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Performance of amplicon and shotgun sequencing for accurate biomass estimation in invertebrate community samples

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Abstract

New applications of DNA and RNA sequencing are expanding the field of biodiversity discovery and ecological monitoring, yet questions remain regarding precision and efficiency. Due to primer bias, the ability of metabarcoding to accurately depict biomass of different taxa from bulk communities remains unclear, while PCR-free whole mitochondrial genome (mitogenome) sequencing may provide a more reliable alternative. Here, we used a set of documented mock communities comprising 13 species of freshwater macroinvertebrates of estimated individual biomass, to compare the detection efficiency of COI metabarcoding (three different amplicons) and shotgun mitogenome sequencing. Additionally, we used individual COI barcoding and de novo mitochondrial genome sequencing, to provide reference sequences for OTU assignment and metagenome mapping (mitogenome skimming), respectively. We found that, even though both methods occasionally failed to recover very low abundance species, metabarcoding was less consistent, by failing to recover some species with higher abundances, probably due to primer bias. Shotgun sequencing results provided highly significant correlations between read number and biomass in all but one species. Conversely, the read–biomass relationships obtained from metabarcoding varied across amplicons. Specifically, we found significant relationships for eight of 13 (amplicons B1FR-450 bp, FF130R-130 bp) or four of 13 (amplicon FFFR, 658 bp) species. Combining the results of all three COI amplicons (multiamplicon approach) improved the read–biomass correlations for some of the species. Overall, mitogenomic sequencing yielded more informative predictions of biomass content from bulk macroinvertebrate communities than metabarcoding. However, for large-scale ecological studies, metabarcoding currently remains the most commonly used approach for diversity assessment.

KEYWORDS
biodiversity, biomass, genome skimming, invertebrates, metabarcoding, metagenomics

1 | INTRODUCTION

The accurate qualitative and quantitative assessment of biodiversity is essential to understand biodiversity and ecosystem function relationships, especially in the face of rapid biodiversity loss (Loreau & de Mazancourt, 2013). However, evaluating the speed and scale of ecosystem degradation is limited by the use of traditional taxonomic approaches that typically require high levels of expertise and are labour-intensive. Moreover, accurate species identification is frequently not possible in cases of damaged or immature specimens (Jackson et al., 2014; Sweeney, Battle, Jackson, & Dapkey, 2011). In biomonitoring, the robust quantification of community composition enables detection of both spatial and temporal variations in the biological community and, by extension, the wider ecosystem (Cranston, 1990). To expedite biomonitoring frameworks, international directives, such as the EU Water Framework Directive (WFD), have been established. Initiatives such as the WFD require ecological status classification of water bodies which are underpinned by the taxonomic identification of organisms from routine monitoring (Collins, Ohandja, Hoare, & Voulvoulis, 2012).

The advent of high-throughput sequencing technologies (HTS) is revolutionizing biomonitoring by increasing the throughput and taxonomic information that can be recovered (Baird & Hajibabaei, 2012). The most commonly used taxonomic groups for such studies include various invertebrate taxa, such as benthic macroinvertebrates for freshwater ecosystems (e.g., Gibson et al., 2014, 2015; Hajibabaei, Shokralla, Zhou, Singer, & Baird, 2011; Pfrender et al., 2010; Shokralla et al., 2015). Similarly, terrestrial invertebrate taxa have been used, from soil or leaf litter (Yang et al., 2014), or from aboveground invertebrate sampling (Malaise traps) (Ji et al., 2013). More recent work encompasses the detection of biodiversity from aqueous environmental DNA (eDNA) (Bista et al., 2017; Ficetola, Miaud, Pompanon, & Taberlet, 2008; Mächler, Deiner, Steinmann, & Altermatt, 2014; Seymour et al., 2018).

