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METHOD

Single metabarcoding multiplex captures community-level freshwater biodiversity and beyond

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Abstract

Cost-effective and accurate quantification of biodiversity is important for biodiversity conservation, resource management, and forecasting. Traditional monitoring approaches have relied on direct observations, remote sensing, and mark-recapture techniques, providing insights into species ecology and the impact of pollution and climate change on indicator species. However, these techniques are typically low throughput, expensive, and can be invasive. In addition, they cannot detect cryptic diversity and are biased toward species that leave identifiable remains. DNA-based methods, such as metabarcoding or single marker gene assays, have enabled high throughput screening of a wide range of taxonomic groups, including ones without well-preserved remains. When compared with traditional techniques, these approaches have high throughput, can resolve cryptic diversity, do not require taxonomic specialist skills, and are non-invasive. However, although they are comparatively cheaper than traditional approaches, they are expensive when applied at the community-level as single marker assays are amplified and sequenced independently. Multilocus approaches in which multiple gene markers are amplified in a single reaction are desirable to deliver community-level assessments in a cost-effective manner. Yet, they are uncommon because of technical challenges that may lead to biases in downstream analyses, such as index hopping and unbalanced representation of taxonomic groups. Here, we developed a highly multiplexed protocol that combines the early pooling of marker genes that target broad taxonomic groups and taxon-specific markers in a single tube reaction. This step is followed by the pooling of up to 384 samples per locus ($N = 15{,}636$ samples) with unique dual-indexed sequencing adapters in a single sequencing run. This approach dramatically reduces the costs of community-level biodiversity quantification and lowers the need for input DNA without compromising output quality. We optimized the multiplex assay on lake freshwater sediment samples and benchmarked the assay on samples from other environmental matrices, demonstrating its direct application to the river and marine communities.

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KEYWORDS
eDNA, freshwater, marine, metabarcoding, multiplex, sediment, singleplex

1 | INTRODUCTION

Biodiversity monitoring is the foundation for effective biodiversity conservation, resource management, and forecasting. Traditional biodiversity monitoring techniques, such as direct observations, remote sensing, and mark recapture techniques have provided valuable insights into species ecology and the impact of pollution and climate change on indicator species (Eveleigh et al., 2007; Ropert-Coudert & Wilson, 2005; Yoccoz, 2012). However, they have significant limitations due to the difficulties associated with the correct identification of (cryptic) species or life stages from the same species. These techniques require specialist taxonomic expertise, are not standardized when it comes to sampling protocols, and can be invasive. Traditional techniques are also typically low throughput and biased towards species that leave identifiable remains (Gillson & Marchant, 2014).

In the last decade, DNA-based methods (e.g., metabarcoding) have revolutionized conventional biodiversity research by enabling high throughput screening from environmental matrices without being limited to taxonomic groups with well-preserved remains (Creer et al., 2016; Cristescu & Hebert, 2018). DNA-based approaches have a higher throughput than traditional approaches, do not require taxonomist specialist skills, can resolve cryptic diversity, and be applied to bulk DNA extracted from environmental matrices ('Environmental DNA' or eDNA; (Cristescu & Hebert, 2018)). Furthermore, large surveys can be conducted in a relatively fast and cost-effective manner, and over large geographic areas, including remote regions, non-invasively (Taberlet et al., 2018). By matching sequence similarity to records in public databases (e.g., NCBI and SILVA), molecular operational taxonomic units (MOTUs) can be identified, enabling the analysis of taxonomic compositional shifts and estimates of species richness.

To date, most metabarcoding studies have used a single locus approach; cytochrome c oxidase subunit I (COI) is commonly used for vertebrates and invertebrates (Krehenwinkel et al., 2018; Leray et al., 2013), the internal transcribed spacer (ITS) is used to identify fungi (Schmidt et al., 2013), plastid DNA (rbcL) is used for plants and primary producers (Chase & Fay, 2009; Tse et al., 2018), and 12S is used for fish (Miya et al., 2015). A multi-locus approach is highly desirable to deliver community-level assessments in a cost-effective manner (Ficetola & Taberlet, 2023). However, thus far, community-level assessments of biodiversity have only been achieved with the integration of results from individual loci (Eastwood et al., 2022, 2023; Li et al., 2023). A multi-locus approach improves the robustness of taxonomic assignment alleviating false negatives caused by random missed amplifications of target genes caused for example by DNA degradation or mutation in primer sites (Zhan et al., 2014). Species detection rate based on multiple loci can be up to 35% more accurate than when using single locus approaches (Zhang et al., 2018). However, highly multiplexed approaches that enable estimates of the community-level biodiversity in a cost-effective manner are largely missing because they can be challenging to optimize (Ficetola & Taberlet, 2023). A step in the right direction is the 2-step PCR protocol, which includes a first-round PCR (PCR1) that amplifies a target DNA locus or marker gene region using universal primers, followed by a second-round PCR (PCR2) that appends sample-specific indexes to marker gene regions (Bohmann et al., 2022). After PCR2, samples are usually combined in a multiplex for high-throughput sequencing. Alternatively, a sample-specific index may be added to PCR1 (Bohmann et al., 2022). This approach improves throughput, but it requires upfront costs (Bohmann et al., 2022; Caporaso et al., 2011; Ushio et al., 2022).

