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<https://doi.org/10.1111/1755-0998.12940>

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RESOURCE ARTICLE

Comparison of environmental DNA and bulk-sample metabarcoding using highly degenerate cytochrome c oxidase I primers

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Abstract

Freshwater biodiversity provides important ecosystem services and is at the core of water quality monitoring worldwide. To assess freshwater biodiversity, genetic methods such as metabarcoding are increasingly used as they are faster and allow better taxonomic resolution than manual identification methods. Either sampled organisms are used directly for “bulk metabarcoding,” or water is filtered and the extracted environmental DNA serves as a proxy for biodiversity via “eDNA metabarcoding.” Despite the advantages of both methods, questions remain regarding their comparability and applicability for routine biomonitoring and stressor impact assessment. Therefore, we compared metabarcoding results from bulk and eDNA samples taken from 19 streams spanning a wide gradient of farming intensities in New Zealand. We performed PCR with highly degenerate cytochrome c oxidase I primers and sequenced libraries on an Illumina MiSeq. The inferred community composition differed strongly between the two methods. More taxa were captured by eDNA than bulk-sample metabarcoding (5,819 vs. 1,483), but more of the commonly used invertebrate bioindicator taxa (mayflies, stoneflies and caddisflies) were found in bulk (47) than eDNA samples (37). Catchment-wide and local land use impacts on communities were detected better by eDNA metabarcoding, especially for non-metazoan taxa. Our findings imply that bulk-sample metabarcoding resembles classical freshwater biomonitoring approaches better, as more indicator macroinvertebrate taxa are captured. However, eDNA metabarcoding might be better suited to infer the impact of stressors on stream ecosystems at larger scales, as many new and potentially more informative taxa are registered. We therefore suggest exploring both methods in future assessments of stream biodiversity.

KEYWORDS

agriculture, biomonitoring, community ecology, ecological genetics, invertebrates

1 | INTRODUCTION

Molecular methods are increasingly used to assess and monitor biodiversity and ecosystem status (Deiner et al., 2017; Hering et al., 2018). This task is of paramount importance as the world is facing a tremendous and potentially threatening loss of biodiversity (Steffen et al., 2015). This is especially true with regard to freshwaters, which are exceptionally diverse ecosystems and of great value for human well-being, yet disproportionately threatened by human activities and anthropogenic stressors such as intensified land use (Vörösmarty et al., 2010). Programmes for monitoring freshwater ecosystem status have therefore been initiated worldwide, for example the European Water Framework Directive (WFD) and the US Clean Water Act. The most commonly used bioindicators in freshwaters are macrophytes (Schaumburg et al., 2004), diatoms (Fore & Grafe, 2002), fish (Coates, Waugh, Anwar, & Robson, 2007) or macroinvertebrates (Verdonschot & Nijboer, 2004) that typically show distinct sensitivities towards environmental conditions. Traditional bioassessment methods largely rely on morphological identification of these taxa and have provided invaluable information for decades. However, it is well known that microalgal and especially invertebrate bioindicators are rarely identified to low taxonomic levels due to a lack of diagnostic characters, scarcity of expert taxonomists or lack of time/funds, which often leads to an underestimation of biodiversity (Jackson et al., 2014). Molecular methods such as DNA barcoding, that is, the amplification and sequencing of a short fragment of a marker gene (Hebert, Cywinska, Ball, & deWaard, 2003), can greatly help with that problem by identifying overlooked taxa. In addition, these methods provide valuable insights into the genetic and ecological diversity of taxa and ecosystems (Bringloe, Adamowicz, Harvey, Jackson, & Cottenie, 2016; Leys, Keller, Räsänen, Gattolliat, & Robinson, 2016) and allow detecting taxon-specific responses to anthropogenic stressors (Macher et al., 2016).

The advent of high-throughput sequencing technologies has given rise to metabarcoding. This technique allows sequencing millions of reads of the cytochrome c oxidase I (COI) barcoding region or other genes (e.g. 12S, rbcL, 16S and 18S) from whole communities, thereby assessing ecosystem biodiversity in yet unmatched detail (Ji et al., 2013; Taberlet, Coissac, Pompanon, Brochmann, & Willerslev, 2012). For freshwater invertebrate diversity, the COI gene has become the standard marker for metabarcoding (Bista et al., 2017; Elbrecht & Leese, 2017; Leray et al., 2013), although alternatives are discussed (Deagle, Jarman, Coissac, Pompanon, & Taberlet, 2014; Elbrecht et al., 2016). Metabarcoding is greatly facilitated by the increasing availability of taxonomic references linked to genetic information through databases such as the “Barcode of Life” database (BOLD, Ratnasingham & Hebert, 2007). In combination with the use of potent, degenerate primers that can amplify a wide range of taxa (Elbrecht & Leese, 2017; Leray et al., 2013), metabarcoding has been shown to reliably assess freshwater ecosystem biodiversity and can also be used in standardized monitoring approaches (Elbrecht, Vamos, Meissner, Aroviita, & Leese, 2017). Metabarcoding has been applied mostly on DNA obtained from

tissue of sampled specimens (i.e. bulk samples, Dowle, Pochon, Banks, & J., Shearer, K., & Wood, S. A., 2016; Elbrecht, Peinert, & Leese, 2017; Elbrecht, Vamos, et al., 2017; Gibson et al., 2015), but recent years have seen an increased use of metabarcoding on environmental DNA (eDNA) samples. eDNA is obtained by extracting DNA from air, sediment or water (e.g. Valentini et al., 2016; Zinger et al., 2016). The technique has been shown to recover the biodiversity of major fractions of whole ecosystems when applied to water samples (Deiner, Fronhofer, Mächler, Walser, & Altermatt, 2016) and has been used for the assessment of mammal (Ushio et al., 2017), amphibian (Secondi, Dejean, Valentini, Miaud, & Audebaud, 2016), fish (Hänfling et al., 2016) and invertebrate communities (Bista et al., 2017).

Direct comparisons of the performance of bulk-sample and eDNA metabarcoding are rare. Several studies have looked at the applicability of either metabarcoding method for biodiversity assessment of rivers but, to our knowledge, to date only one study (Deiner et al., 2016) has directly compared the two techniques, using the standard COI barcoding primers (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994) for amplification. These primers show inferior performance in comparison with highly degenerate COI primers when used for the amplification of whole freshwater invertebrate communities, as they miss many taxa due to primer bias (Elbrecht & Leese, 2017). In addition, the previous studies have shown metabarcoding approaches to be applicable for stressor monitoring in aquatic ecosystems (Laroche et al., 2017), but no study has directly compared the ability of eDNA and bulk-sample metabarcoding to assess the effects of anthropogenic stressors on river ecosystems. For example, high-intensity farming has been shown to negatively affect freshwater communities due to increased fine sediment and nutrient levels in streams (Jones et al., 2011; Macher et al., 2016; Matthaei, Piggott, & Townsend, 2010), and studying the impact of intensive agriculture on freshwater ecosystems is of high importance worldwide (Hering et al., 2015; Vörösmarty et al., 2010). In order to evaluate the potential of eDNA and bulk-sample metabarcoding for biodiversity and stressor assessments, direct comparisons of the two techniques using highly effective primers are urgently needed.