Most studies using HTS for biodiversity assessment of bulk samples to date utilize PCR based amplicon sequencing of one or more marker genes. Amplicon sequencing is often referred to as metabarcoding and is commonly applied for the analysis of bulk/environmental community samples (Creer et al., 2016; Yu et al., 2012). Commonly used markers include the mitochondrial cytochrome C oxidase subunit I (COI) barcode region (Hebert, Ratnasingham, & de Waard, 2003) and ribosomal RNA regions such as 16S (Epp et al., 2012) for animals, or RbcL and ITS2 for plants (Fahner, Shokralla, Baird, & Hajibabaei, 2016). Due to intermediate PCR steps, it is considered that metabarcoding produces bias in relation to accurate taxonomic representation of diversity in bulk samples (Hajibabaei, Spall, Shokralla, & van Konynenburg, 2012; Yu et al., 2012). Specifically, it has been reported that PCR bias might alter the species biomass ratio or produce inaccurate representation of relative abundance of species (Piñol, Mir, Gomez-Polo, & Agustí, 2015), where primer-template mismatches might introduce misrepresentation of particular groups (Clarke, Soubrier, Weyrich, & Cooper, 2014; Elbrecht &

Leese, 2015). On the other hand, correlations between amplicon read number and biomass have also been reported (Elbrecht, Vamos, Meissner, Aroviita, & Leese, 2017; Hiiesalu et al., 2014; Kelly et al., 2014). Optimization of metabarcoding pipelines, use of multiple primer pairs and combination of multiple amplicons from the same region has been suggested to improve species richness recovery and biomass estimations (Gibson et al., 2014; Hajibabaei et al., 2012; Zhan, Bailey, Heath, & Macisaac, 2014). Such improvements to methodological aspects are critical to standardize where possible, not only to improve comparability of independent data sets, but, importantly, also to promote accessibility and eventual uptake of such technologies.

The more recently introduced mitochondrial metagenomics (mitogenomics) uses high-throughput Illumina sequencing of whole mitochondrial genomes from bulk samples (Crampton-Platt, Yu, Zhou, & Vogler, 2016). Some current applications involve characterization of bulk samples for ecological assessment (Tang et al., 2015) and phylogenetic reconstruction of multiple species simultaneously (Gillett et al., 2014). This approach has been suggested as an alternative to PCR-based metabarcoding (Zhou et al., 2013), advocating that the absence of initial PCR amplification step will reduce PCR-related bias. Specifically, it is suggested that mitogenomic sequencing results in more accurate biomass-to-read relationships and possibly more reliable representation of species biomass in bulk samples. The efficiency of this approach could be related to the assembly method used and whether reference sequences are available for mapping (Gómez-Rodríguez, Crampton-Platt, Timmermans, Baselga, & Vogler, 2015).

Here, we provide a direct comparison between metabarcoding and mitogenomics and investigate the ability of each method to depict the richness and biomass presence of macroinvertebrate species in bulk extracted samples. Ten artificial communities were used, comprising 13 common macroinvertebrate species, for which all specimens were individually measured for their biomass. The samples were selected to represent a typical freshwater biomonitoring sample collected for environmental assessment purposes, including various taxonomic orders, while accounting for availability of large numbers of individuals per target species. Additionally, the replicate communities were designed to comprise gradients of biomass, from as low as a single specimen up to multiple specimens depending on the species (e.g., step increase in specimens per community from 1 to 33 for *Potamopyrgus antipodarum* and 1 to 17 specimens for *Radix balthica*), to represent a range of natural densities. DNA was extracted from pooled whole bodies of invertebrates, unlike previous work (e.g., Crampton-Platt et al. (2015); Gómez-Rodríguez et al. (2015)). The community DNA extracts were amplicon sequenced for three fragments of the COI gene on Illumina MiSeq and shotgun sequenced on Illumina HiSeq (see Figure 1 for overview of experimental workflow). Possible limitations and advantages of each method were considered, to evaluate their performance. Furthermore, the overall applicability of each method was assessed while providing suggestions for future improvements. Ultimately, we aim

