The impact of anthropogenic pressures on aquatic ecosystems is ubiquitous and usually leads to alterations of the environment (Scheffer et al. 2001). To mitigate negative human effects, the first step in the improvement of the ecological quality of degraded water bodies is the development of an assessment system to evaluate the current situation. The main European initiative for water quality assessment and improvements, the Water Framework Directive 2000/60/EC (WFD), requires all member states to reach "good" ecological status of lakes, rivers and ground waters based on biological elements including phytoplankton, macrophytes and phytobenthos, benthic invertebrates and fish (CEC 2000).

Fish are widely considered as relevant for detecting and quantifying impacts of anthropogenic pressures on lakes and reservoirs (Argillier et al. 2013). Although most UK WFD ecological tools have been developed and deployed, an effective fish-based assessment tool for lakes remains problematic (Winfield 2002; Kelly et al. 2012). The current fish-based indices (e.g. Argillier et al. 2013) or tools (e.g. "FIL2" in Kelly et al. 2012) rely on semi-quantitative capture-based methods such as electro-fishing or gill-netting to provide information on fish composition and abundance, as well as age-structure of fish populations. Furthermore, the choice of survey methods is heavily dependent on the depth and size of lake. However, both of these sampling methods are relatively laborious and thus expensive, sometimes destructive, taxonomically biased, cannot be deployed in all situations (e.g. in or near dense vegetation, or entire water column in a very deep lake) and have poor sampling accuracy and precision. These drawbacks restrict their ability to meet WFD requirements (Kubecka et al. 2009; Winfield et al. 2009). A future strategy for WFD purposes thus requires a highly cost-effective approach, with a minimum amount of destructive sampling.

A significant "game-changer" in biodiversity monitoring in recent years is the analysis of environmental DNA (eDNA) which is a non-invasive genetic method that takes advantage of

71 intracellular or extra-organismal DNA in the environment to detect the presence of organisms
72 (Lawson Handley 2015; Thomsen & Willerslev 2015; Taberlet et al. 2018). A particularly
73 promising approach is to simultaneously screen whole communities of organisms using
74 eDNA metabarcoding. Several studies have shown that eDNA metabarcoding using High-
75 Throughput Sequencing offers tremendous potential as a complementary method tool to
76 established monitoring methods for ecology and conservation of aquatic species (e.g.
77 Hänfling et al. 2016; Port et al. 2016; Valentini et al. 2016). After reviewing and discussing
78 the potential of eDNA metabarcoding as a tool for WFD status assessment, Hering et al.
79 (2018) suggested that this approach is well-suited for fish biodiversity assessment
80 considering representativeness, sensitivity, precision, comparability, cost-effectiveness, and
81 environmental impact. The mitochondrial COI gene is widely, but not unanimously accepted
82 as the standard metabarcode for metazoans (e.g. Riaz et al. 2011; Clarke et al. 2014).
83 However, with environmental samples, most primers targeting the COI region amplify large
84 proportions of microbial species (e.g. Stat et al. 2017). This fact remains the strongest reason
85 for the use of other genes such as mitochondrial cytochrome b (Cytb) and 12S rRNA (12S)
86 regions. A prototype eDNA tool assayed by Cytb and 12S regions for fish biodiversity
87 assessment was tested in three lakes in the English Lake District, and initial results from this
88 are promising (Hänfling et al. 2016). However, in order to understand factors such as
89 responses to ecological pressures, taxon specific biases, sampling requirements in different
90 lake types and management of low confident occurrence, a much larger dataset from a range
91 of lakes with different ecological characteristics is required. Thus, the objectives of this study
92 were (a) to broaden the lake fish dataset using eDNA-based metabarcoding analysis by
93 collecting and analysing water samples from 14 UK lakes with well described fish faunas; (b)
94 to explore a possible approach to evaluate the confidence of species presence based on site

occupancy and read counts and (c) to use a relative abundance scale to estimate species abundance by comparing the eDNA metabarcoding results to historical data.

Materials and methods

Study sites

The distribution of the 14 lakes including eight Welsh lakes and six Cheshire meres are shown in Figure 1, and the general characteristics are outlined in Appendix S1 Table S1.1 based on data from the UK Lakes Portal (Hughes et al. 2004). In brief, the 14 lakes can be divided into three types according to environmental characteristics including Type 1: three low alkalinity lakes that are broadly upland in character ("CWE" Llyn Cwellyn, "PAD" Llyn Padarn and "OGW" Llyn Ogwen); Type 2: two high alkalinity but shallow lakes ("PEN" Llyn Penrhyn and "TRA" Llyn Traffwll) on the west coast of Anglesey (North Wales) which are close to sea and accessible for migratory fish, and Type 3: nine high alkalinity but shallow lakes that are dominated by coarse fish ("KEN" Kenfig Pool, "LLB" Llan Bwch-llyn, "LLG" Llangorse Lake, "MAP" Maer Pool, "CAM" Chapel Mere, "OSS" Oss Mere, "FEN" Fenemere, "WLF" Watch Lane Flash and "BET" Betley Mere) (Appendix S2).