Since eDNA metabarcoding has been proposed to integrate biodiversity across larger parts of a river catchment (Deiner et al., 2016), this technique might not be suitable for assessing biodiversity and stressor impacts at a specific sampling site of interest; by contrast, this is possible by metabarcoding of bulk samples (Elbrecht, Peinert, et al., 2017; Elbrecht, Vamos, et al., 2017). Further, degenerate COI primers may augment differences between inferred communities in bulk and eDNA metabarcoding samples taken from the same site, as these primers can amplify non-metazoan taxa (Horton, Kershner, & Blackwood, 2017; Siddall, Fontanella, Watson, Kvist, & Erséus, 2009). This might be more often the case in eDNA samples, as in these the template molecules from “classical” stream bioindicator taxa, mainly macroinvertebrates, are expected to be rare in comparison with those of small non-metazoan taxa floating in the water. If degenerate primers amplify a high number of non-metazoan taxa

from eDNA samples, eDNA metabarcoding results may be even less compatible with current stream ecosystem status assessment methods, which largely rely on taxa lists containing benthic macroinvertebrates, diatoms and macrophytes. On the other hand, obtaining a high number of non-metazoan taxa can have advantages for ecological studies and biomonitoring. Diatoms are widely used for stream ecosystem assessment, and previous studies have shown metabarcoding approaches to be suitable for monitoring these algae (Vasselon, Rimet, Tapolczai, & Bouchez, 2017; Zimmermann, Glöckner, Jahn, Enke, & Gemeinholzer, 2015). Other microscopic taxa that are potential bioindicators can also be monitored using metabarcoding (Bik et al., 2012; Pawlowski, Lejzerowicz, Apotheloz-Perret-Gentil, Visco, & Esling, 2016). The COI gene is not frequently used for metabarcoding of non-metazoan taxa due to unsolved questions regarding the ability of the marker to distinguish microbial taxa (Pawlowski et al., 2012) and the lack of reference databases. However, studies have shown that COI might be a good marker for some non-metazoan taxa (diatoms: Yamada et al., 2017; Oomycota: Robideau et al., 2011; Amoebozoa: Nassonova, Smirnov, Fahrni, & Pawlowski, 2010; Rhodophyta: Saunders & McDevit, 2012). As COI is the standard marker for animals (Ratnasingham & Hebert, 2007), highly degenerate COI primers also amplifying non-metazoan taxa might bridge a gap between COI metabarcoding of animals and metabarcoding of non-metazoan taxa and could provide useful complementary information on non-metazoan taxa for ecological studies and biodiversity assessments.

The overall aim of our study was to directly compare results obtained from bulk-sample metabarcoding and eDNA metabarcoding (both performed with highly degenerate COI primers) in the context of ecological stream quality assessment. We surveyed 19 streams spanning a wide gradient of farming intensities in the Southland province of New Zealand to test the following hypotheses:

1. The two metabarcoding methods will infer highly dissimilar communities. This is due to three reasons. First, the nature of sampling differs. eDNA metabarcoding as applied here selects for small, floating organisms, cells and free DNA by straining river water through a filter with very small pore size, whereas bulk sampling focuses on larger, multicellular organisms living on/in the riverbed collected by kick netting. Second, even when only considering bioindicator organisms such as stream macroinvertebrates, we expect differences due to the different spatial scales covered by the two approaches, with bulk metabarcoding capturing the local and eDNA the whole (sub)catchment species pool. Finally, the use of highly degenerate COI primers may further augment differences between communities found in bulk and eDNA metabarcoding samples taken from the same site because they also amplify non-metazoan taxa.
2. Bulk-sample metabarcoding will more accurately detect impacts of anthropogenic stressors present at specific sampling sites on communities, while eDNA metabarcoding will do so for the catchments or at least subcatchments. Consequently, eDNA metabarcoding should be more strongly related to catchment-

level stressors and has been proposed as a tool for studying whole river catchments (Deiner et al., 2016), whereas bulk sample metabarcoding should reveal a clearer association between stream communities and local riparian land use and has been proposed as a tool for studying local biodiversity (Elbrecht, Peinert, et al., 2017; Elbrecht, Vamos, et al., 2017). To test this second hypothesis, we determined the prevalence of high-producing exotic grassland (HPEG) both in the riparian margins of our sampling sites and in their entire upstream catchments and tested whether bulk sample and eDNA metabarcoding detected differences in inferred community structure correlated with land use. In Southland, HPEG areas are used mainly for highly intensive dairy farming, which causes considerable influxes of fine sediment and nutrient into rivers (Drewry & Paton, 2000), two stressors which have been shown to have major detrimental impacts on stream biodiversity (e.g. Matthaei et al., 2010; Matthaei, Weller, Kelly, & Townsend, 2006; Wagenhoff, Townsend, & Matthaei, 2012).

2 | MATERIALS AND METHODS

Bulk samples and water samples were collected over a three-week period in March/April 2016 from 29 streams in the Southland region of New Zealand. The sampling period coincided with a run of dry weather and stable flows, which ensured that biological and water samples accurately represented the environmental conditions in the streams without physical disturbance due to high flows. Community and land use data from 19 of the 29 streams were included in our comparative study, as both eDNA and bulk-sample data were available for those sites (Figure 1). All sampling locations were in fourth-order streams. Sites were selected using the "River Environment Classification New Zealand" data sets (available online: <https://data.mfe.govt.nz/>) in QGIS (v.2.18, QGIS Development team, 2017) to cover a wide gradient of land use intensity (measured as a percentage of high-producing exotic grassland in the catchment). All site coordinates can be found in Supporting Information Table S1.