FIGURE 1 Brief overview of experimental workflow. Arrows indicate workflow steps, including specimen processing, molecular work and sequencing of mock communities. Dashed lines indicate parallel laboratory steps for production of a barcode reference database and reference mitochondrial genomes from individual specimens
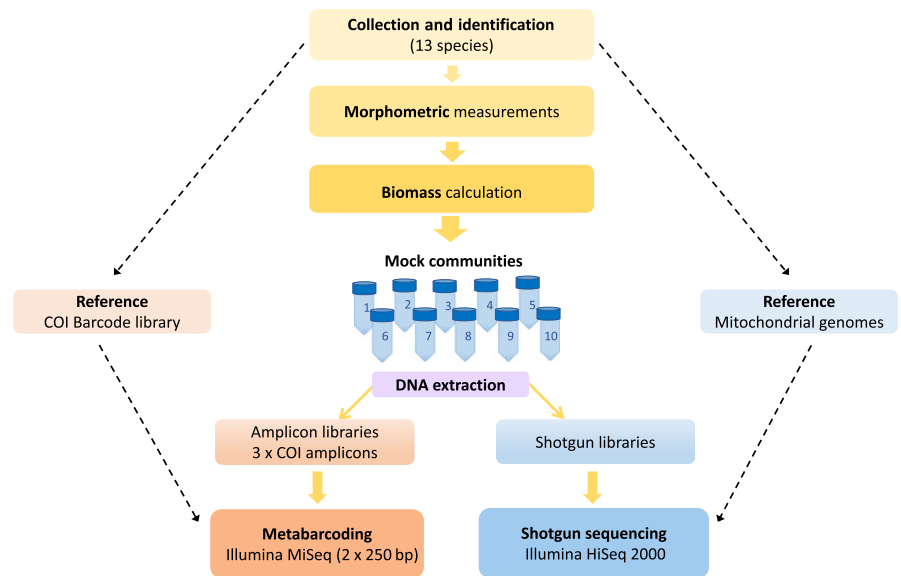
to provide a comprehensive evaluation of the two methods and troubleshoot their future utility for ecological applications in biodiversity and freshwater ecosystem monitoring.

2 | MATERIALS AND METHODS

2.1 | Specimen collection and morphological measurements

Specimens for this work were collected from areas of Somerset and Suffolk, UK (September–October 2014) and were identified to species level (by county surveyors and APEM Ltd, UK). Specimens were preserved in absolute ethanol and stored in a dark and cool environment for up to 6 months until morphological measurements and DNA extraction. In total, 13 species were used for analysis, including eight species of Gastropoda and one of each from the following: Hemiptera, Isopoda, Amphipoda, Ephemeroptera and Coleoptera (Table S1, Fig. S1), which were selected to represent a typical biomonitoring sample.

Body measurements were performed based on published work using the following methodologies: callipers for larger species (*Notonecta glauca*, *Asellus aquaticus*, *Gyrinus marinus* and *Ephemera danica*) and a microscope fitted with an ocular micrometre for smaller species (e.g., *Potamopyrgus antipodarum*). Software Image Pro (Media Cybernetics, Rockville, USA) was used for measurements of the amphipod *Gammarus pulex*, to facilitate accuracy by accounting for the curvature of specimens. Published regressions based on length to mass measurements were used for estimation of biomass for each species (Table S2), taking into account geographic region and ecosystem type wherever possible, as these parameters could produce intraspecific variations in developmental rates (Mährlein, Pätzig, Brauns, & Dolman, 2016). Conversion of length to mass is considered superior to other methodologies, such as determination of biovolume or weighing of specimens, due to increased precision and speed (Benke, Huryn, Smock, & Wallace, 1999). Additionally, direct

weighing of preserved specimens might be inaccurate due to loss of dry mass during preservation (Benke et al., 1999).

2.2 | DNA barcode reference library

Individual specimens were extracted and sequenced for the COI barcode region using universal metazoan primers (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994). Extraction was performed with DNeasy Blood & Tissue kit (Qiagen) (arthropods) and CTAB chloroform extraction protocol (gastropods), due to best suitability of protocols for different tissue types. High-quality barcodes were obtained from all species (Table S1), except *E. danica* (Ephemeroptera), for which barcode sequencing was not successful (for similar findings regarding Ephemeroptera barcoding, see Morinière et al., 2017). Sanger-generated sequences were edited using CodonCode ALIGNER version 3.7.1 (CodonCode Corporation, Massachusetts) and aligned with ClustalW in MEGA version 5 (Tamura, Dudley, Nei, & Kumar, 2007).