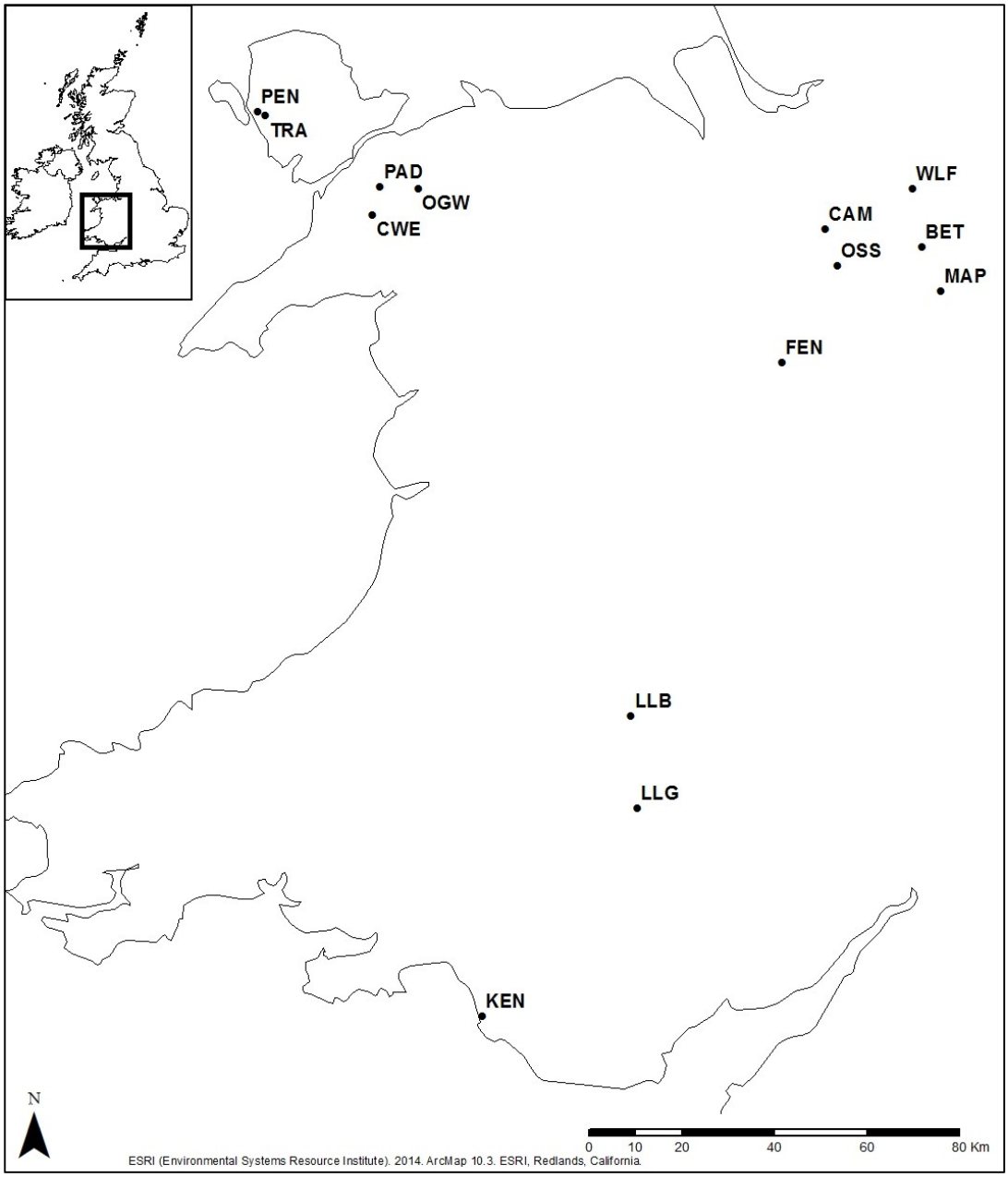


Figure 1 Distribution of sampling lakes. Sampling lake codes: (CWE) Llyn Cwellyn; (PAD) Llyn Padarn; (OGW) Llyn Ogwen; (PEN) Llyn Penrhyn; (TRA) Llyn Traffwll; (KEN) Kenfig Pool; (LLB) Llan Bwch-llyn; (LLG) Llangorse Lake; (MAP) Maer Pool; (CAM) Chapel Mere; (OSS) Oss Mere; (FEN) Fenemere; (WLF) Watch Lane Flash; (BET) Betley Mere. The GPS coordinates of sampling locations in each lake are listed in Appendices S3 & S4.

eDNA capture, extraction, library preparation and sequencing

eDNA capture and extraction

In total, 252 samples were collected from the 14 lakes between 01/12/2015 to 12/07/2016 (Appendix S1 Table S1.2). The detail of sampling strategy can be found in Appendix S5.1. All samples were filtered within 24 hrs of collection. Two litres of each water sample were filtered through a 47 mm diameter 0.45-µm mixed cellulose acetate and nitrate filter (Whatman, UK) using Nalgene filtration units in combination with a vacuum pump (15–20 in. Hg; Pall Corporation, USA). Our previous study demonstrated that the 0.45-µm filters are suitable for fish metabarcoding, with low variation and high repeatability between the filtration replicates (Li et al. 2018a) compared to other filtration methods. The filtration units were cleaned with 10% v/v commercial bleach solution (containing ~3% sodium hypochlorite) and 5% v/v microsol detergent (Anachem, UK), and then rinsed thoroughly with de-ionised water after each filtration run to prevent cross-contamination. For each sampling lake, a filtration blank (2 L deionised water) was filtered before sample filtration in order to test for possible contamination at the filtration stage. After filtration, all filters were placed into 50 mm sterile petri dishes using sterile tweezers, sealed with parafilm and then immediately stored at –20°C until extraction. DNA extraction was carried out using the DNeasy PowerWater Kit (QIAGEN, Germany) following the manufacturer's protocol. The DNA was eluted in 100 µL 10 mM Tris (Solution PW6) and stored at –20 °C freezer.

Library preparation and sequencing

Sequencing libraries were generated from PCR amplicons targeting Cytb and 12S. We previously tested both fragments *in vitro* on 22 UK freshwater fish species and *in situ* on

143 three deep lakes in the English Lake District, and demonstrated their suitability for eDNA
144 metabarcoding of UK lake fish communities (Hänfling et al. 2016).

145 To enable the detection of possible PCR contamination, we included no-template controls
146 (NTCs, i.e. negative control) of molecular grade water (Fisher Scientific, UK) and single-
147 template controls (STCs, i.e. positive control) of cichlid fish DNA (the Eastern happy,
148 *Astatotilapia calliptera,* a cichlid from Lake Malawi, which is not present in natural waters in
149 the UK) within each library. All lake samples (N = 252), together with 14 sampling blanks,
150 14 filtration blanks, 14 NTCs, and 14 STCs were included in each library construction (N =
151 308) for sequencing on an Illumina MiSeq. Three PCR technical replicates were performed
152 for each sample then pooled to minimise bias in individual PCRs. All PCRs were set up using
153 eight-strip PCR tubes in a PCR workstation with UV hood and HEPA filter in the eDNA
154 laboratory at University of Hull (UoH) to minimise the risk of contamination.

155 The Cytb locus, targeting a 414-bp vertebrate-specific fragment, was amplified with the
156 L14912 and H15149 primer pair (Kocher et al. 1989) using a one-step library preparation
157 protocol (Kozich et al. 2013) (see more detail in Appendix S5.2).. To improve clustering
158 during initial sequencing, the final 10 pM denatured Cytb library mixed with 30% PhiX
159 control was sequenced with the MiSeq reagent kit v3 (2×300 cycles) at UoH. The 12S locus
160 targets a 106-bp vertebrate-specific fragment amplified with the 12S_V5_F and 12S_V5_R
161 primer pair (Riaz et al. 2011). Following the consistently lower sequencing yield of the one-
162 step protocol for the 12S compared to the Cytb in previous studies, we decided to switch to a
163 two-step library preparation protocol using nested tagging (Kitson et al. 2018) (see more
164 detail in Appendix S5.2). Since the heterogeneity spacers included in the two-step library
165 preparation protocol will improve the nucleotide diversity, the denatured 12S library (13 pM)
166 was mixed with lower amount of PhiX control (i.e. 10%) compared to the Cytb library, then
167 sequenced with the MiSeq reagent kit v2 (2×250 cycles) at UoH.