Before taking invertebrate bulk samples, eDNA samples were collected by taking 1 L of water from close to the stream bed in sterile bottles (Nalgene, Rochester, USA). Water was filtered on-site using a hand-operated vacuum pump (Nalgene, Rochester, USA), and DNA was captured on nitrate cellulose filters (Nalgene, 0.22 µm pore size). Between 1 and 5 filters per sampling location were used, depending on the level of suspended sediment in the river and how fast filters would clog. Using sterile tweezers, filters were transferred to sterile Eppendorf tubes (2 ml) containing 96% molecular grade ethanol and transported to the laboratory at 4°C in a cooling box. Bulk invertebrate samples were taken following the standard kick sampling method for wadeable streams in New Zealand (Stark, Boothroyd, Harding, Maxted, & Scarsbrook, 2001) using a D-shaped kick net (0.5 mm mesh size). Streambed substratum was disturbed in ten locations per site via kicking for 30 s each, thus standardizing sampling effort per site (5 min total). Samples were preserved in 70% ethanol in the field, transported to the laboratory, rinsed carefully to remove sediment without losing any invertebrates, transferred to

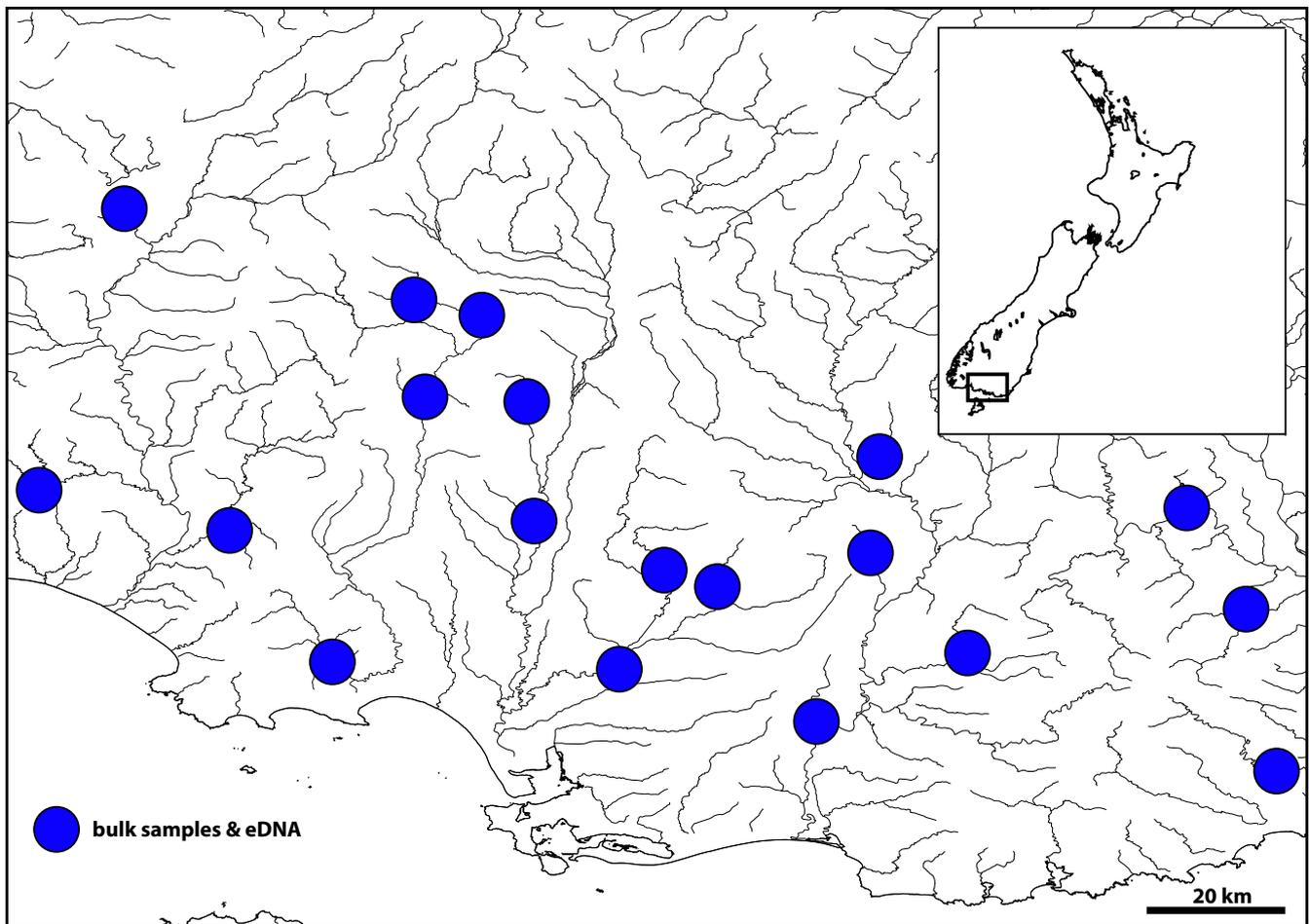


FIGURE 1 Location of sampling sites in Southland New Zealand used for comparison of bulk-sample and eDNA metabarcoding. Map based on freely available LINZ “NZ River Centrelines (Topo, 1:500 k)” map (data.linz.govt.nz) [Colour figure can be viewed at wileyonlinelibrary.com]

96% ethanol and stored at 4°C. Bulk invertebrate samples and filters containing eDNA were express-shipped to Germany for further analyses within 14 days of collection.

Invertebrates from bulk samples were dried under sterile conditions in petri dishes and weighed using a Mettler Toledo microscale (Columbus, USA). Specimens >5 mm in length were removed from each sample, dried and weighed separately. This size-sorting step was performed in order to reduce the probability of large specimens capturing a disproportionately high number of sequenced reads, a problem outlined in Elbrecht, Peinert, et al. (2017), Elbrecht, Vamos, et al. (2017). Samples were ground to fine powder using a disperser (ULTRA-TURRAX® Tube Drive control, Ika, Staufen, Germany) at 4,000 rpm for 30 min. After grinding, homogenized powder corresponding to 3% of the original dry weight of the smaller size fraction was taken from each sample for DNA extraction. From the fraction containing the >5 mm specimens, powder corresponding to 1% of the weight was taken with the tip of a spatula and mixed with the powder taken from the smaller size fraction. DNA was extracted using a modified salt extraction protocol (Sunnucks & Hales, 1996; Weiss & Leese, 2016) under sterile laboratory conditions.

Filters containing eDNA were dried, ripped into small pieces using sterile tweezers and extracted in a PCR-free laboratory using

the same salt extraction protocol, but with an increased proteinase K digestion time of 12 hr. After extraction, DNA from all filters per sampling site was pooled. RNA digestion was performed by adding 1 µl of RNase A (Thermo Fisher Scientific, Waltham, USA) to 25 µl of eluted DNA solution. Afterwards, samples were cleaned using the Qiagen MinElute PCR Purification Kit (Qiagen, Venlo, Netherlands). All persons involved in handling eDNA samples wore full-body protective clothing and surgical masks as breathing protection. The laboratory is frequently cleaned with hydrogen peroxide solution and irradiated with high-intense UV light for one hour every night. Further, samples were only handled under sterile UV hoods.