We developed a highly multiplexed protocol that combines pooling of samples amplified with a multiplex approach at the PCR1 stage, including four loci, and pooling after PCR2 at the sequencing stage of up to 384 samples per locus, significantly reducing costs of metabarcoding and lowering the amount of input DNA required to capture community-level biodiversity. We optimized this protocol for lake freshwater communities because these communities support humans and wildlife (Darwall et al., 2018), and have high conservation value, delivering important ecosystem services (e.g., clean water, food provision, and recreation) (Dudgeon et al., 2006; Ruckelshaus et al., 2020). The samples used for this protocol optimization were a subset of samples isolated from a well-characterized sedimentary archive (see (Eastwood, 2023) for details). In this previous study, a traditional single locus approach was applied to eDNA extracted from sediment samples, providing reassurance on the quality of the input DNA used in this study. Here, we combined three metabarcoding primer pairs that target 18S and 16S loci, broadly capturing prokaryotes and eukaryotes, with a taxon-specific locus (rbcL) used by regulators to determine water quality in both rivers and lakes. By combining four loci, we capture community-level biodiversity in a single tube reaction.

We benchmarked the multiplex by comparing taxonomic detection rates and accuracies of the multiplex with single locus metabarcoding assays on the same samples. We validated the multiplex on independently sampled material originating from diverse environmental matrices, including river water, soil, peatland, coastal, and offshore marine environments. The developed multiplex approach has the potential to significantly improve the capacity for both biodiversity routine monitoring and research discoveries.

2 | MATERIALS AND METHODS

2.1 | Multiplex optimization on freshwater *seda*DNA samples

A standard 2-step PCR protocol includes a primary reaction for the target locus or marker gene regions in PCR1, in which primers with

5′ sequence overhangs are added to the marker gene of choice, and a PCR2, which carries sequencing adapters and indices to be attached to cleaned PCR1 products (Bohmann et al., 2022; Ushio et al., 2022). PCR2 libraries are then pooled for sequencing on an Illumina or Illumina-compatible platform, following removal of excess primers. We modified this protocol by multiplexing four loci in PCR1 (Figure 1). The multiplex protocol was optimized on randomly selected bulk environmental DNA (eDNA) samples extracted from a freshwater lake sediment core from a previous study (Eastwood et al., 2023) using DNeasy PowerSoil kit (Qiagen) (*seda*DNA), following the manufacturer instructions, in a PCR free environment. Extraction and PCR blanks were used to monitor for contamination. The metabarcoding loci used in the multiplex were: two regions targeting eukaryotes broadly [(18SV1V2) (Hadziavdic et al., 2014) and (18SV8V9) (Bradley et al., 2016)], and prokaryotes (16SV4) (Caporaso et al., 2011), plus a taxon-specific marker targeting diatoms (rbcL) (Kelly et al., 2018). Triplicate samples were amplified in PCR1 using Q5 HS High-Fidelity Master Mix (New England Biolabs) following the manufacturer's instructions. To protect commercially sensitive information, the amplification parameters of this step will not be disclosed. After removing excess primers with High Prep PCR magnetic beads (Auto Q Biosciences), cleaned PCR1 products were pooled in a second PCR in which unique dual-indexed sequencing adapters allowed the pooling of up to 384 samples per locus in a single sequencing run ($N = 1536$ samples per run). Unique dual indices were used to reduce index-misassignment and index-hopping between samples (MacConaill et al., 2018). PCR2 amplicons were purified using High Prep PCR magnetic beads (Auto Q Biosciences) and quantitated using a 200 pro plate reader (TECAN) using qubit dsDNA HS solution (Invitrogen). A standard curve was created by running standards of known concentration on each plate against which sample concentration was determined. To confirm that all amplicons were equally represented in the PCR1 multiplex, we performed single locus or gene marker PCRs on the cleaned PCR1 products (Figure S1). This approach was adopted because the amplicons had overlapping lengths and could not be distinguished based on gel migration alone (Figure S1). PCR2 libraries were mixed in equimolar quantities (at a final concentration of 12 pmol) using a biomek FXp liquid handling robot (Beckman Coulter). The final molarity of the pools was confirmed using an HS D1000 tapestation screentape (Agilent) prior to 250 bp paired-end sequencing on an Illumina MiSeq platform.