Data analysis

Collation of fish data

Existing fish data for the 14 lakes was collated using a range of data sources (see the detail in Appendix S2). These included site-specific surveys carried out by Natural Resources Wales or the Environment Agency and their predecessor bodies, third party fishery surveys, and data published in the literature and *ad hoc* records stored on databases such as the National Biodiversity Network Atlas (https://nbnatlas.org). Based on existence of survey data and expert opinion (TWH-E and GP), fish species were placed into four general categories with confidence of species presence/absence for each lake (Table 1), reflecting both the available data and the uncertainty around it. Assessing relative abundance *a priori* was more challenging. For the Welsh lakes, an abundance category approach was used, with each species being placed on DAFOR scale (D = Dominant; A = Abundant; F = Frequent; O = Occasional; R = Rare) (Tansley 1993) using available data where possible and/or TWH-E's expert opinion. All records where no abundance was recorded were assumed to be rare. For Cheshire meres, summaries of fish community composition and fish density per unit area (ind. ha^{-1} and kg ha^{-1}) were produced from the Ecological Consultancy Ltd (ECON) fishery survey using point abundance sampling by electro-fishing (PASE) and seine netting in the same period as water samples collection with this study in 2016 (Appendix S1 Tables S1.3 & S1.4). The ECON fishery survey in 2016 was undertaken by ECON and commissioned by Natural England as part of an investigation of the impacts of fisheries on Sites of Special Scientific Interests (SSSIs) condition.

190 Table 1 Criteria for assessing the confidence of species presence/absence using existing data.

Category	Confidence in Presence	Description
Probably absent (Pa)	Very low	No records and at least one of outside biogeographic range/habitat unsuitable.
Possibly present (Po)	Low	Either not recorded since 1977 or not recorded from the lake, but present in connected water body or diadromous species with direct access to the sea.
Probably present (Pr)	Moderate	Either multiple records but not recorded since 1997 or only one record since 1997. For Welsh lakes, this category has also been used where the current status of a population is uncertain, even if records data meet the criteria for Established.
Established (E)	High	For Welsh lakes, multiple records including at least one since 2000. For Cheshire meres, records in the Ecological Consultancy Ltd (ECON) survey.

191

192 ## Bioinformatics analysis

193 Raw read data from Illumina MiSeq sequencing have been submitted to NCBI (BioProject:
194 PRJNA454866). Bioinformatics analysis was implemented following a custom reproducible
195 metabarcoding pipeline (metaBEAT v0.97.9) with custom-made UK freshwater fish
196 reference databases (Cytb and 12S) as described in our previous study (Hänfling et al. 2016).
197 Sequences which the best BLAST hit had a bit score below 80 or had less than 95%/100%
198 identity (Cytb/12S) identity to any sequence in the curated databases were considered non-
199 target sequences. The different level of similarity for each locus depended on the length of
200 metabarcode and the knowledge of intraspecific diversity of the studied taxon. To assure full

201 reproducibility of our bioinformatics analysis, the custom reference databases and the Jupyter
202 notebooks for data processing have been deposited in an additional dedicated GitHub
203 repository (https://github.com/HullUni-
204 bioinformatics/Li_et_al_2018_eDNA_fish_monitoring). The Jupyter notebook also performs
205 demultiplexing of the indexed barcodes added in the first PCR reactions.
206

207 Low-frequency noise threshold

208 Filtered data were summarised into the number of sequence reads per species/sample for
209 downstream analyses (Appendixes S3 & S4). After bioinformatics analysis, the low-
210 frequency noise threshold (proportion of STC species read counts in the lake sample) was set
211 to 0.07% and 0.3% for Cytb and 12S respectively to filter high-quality annotated reads
212 passing the previous filtering steps that have high-confidence BLAST matches but may result
213 from contamination during the library construction process or sequencing (De Barba et al.
214 2014; Hänfling et al. 2016; Port et al. 2016). The low-frequency noise threshold was guided
215 by the analysis of sequence data from STCs under several thresholds. Filtered data were
216 summarised in two ways for downstream analyses: (a) the number of sequence reads per
217 species at each lake (hereafter referred to as read counts) and (b) the proportion of sampling
218 locations in which a given species was detected (hereafter referred to as site occupancy).
219

220 Estimating abundance with eDNA

221 Based on our previous results (Hänfling et al. 2016), site occupancy is a better proxy for
222 estimates of abundance than read counts. However, read counts should not be completely
223 ignored because they still contain important abundance information. The maximum site
224 occupancy across both loci was used as a score for species abundance (Table 2). In addition

225 to site occupancy score, the maximum relative read count (i.e. proportion of read counts per
226 species at each sampling lake) across both loci, and the number of loci with which the species
227 was detected were used as confidence indicators to assign fish species into the same general
228 categories which were used for non-eDNA survey data (Table 3). Those species that were
229 assigned the lowest confidence scores (probably absent) were excluded from downstream
230 analysis (i.e. roach *Rutilus rutilus* in CWE, bream *Abramis brama* and bullhead *Cottus gobio*
231 in OGW, pike *Esox lucius* in PEN, bullhead in TRA, gudgeon *Gobio gobio* in BET). Each
232 retained species was assigned a corrected abundance score by multiplying site occupancy
233 score × confidence score. The corrected abundance score was used to assign each species to a
234 relative abundance DAFOR scale (Table 4) (For instance, bream in LLB: 2 × 5 = 10 resulting
235 in "Frequent" category in DAFOR scale).
236

237 Table 2 Site occupancy score based on maximum site occupancy across Cytb and 12S.

Site occupancy score	**Maximum site occupancy (across Cytb and 12S)**
1	> 0 and ≤ 0.1
2	> 0.1 and ≤ 0.3
3	> 0.3 and ≤ 0.6
4	> 0.6 and ≤ 0.8
5	> 0.8

238

239 Table 3 Criteria for assessing confidence of species occurrence using eDNA data.

Category	**Confidence score**	**Description**		
		Locus	**Site occupancy score**	**Maximum relative read count (across Cytb and 12S)**
Probably absent (PrA)	Very low (-1)	Any	= 1	> 0 and ≤ 0.005

Possibly present (PoP)	Low (1)	Any	> 1	> 0 and ≤ 0.005
		One	≥ 1	> 0.005 and ≤ 0.05
Probably present (PrP)	Moderate (3)	Both	≥ 1	> 0.005 and ≤ 0.01
		Both	= 1	> 0.01 and ≤ 0.1
		One	≥ 1	> 0.05 and ≤ 0.1
		One	= 1	> 0.1 and ≤ 0.5
Established (E)	High (5)	Both	> 1	> 0.01
		Both	= 1	> 0.1
		One	> 1	> 0.1
		One	= 1	> 0.5

Table 4 The relative abundance DAFOR scale based on eDNA data.