2.3 | Design of mock communities

Ten communities were created containing either 13 or 14 species (including control species, see below). Exception was made for two species which were present in only six (*N. glauca*) or nine (*P. fontinalis*) of the communities, due to low number of individuals available. Specimens ranged from 139 to 157 individuals, each at a gradient of biomass presence from a single to multiple specimens (Tables 1, 2). The total sum of specimens across 10 communities was 1479. Two positive controls were used to assess the quality of sequencing performance across mock communities. First, three specimens of *Drosophila melanogaster* were added in each community, to assess extraction efficiency. Second, DNA of the butterfly *Mycalesis mineus*, which was separately extracted (previously assembled by Tang et al., 2014), was added in each community extract at 1% concentration (at BGI-Shenzhen, China), to account for variability in DNA subsampling before

shotgun sequencing. The two control species were selected due to existing reference mitogenome information.

did not use any mtDNA enrichment method to avoid skewing the biomass proportions of species between samples.

2.4 | DNA extraction for reference mitogenomes and mock communities

For the construction of individual shotgun reference genomes for each species, high-quality genomic DNA was extracted from leg or muscle tissue of a single specimen using the Qiagen DNeasy Blood and Tissue extraction kit [final elution in 50 μl PCR-grade water (Roche)] and DNA concentration and quality were assessed with Qubit dsDNA assays and agarose gel electrophoresis. A minimum amount of 2.5 μg total DNA was used for shotgun sequencing.

For the mock communities, DNA was extracted from the entire bodies of invertebrates, in bulk. First, ethanol preservative was removed and then specimens were allowed to dry at 37°C for 2 h. The dried specimens were ground using sterile mortar and pestle sets, then transferred into 50-ml Power Bead tubes from the Power Max Soil DNA Isolation Kit (MO-BIO) and vortexed at high speed for 5 min. Lysis was performed by incubation for 3 h at 65°C in a shaker at medium speed, with the addition of 450 μl of proteinase K (20 mg/ml) (Sigma-Aldrich). Subsequent steps followed manufacturer's instructions. For final elution (total volume 4 ml), the columns were incubated for 30 min at room temperature and centrifuged at 2500 g for 5 min. The elution step was repeated a second time to maximize yield (Fig. S2, Table S3). For the purposes of this work, we

2.5 | Metabarcoding-primer selection and amplicon library preparation

Metabarcoding was performed through sequencing of three amplicons of the COI barcode region using the following primer pairs: (i) amplicon FFFR using the universal Folmer primers (Folmer et al., 1994) (658 bp); (ii) amplicon FF130R using the Folmer forward primer and the I-130R reverse primer (130 bp); and (iii) amplicon B1FR using I-B1 forward primer and Folmer reverse (450 bp) (I-B1 primer was modified from Hajibabaei et al. (2012), and I-130R was modified from Meusnier et al. (2008) both degenerate, modified for use with macroinvertebrate communities) (Figure 2 and Table 3). These primers were selected based on visual comparison with aligned barcode sequences (Appendix I, sequence alignment of COI barcodes and primers I-B1 and I-130R) and amplification performance tests on mock community extracts. Libraries were prepared using a two-step PCR protocol with final indexing step, to minimize the effects of variant index sequences on the amplification efficiency of each community (Berry, Mahfoudh, Wagner, & Loy, 2011; O'donnell, Kelly, Lowell, & Port, 2016). Round 1: amplification was performed using only the target-specific COI primer; Round 2: purified PCR product from round 1 was used as template using the target-specific primers with added Illumina tails, and a final step was used for indexing of all amplicons from round two with Illumina indexes.

TABLE 1 Design of mock communities. See columns for the detailed contents of each community (1–10), with numbers referring to number specimens from each species used. Last column: total number of specimens/species; bottom line: total number of specimens/community and number of species/community. Highlighted the species with lowest abundance (dark grey) and highest abundance (light grey) in each community. *Drosophila melanogaster* was used as positive control for each mock community

Number	Species	Community										Specimens per species
		1	2	3	4	5	6	7	8	9	10	
1	*Anisus vortex*	35	40	45	5	25	20	15	10	30	1	226
2	*Asellus aquaticus*	1	4	8	10	14	17	19	21	24	24	142
3	*Bathyomphalus contortus*	14	13	12	11	10	8	6	1	2	4	81
4	*Bithynia tentaculata*	24	10	6	25	26	1	27	15	20	26	180
5	*Ephemera danica*	16	3	1	6	8	12	10	18	14	20	108
6	*Gyrinus marinus*	2	1	3	10	4	8	5	9	6	7	55
7	*Planorbis planorbis*	24	25	19	22	1	4	7	10	13	16	141
8	*Potamopyrgus antipodarum*	10	32	28	25	21	33	14	17	1	5	186
9	*Radix balthica*	3	15	5	17	16	10	12	1	9	6	94
10	*Physa fontinalis*	1	3	4	6	8	10	12	13	13	0	70
11	*Notonecta glauca*	10	0	0	4	2	1	0	6	0	8	31
12	*Bithynia leachi*	12	3	5	1	9	11	8	7	13	14	83
13	*Gammarus pulex*	2	5	6	4	8	8	1	8	3	7	52
14	*Drosophila melanogaster*	3	3	3	3	3	3	3	3	3	3	30
	Total specimens	157	157	145	149	155	146	139	139	151	141	1479
	Total number of species	14	13	13	14	14	14	13	14	13	13	