2.2 | Single and multiplex performance on freshwater sediment samples

The sequenced reads were demultiplexed per locus using cutadapt v4.1 (Martin, 2023), and analyzed with QIIME2 v2022.8 (Bolyen et al., 2019). Trimming, filtering, merging and denoising of reads was done using the QIIME2 DADA2 module (Callahan et al., 2016) with pooling-method set to 'pseudo' and all other parameters set to default. Taxonomy assignment was completed with the QIIME2 feature-classifier module (Bokulich, Kaehler, et al., 2018) with naive-bayes taxonomic classifiers trained using different reference databases, depending on the marker gene: the SILVA v138 database was used for the assignment of the 16S and 18S reads (Yilmaz et al., 2014); and the diat.barcode v9.2 was used for the assignment of rbcL reads (Rimet et al., 2019). The cleaned reads were rarefied and diversity indices (e.g., alpha and beta diversity) were calculated using the QIIME2 diversity module.

The performance of single and multiplex assays was assessed by comparing alpha and beta diversity, using the rarefied reads. Alpha diversity was measured as Pielou evenness and Shannon diversity, supported by Kruskal-Wallis (Kruskal & Wallis, 1952) using the function alpha-group-significance in the QIIME2 diversity module. Beta diversity was measured as Bray-Curtis distance and significant differences between single and multiplex assays assessed with a PERMANOVA test (999 permutations) using the funciton beta-group-significance in the QIIME2 diversity module.

2.3 | Benchmarking the multiplex on eDNA from other environmental matrices

We benchmarked the multiplex using eDNA samples extracted from different environmental matrices and including grassland, marine coastal and marine offshore water, marine coastal and marine

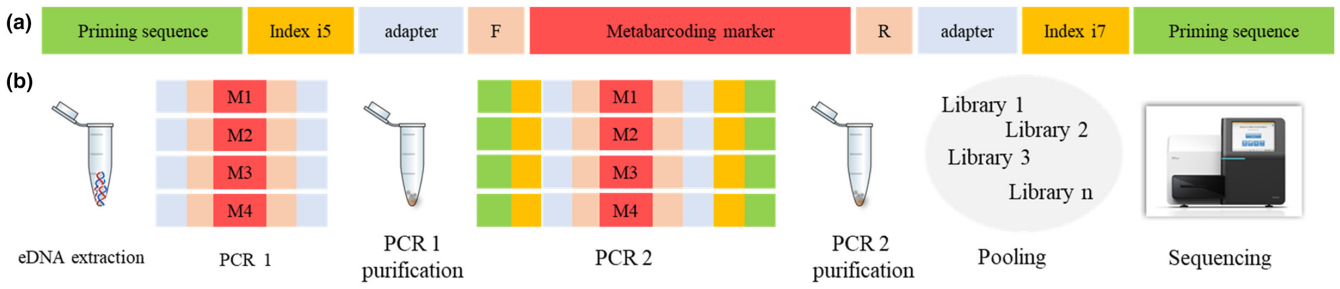

FIGURE 1 (a) The composition of a dual-indexed metabarcoding Illumina library sequence, including a gene marker or locus, forward (F) and reverse (R) primers, sequence adapters, Illumina indexes (i5 and i7) and sequences used to prime to sequencing flow cell. (b) Multiplex key steps are shown, including eDNA extraction, multiplexed PCR1 with 4 metabarcoding markers plus a cleaning step to remove excess primers, PCR2 plus a second cleaning step and a final pooling step before sequencing on an Illumina or Illumina compatible platform.