Abundance rank number	**DAFOR scale**	**Corrected abundance score (site occupancy score × confidence score)**
0	None	= 0
1	Rare	≥ 1 and < 4
2	Occasional	≥ 4 and < 9
3	Frequent	≥ 9 and < 15
4	Abundant	≥ 15 and < 25
5	Dominant	= 25

Ecological and statistical analyses

All downstream analyses were performed in R v3.3.2 (R_Core_Team 2016) and graphs plotted using GGPLOT2 v2.2.1 (Wickham & Chang 2016). Before investigating species detection and abundance estimate with eDNA, we first evaluated whether Cytb and 12S datasets produced consistent results by calculating the Pearson's product-moment correlation

248 coefficient for both read counts and site occupancy. Spearman's rank correlations were
249 performed between historical data and eDNA data – firstly in terms of confidence of species
250 presence and secondly in terms of relative abundance. To investigate differences in fish
251 communities between sampling lakes, hierarchical clustering dendrogram was used to assess
252 the existence of distinct community types using the function *fviz_dend* in FACTOEXTRA
253 v1.0.4 (Kassambara & Mundt 2017) to extract and visualize the results calculated from the
254 function *hclust* using Canberra distances method based on site occupancy. Furthermore, non-
255 metric multidimensional scaling (NMDS) based on read counts in individual sampling
256 location of lakes, allied with analysis of similarities (ANOSIM), were performed using the
257 abundance-based Bray-Curtis dissimilarity index with the function *metaMDS* and *anosim*
258 respectively in VEGAN v2.4-4 (Oksanen et al. 2017). The sequencing depth and sample-
259 based rarefaction was performed using the *rarecurve* and *rarc* function in VEGAN v2.4-4
260 (Oksanen et al. 2017), respectively. The full R script is available on the GitHub repository
261 (https://github.com/HullUni-
262 bioinformatics/Li_et_al_2018_eDNA_fish_monitoring/tree/master/R_script)
263

264 # Results

265 The Cytb and 12S libraries generated 17.15 and 15.97 million reads, respectively, with
266 15.91 and 13.4 million reads passing filters (including 49.01% or 11.07% PhiX). After
267 quality filtering and removal of chimeric sequences, the average read count of lake sample
268 was 12,503 for Cytb and 21,703 for 12S. After BLAST searches for taxonomic assignment,
269 59.47% ± 35.56% and 26.46% ± 19.60% reads in each lake sample were assigned to fish for
270 Cytb and 12S, respectively. Rarefaction analysis revealed our sequencing depth to be
271 sufficient to identify all of the amplified fish species present in the 14 lakes for both loci
272 (Appendix S1 Figure S1.1).

273 Significant correlations were found between site occupancy and average read counts, for
274 both loci and all sampling lakes apart from OGW (Pearson's $r = 0.81$, $df = 4$, $p = 0.05$) and
275 LLB (Pearson's $r = 0.94$, $df = 2$, $p = 0.06$) for Cytb (Appendix S1 Figure S1.2a, b). Data from
276 the two loci were significantly correlated (Pearson's r consistently $p < 0.05$) for site
277 occupancy for each lake (Appendix S1 Figure S1.2c), and for average read counts apart from
278 in three lakes: PAD (Pearson's $r = 0.55$, $df = 7$, $p = 0.12$), TRA (Pearson's $r = 0.59$, $df = 5$, p
279 $= 0.16$) and LLB (Pearson's $r = 0.89$, $df = 2$, $p = 0.11$) (Appendix S1 Figure S1.2d). The more
280 detail of comparisons between these two loci in detection probabilities can be found in
281 Appendix S5.3.
282

283 ## Comparison of eDNA data and expected results

284 In total, 24 species with 111 species occurrences in 14 lakes were detected across Cytb
285 and 12S. Perch *Perca fluviatilis* was the most common species which were detected in all
286 lakes. The second most frequently occurring species was roach with detections in 10 lakes. In
287 addition to these two species, pike were found in all nine coarse fish lakes (Table 5; Figure 2;
288 Appendix S1 Figure S1.3). There was a significant positive correlation between expected
289 faunas and eDNA data in terms of confidence of species occurrence (Spearman's $r = 0.74$, df
290 $= 109$, $p < 0.001$). A total of 73 "established" species occurrences have been recorded across
291 all lakes according to historical data. Of these, 72 (98.6%) were detected with eDNA (Table
292 5). The only false negative in the eDNA datasets was tench *Tinca tinca* in PEN. Tench were
293 detected by 12S in PEN but at a read count less than the low-frequency noise threshold
294 (Appendix S4; site occupancy = 0.3, average read counts = 3.9). Tench are likely to be very
295 rare in PEN: only two tench were recorded by the 2016 Royal Society for the Protection of
296 Birds survey and small numbers have also been detected in previous years (Appendix S2.4).

297 Twenty-five of the 111 occurrences were "probably or possibly present", and 13 were "very
298 low confidence present" (i.e. probably absent records based on historical data) (Table 5).
299 There were consistent positive correlations between DAFOR scale based on eDNA data
300 and expected DAFOR scale based on both historical data for Welsh lakes and fish density per
301 unit area from the ECON survey of Cheshire meres (Figure 3). The correlations were
302 significant in three out of eight Welsh lakes (PAD, OGW and KEN). Significant correlations
303 were observed in three (MAP, CAM and BET) and four (MAP, CAM, FEN and WLF)
304 Cheshire meres based on individual density (ind. ha^{-1}) and biomass density (kg ha^{-1}),
305 respectively (Figure 3; Appendix S1 Figure S1.4).
306 Furthermore, the rigorous sampling of shore and offshore locations is carried out to
307 compare the suitability of the different spatial sampling location (Appendix S1 Table S1.2).
308 The more detail of comparisons between shore and offshore samples can be found in
309 Appendix S5.4.
310

311 ## Characterisation of fish assemblages using eDNA data

312 The hierarchical clustering dendrograms based on site occupancy for two loci indicated
313 that there were four distinct community types compared to three pre-defined lake types
314 according to environmental characteristics. Specifically, the clustering dendrograms accorded
315 with the pre-defined lake Type 1 (CWE, OGW and PAD) and Type 2 (TRA and PEN), but
316 indicated that WLF and BET can be divided from the pre-defined lake Type 3 (Figure 4a1,
317 b1). The NMDS ordination allied with ANOSIM based on read counts for two loci confirmed
318 the clustering dendrograms results that there were four distinct community types according to
319 the predominant groups of fish including Community 1: salmonids and minnow *Phoxinus*
320 *phoxinus*; Community 2: mixed diadromous fish; Community 3: coarse fish, and Community
321 4: bream-dominated coarse fish (Figure 4a2, b2). The *R* statistic in the ANOSIM global test

with these four different communities were high (Appendix S1 Table S1.5, 0.75 ± 0.02) supporting statistical differences between communities (Appendix S1 Table S1.5, $p = 0.001$). The overall distance pattern of these four fish communities was that both coarse fish communities (Communities 3 & 4) were close to each other; the mixed diadromous fish community (Community 2) was between the coarse fish communities and the salmonids and minnow community (Community 1) (Figure 4; Appendix S1 Table S1.5).