Extracted DNA was quantified on a Fragment Analyzer with the Standard Sensitivity Genomic Kit (Advanced Analytical, Oak Tree, USA), and 15 ng of DNA per sample was used for PCR. A two-step protocol was followed: For the first PCR, primers BF2 and BR2 (Elbrecht & Leese, 2017) without tails were used for amplification using illustra PuReTaq Ready-to-go PCR beads (GE Healthcare, Little Chalfont, UK). After an initial denaturation for 3 min at 94°C, 25 cycles at 94°C for 30 s, 50°C for 30 s and 72°C for 2 min were performed, followed by a final elongation at 72°C for five minutes. For the second PCR, 1 µl of the product was used with individually tagged BF2/BR2 primers (Elbrecht & Leese, 2017; combinations:

Supporting Information Table S2). The PCR protocol remained the same, but 15 cycles were used. Two independent PCRs were run for each sample, resulting in two technical replicates per sample. PCR products were cleaned up using the MinElute PCR Purification Kit, quantified on the Fragment Analyzer using the NGS High Sensitivity Kit, size selected using SpriSelect beads (Beckman Coulter, Brea, USA) and equimolarly pooled. Negative controls (1 L of sterile water) were filtered, extracted and amplified together with eDNA samples, checked for DNA content on the Fragment Analyzer and were added to the library so that they made up 10% of the total library volume. The final DNA library was again cleaned using the Qiagen MinElute kit and sent for sequencing on the Illumina MiSeq platform (v2 Kit, 2× 250 bp reads, bulk samples: 1 run, eDNA: 2 runs) at GATC Biotech (Constance, Germany).

Bulk-sample and eDNA sequences were bioinformatically processed together in order to be comparable. All raw reads were processed with *R* scripts as in (Elbrecht et al., 2016). Reads were demultiplexed, and paired-end reads were merged using USEARCH (v.8.1.1756, Edgar, 2010) with the maximum error rate set to $\text{fastq_maxee}=1$. Primers were removed with CUTADAPT (v.1.9, Martin, 2011). Sequences were dereplicated, singletons and chimeras were removed and the UPARSE pipeline (Edgar, 2013) was used to cluster OTUs at 97% similarity, which is a common approach in invertebrate metabarcoding studies relying on the barcoding gap approach (Bista et al., 2017; Elbrecht, Peinert, et al., 2017; Elbrecht, Vamos, et al., 2017). Subsequently, only OTUs that had read abundances over 0.005 per cent per sample were retained, while all other OTUs were discarded. This is a suitable alternative to rarefaction (Elbrecht & Leese, 2015). Only OTUs that were present in both sequencing replicates per sampling site were included in further analyses.

Taxonomy was assigned to reads by MEGAN (Huson, Auch, Qi, & Schuster, 2007); $-\text{evalue } 1\text{e-}60$, $-\text{max_target_seqs } 10$), using a custom-made database of all COI reads downloaded from NCBI GenBank (23-06-2017, max sequence length 5,000 bp). Reads were dereplicated using versusearch (Rognes, Flouri, Nichols, Quince, & Mahé, 2016) and quality checked in GENEIOUS (v.8.1.8, Kearse et al., 2012) in order to prevent overrepresentation of sequences. To assign taxon names to taxa known to be underrepresented in the NCBI nucleotide database (i.e. all taxa apart from Metazoa), MEGAN was used to assign taxonomic names to sequences in two ways. First, MEGAN was run with $\text{min_score}=300.0$, corresponding to ~80% identity in this data set, and taxonomy was assigned to OTUs at kingdom level (NCBI taxonomy). Second, a min_score of 700 (corresponding to ~97% identity) was used in order to achieve a reliable taxonomic assignment for Metazoa, EPT taxa and Stramenopiles. This was done in order to account for any uncertainty and possible wrong database entries. The University of Ghent Venn diagram creator (available online: <https://bioinformatics.psb.ugent.be/webtools/Venn/>) was used to visualize species number and number of shared species between bulk and eDNA metabarcoding samples.

Mean species richness across all 19 sampling sites was calculated separately for bulk and eDNA samples using the *R* (v. 3.3.3, R Development Core Team, 2017) package *vegan* (Oksanen et al., 2007).

This was also employed to run a *t* test to determine potential differences between techniques and for calculating Beta diversity (Sørensen index, indicating dissimilarity between sites) across eDNA and bulk samples.

Differences in inferred community composition as a function of the used sampling technique were analysed further in two ways, again using the *vegan* package in *R*. First, analyses were performed on the presence/absence OTU data, and the *vegdist* function was employed to calculate Jaccard distances. Based on the latter, communities were clustered with an average-linkage algorithm (*hclust* function) as in Burdon et al. (2016). We then performed PERMANOVAs with the sampling technique as predictor and the Jaccard distances as response variables using the *adonis* function as implemented in the *vegan* package. These analyses were conducted for all OTUs, Metazoa, the pollution-sensitive EPT taxa (Ephemeroptera, Trichoptera, Plecoptera), and Stramenopiles (diatoms, brown algae, golden algae and oomycetes). For Stramenopiles, sites 9 and 17 had to be removed from the data set because no Stramenopiles were found in bulk samples at these sites. We regarded significant PERMANOVA results with $R^2 > 0.09$ (equivalent to $r = 0.30$) as moderate and $R^2 > 0.25$ ($r = 0.50$) as strong, following Nakagawa and Cuthill (2007) and Cohen (1988), who both categorized correlation coefficients of $r = 0.3$ and 0.5 as moderate or strong effects, respectively. However, we acknowledge that these effect size categories, although widely used, are subject to discussion (Nakagawa & Cuthill, 2007).

Second, we calculated Sørensen dissimilarities (as a measure of Beta diversity) and compared them between communities inferred via eDNA metabarcoding versus bulk-sample metabarcoding. For all OTUs, Metazoa, EPT taxa and Stramenopiles, inferred community dissimilarity was calculated within each sampling site and also between sampling sites.

The Land Cover Database for New Zealand (v4.1, LCR Informatics Team, 2012; latest available version) was used to identify the percentage of high-producing exotic grassland (HPEG) in the entire upstream catchment and in a radius of 200 m around each sampling site. In Southland, HPEG areas are used mainly for highly intensive dairy farming, which causes most of the nutrient and sediment influxes into New Zealand rivers (Monaghan et al., 2007). Therefore, the percentage of HPEG represents a proxy of farming intensity. Percentages of land covered by HPEG per upstream catchment and in a radius of 200 m around each sampling site ranged from 9.8% to 96.6% in the upstream catchment and 0.1% to 100% in the local area (see Supporting Information Table S6 for details).

Analyses of inferred community composition as a function of farming intensity in the catchment (HPEG_catchment) or the area surrounding the sampling sites (HPEG_local) were performed separately using PERMANOVAs and Jaccard distances as response variables as described above, but this time with either of the two HPEG land cover variables modelled as a categorical predictor with four levels (1:0%–25%; 2:25%–50%; 3:50%–75%; and 4:75%–100% cover). As above, these analyses were conducted for all OTUs, Metazoa, EPT taxa and Stramenopiles.