offshore sediment, river water, peatland and woodland (Table S1). These samples were provided by NatureMetrics (www.naturemetrics.co.uk) and are therefore anonymised to adhere to data protection requirements. eDNA from water samples (freshwater and marine) was extracted using a DNeasy Blood and Tissue Kit (Qiagen) following Spens et al. (2017). The original method was modified as described in Egeter et al. (2023). Briefly, proteinase K was added directly to the disc filters on which water was filtered and stored. 1 mL of the lysate was carried forward for extraction with the DNeasy Blood and Tissue Kit (Qiagen). eDNA was extracted from soil and sediment samples using DNeasy PowerSoil Kit (Qiagen). An extraction blank was processed with each batch of extractions to assess potential contamination in the DNA extraction process. DNA was purified to remove PCR inhibitors using a DNeasy PowerClean Pro Cleanup Kit (Qiagen). Purified DNA extracts were quantified using a Qubit dsDNA HS Assay Kit on a Qubit 3.0 fluorometer (Thermo Scientific). The DNA concentration was quantified using a Qubit DNA broad-range kit.

Some samples extracted from woodland, grassland, and peatland did not generate a visible PCR1 product on agarose gel. We suspected that PCR inhibitors (e.g., humic substances) were responsible for these failures. Therefore, we tested the single and multiplex assays with the addition of bovine serum albumin (BSA) (Ramalingam et al., 2017) and compared the performance of these assays with the regular assays described above. The samples collected from different environmental matrices were amplified with the same four amplicons used in the *seda*DNA samples above, both in single and multiplex assays. The metabarcoding libraries and sequencing strategy were the same as above. The sequenced reads were demultiplexed and analyzed with QIIME2 v2022.8 (Bolyen et al., 2019), as above. The taxonomic assignment followed the same strategy used for the *seda*DNA samples described above.

The performance of the benchmarking samples used in single and multiplex assays was assessed by comparing overall alpha and beta diversity, as above. Significant differences in beta diversity (Bray-Curtis distance) between single and multiplex assays was assessed with a PERMANOVA test (999 permutations) with sample type as strata using the function pairwise.adonis2 (v0.0.1) (Arbizu, 2017), wrapping the package vegan (v2.5–7) (Oksanen et al., 2020) in R (v4.0.2) (R Core Team, 2020). In addition, we assessed alpha diversity (Pielou evenness and Shannon diversity) of sample types (e.g., river water, marine sediment) between single and multiplex using a Wilcoxon signed rank test with Benjamini & Hochberg correction for multiple testing using the pairwise-distances function in the longitudinal module in QIIME2 (Bokulich, Dillon, et al., 2018). PCoA of Bray-Curtis distance was used to visualize the similarity between single and multiplexed samples, plotted using ggplot2 (v3.4.0) (Wickham, 2016) in R (v4.0.2) (R Core Team, 2020). A Venn diagram was used to visualize the overlap of species and ASVs between the single and multiplex assays for the total number of features (100%), as well as for the topmost abundant features making up 85% and 70% of the reads in the two assays, plotted using the package ggven (v0.1.10) (Yan, 2023) in R (v4.0.2) (R Core Team, 2020). This approach was used to determine whether discrepancies, if any, between assays could be explained by the capture efficiency of rare species. To assess whether sequencing effort would lead to a convergence in the number of ASVs detected by the two assays, we performed a rarefaction analysis with resampling strategy, with the function rarecurve in the package vegan (v2.6–4) (Oksanen et al., 2020) using R (v4.0.2) (R Core Team, 2020), plotted with ggplot2 (v3.4.0) (Wickham, 2016).

3 | RESULTS

3.1 | Single and multiplex assays on *seda*DNA samples

The rarefaction depth for the *seda*DNA samples was as follows: 16SV4 = 8245; 18SV1V2 = 12,584; 18SV8V9 = 17,703; and rbcL = 6372. The alpha diversity measured on *seda*DNA samples, both as Pielou evenness and Shannon diversity, did not significantly differ between single and multiplex assays (Table 1). The beta diversity measured as Bray-Curtis distance across the four loci or gene markers did not differ significantly between single marker and multiplex assays (Table 2).

3.2 | Benchmarking the newly developed multiplex in other environmental matrices

The rarefied sequence depth for the multiplex benchmarking samples were as follows: 16SV4 = 5549; 18SV1V2 = 7734; 18SV8V9 = 10,900; rbcL = 1590. The Pielou evenness index (alpha diversity) measured across all samples extracted from different environmental matrices did not differ significantly between single marker genes and multiplex assays for the 16S and both the 18S loci (Table 3; Figure 2). A significantly different Pielou index was observed between single and multiplex assays for the rbcL locus, for which some samples in the single and multiplex assays showed dissimilar evenness (Table 3; Figure 2). The Shannon index showed more variability than the Pielou evenness with three out of four indices showing significant difference between single and multiplex (Table 3; Figure 2). The addition

TABLE 1 Kruskal-Wallis test on Pielou's evenness and Shannon diversity calculated between single and multiplex assays for four loci (18SV1V2; 18SV8V9; rbcL; and 16SV4) sequenced on the *seda*DNA samples.