The salmonids and minnow community (Community 1) is characteristic of three low alkalinity upland lakes (CWE, OGW and PAD). These sites were generally dominated by brown trout *Salmo trutta*, rainbow trout *Oncorhynchus mykiss* or Arctic charr *Salvelinus alpinus*, but also included minnow and Atlantic salmon *Salmo salar* (Figure 2; Appendix S1 Figure S1.3). CWE and PAD are designated as SSSIs under the Wildlife and Countryside Act 1981 (as amended) to conserve Arctic charr, and CWE, a oligotrophic lake, is also protected as a Special Areas of Conservation under the EU Habitats Directive. The eDNA data showed that PAD contained a small number of Arctic charr, whereas this species was dominant in CWE (Figure 2; Appendix S1 Figure S1.3). Rainbow trout were only detected in OGW, where they are regularly stocked by the local angling club (Appendix S2.3). Some of these sites may also contain low density of other species; usually diadromous species were detected using eDNA as well such as European eel *Anguilla anguilla* in all three upland lakes and three-spined stickleback *Gasterosteus aculeatus* in PAD. Perch have recently been recorded from PAD for the first time using captured-based survey methods (Appendix S2.2). Surprisingly, this species was detected using eDNA (though shown to be rare), in all of these three lakes (Figure 2; Table 5). The diadromous fish community (Community 2) is characteristic TRA and PEN which were dominated by European eel and three-spined stickleback. Other species included brown trout, nine-spined stickleback *Pungitius pungitius*, roach, perch and rudd *Scardinius erythrophthalmus* (Figure 2). The coarse fish communities

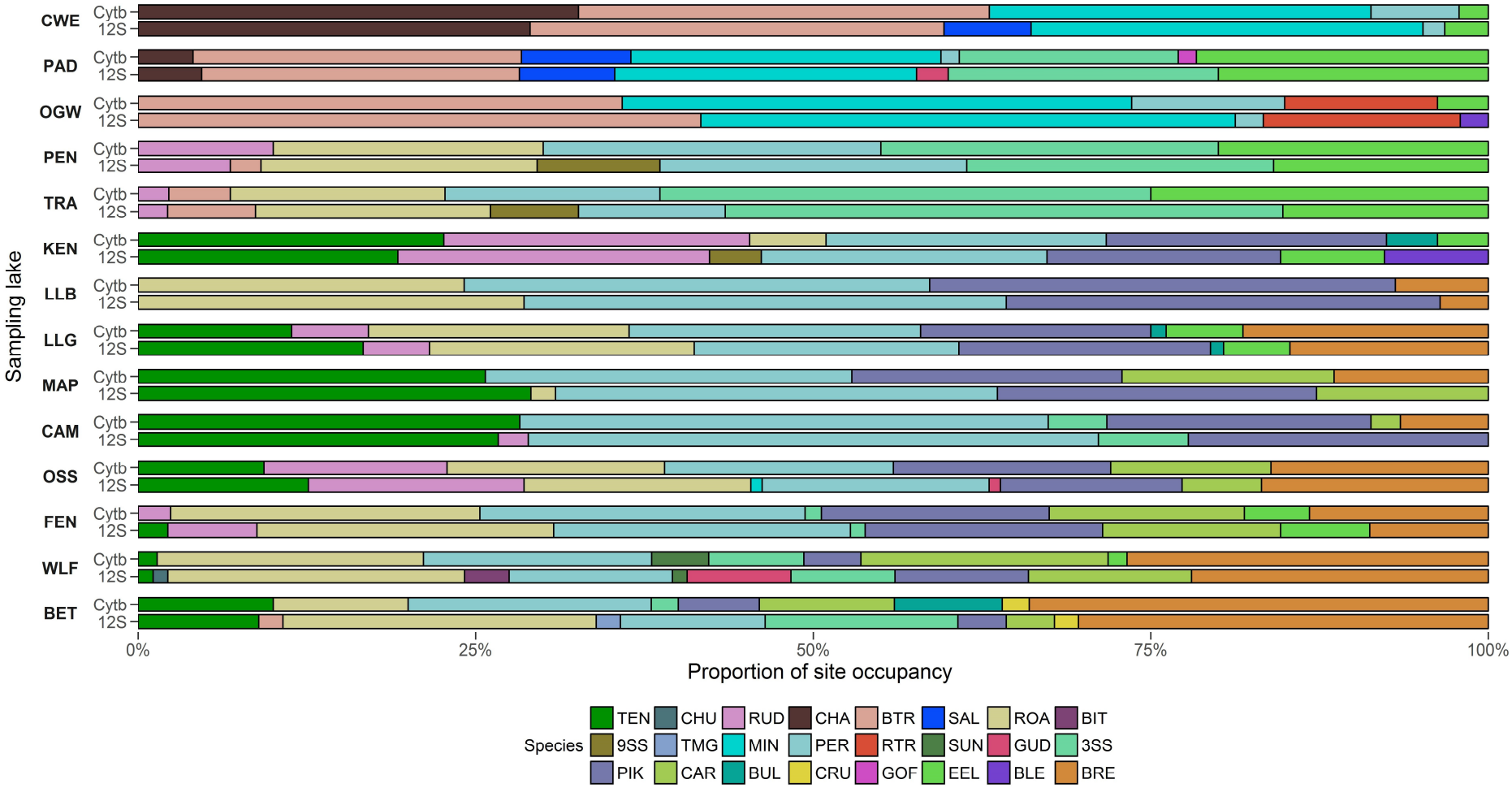
consisted of bream, common carp *Cyprinus carpio*, pike, perch, roach, rudd and tench, plus some additional species (e.g. European eel, bullhead, three-spined stickleback and gudgeon). Pike, perch, roach and tench were dominant in KEN, MAP, CAM, LLB, LLG, OSS and FEN (Community 3); however, the dominant species in WLF and BET (Community 4) was bream (Figure 2; Appendix S1 Figure S1.3).

Table 5 Correspondence of confidence of species occurrence between predicted and eDNA data in 14 lakes.

