A tool for classifying the ecological status of lake fish in Britain based on eDNA metabarcoding

Report to Scottish Environment Protection Agency

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1. Introduction

1.1. The EU Water Framework Directive (WFD) requires that member states develop robust methods for assessing the ecological status of freshwater resources using different biological quality elements (BQEs). Methods have now been developed and successfully intercalibrated for most BQEs in most water body types. However, a suitable and cost-effective method for monitoring and assessing status of fish in lakes has yet to be developed in Britain, where established invasive methods such as gill-netting are strictly controlled and unfeasible in some areas. The WFD normative definitions stipulate that methods should consider aspects of fish species composition, abundance and age structure of the community that show changes in response to anthropogenic impacts.

1.2. In Ireland a gill netting based method, FIL2, was developed and successfully intercalibrated against the lake fish assessment method of Finland. FIL2 has been adopted for use in Northern Ireland (Kelly et al., 2012). Applying the same principles, a gill netting-based method (SFINX) was subsequently developed for use in comparable lakes in NW Britain. This uses total fish individuals per unit effort (CPUE), total fish biomass per unit effort (BPUE), brown trout CPUE, euryhaline species CPUE and number of piscivorous species as the basis for classification (Allen et al., 2016).

1.3. Recent advances in molecular techniques have enabled rapid developments in the field of biodiversity monitoring, particularly through analysis of environmental DNA (eDNA) (Pawlowski et al., 2018). Research in Britain funded mainly by the EA and SEPA has demonstrated that eDNA metabarcoding provides both qualitative and to some degree quantitative information on fish communities in large lakes, outperforming established methods in terms of the number of species detected (Hänfling et al., 2016; Li et al., 2019). Reviewing the potential contribution of eDNA-based approaches to bioassessment Hering et al. (2018) considered this approach especially well-suited to fish assessment on the grounds of representativeness, sensitivity, precision, comparability, cost-effectiveness, and environmental impact.

1.4. To date this method has been tested on a limited number of lakes of different size, nutrient status and fish density, in NW England, Scotland and Wales. The current project expands this testing to cover the full range of lakes and associated fish communities represented in Britain and uses the supporting analysis to develop an eDNA-based classification tool suitable for reporting the status of lake fish for the WFD. Given the limitations of the available data, at this point the proposed tool only provides sensitivity to eutrophication, the dominant pressure on European lakes.

2. Methods

2.1. Biological data collection

At each lake 20 water samples (2L volume) were collected at roughly equidistant points around the perimeter. Samples were collected from surface water (<0.3m depth) and location was recorded using GPS. In some smaller lakes 10 samples were collected and occasionally 17-21 samples were collected for logistical reasons. Water samples were collected from a total of 101 water bodies. Samples were transported in insulated cool boxes and filtered through 0.45 µm cellulose nitrate filters (47 mm diameter; Whatman, GE Healthcare, UK) within 24 hours of collection. Filters were stored at -80^{o}C under sterile conditions prior to eDNA extraction in a dedicated laboratory at University of Hull and were assayed by eDNA metabarcoding of the mitochondrial 12S region (see Appendix 1 for protocol). The data presented here and used as the basis for the classification tool relate exclusively to shore-based samples collected during the winter (Dec-March). An evaluation of the merits of different sampling effort and onshore vs offshore sampling is provided in Appendix 2.

2.2. Biological data set characteristics

Forty fish taxa were recorded across the 101 water bodies based on metabarcoding of eDNA extracted from water samples. The raw data comprised the number of sequence reads per taxon per sample per lake. Of these 40 taxa only 15 occurred widely (>20% of water bodies) with 16 taxa confined to <5% of sites. The most widely recorded taxa were brown trout, European eel, common perch, three-spined stickleback, minnow and pike. Most taxa were resolved to species level but due to the limitations of the 12S marker *Coregonus* and *Salvelinus* could not be resolved beyond genera, while two members of the family Percidae, *Perca fluviatilis* (common perch) and *Sander lucioperca* (zander), also could not be separated. It is highly unlikely that *S. fontinalis* (brook trout) occurred at any of the sites studied so *Salvelinus* can be assumed to refer to *S. alpinus* (arctic charr). Zander, if present at all, would have been restricted to one or two sites in central or eastern England so the label Percidae can be assumed to refer exclusively to *Perca fluviatilis* in almost all cases.

2.3. Environmental data set characteristics

Sites were matched to their unique WFD waterbody ID and linked to data on background environmental characteristics (area, elevation, depth, alkalinity, catchment area, connectivity (i.e. length of water courses and area of standing waters within the upstream catchment) and catchment land cover via the UK Lakes Portal (eip.ceh.ac.uk/apps/lakes). They were also linked to annual mean concentrations for chlorophyll a, TP and TON via national agencies data (typically based on the last 5 years data) and to the WFD classifications reported in previous cycles for a range of biological quality elements (BQEs) and relevant physicochemical variables. We used classifications based on multiple cycles since 2009, not only the most recent one, on the assumption that, for long-lived organisms such as fish, the biological signal will reflect classifications over the last 10 years not only the last 2-3 years. In 10% of cases a specific lake had not been classified so we were obliged to use the status of an adjacent, comparable, directly assessed water body. Given the

low variation in status among surrounding water bodies in such situations we assume that this is a minor issue and that the reported class would be representative of the target water body.

2.4. Based on the available classifications we defined a 'typical' class for a water body based on the median class reported for the elements listed above across multiple cycles, as well as a worst-case class applying the one-out, all-out rule (1oAo) to the same data for each cycle. If the worst case changed between cycles we used the one most commonly observed, or, in the rare case, of equal numbers of different classes, the lowest one. We also created a simple continuously scaled index of status based on pre-existing classifications using the principle that H=5 through to B=1, and weighting by the number of times each class was reported across all the elements considered (i.e. a score of 5 would imply that all elements in all cycles were classified as High, while a score of 4.5 implies that 50% of reported classifications were High and 50% were Good).