	Pielou evenness		Shannon diversity	
	H	*p*	H	*p*
16SV4	0.0	1.0	2.4	0.12
18SV1V2	1.8	0.18	0.2	0.65
18SV8V9	1.5	0.22	0.0	1.0
rbcL	0.6	0.44	0.6	0.44

of BSA to the PCR reactions improved the amplification results, but did not significantly change the sequencing results, as the statistical tests comparing multiplex assays with and without BSA showed (Table 3). No significant (Wilcoxon signed rank *p* adj. <=0.05) difference in alpha diversity (both Pielou evenness and Shannon diversity) was observed between individual sample types that successfully amplified with both the single plex and multiplex (Table 4).

The beta diversity, measured as Bray-Curtis distance, did not significantly differ between single and multiplex across all marker genes (Table 5; Figure S2). There was no significant difference in beta diversity between multiplex assays with and without BSA (Table 5).

The percentage of ASVs identified by both single and multiplex assays ranged between 44.9% in 18Sv1v2 and 21.1% in rbcL when 100% of the features were considered (Figure 3). The performance of the two assays converged when the top 85% most abundant features were included in the analysis (Figure 3). The similarity increased more evidently for the 18S regions (97.2% in 18Sv1v2, 97.4% in 18Sv8v9), whereas it increased to a lesser, but still considerable, extent in 16Sv4 and rbcL (82.1% and 80.0% respectively; Figure 3). When the 70% most abundant features were considered, the ASVs identified by single and multiplex assays overlapped 98.9% in 18Sv1v2, 98.9% in 18Sv8v9, 96.4% in the 16Sv4 and 97.9% in the rbcL gene marker (Figure 3). The single and multiplex assays showed similar performance when overlap was studied at species rather than ASV level (Figure S3).

The rarefaction analysis, aimed at understanding whether a higher sequencing effort would lead to more congruence between single and multiplex assays, showed that both assays had plateaued at the rarefied number of reads used in our analyses (Figure S4), suggesting that a higher sequencing effort would not increase the number of ASVs or species detected.

TABLE 2 Permutational Multivariate Analysis of Variance (PERMANOVA) using Bray-Curtis distance (F) assessing differences between single plex and multiplex assays on *seda*DNA samples (999 permutations) across four loci (18SV1V2; 18SV8V9; rbcL; and 16SV4).

Locus	F	*p*
16SV4	0.52	0.65
18SV1V2	0.53	0.74
18SV8V9	0.37	0.69
rbcL	0.18	0.68

4 | DISCUSSION

Holistic approaches that enable the quantification of community-level biodiversity are critical to research and monitoring efforts. Because environmental change affects taxonomic groups differently, ignoring the biotic interactions of a species within its food web can lead to wrong estimation of effects (Fricke et al., 2022; Urban et al., 2016). Only by capturing the response of entire communities to environmental change, can we begin to understand the diagnostic links between environmental drivers and loss of biodiversity (Eastwood et al., 2022, 2023; Li et al., 2023; Urban et al., 2016).

Highly multiplexed metabarcoding approaches have the potential to meet the challenge of capturing community-level biodiversity and help identify the causes of biodiversity loss, at comparable efforts and costs than required by single gene markers (e.g., Balint et al., 2018). However, they have technical challenges that may lead to biases in downstream analyses. One of the most common challenges of multiplexing is finding suitable DNA regions and achieving a balanced amplification of all regions, avoiding potential competitive PCR amplifications. It is often challenging to obtain an even amplification success rate across diverse taxonomic

TABLE 3 Kruskal-Wallis test on Pielou's evenness (H) and Shannon diversity calculated between single and multiplexes (both regular and BSA), and between the two multiplexes assays in the benchmarking samples.