3 | RESULTS

Bulk-sample metabarcoding yielded 13,801,156 raw reads (1 MiSeq run) and eDNA metabarcoding 16,426,154 raw reads (2 MiSeq runs, 8,112,661 and 8,313,493 reads, respectively). Reads will be deposited in the Short Read Archive upon publication. 11,948,880 reads were retained after quality filtering (eDNA: 6,606,846, bulk sample: 5,340,554). All replicates with read numbers <20,000 were excluded from further analyses (read numbers per replicate: Supporting Information Table S3). 908 reads comprising 370 OTUs were found in negative controls; 580 of these reads (comprising 291 OTUs) were found in eDNA-negative controls and 328 (85 OTUs) in bulk-sample negative controls. Overall, this read number corresponds to 0.08% of all reads. Such a small proportion of reads in negative controls is commonly found on Illumina sequencing platforms and presumably caused by tag switching (Schnell, Bohmann, & Gilbert, 2015); thus, no contamination was suspected in our study. Six thousand seven hundred sixty-four OTUs were retained after removing OTUs that were not present in both replicates per sample. For direct comparison of eDNA and bulk-sample metabarcoding, the data set was further reduced to data from the 19 sampling sites for which both eDNA and bulk-sample data were available. In these samples, 6,451 OTUs (bulk samples: 1,483, eDNA: 5,819) were present.

3.1 | Sampling technique and taxonomic composition

When assigning taxonomy to OTUs with a minimum bit score ("min_score") of 300 (i.e. ~80% identity) in MEGAN, 2,548 OTUs (39.4% of the total) could be assigned to a taxonomic name at kingdom level (eDNA: 2,147 [36.9%], bulk sample: 972 [65.5%]). When assigning taxonomy with a minimum identity of 97% in MEGAN, 187 (3.0%) of all OTUs were identified as Metazoa, of which 51 (27.3%) were identified as EPT taxa (Ephemeroptera, Plecoptera, Trichoptera). Eighty-eight (1.4%) of all OTUs were identified as Stramenopiles, of which 30 (34.1%) were identified as Pythiales (Oomycetes), 20 (22.7%) as Peronosporales (Oomycetes) and 11 (12.5%) as Naviculales (Bacillariophyceae).

In eDNA samples, the majority of identified taxa identified at kingdom level were Stramenopiles (1,026 OTUs; 47.8%), followed by Metazoa (453 OTUs; 21.1%; Figure 2a). In bulk samples, the majority of identified taxa were Metazoa (485 OTUs; 49.9%), followed by Stramenopiles (209 OTUs; 21.5%; Figure 2b; number and proportion of OTUs assigned to taxonomic names at kingdom level: Supporting Information Table S4; full OTU table: Supporting Information Table S5).

Out of the total number of 6,451 OTUs, 77% were exclusively found in eDNA samples, 10% were exclusively found in bulk samples, and 13% were found with both techniques (Figure 3a). Of the 187 metazoan OTUs identified with the strict filtering approach, 24% were found only in bulk samples, 26% only in eDNA samples and 50% with both techniques (Figure 3b). Of the 51 EPT taxa, 8% were found only in eDNA samples, 28% only in bulk samples and 65% with both techniques (Figure 3c). Most Stramenopiles (65%) were exclusively found in eDNA samples, whereas only 1% was found only in bulk samples and 33% with both techniques (Figure 3d).

The mean number of OTUs was significantly higher in eDNA samples than in bulk samples for all OTUs (785.3) and Stramenopiles (21.5), while significantly more metazoan taxa were found in bulk (39.6) than in eDNA (31.9) samples (*t* tests; see Table 1 for all means and *p*-values). Further, significantly more EPT taxa were found in bulk (16.3) than in eDNA (8.3) samples.

3.2 | Sampling technique and inferred community composition

Beta diversity (Sørensen dissimilarity) across all sampling sites was higher in bulk samples than in eDNA samples for all OTUs (0.75 vs. 0.73), Metazoa (0.66 vs. 0.63) and Stramenopiles (0.86 vs. 0.52), whereas for EPT taxa, it was higher in eDNA samples (0.58 vs. 0.69; Table 2).

When comparing Sørensen dissimilarities for communities inferred via eDNA and bulk-sample metabarcoding, values were significantly lower for communities sampled at a given site for all OTUs,

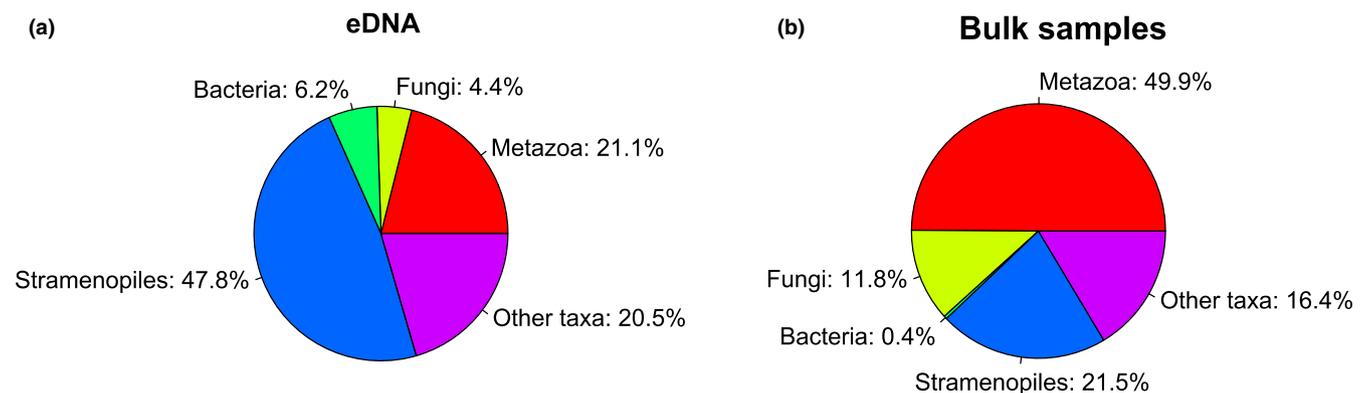


FIGURE 2 Proportions of OTUs (Metazoa, Stramenopiles, bacteria, fungi and other taxa) found in eDNA and bulk samples, respectively. (a) eDNA samples and (b) bulk samples [Colour figure can be viewed at wileyonlinelibrary.com]