Community type		**1**			**2**		**3**							**4**		**Number of lakes**
Scientific name	**Code**	**CWE**	**PAD**	**OGW**	**PEN**	**TRA**	**KEN**	**LLB**	**LLG**	**MAP**	**CAM**	**OSS**	**FEN**	**WLF**	**BET**	
Abramis brama	BRE							PrP/E	E/E	PoP/PoP	PoP/PoP	E/E	E/E	E/E	E/E	8
Alburnus alburnus	BLE			PrA/PoP			PrA/PoP									2
Anguilla anguilla	EEL	E/PrP	E/E	PoP/PrP	E/E	E/E	E/E		E/E				E/E	PoP/PoP		9
Carassius auratus	GOF		PrA/PrP													1
Carassius carassius	CRU														Po/Pr	1
Cottus gobio	BUL						PrA/PoP		PoP/PrP						Po/Po	3
Cyprinus carpio	CAR									E/E	PoP/PoP	E/E	E/E	E/E	E/E	6
Esox lucius	PIK						E/E	E/E	E/E	E/E	E/E	E/E	E/E	E/E	E/E	9
Gasterosteus aculeatus	3SS		E/E		E/E	E/E					PoP/E		E/PrP	PoP/E	Po/Pr	7
Gobio gobio	GUD		PrA/PoP									PoP/PoP		E/PoP		3
Leucaspius deliniatus	SUN													E/E		1
Oncorhynchus mykiss	RTR			E/E												1
Perca fluviatilis	PER	PrA/E	PrP/PoP	PrA/PrP	E/E	PrA/E	E/E	E/E	E/E	E/E	E/E	E/E	E/E	E/E	E/E	14
Phoxinus phoxinus	MIN	E/E	E/E	E/E								PrA/PoP				4
Pseudorasbora parva	TMG														Pa/Po	1
Pungitius pungitius	9SS				PoP/PrP	PoP/PoP	PoP/PoP									3
Rhodeus amarus	BIT													PoP/PoP		1
Rutilus rutilus	ROA				E/E	E/E	PoP/PoP	E/E	E/E	PrA/PoP		E/E	E/E	E/E	E/E	10
Salmo salar	SAL	E/PoP	E/E													2
Salmo trutta	BTR	E/E	E/E	E/E	PoP/PoP	PoP/E									Po/Po	6
Salvelinus alpinus	CHA	E/E	E/PrP													2
Scardinius erythrophthalmus	RUD				E/E	PoP/PrP	E/E		E/E		PrA/PoP	E/E	E/E			7
Squalius cephalus	CHU													PrA/PoP		1
Tinca tinca	TEN				E/PrA		E/E		E/E	E/E	E/E	E/E	PoP/PoP	PoP/PrP	E/E	9

Number of species	6	9	6	8	7	9	4	8	6	7	9	9	12	11	111 in total

Notes: Categories of presence confidence ("PrA" probably absent, "PoP" possibly present, "PrP" probably present and "E" established) are described in Table 1 showing as based on predicted/eDNA data. The "Established" species occurrences based on historical data are shown in blue background (*N* = 73). Solid vertical lines indicate community types (Community 1–3) with similar faunas. Dashed line indicates division between whether bream *Abramis brama* is dominant species in the lake (Community 4). Sampling lake codes are given in Figure 1.



358

359 Figure 2 Species composition of site occupancy for Cytb and 12S. Species three letter codes and sampling lake codes are given in Table 5 and Figure 1,
360 respectively.

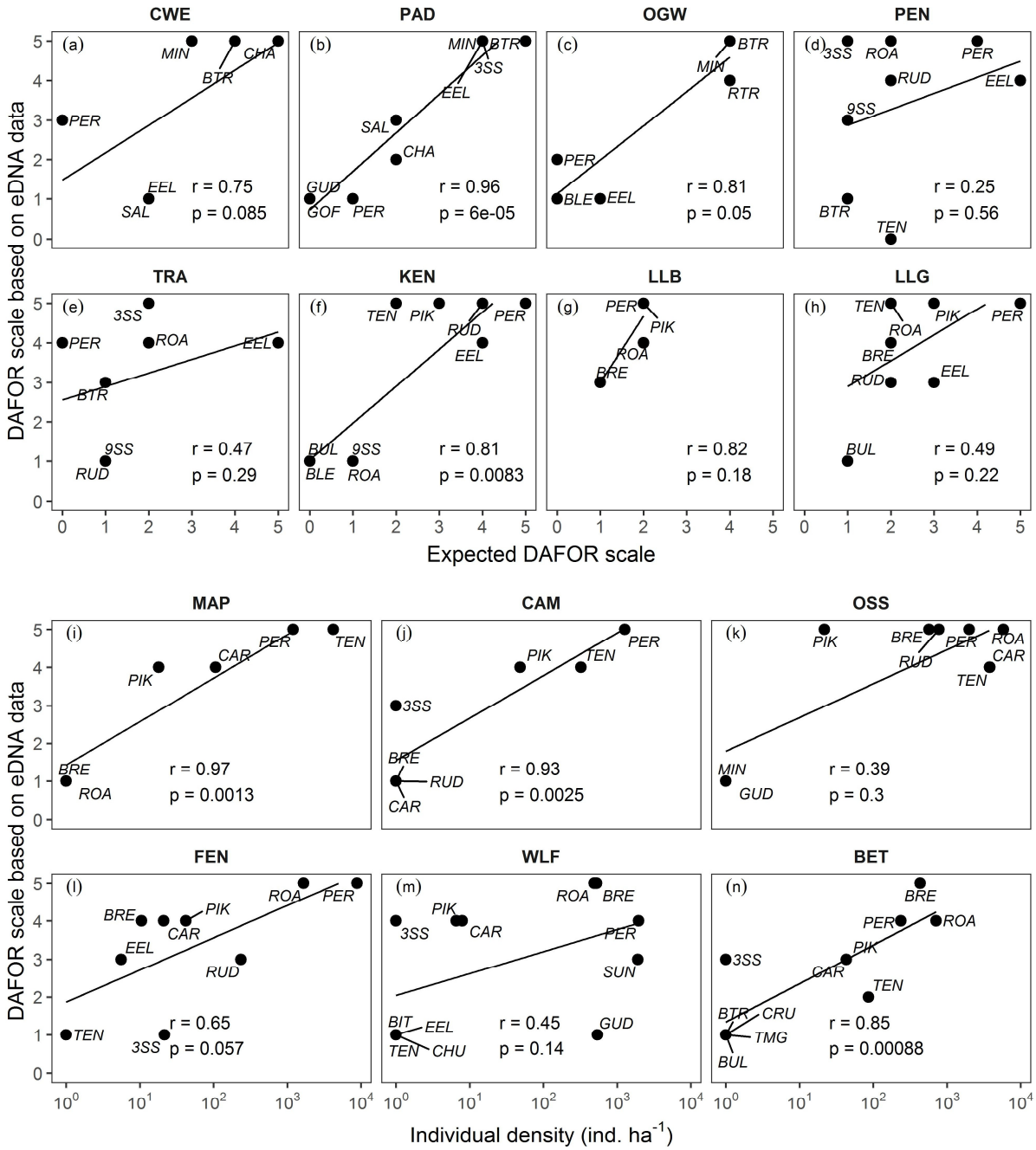


Figure 3 Correlations between DAFOR scale based on eDNA data and expected DAFOR scale based on existing data of Welsh lakes or individual density from the ECON survey of Cheshire meres. Sampling lake codes, abundance rank numbers to DAFOR scale and species three letter codes are given in Figure 1, Table 4 and Table 5, respectively.