2.5. The morpho-edaphic index (MEI=log (alkalinity (meqL)/mean depth (m)) was calculated for all sites to provide a summary measure of background productivity. High values of MEI (>0) are associated with base-rich, shallow lakes that are naturally productive due to their catchment geology, water column mixing and ability of light to reach the lake bed. Low values (<-1.5-2.0) are associated with base-poor, deep lakes that are naturally unproductive. A synthetic pressure index for eutrophication was constructed using the first axis of a PCA of lake chlorophyll, TP and TON concentrations and catchment % agriculture and %urban land covers (derived from CEH Land Cover Survey 2015).

2.6. A subset of 28 'reference' sites was identified by reference to pressure data and classifications previously reported for TP, phytoplankton, macrophytes, diatoms and littoral invertebrates (Appendix 3). Reference sites had low pressure on the eutrophication axis (Fig. 1) and were consistently classified as high, or in a small number of cases, good status. The term 'reference' is used loosely here; fixed or type-specific thresholds for pressure were avoided to ensure that sites spanned the MEI gradient and these sites simply represent a pool of the best available sites ('good' sites) within the dataset, assessed independently of information on fish. The one exception to this was to exclude six sites with a recent history of rainbow trout stocking from consideration as reference sites. These 'good' sites were contrasted in analyses below with a subset of sites with high pressure that were consistently classified at below good status ('not good' sites). Other sites, typically classified as moderate, good to moderate, or with classifications that varied widely between elements were regarded as being of 'uncertain/intermediate' quality for the purposes of the analysis.

2.7. Testing and choice of fish metrics

A suite of 40+ metrics was derived from the fish eDNA data representing the occupancy (number of samples in which a taxa was detected as a proportion of the total samples collected per lake), share of reads per site (total number of read counts per taxa in a lake as a proportion of the summed read counts across all fish taxa detected in the lake), or mean share of reads per sample (read counts per taxa

in a sample as a proportion of the summed read counts in that sample, averaged across all samples collected from that lake) for individual species or combinations of species (e.g. piscivores, benthivores, salmonids, common bream + common carp, rudd + tench). Since some freshwater fish species (perhaps most notably perch) display strong ontogenic shifts in diet and/or distribution, guild-based approaches may be of limited utility with eDNA-based data since age/size-related information on individual taxa is not available. Additional metrics were also prepared based on total recorded fish richness and the share of non-native fish species; a community-based index was also derived based on the occupancy-weighted optima of each commonly recorded species (the 24 species with occurrence in >5 lakes) on the eutrophication pressure axis (analogous to an invertebrate ASPT score for fish). Further details of this index are given in Appendix 4.

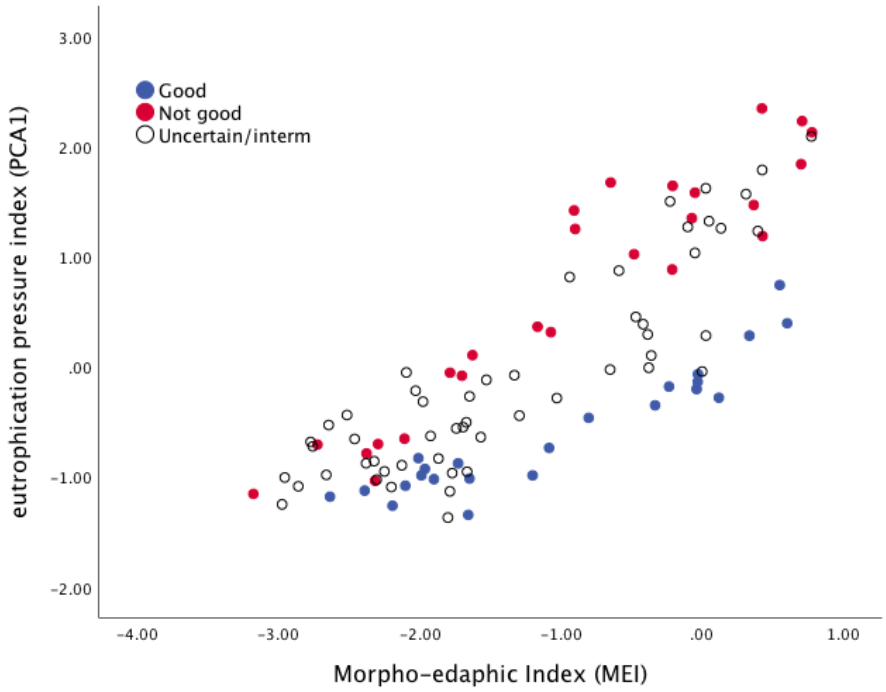


Figure 1. Distribution of the sites in the fish eDNA dataset in relation to the lake morpho-edaphic index (MEI; low values = low productivity) and the eutrophication pressure index (a PCA composite axis based on TP, chlorophyll and TON concentrations, and agricultural and urban catchment land covers), showing the allocation of sites to good (blue), not good (red) and uncertain/intermediate (open black) quality used in the exploration of fish metrics.

2.8. When using the eDNA data we have taken the view that, given the method and the sampling effort, not detecting a species equates to genuine evidence of absence rather than simply absence of evidence. In all cases we have treated the data at 'face value' rather than assigning and excluding potential 'false positives' (i.e. the DNA of species detected, due to inputs from external sources (e.g. avian deposition, import of dead bait by anglers, wastewater discharges), that in reality are absent from the ecosystem). Examination of the raw data suggested that <2% of fish taxon x lake occurrences could be false positives if simple, arbitrary read count and occupancy

thresholds are applied and those species plausibly indigenous to a lake are excluded. We also took no specific account of known (re)introductions of native fish, such as coregonids, on the basis that such knowledge is highly incomplete and selective; using this information could imply that all other cases derive from natural colonization events which would not be a valid assumption.

2.9. Metrics were screened for their utility in classification using logistic regression with MEI as the main environmental covariate and *a priori* quality (good, not good or intermediate) as a factor and the metric as the response. Metrics were considered of potential value where there was a significant difference in the parameter estimates for the *a priori* quality, confirming effective separation of the response between good versus not good sites.