TABLE 2 Estimated biomass for each species included in the mock communities. Values are presented in milligrams (mg)

Species	Community									
	1	2	3	4	5	6	7	8	9	10
Anisus vortex	5.08	5.75	6.44	0.70	3.41	2.84	2.10	1.42	4.21	0.13
Bathyomphalus contortus	0.79	0.70	0.65	0.56	0.47	0.40	0.31	0.05	0.10	0.22
Planorbis planorbis	8.87	9.04	6.75	7.66	0.37	1.56	3.10	4.53	4.77	5.55
Bithynia leachi	11.97	3.19	4.52	0.94	8.95	10.30	7.45	6.61	12.50	14.54
Bithynia tentaculata	115.60	46.33	26.58	113.80	128.77	4.01	135.45	79.47	104.71	124.64
Physa fontinalis	1.16	4.14	4.78	7.91	10.81	13.10	16.42	17.02	18.70	**0.00**
Potamopyrgus antipodarum	3.82	13.37	11.66	9.89	8.10	13.87	5.27	6.46	0.36	1.74
Radix balthica	20.47	89.72	31.37	106.12	101.69	57.27	70.67	6.08	53.80	35.91
Notonecta glauca	77.38	**0.00**	**0.00**	33.88	16.09	7.47	**0.00**	46.54	**0.00**	62.09
Asellus aquaticus	1.00	4.92	9.00	13.02	19.50	22.01	22.68	27.56	30.73	37.72
Gammarus pulex	30.49	54.70	75.11	51.18	90.38	104.72	10.13	128.80	42.39	83.34
Ephemera danica	71.92	14.24	4.18	28.62	35.93	54.17	46.02	80.35	62.88	86.31
Gyrinus marinus	21.69	9.36	30.00	104.94	40.35	85.77	52.20	98.29	60.07	72.01
Total (mg)	370.25	255.46	211.02	479.23	464.82	377.48	371.80	503.18	395.20	524.22

The samples were sequenced on an Illumina MiSeq using MiSeq V2 reagents (500 cycles) following the (2 × 250) paired-end protocol (PE), at the Biodiversity Institute of Ontario, University of Guelph (Canada). Detailed PCR protocols are available in Appendix II. Throughout the library preparation, appropriate laboratory precautions were taken to establish clean conditions for the analysis of community DNA. Protocols included sterilization of equipment by autoclaving or bleaching, and PCR set-ups were performed in a dedicated clean cabinet.

2.6 | Amplicon data analysis

Quality control and analysis of the Illumina MiSeq sequences were performed according to Bista et al. (2017), using a USEARCH version 7 (Edgar, 2010) custom pipeline for filtering, sorting, dereplication and merging of sequences. For amplicons 2 (FF130R, 130 bp) and 3 (B1FR, 450 bp), the forward and reverse reads were merged with a 25 bp minimum overlap. For amplicon 1 (FFFR, whole barcode region 658 bp), only the forward reads were used (truncation at 230 bp), based on FastQC (www.bioinformatics.babraham.ac.uk) results (Phred score >25). The truncation strategy was selected