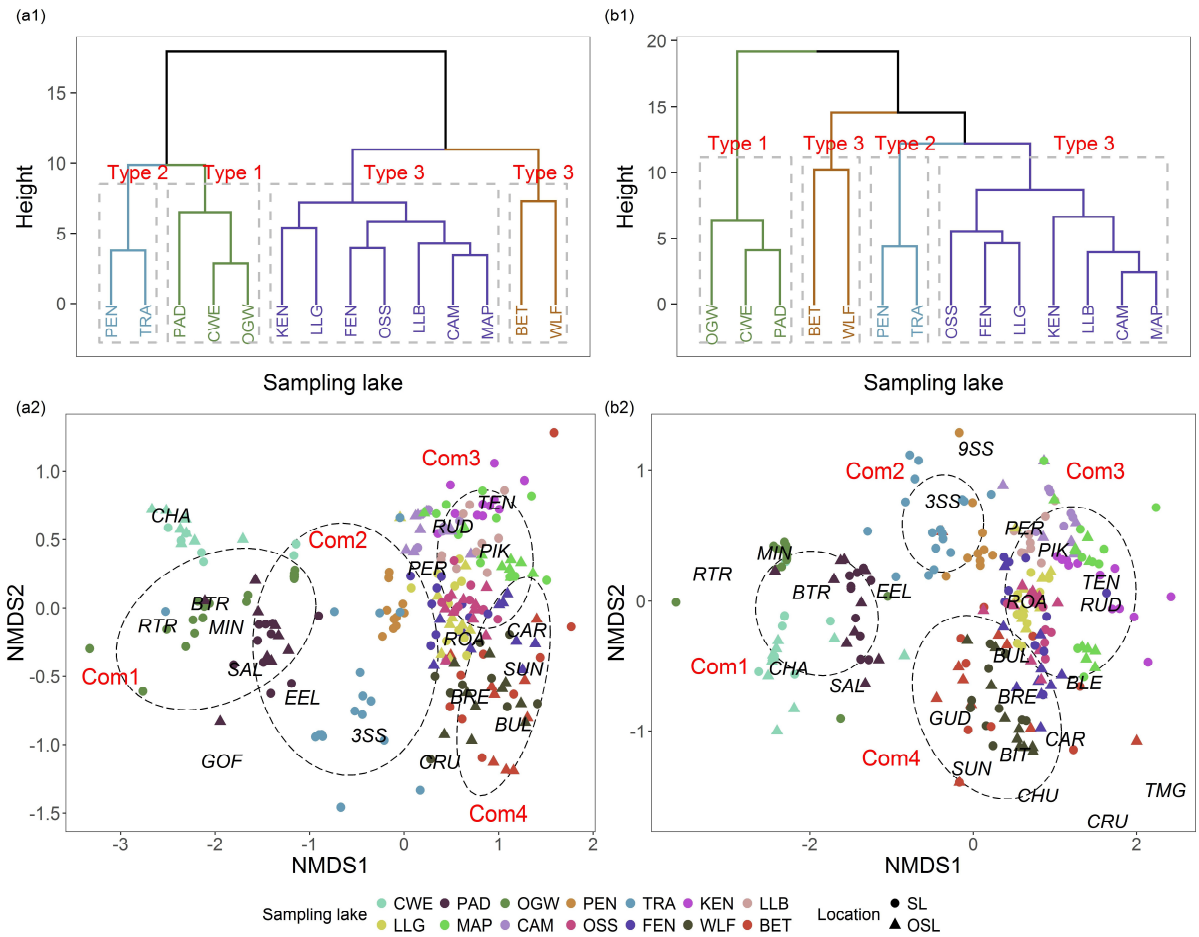


Figure 4 Hierarchical clustering dendrograms and non-metric multidimensional scaling (NMDS) ordination of the sampling lakes. Dendrograms using Canberra distances method based on site occupancy for (a1) Cytb and (b1) 12S. The dashed frames are drawn around each cluster in dendrograms. The three pre-defined lake types according to environmental characteristics. NMDS in individual sampling location of lakes using Bray-Curtis dissimilarity index based on read counts for (a2) Cytb and (b2) 12S. Each symbol corresponds to a sampling lake, with circles corresponding to shore samples ("SL") and triangles corresponding to offshore samples ("OSL") in NMDS ordination. The ellipse indicates 70% similarity level within each community type in ordinations. Scores for species taxa are plotted on the same axes to better visualise the ordination in space between species and samples. Species three letter codes and community type (Com 1–4) with individual lake codes are given in Table 5 and Figure 1, respectively.

381 # Discussion

382 This study confirmed key results from our previous study in that there was (a) a consistent,
383 strong correlation between Cytb and 12S in terms of read counts and site occupancy; (b) a
384 consistent, strong correlation between site occupancy and read counts and (c) site occupancy
385 was consistently better than read counts for estimating relative abundance, for both Cytb and
386 12S datasets (Hänfling et al. 2016).

387

388 ## Comparison of eDNA and existing data for species detection

389 A total of 73 "established" species occurrences were recorded across the 14 lakes
390 according to existing and historical fish data. Of these, 72 (98.6%) were detected with eDNA,
391 which demonstrated that eDNA metabarcoding outperformed the conventional surveys and
392 observations using a range of sampling methods over date ranges spanning several decades in
393 species richness estimates. This result is consistent with previous studies that have
394 demonstrated that eDNA metabarcoding produced a more comprehensive species list than
395 alternative survey techniques with a similar effort in both marine and freshwater ecosystems
396 (Hänfling et al. 2016; Port et al. 2016; Valentini et al. 2016).

397 Moreover, there was a significant positive correlation between historical data and eDNA
398 data in terms of confidence of species presence (Spearman's $r = 0.74$, $df = 109$, $p < 0.001$).
399 Species occurrences that were assessed as "probably or possibly present" (25 of the 111
400 species occurrences) and "probably absent" (13 of the 111 species occurrences) based on
401 distribution data or anecdotal evidence also fitted the eDNA criteria for lowered confidence
402 levels. In most cases, the "probably absent" occurrences were at very low site occupancy (≤
403 0.1) and read counts (~0.5%), just above our threshold for accepting a positive record. These
404 records could be genuine detections of species that have previously been missed. For example,

405 perch were recorded with both Cytb and 12S in CWE, OGW and TRA. The confidence of
406 species present was either "established present" or "probably present" according to eDNA
407 data. The recent appearance of this species in nearby lakes PAD and PEN (see detail in
408 Appendix S2) suggest that the species is spreading in North Wales either by natural means or
409 as a result of illegal introductions. In addition, the other "probably absent" occurrences could
410 be either false positives from sequencing error, laboratory or environmental contamination.
411 For instance, bleak *Alburnus alburnus* in OGW, goldfish *Carassius auratus* and Gudgeon in
412 PAD, rudd in CAM, roach in MAP, topmouth gudgeon *Pseudorasbora parva* in BET and
413 chub *Squalius cephalus* in WLF are most likely explained by low-level cross contamination
414 or sequencing barcode misassignment since these species were only detected by either Cytb
415 or 12S in single sample of the lake.