3. Results

3.1. Candidate metrics

For the fish community as a whole, and most of the common component species MEI was the major determinant of distribution. Several common species (e.g. three-spined stickleback, European eel) had a ubiquitous distribution, irrespective of MEI and the *a priori* quality. Others showed strong variation in distribution with respect to MEI but were insensitive to quality (e.g. minnow). Six taxon-based metrics using roach occupancy (occ), Percidae occ, common carp occ + common bream occ, brown trout occ, and other salmonids occ (salmon occ + arctic charr occ + coregonid occ) and read share of brown trout, showed potential value for use in classification, being able to discriminate between different *a priori* quality (Fig 2). Generally, occupancy-based models showed a stronger relationship to MEI and site quality than metrics based on read share.

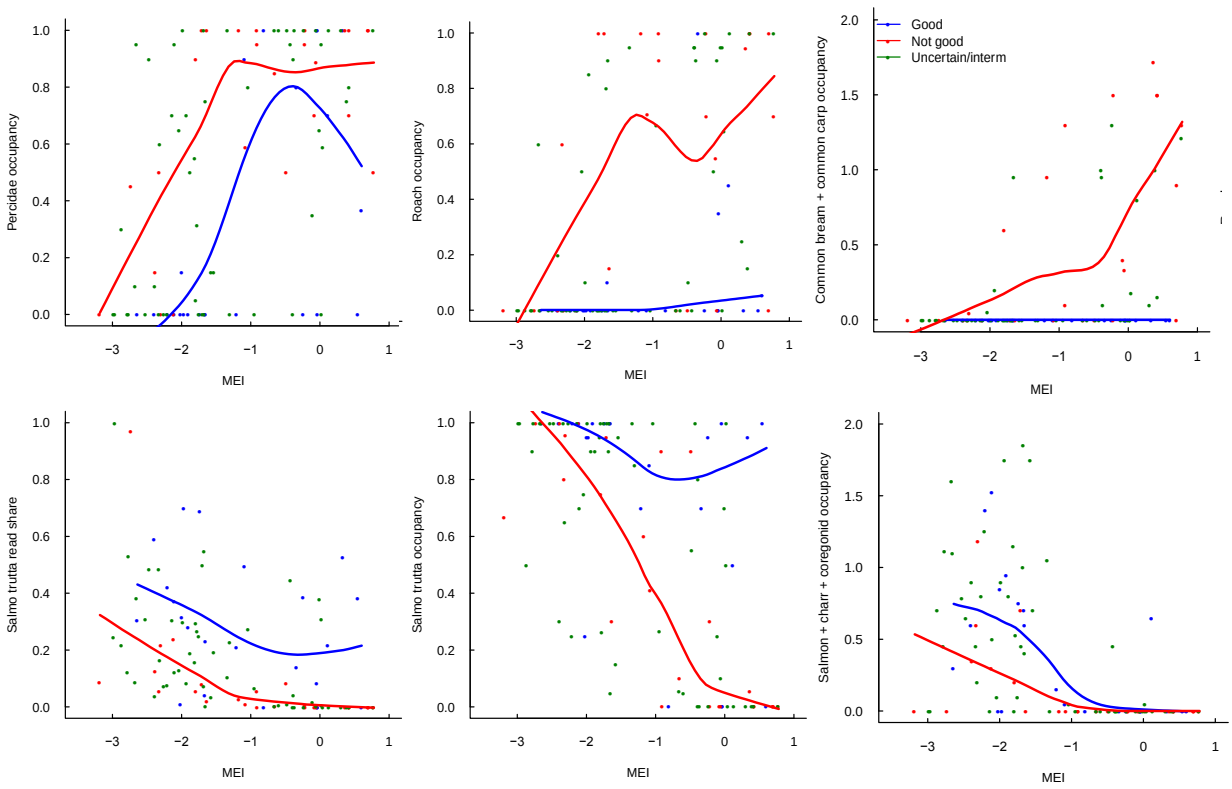
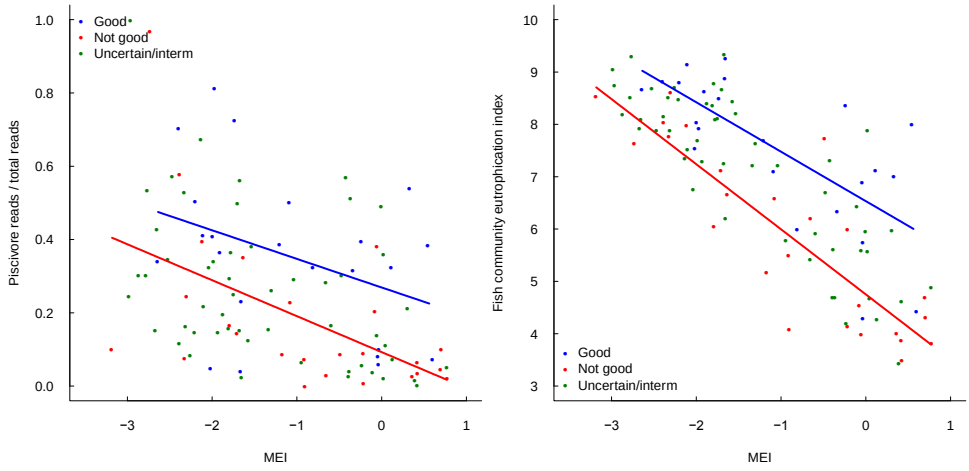


Figure 2 Distribution of occupancy values for selected fish metrics in relation to MEI showing effective separation of *a priori* good (blue) and not good (red) quality sites. Lines are illustrative of relationships and fitted via locally weighted smoothing. Upper panels show negative indicators (i.e. those that increase with pressure for a given MEI), lower panel shows positive indicators (i.e. those that decrease with pressure for a given MEI).