because the length of the original amplicon (658 bp) did not allow sufficient overlap between the forward and reverse reads due to the current limitations of Illumina 2 × 250 bp MiSeq chemistry. Chimeras were removed with the de novo option in USEARCH, and a 97% similarity level was used for OTU clustering and generation of an OTU table in USEARCH (the dominant OTU centroids in this case are selected through the most abundant sequences). The OTU level of similarity was used as an approximate average value for characterization of the diverse taxa present in the community samples. Similarly, taxonomic assignment was performed as in Bista et al. (2017), using Quantitative Insights In Microbial Ecology (QIIME) (Caporaso et al., 2010) and BLAST+ (megablast) (Camacho et al., 2009) with ≥98% similarity cut-off, against the downloaded NCBI COI barcodes and our custom-generated COI barcodes. All analyses including USEARCH, QIIME and BLAST were performed using High Performance Computing (HPC) Wales systems. The identified OTUs were further checked for stop codons and insertions in MEGA6 (Tamura et al., 2007) and through phylogenetic analysis using a neighbour-joining (NJ) method (Saitou & Nei, 1987). Metabarcoding library preparation and data analysis were performed at Bangor (UK), and MiSeq sequencing was run at BIO Guelph (Canada).
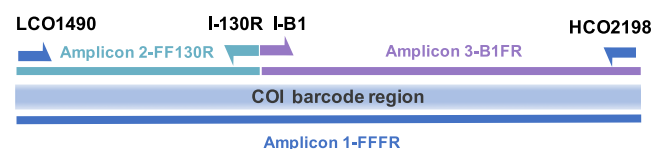
FIGURE 2 Positions of primers and sequenced amplicons along the COI barcode region. Arrows indicate position and name of the primers used. (1) FFFR, 658bp (dark blue), primers LCO1490 – HCO2198, (2) FF130R, 130bp (light blue) LCO1490 – I-130R, (3) B1FR, 450bp (purple) I-B1 – HCO2108

2.7 | Construction of reference mitogenomes and mitogenome skimming

Genomic DNA extracted from individual specimens was used for generation of reference mitogenomes. For each species, a library with insert size of 200 bp was constructed following manufacturer's instructions (Illumina, Nextera). The 12 individual species libraries were pooled and 100 bp PE sequenced on a whole lane of Illumina HiSeq 2000. Library construction and assembly of reference mitogenomes as well as mitogenome skimming and analysis of mock

TABLE 3 COI primers used for metabarcoding

Primer name	Primer sequence	Direction	Citation
LCO1490	GGTCAACAAATCATAAAGATATTGG	F	Folmer et al. (1994)
HC02198	TAAACTTCAGGGTGACCAAAAAATCA	R	Folmer et al. (1994)
I-B1	CCHGATATAACITTYCCICG	F	Hajibabaei et al. (2012) (modified)
I-130R	GAAAATYATAAIGAAIGCRTGAGC	R	Meusnier et al. (2008) (modified)

community samples were performed at BGI, Shenzhen, China. For reference mitogenomes, raw data from each species were filtered as previously described in Zhou et al. (2013), Tang et al. (2014, 2015), removing reads with low quality or adaptor contamination. Clean data were assembled into scaffolds using SOAPdenovo-Trans (-K 71) (Xie et al., 2014) and IDBA-UD (Peng, Leung, Yiu, & Chin, 2012). Assembled sequences were annotated following Tang et al. (2015), to identify candidate mitogenome sequences, which were used for mitogenome reference construction. Subsequently, manual correction and checking were carried out as described by Tang et al. (2014). Thirteen protein-coding genes (PCG) were extracted from all mitogenomes, and each of them were aligned with corresponding reference protein-coding genes from four arthropod species (*Macrogyrus oblongus*, *Gammarus duebeni*, *Ligia oceanica* and *Siphlonurus immanis*) and three mollusc species (*Biomphalaria tenagophila*, *Physella acuta* and *Oncomelania hupensis*) using CLUSTALW 2.1 (Thompson, Higgins, & Gibson, 1994). The translation frame was checked in MEGA6 (Tamura et al., 2007) to correct gap length generated inside protein-coding genes by the assembly program when constructing scaffolds based on PE reads. In addition, the original read mapping was done and monitored using BWA 0.6.2 (Li & Durbin, 2009) and SAMTOOLS 0.1.19 (Li et al., 2009), respectively, following Tang et al. (2014, 2015).

Each of the 10 community DNA samples was used for construction of 200 bp insert size libraries, which were 100 bp PE sequenced on two lanes of a HiSeq 2000 (ca. 2–3 Gb per sample), according to manufacturer's instructions. Filtered data were aligned onto the 12 previously constructed reference mitogenomes by BWA, and reads that uniquely mapped onto the references with 100% read coverage and ≥99% identity were considered as reads from the focal species.