416 Barcode misassignment and tag jumps have been proved in metabarcoding studies (e.g.
417 Deakin et al. 2014; Schnell et al. 2015). To minimise contamination or false assignments,
418 further work needs to be done to optimise each step, as well as the incorporation of robust
419 controls to identify contamination when it occurs. Together with a growing number of studies
420 (Stat et al. 2017; Li et al. 2018b), the results demonstrate the importance of using two (or
421 more) markers for metabarcoding to avoid problems due to primer bias (Clarke et al. 2014),
422 gene copy number, PCR or sequencing artefacts (Schloss et al. 2011) and/or contamination.
423 Other strategies such as using consistency of presence across technical replicates as used by
424 Port et al. (2016) might be a more suitable approach to control for false positives if rare
425 species are of particular interest. Where verification of rare species detections is considered a
426 high priority, additional confirmation from targeted species approaches (e.g. quantitative
427 PCR) and/or field surveys may be required.

428

429 ## Use of eDNA for assessing relative abundance of fish

430 WFD specifies that abundance should be considered when determining ecological status;
431 hence, current WFD approaches include estimates of abundance (often as abundance classes).
432 For instance, a five-level scale, adapted from DAFOR scale, is accepted for Austrian standard
433 and national monitoring techniques applied under WFD for surveying aquatic macrophytes
434 (Pall & Moser 2009). Here, we used DAFOR scale to estimate fish relative abundance in the
435 present study based on DNA-based identification to facilitate integration into current WFD
436 approaches.

437 There were consistently positive correlations in terms of relative abundance between the
438 eDNA data and historical data. However, these correlations were not always statistically
439 significant. The correlations were not significant in TRA and PEN probably because three-
440 spined stickleback were more abundant based on eDNA data than expected. This species is
441 often under-represented or overlooked in surveys using established fish capture methods
442 (Hänfling et al. 2016). Moreover, tench were not detected by eDNA in PEN. LLB is a
443 species-poor lake with only four species detected, which could reduce the statistical power.
444 The non-significant correlation in WLF could be attributed to under-representation of three-
445 spined stickleback in previous fish surveys and reduced detection probability of gudgeon with
446 Cytb. Although these results are generally encouraging, further work is critical to obtain
447 enough statistical power to directly test the relationship between abundance estimates from
448 eDNA and surveys using other methods. It is also important to investigate taxon-specific
449 detection probabilities and abundance estimates so that a pressure-sensitive tool can be
450 developed.

451

452 Using eDNA to describe ecological communities

453 According to environmental characteristics, there were three pre-defined lake types.
454 Encouragingly, four distinct community types could be identified based on clustering
455 dendrograms and NMDS ordinations using eDNA data. Basically, the Community 1 and
456 Community 2 agreed with the pre-defined lake Type 1 and Type 2, respectively. The pre-
457 defined coarse fish lakes (Type 3) can be further divided into Community 3 and Community
458 4 based on whether bream was dominant species in the lake. These findings indicated that
459 eDNA metabarcoding has great potential as fish-based assessment tool for WFD lake status
460 assessment.

461 Specifically, our results showed that the low alkalinity lakes within a predominantly
462 upland catchment were mainly dominated by salmonids reflecting their relatively deep and
463 oligotrophic nature. Except from salmonids, all these sites contained minnow, most likely
464 introduced by anglers using them as live bait (Hatton-Ellis 2005). Compared to the salmonids
465 and minnow lakes (Community 1), perch, pike and cyprinids (such as bream, common carp,
466 roach and tench) were prevalent in the coarse fish lakes (Communities 3 & 4) reflecting their
467 relatively shallow and eutrophic nature. These findings support that eDNA profiles are
468 suitable to reflect the eutrophic conditions of Lake Windermere in which species that prefer
469 less eutrophic conditions (Atlantic salmon, brown trout, Arctic charr, minnow and bullhead)
470 were more abundant in the mesotrophic North Basin, and species associated with eutrophic
471 conditions (roach, tench, rudd, bream and European eel) were more common in the eutrophic
472 South Basin (Hänfling et al. 2016). Furthermore, both of the diadromous fish lakes
473 (Community 2) contained a different fauna including various combinations of mostly
474 diadromous species such as European eel, three-spined stickleback, nine-spined stickleback
475 and brown trout. Alongside these, several coarse fish species such as roach, perch, and rudd
476 also occurred in these lakes. However, of greater conservation interest is the distribution of

477 nine-spined stickleback. This species occurs only locally in Wales, mainly at lowland sites
478 close to the sea (Davies et al. 2004; Hatton-Ellis 2005).
479

480 # Conclusions

481 In this study, we have extended the geographical, ecological and taxonomic extent of the
482 lake fish eDNA-based metabarcoding dataset, including 14 UK lakes with well described fish
483 faunas. Four eDNA communities are characterised, which is consistent with earlier
484 assessments and ecological interpretations. Moreover, eDNA metabarcoding outperformed
485 established survey techniques in terms of species detection, relative abundance using the
486 standard five-level classification scale and characterisation of ecological fish communities,
487 suggesting eDNA metabarcoding has great potential as fish-based assessment tool for WFD
488 lake status assessment. Further optimisation and development such as understanding taxon-
489 specific biases, abundance estimates and management of low confident occurrence are
490 strongly recommended with a larger dataset, to improve the accuracy, effectiveness and
491 applicability of WFD monitoring tool.
492

493 # Authors' contributions

494 JL wrote the manuscript; BH, TWH-E, and GP conceived and designed the study; TWH-E
495 and GP prepared historical fish data; HSK, MB, and JL carried out the fieldwork; MB
496 prepared the sampling map; JL conducted laboratory work and performed bioinformatics
497 analyses; JL, LLH, and BH performed the statistical analyses. All authors commented the
498 final manuscript.
499

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Data accessibility

Data is available from the GitHub repository (https://github.com/HullUni-bioinformatics/Li_et_al_2018_eDNA_fish_monitoring). The repository is archived with Zenodo (https://doi.org/10.5281/zenodo.1462898).

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