3.2. Three other derived metrics also showed potential value in classification; summed read counts of apex piscivores (i.e. brown trout + pike) expressed as a proportion of the total reads of all fish taxa, community eutrophication pressure index, and total recorded fish taxon richness (Fig 3).



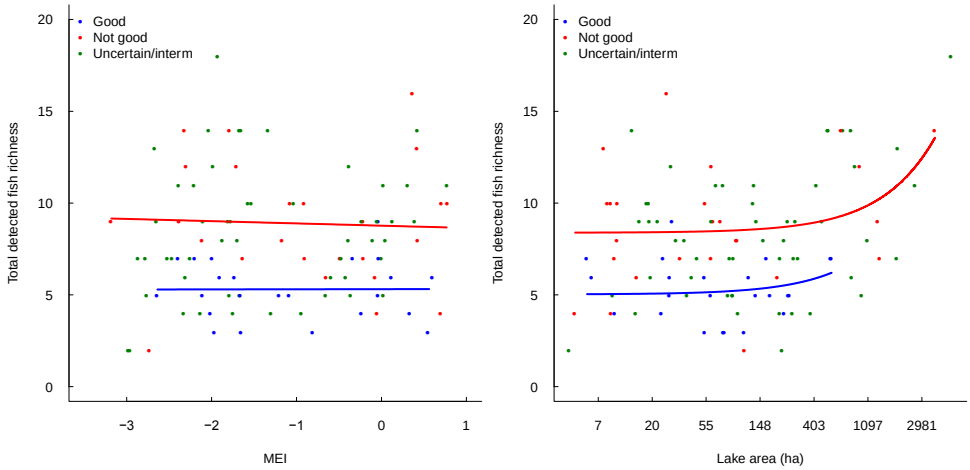


Figure 3. Variation in piscivore share (left) and fish community eutrophication index (right) in relation to MEI, and total detected fish richness in relation to MEI and lake area (lower panels) showing effective separation of *a priori* good (blue) and not good (red) quality sites.

3.3. Calculation of expected metric values

For each of the nine selected metrics the observed values in the subset of good quality sites were modelled with MEI and other key environmental variables (lake area, altitude, distance to sea) as covariates. For the occupancy- and read share-based models we used logistic regression, for the community pressure index a general linear model and for fish richness a generalized linear model with a poisson log link function. These models served to generate the expected metric values for all sites assuming no or low pressure.

3.4. Calculation of raw metric EQRs

The principle of the Observed (O) to Expected (E) ratio was followed in the calculation of metric EQRs. For positive indicators, where a higher value of the metric indicates higher quality (e.g. brown trout occupancy), EQR = O/E is used, whereas for negative indicators, where a higher value of the metric indicates lower quality (e.g. roach occupancy), EQR = (worst – O)/(worst – E) is used. The MEI value for each water body is used in the logistic regression function to generate the predicted (E) values for each metric. For example:

Loch Lomond (South) MEI = -1.92 (based on alkalinity of 0.232meq/L and mean depth of 19.5m, MEI = log (0.232/19.5) = -1.92)

Observed (O) occ of brown Trout = 0.9
Expected (E) occ of brown trout = 1/(1+(2.103*(7.451^-1.92))) = 0.96
Raw EQR = O/E = 0.9/0.96 = 0.94

Observed (O) occ of roach = 0.85
Expected (E) occ of roach = =1/(1+(73.702*(0.334^-1.92))) = 0.002
Raw EQR = (worst – O)/(worst – E) = (1-0.85)/(1-0.002) = 0.15

3.5. Transformation and combination of raw metric EQRs

The candidate list of nine separate metric EQRs was reduced to five (occupancy of (i) roach (ii) common carp + common bream (iii) Percidae (iv) brown trout (v) salmon + arctic charr + coregonids) by assessing redundancy among metrics and the effect of varying combinations of metrics on the strength of the pressure response relationship, agreement with independent classifications of ecological status based on other BQEs, and ability to discriminate between different *a priori* quality. Prior to being combined each metric raw EQR series was normalized to position values on a cumulative probability frequency curve (thus ranging from 0 to 1) defined by the mean and SD of that series. Where necessary the raw EQR values were first log transformed to remove the influence of outliers. Taking the above example for brown trout in Loch Lomond (south)

Log transform the raw EQR series = Log (0.94 +1) = 0.286

Based on the population of log transformed EQR values for brown trout occ the overall mean = 0.230 and SD = 0.178 (n=101).

Position the log transformed EQR on a normal cumulative distribution (values scaled from 0 to 1) =NORM.DIST(0.286,0.230,0.178,TRUE) = 0.627

3.6. The final combination rule to generate the 'overall fish EQR' was a simple averaging of the five separate normalized metric EQRs. Alternative weightings of variables or averaging various subsets of variables was found to have only a small effect on the relationship with the pressure index or pre-existing classifications (Fig 4) compared to the gain in complexity. A worked example showing calculation of the overall fish EQR is given in Appendix 5.