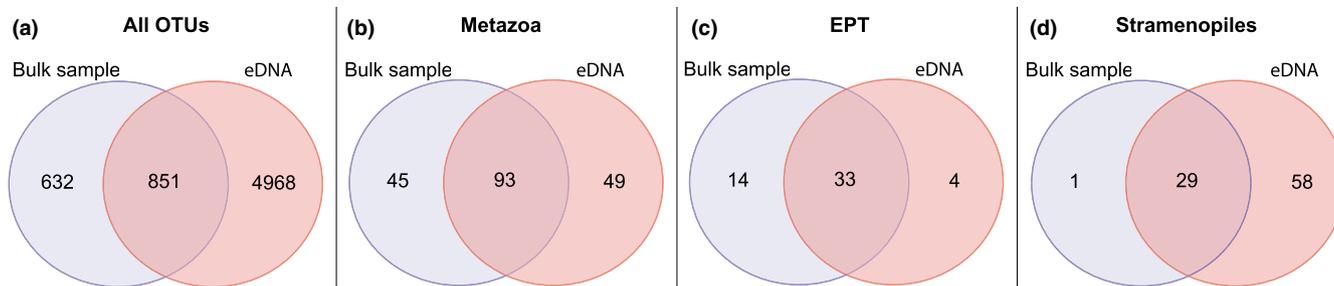


FIGURE 3 Venn diagrams showing the OTUs exclusively found in the eDNA and the bulk-sample data set, respectively, and the OTUs found with both sampling techniques. (a) All OTUs, (b) Metazoa, (c) EPT taxa and (d) Stramenopiles [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Mean OTU numbers (standard errors) for all OTUs, Metazoa, EPT and Stramenopiles in bulk samples and eDNA samples, respectively, and *p*-values of *t* tests comparing the two metabarcoding methods

Response variable	Bulk samples	eDNA samples	<i>p</i> -value (t tests)
arAll OTUs	193.7 (15.382)	785.3 (56.174)	<0.001
Metazoa	39.6 (3.192)	31.9 (3.136)	0.024
EPT	16.3 (1.035)	8.3 (1.368)	<0.001
Stramenopiles	3.6 (0.374)	21.5 (1.466)	<0.001

Notes. Significant differences between methods are indicated in bold print. Sample size was *n* = 19 except for Stramenopiles where *n* = 17.

TABLE 2 Beta diversity (Sørensen dissimilarity) calculated for all OTUs, Metazoa, EPT and Stramenopiles in bulk and eDNA samples, respectively

Response variable	Beta diversity (Sørensen dissimilarity)	
	Bulk samples	eDNA samples
All OTUs	0.75	0.73
Metazoa	0.66	0.63
EPT	0.50	0.69
Stramenopiles	0.86	0.52

Metazoa, EPT and Stramenopiles compared to the between-site comparisons (Table 3).

PERMANOVA results for Jaccard distances showed that across all sampling sites, sampling technique had moderate but significant effects on inferred community composition in all OTUs ($R^2 = 0.22$, $p < 0.001$), metazoans ($R^2 = 0.17$, $p < 0.001$), EPT taxa ($R^2 = 0.13$, $p < 0.001$) and Stramenopiles ($R^2 = 0.18$, $p < 0.001$).

3.3 | Land use and inferred community composition

PERMANOVA results showed that in the bulk-sample data set, the percentage of HPEG surrounding each sampling site (HPEG_local) was unrelated to the communities of all OTUs, metazoans, EPT taxa or Stramenopiles (Table 4). In the eDNA data set, by contrast, HPEG_local had moderate effects on communities of all OTUs, metazoans, EPT taxa and Stramenopiles. In the bulk-sample data set,

TABLE 3 Mean beta diversity (Sørensen dissimilarity; standard errors) between communities inferred via eDNA and bulk-sample metabarcoding, calculated for within sampling sites versus between sampling sites for all OTUs, Metazoa, EPT and Stramenopiles

Response variable	Beta diversity (Sørensen dissimilarity)		<i>p</i> -value (t test)
	Within sampling sites	Between sampling sites	
All OTUs	0.83 (0.014)	0.90 (0.004)	<0.001
Metazoa	0.55 (0.027)	0.68 (0.011)	<0.001
EPT	0.58 (0.046)	0.69 (0.021)	0.037
Stramenopiles	0.77 (0.026)	0.86 (0.016)	0.005

Notes. Significant differences (*t* tests) are indicated in bold print. Sample size was *n* = 19 except for Stramenopiles where *n* = 17.

the percentage of HPEG in the catchment (HPEG_catchment) showed a weak relationship with communities when considering all OTUs inferred through bulk-sample metabarcoding, whereas it was unrelated to metazoans, EPT taxa or Stramenopiles. In contrast, for the eDNA data, HPEG_catchment had moderate effects on communities when considering all OTUs, metazoans, EPT taxa and Stramenopiles (Table 4).

4 | DISCUSSION

We compared metabarcoding of eDNA from water samples to bulk-sample metabarcoding of specimens. Specifically, we determined to which extent the inferred communities differed between the two techniques, and we also compared the techniques' ability to detect the impact of land use on community composition.

4.1 | Differences in community composition inferred through eDNA and bulk-sample metabarcoding

We had hypothesized that bulk-sample metabarcoding and eDNA metabarcoding would infer highly dissimilar stream communities, due to the different spatial scales covered with the two approaches and the different nature of sampling (eDNA selects for small, floating organisms, cells and free DNA, whereas bulk sampling focuses on

TABLE 4 PERMANOVA results (R^2 -values and p -values) showing the relationships of either local HPEG land cover or catchment-scale HPEG land cover (both modelled as a categorical predictor with four levels: 1:0%–25%; 2:25%–50%; 3:50%–75%; and 4:75%–100% cover) with the Jaccard distances calculated for communities comprising all OTUs, Metazoa, EPT or Stramenopiles, respectively

Response variable	Bulk samples		eDNA samples	
	HPEG_local	HPEG_catchment	HPEG_local	HPEG_catchment
All OTUs	$R^2 = 0.06$ $p = 0.26$	$R^2 = \mathbf{0.08}$ $p = \mathbf{0.048}$	$R^2 = \mathbf{0.11}$ $p = \mathbf{0.001}$	$R^2 = \mathbf{0.12}$ $p = \mathbf{0.001}$
Metazoa	$R^2 = 0.06$, $p = 0.37$	$R^2 = 0.09$ $p = 0.08$	$R^2 = \mathbf{0.12}$ $p = \mathbf{0.001}$	$R^2 = \mathbf{0.15}$ $p = \mathbf{0.001}$
EPT	$R^2 = 0.08$ $p = 0.27$	$R^2 = 0.07$ $p = 0.28$	$R^2 = \mathbf{0.15}$ $p = \mathbf{0.002}$	$R^2 = \mathbf{0.16}$ $p = \mathbf{0.004}$
Stramenopiles	$R^2 = 0.05$ $p = 0.77$	$R^2 = 0.07$ $p = 0.40$	$R^2 = \mathbf{0.18}$ $p = \mathbf{0.003}$	$R^2 = \mathbf{0.17}$ $p = \mathbf{0.002}$

Note. Significant relationships are indicated in bold print.

larger, multicellular organisms living on the riverbed). Our results clearly demonstrate that the sampling technique can greatly impact the inferred stream community composition. This has previously been shown for other taxonomic groups and ecosystems, for example soil meiofauna (Creer et al., 2010). In our study, only 13% of all OTUs detected were found in both eDNA and bulk samples. On average, significantly more OTUs were found in eDNA than in bulk samples. Metazoan OTU richness was similar for both techniques (bulk: 40; eDNA: 32), and Sørensen dissimilarity was similar high in both bulk (0.66) and eDNA data sets (0.63), indicating that communities strongly differed between sampling sites, regardless of the sampling technique. The percentages of OTUs found with both techniques increased when focussing on specific taxonomic groups: 33% for Stramenopiles, 50% for metazoan OTUs and 65% for the pollution-sensitive stream EPT taxa. Nevertheless, on average twice as many EPT taxa were found in bulk than in eDNA samples, and Sørensen dissimilarity between sites was higher in the eDNA data set compared to the bulk samples. Our results are similar to those of Deiner et al. (2016), who reported that 33 of 78 detected macroinvertebrate families (42%) were found with both sampling techniques.