			Pielou evenness		Shannon diversity	
	Group 1	Group 2	H	*p* adj value	H	*p* adj value
16Sv4	bsa	mplex	0.003	0.955	0.079	0.779
	bsa	splex	1.228	0.402	7.277	**0.011**
	mplex	splex	1.271	0.402	7.226	**0.011**
18Sv1v2	bsa	mplex	0.103	0.749	0.053	0.817
	bsa	splex	1.473	0.337	5.975	**0.022**
	mplex	splex	1.920	0.337	7.013	**0.022**
18Sv8v9	bsa	mplex	0.127	0.973	0.062	0.803
	bsa	splex	0.001	0.973	1.276	0.388
	mplex	splex	0.015	0.973	1.830	0.388
rbcL	bsa	mplex	0.006	0.937	0.002	0.968
	bsa	splex	11.590	**0.001**	16.388	**0.000**
	mplex	splex	12.650	**0.001**	17.614	**0.000**

Note: *p*-values are Benjamini & Hochberg corrected. Significant adjusted *p*-values are in bold. Splex – single plex; mplex – multiplex (regular protocol); bsa – multiplex with addition of BSA.

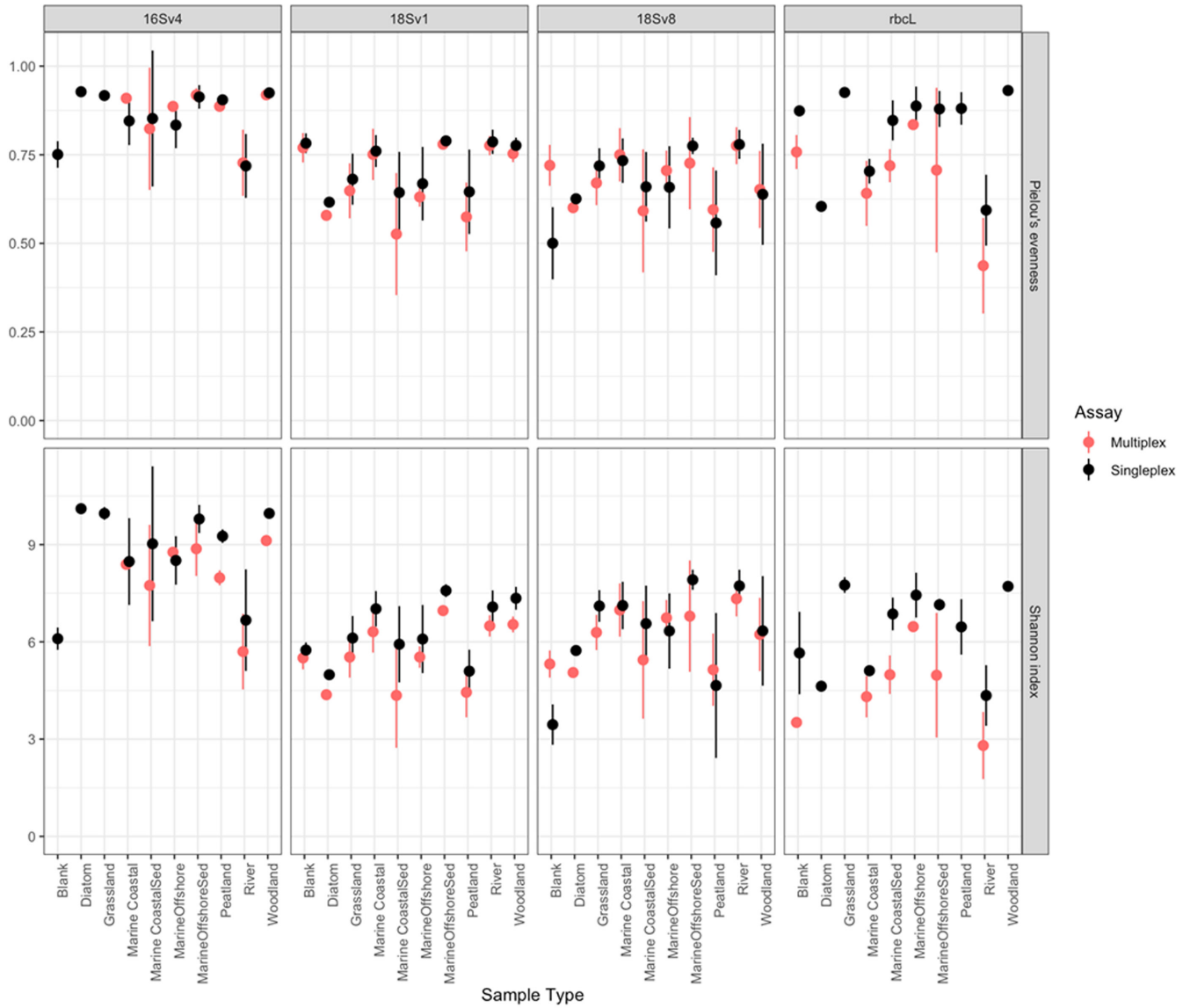

FIGURE 2 Alpha diversity mean and standard error for the benchmarking samples listed in Table S1 measured in single (black) and multiplex (red) assays. For each sample type mean and standard deviation are shown. Lack of a data point indicates that the specific sample/plex failed.