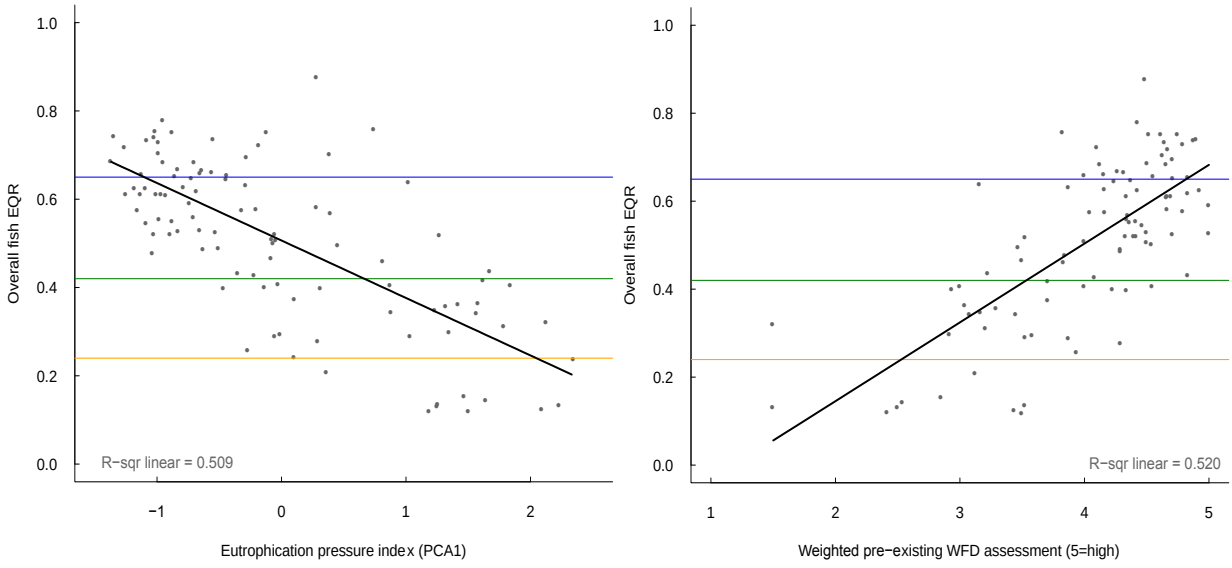


Figure 4. Relationship between the overall lake fish EQR based on eDNA data and the eutrophication pressure index (left) and a weighted average of the relevant pre-existing WFD assessments (right). Reference lines show HG (blue), GM (green) and MP (orange) class boundaries on fish EQR scale as defined in section 3.10.

3.7. These metrics represent a minimum adequate subset that most effectively exploits the eDNA metabarcoding data for use in ecological classification. The key principle is that sites are 'rewarded' (higher EQR) when species are detected that are expected to be there in the absence of pressure (e.g. arctic charr or coregonids in low productivity lakes) or are absent where expected to be absent (e.g. arctic charr in high productivity lakes), but are 'penalized' (lower EQR) where species are absent where they should be present (e.g. brown trout in moderate productivity sites) or are present where they should be absent (e.g. roach in low productivity lakes or roach at high occupancy in more productive lakes). Trial inclusion of EQR values for other metrics that suggested some with potential value in classification, such as pike occupancy, or rudd + tench occupancy, were found to weaken the performance of the tool in relation to pressures and the classifications of other BQEs.

3.8. In cases where observed values of *all* five component metrics were zero (i.e. brown trout, salmon, coregonids, arctic charr, roach, perch, bream and common carp were all completely absent from a lake) the tool would not be appropriate to classify ecological status. In our dataset values were positive for two or more metrics in 88% of sites and the scenario of all scoring species being absent never arose. A much larger dataset would allow an assessment of the risk of this occurring, and, perhaps, an interpretation of any underlying environmental basis. However, given the diverse population of lakes considered in our study, we regard it as exceptionally unlikely that none of the scoring fish taxa would be detected in a lake where this tool would be expected to be applied.

3.9. Sensitivity of tool

The overall fish EQR displays a highly significant relationship with the eutrophication pressure index (r^2=0.51; p<0.001, Fig 4) that is of a comparable strength to the relationship that other BQEs exhibit with eutrophication indicators in lakes (e.g. chlorophyll a or macrophytes vs TP (Lyche-Solheim et al., 2013)). Having selected metrics that distinguish between groups of sites defined by their contrasting nutrient pressure it is unsurprising to find that the overall fish metric calculated from the same dataset is related to a eutrophication gradient, but the strength of the relationship is still encouraging. As would be expected there was also a strong correlation between the overall fish EQR and a continuous index of ecological status (Fig 4) based on other lake BQEs and physico-chemical variables (weighted average where values reported as high status score 5, through to 1 for Bad status).

3.10. There was no evidence of sensitivity to hydromorphic pressures. This lack of sensitivity may reflect limited size of species pool in low productivity lakes where access or hydromorphic pressures are relatively more pronounced, or that such pressures are concealed if impacted sites contain relic populations of arctic charr or introduced coregonids. Alternatively, training the tool towards assessment of a dominant pressure in the form of eutrophication may constrain its sensitivity to other pressures, or non-migratory taxa that dominate may simply show low sensitivity to hydromorphological pressures. Fish would also be expected to demonstrate sensitivity to acidification but the number of sites in the present dataset known to be affected by acidification was too small to confirm this, or to develop separate metrics to reflect this impact. Age/size structure for populations of

a widespread focal species such as brown trout may also offer a better basis for assessing acidification pressure.

3.11. Class boundary placement.
The overall fish EQR was compared against the median class for a site based on existing WFD classifications using TP, chlorophyll, macrophytes, diatoms and CPET across available reporting cycles. Insufficient potential bad status sites were available for these to be considered separately so they were pooled with poor status (arguably, in the case of fish, bad status might be reserved for lakes lacking fish altogether, or perhaps those containing only non-native species, but neither of these scenarios were close to arising in our data set). There was a highly significant relationship between median class and overall fish EQR (GLM, $r^2 = 0.54$, $p<0.001$; Fig. 5), with highly significant ($p<0.005$) pairwise contrasts between all classes except M vs P/B ($p=0.11$). This indicates that the overall fish EQR can distinguish effectively between pre-existing WFD classes based on metrics sensitive to the same suite of pressures. The separation between sites classified <G or ≥G was especially strong.