Our findings also show that communities differed strongly between sampling sites, presumably as a result of the large geographical area sampled and the selected streams spanning a wide gradient from pristine to strongly impacted conditions. Previous studies have shown that communities in river networks can differ because of network connectivity, ecological selection and trophic interactions (Heino, 2005; Liu, Soininen, Han, & Declerck, 2013; Seymour, Deiner, & Altermatt, 2016), and these factors most likely contributed to the between-site patterns found in our study. Beta diversity analyses (Sørensen dissimilarity) showed that communities inferred through eDNA and bulk-sample metabarcoding differed significantly within sampling sites for all analysed taxonomic groups. However, significantly greater differences were found between sampling sites, indicating that both methods can detect site-specific communities. As expected, the strongest differences within and between sampling sites were found for all OTUs and Stramenopiles. The high diversity of microbial taxa is the main reason for this high

dissimilarity, and the differences are further augmented due to the fact that eDNA sampling selects for these taxa, whereas bulk sampling selects for larger organisms.

Our findings further imply that bulk-sample metabarcoding is superior to eDNA metabarcoding when the goal is to assess the benthic macroinvertebrate groups of primary interest for current stream biomonitoring, at least when limited by sequencing depth, as eDNA sampling selects for small, floating organisms and DNA, which then outcompetes target taxon DNA during PCR and sequencing. Four of the 51 EPT taxa (8%) were found exclusively in eDNA samples, and 14 (28%) were detected solely in bulk samples. EPT taxa found only in eDNA samples were *Zelandoperla fenestrata*, a sub-alpine stonefly (McCulloch, Wallis, & Waters, 2009), *Costachorema callistum*, a caddisfly, known from fast flowing, montane rivers (McCulloch et al., 2009; Smith, 2002), *Hudsonema alienum*, a caddisfly common in many New Zealand streams (Collier, Smith, & Halliday, 2004; Matthaei et al., 2006), and *Triplectides dolichos*, a caddisfly known to be abundant in native forest (Collier, Smith, & Baillie, 1997). Of the taxa found only in bulk samples, several are known to occur in standing or slow-flowing water (e.g. the caddisfly *Oecetis unicolor*: Talbot & Ward, 1987), be relatively tolerant to stressors (e.g. the caddisfly *Hudsonema amabile*, Collier et al., 1997; Townsend, Sebastian Uhlmann, & Matthaei, 2008) or be common in many streams (e.g. the caddisfly *Olinga feredayi*). The respective habitat preferences of these EPT taxa suggest that eDNA may capture more taxa from upstream, pristine sites, whereas bulk samples are more likely to depict the local community at the somewhat more degraded downstream sites. However, the low number of taxa detected only by one of the two methods, and the general lack of knowledge regarding the autecology of many New Zealand stream invertebrate species do not allow drawing any firm conclusions regarding this theory. For future studies aiming to detect mostly metazoan taxa but using eDNA, it might be useful to use filters with larger pore sizes during field sampling, as this might help reduce the number of captured small, non-metazoan taxa.

Many more Stramenopiles were found in eDNA samples (21.5 on average) than in bulk samples (3.5). Moreover, dissimilarity in

inferred Stramenopiles community composition across sampling sites (based on the Sørensen index) was very high in bulk samples (0.86), whereas community composition inferred through eDNA metabarcoding (0.52) was more consistent. Only one of the taxa identified as Stramenopiles (1%) was exclusively found in bulk samples, while 58 (66%) were detected only in eDNA samples. We assume that most non-metazoan taxa found in bulk samples were adherent to macroinvertebrate bodies or were food organisms that were co-extracted from stomach contents of macroinvertebrates. As we did not target microbial taxa with bulk samples but selected for macroinvertebrates, these differences would most likely not be found when bulk samples targeting microbial taxa would have been taken.

Overall, our results suggest that bulk sample metabarcoding is better suited than eDNA metabarcoding for studies focusing on benthic macroinvertebrates. However, eDNA metabarcoding could still be an alternative to bulk-sample metabarcoding in cases where kick sampling of macroinvertebrates is not feasible, for example in rivers with very coarse substrata or in large, non-wadeable rivers.

4.2 | Amplification of non-metazoan taxa by highly degenerate COI primers

The highly degenerate BF2/BR2 COI primers specifically designed for freshwater macroinvertebrates amplified not only macroinvertebrates, the “classical” stream bioindicators, but also many non-metazoan groups that could potentially be used as new bioindicators. eDNA metabarcoding recovered more than four times the number of OTUs found in bulk samples, and only 21% of identified OTUs were Metazoa. In bulk samples, 50% of identified OTUs were Metazoa. This difference occurred because in eDNA samples, many small organisms, individual cells or DNA of higher organisms are present, allowing one to capture more taxa with a given number of sequences on a HTS platform (Barnes & Turner, 2015). By contrast, just a few taxa can contribute the overwhelming proportions of biomass and DNA in bulk samples, limiting the chances of discovering small and rare taxa. This problem can be solved to some extent by size sorting (Elbrecht, Peinert, et al., 2017), and rare taxa with few reads can be excluded from analyses by using a higher minimum read count threshold during bioinformatic processing of data sets.

The larger quantity of OTUs obtained through eDNA metabarcoding does not necessarily mean that these data are also of higher quality. In our study, only 37% of all OTUs detected could be assigned to a taxonomic name when using relaxed settings for identification (80% identity to reference sequences). This result highlights two problems: (a) New Zealand taxa are underrepresented in COI reference libraries compared to taxa from North America and Europe; and (b) the highly degenerate COI primers captured many non-metazoan taxa (eDNA: 79% non-metazoan OTUs and 48% Stramenopiles; bulk samples: 50% non-metazoan OTUs and 21% Stramenopiles) for which few reference database entries comprising COI sequences exist. Identification of OTUs was thus challenging and often impossible. These findings suggest that the use of highly degenerate primers in running waters should be considered carefully,

as has been shown for other ecosystems (Horton et al., 2017; Siddall et al., 2009), and their usefulness may largely depend on the taxonomic groups of interest.