groups (Bohle & Gabaldon, 2012). A step in the right direction are recent efforts that successfully apply early pooling strategies after PCR1 in a 2-step traditional PCR protocol (e.g., application to fish communities (Ushio et al., 2022)). Yet, these strategies are costly because each sample is tagged with a unique string of nucleotides to make the assignment of sequences to samples more robust. We overcame the limitations of combining primers with different length and amplification performance by balancing the concentration of each primer according to its amplification performance, at the same annealing temperature.

A second challenge common to multiplexing individual gene markers is the wrong assignment of reads to samples and barcodes, a phenomenon known as index hopping (MacConaill et al., 2018; Taberlet et al., 2018). We used a paired end strategy with unique 384 × 384 dual tag barcoding to reduce crosstalk between samples in downstream analyses. Furthermore, we adopted downstream bioinformatics tools to reduce the number of false positives due to index-hopping and PCR and sequencing errors (Bolyen et al., 2019).

A third challenge affecting multiplexing assays is the lower accuracy in detecting taxa in each DNA sample. Singleplex metabarcoding is expected to have higher accuracy than multiplex metabarcoding because a single target sequence is included in each reaction. Working with individual gene-markers can reduce the risk of cross-contamination between samples and the error rate introduced during amplification and sequencing of a pool of barcodes (Caroe & Bohmann, 2020). Accuracy and low cross-contamination are particularly important when working with low abundance or endangered species critical for conservation efforts (Giebner et al., 2020). We showed that alpha diversity measured

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TABLE 4 Wilcoxon signed rank test on Shannon Diversity and Pielou Evenness calculated between samples successfully amplified with both single plex and multiplex (regular only) assays for the benchmarking samples.

		Shannon diversity		Pielou evenness	
Locus	Sample type	W score	*p* adj value	W score	*p* adj value
16Sv4	Marine Coastal	0	1	0	1
	Marine CoastalSed	1	0.21875	4	0.765625
	MarineOffshore	0	1	0	1
	MarineOffshoreSed	0	0.875	1	1
	Peatland	0	1	0	1
	River	0	0.58333333	2	1
	Woodland	0	0.58333333	0	0.875
18Sv1v2	Diatom	0	1	0	1
	Grassland	0	0.375	0	0.45
	Marine Coastal	0	0.375	3	1
	Marine CoastalSed	0	0.28125	0	0.28125
	MarineOffshore	0	0.64285714	1	1
	MarineOffshoreSed	0	1	0	1
	Peatland	0	0.375	0	0.45
	River	0	0.375	0	0.45
	Woodland	0	0.375	0	0.45
18Sv8v9	Diatom	0	1	0	1
	Grassland	0	0.75	0	0.75
	Marine Coastal	0	0.75	0	0.75
	Marine CoastalSed	0	0.28125	1	0.5625
	MarineOffshore	1	1	0	0.9
	MarineOffshoreSed	0	0.9	1	1
	Peatland	3	1	3	1
	River	0	0.9	0	0.9
	Woodland	3	1	2	1
rbcL	Marine Coastal	0	0.41666667	1	0.625
	Marine CoastalSed	0	0.3125	0	0.3125
	MarineOffshore	0	1	0	1
	MarineOffshoreSed	0	0.625	0	0.625
	River	0	0.41666667	0	0.625

Note: *p*-values are Benjamini & Hochberg corrected.

TABLE 5 Pairwise PERMANOVA on Bray-Curtis distance between single plex (single) and multiplex (multi, both regular and with addition of BSA) in the benchmarking samples following 999 permutations, with strata set to sample type.

Locus	Single/multi (*p* val)	Single/multiplex + BSA (*p* val)	Multi/multix + BSA (*p* val)
16Sv4	0.268	0.733	0.989
18Sv1v2	0.923	0.954	1
18Sv8v9	0.995	1	0.999
rbcL	0.597	0.602	0.978

Note: Beta diversity was also tested between regular multiplex and multiplex with addition of BSA.

with Pielou evenness and beta diversity did not significantly differ between single and multiplex assays across different sample types.