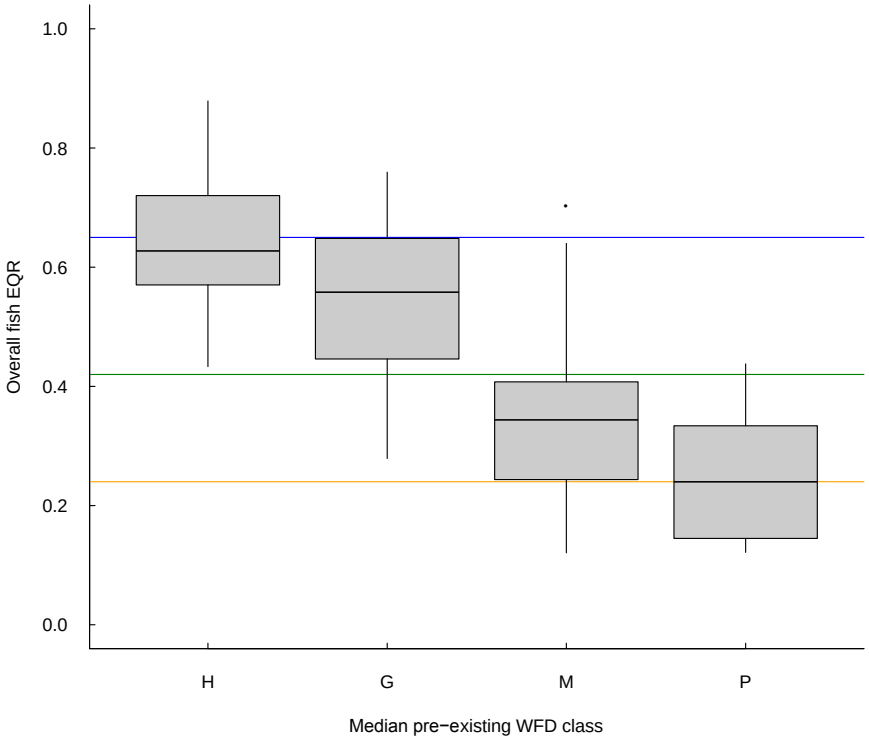


Figure 5. Distribution of fish EQR values according to the typical pre-existing WFD assessment of sites based on eutrophication relevant metrics. Class boundaries shown (HG=blue, GM=green, MP=orange) maximise the agreement between the fish classification and other classifications and minimize the classification bias.

3.12. Class boundaries were optimized using a matrix of fish-based class versus the median class. The median class represents the consensus view of a site with agreement between the fish class and the median class representing the 'least surprising' classification. Starting with the average between the lower CL of the fish EQR in the upper median class and the upper CL of the next lower median class the class

boundaries were changed iteratively to minimize the class bias and rate of misclassification. This indicated boundaries should be placed at HG=0.65, GM=0.42 and M/P=0.24 (Fig 5). Using these class boundaries 54% of sites were classified using fish the same as the median class, with 99% of sites being within one class of the median class (Table 1). The fish classification was 0.15 classes more precautionary than the median class. The classifications of the 101 lakes are given in Appendix 3.

Table 1. Classification of sites according to the overall fish EQR versus the typical pre-existing WFD classification based on eutrophication sensitive BQEs or physiochemical variables (left) and the 1oAo classification (right) inferred from the same parameters. This analysis refers to classes across multiple reporting cycles not only the most recent. Yellow cells = exact agreement. Blue cells =classifications differing by more than 1 class.

		fish class				
		H	G	M	P/B	sum
WFD median class	H	18	19	0	0	37
	G	7	18	7	0	32
	M	1	4	14	6	25
	P/B	0	1	2	4	7
	sum	26	42	23	10	101

		fish class				
		H	G	M	P/B	sum
WFD 1oAo class	H	1	3	0	0	4
	G	17	9	1	0	27
	M	8	29	14	4	55
	P/B	0	1	8	6	15
	sum	26	42	23	10	101

3.13. Of the 101 sites considered here 67 % were classified as being at high or good status. The bias towards high and good status is somewhat to be expected since 70% of sites were of low or moderate alkalinity types and 69% had a typical pre-existing WFD classification of high or good status. Of the low alkalinity lakes the vast majority are classified between the middle of good to high status (Fig. 6). Moderate alkalinity lakes cover the full status gradient. Most of the high alkalinity lakes are classified as Moderate or Poor status, the notable exceptions being those on hard limestone such as the Roman Wall Loughs.

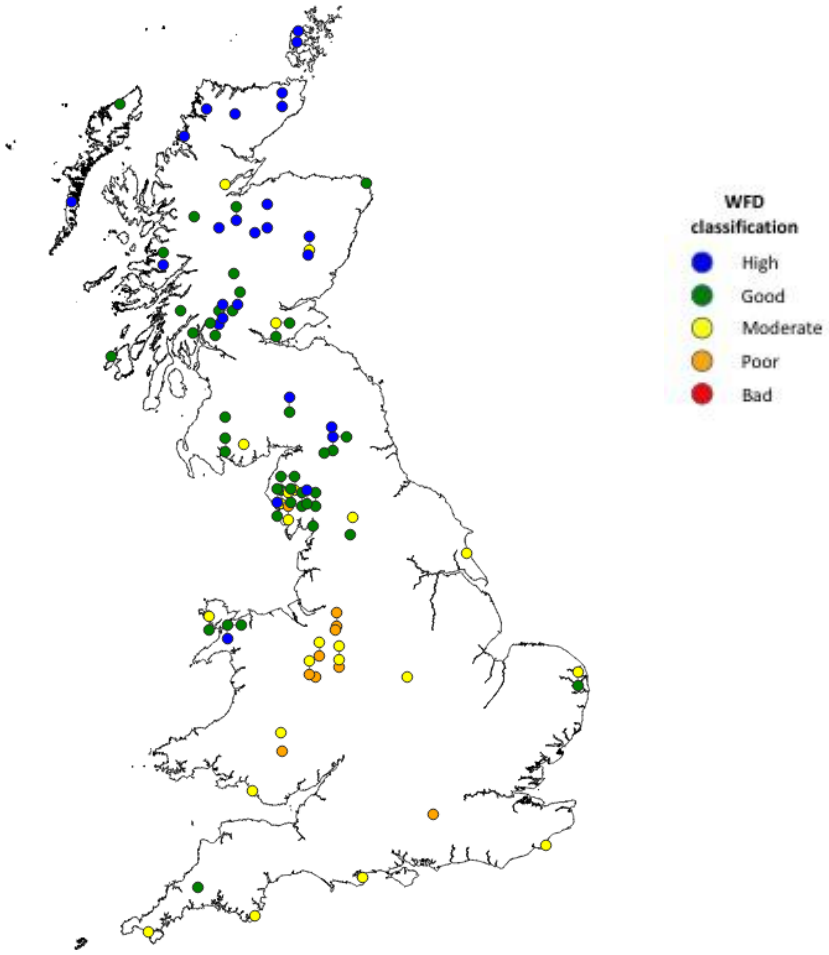


Figure 6. Distribution of the 101 water bodies based on their classification using fish eDNA. The position of some markers has been moved slightly to improve visibly.