Nevertheless, degenerated COI primers have considerable potential as research tools in running waters because they can help discovering OTUs suitable for stream health bioassessment and ecological research in general, for example studies on species co-occurrence or stream food webs (Apothéloz-Perret-Gentil et al., 2017; Pawlowski et al., 2016). Our study demonstrates that degenerate BF2/BR2 COI primers can simultaneously capture not only metazoans, but also Stramenopiles (which include diatoms, an important bioindicator group) and to a lesser extent other non-metazoan taxa in both eDNA samples and bulk samples. This approach of “one marker for everything” is routinely used for metabarcoding entire non-metazoan communities (18s rRNA: Chain, Brown, MacIsaac, & Cristescu, 2016; Markmann & Tautz, 2005), and many microbial taxa are routinely metabarcoded (Pawlowski et al., 2016; Wurzbacher et al., 2017) and hold great potential for bioassessment. COI is widely accepted as the standard barcoding marker for Metazoa, and the possibility to complement the widely available COI data for Metazoa with data of non-metazoans holds great promise. However, we acknowledge that COI, as all genetic markers, has important limitations. Although it has been found to be a suitable marker for several non-metazoan taxa (diatoms: Yamada et al., 2017; Oomycota: Robideau et al., 2011; Amoebozoa: Nasonova et al., 2010, Rhodophyta: Saunders & McDevit, 2012), care should be taken when interpreting data and further research is needed in order to fully understand the usability of this marker for non-metazoans. This applies especially to Stramenopiles, as open questions remain regarding the identity of species in this highly diverse non-monophyletic taxon and the resolution that can be achieved by using COI (Pawlowski et al., 2012), and COI references are scarce compared to metazoan taxa. Still, these problems might be solved to some extent by the use of recently developed machine-learning approaches, which allow the use of OTUs with unknown taxonomy or ecological traits for ecosystem assessment (Cordier et al., 2017). In any case, analysing COI data not only of Metazoa but also for other Eukaryota and microbial taxa could provide very useful information for ecological studies and biodiversity assessments.

4.3 | Stressor assessment through eDNA and bulk-sample metabarcoding

Our second hypothesis had predicted that bulk-sample metabarcoding should reveal a clearer association between stream communities and local riparian land use than eDNA metabarcoding, due to the fact that eDNA should better reflect catchment rather than local biodiversity (Deiner et al., 2016). Our results do not support this hypothesis, because eDNA metabarcoding actually performed better as a tool for detecting land use impacts on stream communities. The community of all OTUs inferred through bulk-sample metabarcoding was only weakly related to HPEG landcover in the upstream catchment, and this relationship was nonsignificant for Metazoa, EPT taxa

and Stramenopiles. Moreover, none of these four community variables were significantly related to the percentage of HPEG surrounding the sampling sites. In contrast, for eDNA metabarcoding, all four analysed communities were significantly related to HPEG land cover at both spatial scales. The relatively strongest relationships were found for EPT taxa and Stramenopiles. These results correspond well with previous studies using traditional morphological identification methods that found EPT taxa and diatoms (an important group within the Stramenopiles) to be especially sensitive to environmental stressors (Fore & Grafe, 2002; Kaller & Hartman, 2004; Matthaei et al., 2006; Wagenhoff, Lange, Townsend, & Matthaei, 2013). In the Southland region of New Zealand where our sampling sites are located, intensive dairy farming leads to elevated fine sediment and nutrient levels in streams, and both sediment and nutrients have been shown to be major stressors for stream communities (Wagenhoff et al., 2013; Wagenhoff, Townsend, Phillips, & Matthaei, 2011).

While our results imply that eDNA metabarcoding might be better suited for assessing stressor impacts on stream communities than bulk-sample metabarcoding, this conclusion remains tentative because our study is correlative and needs to be complemented by future manipulative experiments. Further, the proportion of explained variance in our statistical models is quite small ($R^2 \leq 0.17$), indicating substantial unexplained background variation. Despite these uncertainties, our results suggest that eDNA metabarcoding is a suitable technique for studying stressor impacts on stream communities, especially in combination with degenerate primers. It is important to note that the eDNA bioassessment will likely induce a paradigm shift away from traditional metazoan indicator taxa with well-known ecological preferences such as EPT (Schmidt-Kloiber & Hering, 2015) towards indicators integrating from microbial to macroorganisms. At present little is known about the relationships of most microbes to environmental stressors, but capable machine-learning algorithms may lead to the generation of integrated and powerful new indices directly from the data (Apothéloz-Perret-Gentil et al., 2017; Keck, Vasselon, Tapolczai, Rimet, & Bouchez, 2017). While these changes are unlikely to be implemented in routine freshwater biomonitoring in the near future, the potential of metabarcoding techniques for bioassessment needs to be explored further (Hering et al., 2018).

5 | CONCLUSIONS

We found that both bulk-sample and eDNA metabarcoding hold great potential for future application in biomonitoring and ecological research in running waters. In addition, the use of well-designed, highly degenerate COI primers might lead to a more universal biodiversity assessment, as the BF2/BR2 primers also captured microbial taxa that hold considerable promise for use as future bioindicators in streams. Both techniques have been shown to produce valuable data. Nevertheless, inferred community compositions differed substantially, and interestingly community composition inferred via eDNA better reflected catchment-wide as well as local stressor regimes than bulk-sample metabarcoding. Bulk-sample metabarcoding, however, provided a better picture on the classical macroinvertebrate indicator

taxa. Thus, care should be taken when planning future biodiversity assessment and ecological studies. On balance, we suggest using eDNA metabarcoding with degenerate primers for the assessment of whole-ecosystem taxonomic diversity, but using bulk-sample metabarcoding for studies targeting specific groups of benthic macroinvertebrates. Often, combining the methods of bulk-sample and eDNA metabarcoding may offer the best solution, as this would allow assessing the majority of a stream ecosystem's biodiversity.

ACKNOWLEDGEMENTS

We thank Romana Salis, Cristina Hartmann-Fatu and Bianca Peinert for help in the laboratory. The Herzog-Sellenberg Foundation is thanked for financial support. The Aquatic Ecosystem Research Journal Club provided helpful comments.

AUTHOR CONTRIBUTIONS

J.N.M. and F.L. designed the study; A.V. and F.C.C. performed the sampling; J.N.M. performed laboratory work and bioinformatics; J.N.M., F.L., C.D.M., J.J.P., A.V. and F.C.C. wrote the manuscript.

DATA ACCESSIBILITY

Raw data have been deposited in the Sequence Read Archive (SRA), accession number: SRP152832.

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SUPPORTING INFORMATION

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How to cite this article: Macher J-N, Vivancos A, Piggott JJ, Centeno FC, Matthaei CD, Leese F. Comparison of environmental DNA and bulk-sample metabarcoding using highly degenerate cytochrome c oxidase I primers. *Mol Ecol Resour.* 2018;18:1456–1468. <https://doi.org/10.1111/1755-0998.12940>