However, our results showed significant difference between single and multiplex assays when alpha diversity was measured with the Shannon index. This may be explained by the Pielou evenness index accounting for species relative abundance, as opposed to an overall assessment of richness measured by the Shannon index, and a higher sensitivity of the Shannon index to species abundance (Johnston & Roberts, 2009). This is supported by the non-significant difference between single and multiplex assay for individual sample types successfully amplified in both assays, even if this analysis

FIGURE 3 Venn diagrams showing ASVs shared between single (blue) and multiplex (red) assays, as well as unique to either assay for the total number of detected features (100%), the top 85% and 70% features.

could not be completed on all paired samples due to amplification failure of some samples. Furthermore, higher sequencing effort of the single plex when compared with multiplex assays may have resulted in a skewed estimate of richness, affecting the Shannon index more pronouncedly than Pielou evenness.

We also showed that the ASVs and species captured by the two assays largely overlapped. This overlap was higher for genes targeting a wide range of taxonomic groups than for taxon-specific genes. This is expected, given the variable performance of the taxon-specific gene rbcL with different sample types. For example, the rbcL performed poorly with samples originating from soil, peatland and woodland, in which freshwater diatoms are not expected. It is possible that nonspecific amplification affected single and multiplex differently for this locus. The congruence between single and multiplex assays improved for all marker genes when rare ASVs/species were excluded from the analyses, suggesting that rare species were less efficiently captured in multiplex assays. This could be explained by the lower depth of sequencing of the multiplex when compared with single plex assays. However, the rarefaction and resampling approach we used to determine whether higher sequencing depth of the multiplex assay would capture more species, showed that both assays had already plateaued at the depth of sequencing used for the data analysis (rarefied reads). This suggests that a higher sequencing effort alone is not likely to increase the capture of rare ASVs/species in multiplex assays. The likely strategy needed to capture rare species involves a higher eDNA input and/or a higher number of biological replicates in PCR1. In particular, a higher number of replicates has been previously shown to reduce errors and biases, such as the missed amplification of rare species due to preferential amplification of abundant species (Bohmann et al., 2014, 2022).

In conclusion, we have shown that multiplexing gene markers in the same reaction improves throughput, reduces costs and enables the amplification of community-level biodiversity with limited input material. The cost savings are at the metabarcoding library construction stage, where a single tube reaction on four loci reduced the cost to a fourth. Further savings are achieved in the pooling of 1536 samples in a single sequencing run. In addition, the choice of sequencing platform (MiSeq, HiSeq and BGISeq) can result in 40% cost saving. Our multiplexing approach is a significant advancement

over previous studies using multiple primers to improve the amplification of longer marker regions (e.g., COI-5P gene (Govender et al., 2022)) and the detection capacity of target taxonomic groups [e.g., zooplankton (Zhang et al., 2018) and fish (Ushio et al., 2018)]. Multiplexing four loci at PCR1, combined with sample-specific dual indexes, and pooling of PCR2 libraries, provided significant savings without compromising quality and accuracy and reducing requirements on input DNA. The multiplex optimized for freshwater *seda*DNA performed comparably well on samples extracted from rivers, marine coastal and marine offshore samples (water and sediment). As expected, the multiplex performed poorly when applied to peatland, soil and woodland samples in which phytoplankton (e.g., diatoms) are not expected to occur. This seemingly negative result increases confidence on the specificity of the multiplex. A potential limitation of our assay was the lower detection of rare species. We suggest that this limitation can be overcome with a higher number of biological replicates or input eDNA. It is noteworthy that the multiplex assay presented here shows a high congruence with single marker genes, especially when targeting a wide range of taxonomic groups, which was the intended use for this tool.

AUTHOR CONTRIBUTIONS

Niamh Eastwood and Stephen Kissane optimized the experimental conditions for the multiplex. Niamh Eastwood completed the data analysis. Lewis Campbell, Andrew G. Briscoe, and Bastian Egeter provided materials and protocols. Luisa Orsini conceived the study and supervised data analysis. Luisa Orsini and Niamh Eastwood drafted the manuscript. All authors contributed to the manuscript writing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The metabarcoding sequences generated for this project are available at Biosample ID SAMN36027245 – SAMN36027412 (benchmarking samples) and SAMN36027413 – SAMN36027427 (*seda*DNA samples).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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