2.8 | Statistical and community analysis

To allow statistical comparisons between samples, we performed normalization of sequencing data per mock community, while accounting for variation of sequencing depth. For the shotgun data, normalization was performed by mitogenome length and mito-ratio (MitoNorm), and as proportion of mitochondrial reads on total reads per mock community (pShotgun) (see detailed information in Tang et al. (2015)). For the amplicon data, normalization was performed as amount of OTU reads from the total number of reads per mock community, for each amplicon (target_species_reads/total_community_reads). To select the best model explaining the relationship between number of reads and biomass (mg), linear and logistic

models were compared for each species and sequencing methods. The best model was selected using Akaike information criterion (AIC) (Hu, 1987). All statistical analyses, including calculation of model parameters, were performed using the program R Core Team (2015).

To visualize community variation across sequencing treatments for amplicon data, nonmetric multidimensional scaling was performed (nMDS), using the metaMDS function in the VEGAN package in R (version 3.3.0), based on calculation of the Bray–Curtis dissimilarity index as a measure of relative abundance, based on species-level data. Additionally, the function "ORDISPIDER" in package vegan was used to connect the same communities (resulting from different sequencing treatments) on the ordination plot. The software PRIMER-E version 6 (Clarke & Gorley, 2006) was also used to examine differences in community composition between sequencing methods (nMDS, Bray–Curtis).

3 | RESULTS

3.1 | Sequencing results

For metabarcoding data, the total number of sequencing reads obtained after quality control was 1,430,531, sequenced on a fraction of an Illumina MiSeq lane. Each amplicon produced the following total number of reads (Mean), (i) FFFR1: 248,776 (24,878), (ii) FF130R: 1,004,530 (100,453) and (iii) B1FR: 177,225 (17,722.5) (Table S4). After OTU clustering, for each amplicon, we obtained 49 (FF130R), 20 (FFFR) and 14 (B1FR) OTUs, respectively. Collapsing of multiple OTUs per species was used to account for intraspecific diversity in our data although the observed intraspecific diversity among OTUs assigned to the same species was generally low (Table S5).

We sequenced the reference mitochondrial genomes for 12 of 13 species used in this experiment; species *A. vortex* was not included in the run due to low quality of extracted DNA. Total length of mitochondrial DNA assemblies ranged between 13,326 and 16,159 bp, with three species also achieving circular genomes (*Notonecta glauca*, *Physa phontinalis* and *Gyrinus marinus*) (Table S6). The average mitochondrial genome length was 14,760 bp.

The amount of data attributed to mitochondrial reads compared to the total reads per species (mito-ratio) varied largely between species, ranging between 0.011% (*G. pulex*) and 0.692% (*E. danica*), with an average mito-ratio of 0.19%. The average sequencing depth per mitogenome was 177.47X (min depth: 6.4X—*G. pulex*, max depth: 670.4X—*E. danica*) (Table S6). Shotgun sequencing of the mock

community samples returned 23,984,200,200 total number of reads with an average of 2,398,420,020 reads (Fig. S3, Table S4).

For the two positive controls, *D. melanogaster* returned an average of 344.2 reads, and for *M. mineus*, an average of 787.3 reads (Fig. S4) (read numbers for mitochondrial genomes only). The latter was significantly correlated with the number of reads achieved per sample ($R^2 = .717$, $p = .002$), while no significant relationship was found for the *D. melanogaster* read number vs. total number. For amplicon sequencing, only the *D. melanogaster* positive control was used. Significant relationships between the positive control sample and the total number of reads were found for two of the amplicons (B1FR: $R^2 = .939$, $p = 0$) (FF130R: $R^2 = 0.610$, $p = .008$), but not for the whole COI region amplicon (FFFR1).

3.2 | Detection rates per species

Cases of false negatives and false positives were found for both sequencing methods, based on a lower cut-off of reads present (defined as zero for indicating the presence/absence of species). The proportion presence of false negatives is reported here based on number of expected (known) occurrences (presence in sample) for each species in the communities. Occurrences were calculated normally as 10 per species (10 communities), except for species *P. fontinalis* (nine occurrences) and *N. glauca* (six occurrences) [(11 sp. × 10) + (1 sp. × 6) + (1 sp. × 9) = 125 total occurrences/cases] (based on counts from Table 1). For this step, we were considering the number of species across all 10 communities to increase statistical power of calculations.