3.14. On the basis of the proposed boundaries 8 sites would have a lower 1oAo classification than prior to the inclusion of the fish tool, although only one site (Loch Ussie) would move from above to below good status. The fish classification was 0.63 classes more relaxed than the 1oAo classification (any single method, regardless of BQE, will be more relaxed than the 1oAo classification unless that BQE was systematically the most precautionary one) with 30% of sites classified as the same 1oAo class and 92% of sites classified as within one class (Table 1). Using the fish classification nine sites were placed two classes more relaxed than the 1oAo classification, although most of these were close to a class boundary.

3.15. Intercalibration of proposed method

Since the Irish gill netting method, FIL2, has already been intercalibrated in Northern GIG, one option is to apply this method to sites in the current dataset that also have gill-netting data. This data was also used in the development of a gill-netting based method specific to Scotland, SFINX. FIL2 was applied to gill netting data for 27 sites in the present data set to generate an EQR. The SFINX EQR was also available for 18 of these sites.

3.16. The proposed eDNA method was weakly correlated with SFINX (r^2 = 0.27; p = 0.03) and slightly less well correlated with FIL2 (r^2 = 0.21; p = 0.06). However, SFINX itself and FIL2 were also very poorly correlated (r^2 = 0.12; p = 0.16), despite both being based on gill netting data and being developed by the same authors. This raises questions over the compatibility of gill netting data from Scotland and NW England with that from Ireland, or in the comparability of their fish communities. While both FIL2 and the eDNA tool had a similar relationship with the pressure axis over the common subset of 27 sites (FIL2: r^2 = 0.29; p = 0.004, eDNA: r^2 = 0.31; p = 0.002) most of these inter-relationships would fall below the thresholds needed for a successful intercalibration.

4. Discussion & Recommendations

4.1. Freshwater fish are not the easiest of organisms to use in ecological classification, being species poor, hard to sample and having a distribution that is sensitive to natural or artificial barriers or widespread alteration through introductions. The present approach, based on eDNA metabarcoding, offers an effective and non-invasive alternative for classification of lake ecological status, which generates classifications that are largely compatible with those provided by other WFD measures and is sensitive to the major pressures affecting lakes.

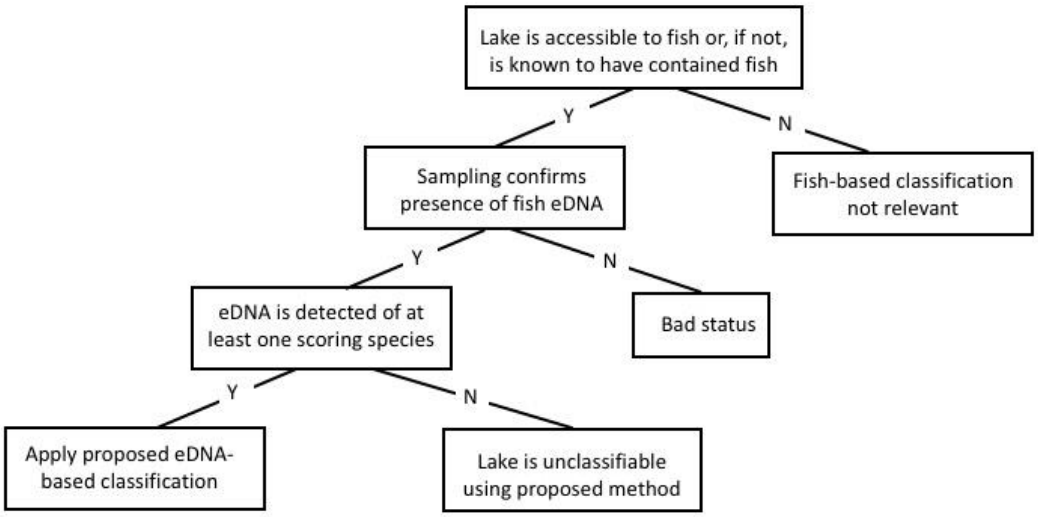


Figure 7. Decision tree illustrating when it is valid to apply the proposed classification method.

4.2. Figure 7 provides a decision tree to assess when application of lake fish classification in general, and the proposed method specifically, is appropriate. In summary a lake should have the potential to contain fish based on its connectivity, or, if not, be known to have contained fish, for fish classification methods in general to be applicable. Assuming that this applies then water samples for eDNA metabarcoding should be collected using compliant approaches. In the event that no fish eDNA is detected in these samples the default position is that the site would be classed as Bad status. We expect this scenario to be extremely rare and to only apply in lakes

with well-known problems that are also captured by other BQEs (e.g. history of chronic acidification, hypertrophy leading to regular fish kills, or industrial pollution (chemical or thermal)). If eDNA is detected from one or more of the species that contribute to the metrics used in the described method then eDNA-based classification can be applied and the resulting classification used. If not, the site is unclassifiable (but Bad status cannot be assumed).

4.3. The sensitivity of the tool in isolated low productivity lakes, where the fish community may be naturally restricted to brown trout, plus a few other catholic species, such as three-spined stickleback or eel, may be attenuated, but the same problem will confront any fish-based tool. For some metrics it is also possible that occupancy is influenced by the abundance of other fish taxa that are not themselves used as indicators and which vary independently of pressure. For example, large populations of pike might potentially reduce brown trout populations in lower productivity lakes, or prevent their re-establishment in higher productivity lakes, thereby lowering ecological status. However, a larger dataset would be required to investigate this.

4.4. The metrics required in the proposed method are easily generated from summary level eDNA-based data, do not require interrogation of sample level data, and, being based on occupancy, should be relatively insensitive to the influence of false positives. Previous studies have confirmed the value of eDNA based approaches for fish monitoring, with a close match being found with contemporary gill netting data plus detection of additional species not recovered by conventional methods (Hänfling et al., 2016; Li et al., 2019). eDNA-based assessments also offer the advantages of highly standardisable field survey effort with lower sampling and surveyor bias and lower inter-annual variation. These features should be reflected in increased confidence of classification. Although not considered as an integral part of the present method eDNA-based sampling should also be effective for detecting high or low impact invasive non-native fish species at low abundance and may therefore contribute to classification over-rides.

4.5. The proposed tool is rather weakly correlated with a gill netting based method, FIL2, adopted for use in the island of Ireland. It would be possible to manipulate the selection of metrics to optimize the correlation of the eDNA-based tool with FIL2. Since this would be at the expense of the performance of the tool in its intended region of application it is not advised. Given that the eDNA tool displays close agreement with typical classifications of water bodies based on methods which are themselves intercalibrated it is suggested that the resultant classifications are fit for purpose. FIL2 is a type-specific method that uses lake types that are potentially too coarse to be compared with the present tool. It is also heavily reliant on BPUE data for the total fish community, native species and perch which may limit its comparability with classifications based on eDNA data.

4.6. The dataset involved in the development of this tool is relatively small and therefore more subject to the influence of atypical sites. Supplementing it with data from very large high status lakes in Scotland and better quality sites in unglaciated regions of central or southern England would help to expand the envelope of conditions in

reference sites. This would allow more effective testing and any necessary refinement. Similarly, including more low quality sites (e.g. ultra-acidified, industrially polluted, or hypertrophic and prone to fish kills) would be useful in resolving the position of the P/B boundary. Applying the fish classification to an independent set of test sites covering a similar MEI and pressure gradient would also be advantageous.

4.7. The morpho-edaphic index naturally covaries with a NW-SE gradient across Britain and will therefore also covary with human population density and pressures additional to eutrophication. Diagnosing reduced fish EQR as being specifically due to nutrient pressures is therefore not possible. The overall pool of fish taxa also diminishes from south to north reflecting the post-glacial history of Britain. This may increase the risk of failure in more southerly or highly connected lakes compared to northerly or isolated ones, over and above the intrinsically higher levels of pressure at these sites. However, the pool of lakes in this study from unglaciated regions was small, especially in terms of reference sites, and, even with more data, some of these elements of covariation are intractable.

4.8. Determining the origin or native status of fish in a lake is not always straightforward, even where supporting information exists. Much information on introductions beyond normal ranges or translocations within ranges is incomplete or anecdotal and it is difficult to utilize such information in ecological classification, other than subjectively. We have taken the pragmatic view that if a population of a fish species survives long-term in a lake then the environmental conditions there must be conducive to its survival, regardless of its origin.

4.9. False positives, arising from external inputs of the DNA of species not found in the water body, are a legitimate concern in all eDNA-based monitoring. Avian deposition, especially by piscivores such as cormorants, wastewater inputs from STW, hatcheries, camping or waterside restaurants, and inflows from low order feeder streams are all relevant to fish in lakes. A source of false positives of perhaps unique relevance to lake fish concerns the use of dead bait or commercially available feed and synthetic oils by anglers. Inputs from the above wide range of sources could therefore contribute towards the pattern shown in Figure 3 where ‘not good’ lakes have significantly higher richness than ‘good’ lakes of an equivalent size and MEI (although the pattern shown was largely preserved if rare taxa were removed).

4.10. False positives, such as marine taxa detected far inland, are easily excluded, but it is more difficult to prove that eDNA-inferred abundance or occupancy of species that plausibly exist as live fish in a lake is not influenced by false positives. For example, brown trout occupancy might potentially be inflated by false positives in lakes with a high density of feeder streams, or after periods of heavy rainfall. In the present study rainbow trout are the only species that offer a potential cause for concern in terms of the use of eDNA for fish assessment. This species was found in 23 sites. Six of these were known to have been stocked and this was consistent with a high read share of rainbow trout (>15% of reads) in these sites. In most other sites the read share of rainbow trout was negligible (<1%) but a further 6 sites with no history of stocking contained rainbow trout DNA at read shares of 1-5%, occasionally combined

with quite high occupancy (>0.5). The location of these lakes was consistent with hatchery, wastewater or angler-related inputs. Using occupancy-based, as opposed to read share-based metrics avoids the direct influence of one species on the calculation of the share of another, so false positives of rainbow trout are not a serious issue. However, one should be aware that certain species, especially those in the human food chain, could affect the eDNA signal at some sites.

4.11. In its current form this tool is suitable for reporting the status of fish in water bodies where eutrophication is the dominant pressure. The focus of the tool is on metrics related to species composition and abundance, where, at least in terms of composition this approach consistently outperforms conventional capture methods. Information on age/size structure is not currently available through eDNA-based approaches. Obtaining a surrogate for this may be possible but would likely require significantly increased sampling effort (e.g. temporal replication to identify seasonal aggregations or changes in distribution consistent with spawning). However, elsewhere in Europe, most lake fish classification methods based on conventional capture methods (Ritterbusch et al., 2017) incorporate some aspect of population size/age only as a relatively minor component of their classification alongside species composition and abundance, implying that this aspect carries less weight in the final classification. As such, omitting age/size aspects from an eDNA-based classification should not prove overly critical.

5. References

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6. Appendices

Appendix 1: eDNA extraction and processing method

Appendix 2: Sampling method and evaluation

Appendix 3: Face value water body classifications for eDNA-based fish tool, plus a priori quality designations and observed fish metrics

Appendix 4: Scores for fish eutrophication index

Appendix 5: Calculator spreadsheet and worked example showing calculation of metric EQRs and the overall fish EQR