The shotgun approach failed to detect the presence of species in the community samples in seven of 125 cases (5.6%), for five species. Generally, the false negatives with this method occurred only for the lowest and second lowest amount of biomass present for the rare species. For the three amplicons, false negatives occurred in (i) three cases (2.2% in three species) for FFFR, (ii) six cases (4.5% in three species) for FF130R and (iii) seven cases (5.6% in five species) for B1FR (Table 1, Table S7). Here, false negatives appeared not only for the lowest biomass of species, but also when up to 10 (FF130R, FFFR), 13 (FFFR) or 17 (B1FR) specimens were known to be present in that community. Overall, false negatives mostly came from gastropod species except, *G. pulex* (amphipod, two cases) and *E. danica* (mayfly, one case). False positives were detected for species *N. glauca* in two cases (FFFR amplicon), with 111 and 34,511 reads detected in communities 7 and 9, respectively, and for species *P. fontinalis* with 1,204 reads in community 10 (FFFR amplicon). The presence of false positives here could be attributed to cross-contamination due to carry over from species during common storage of specimens.

3.3 | Associations between biomass and number of reads

Statistical model investigations suggested that both logistic and linear models were appropriate for characterizing the number of

read-to-biomass relationships, with the model type generally linked to species across the different sequencing methods (Table 4). The relationship of reads with biomass was examined individually for each sequencing treatment (three COI amplicons, sum of all amplicon data and shotgun data) and each species and plotted with the appropriate best-fit model (Figures 3, 4, S5–S8). Additionally, the total data obtained for each community were simultaneously plotted against specimen biomass to visualize overall trends per community (shotgun and sum of amplicon data, Figs S9–S10).

Positive and mostly significant relationships were found between biomass and shotgun reads (11 of 12 species with one trending towards significance; $p = .08$). Metabarcoding results varied across amplicons, but we found significant positive read–biomass relationships for 4–8 of 13 species (Table 4). All species presented positive relationships, with the exception of *E. danica* which presented negative read–biomass relationship, but only for the FFFR amplicon generated using the universal Folmer primers (Fig. S7). We also investigated the relationships obtained when adding up the reads obtained from all three amplicons vs. known biomass, as sum of amplicon data (Table 4, Figure 4). In this case, the detection rates improved, by removing false negatives. Nevertheless, significant read–biomass relationships were found in only eight species, which was an improvement in comparison with using only FFFR (Folmer) amplicon data, but not in comparison with the other two amplicons (Table 4).

3.4 | Community analysis

Comparison between the three COI amplicons on the nMDS showed grouping of the same communities along the vertical axis with the exception of communities 9 and 10 (Fig. S11). Such findings suggest a qualitatively similar community composition in the results obtained by the different amplicons. Simultaneous plotting of amplicon and shotgun data (Fig. S12a) shows each sequencing treatments separated along the horizontal axis. When the amplicon data were plotted as a sum (SumAmplicon) (Fig. S12b) against the shotgun reads, we could again only observe vertical separation of the groups, although in this case, much clearer than when the individual amplicons were plotted. Moreover, the similarity ranking of communities was almost identical for the two types of sequencing (see order of communities as B, C, G, I, etc.).

4 | DISCUSSION

Here, we used two HTS methods for DNA based biodiversity analysis, metabarcoding and shotgun mitogenomics, to characterize species composition and biomass of bulk invertebrate samples. Unlike previous work (Crampton-Platt et al., 2015; Gómez-Rodríguez et al., 2015; Yu et al., 2012), we extracted whole bodies in bulk and also used exact biomass measurements for each specimen used, through a structured design of replicates which allowed testing of different biomass scenarios (from single specimen to multiple per species).

TABLE 4 Summary table of significance of reads to biomass correlations, for each sequencing treatment. Amplicon data ("Amplicon", B1FR: 450 bp, FF130R: 130 bp, FFFR: 658 bp, SumAmplicon: sum of all amplicon data per species) and shotgun data ("Shotgun", pShotgun: proportion of reads, MitoNorm: mito-ratio normalized). The type of model used is indicated by shading (light grey: logistic, white: linear). Shotgun data not shown for species *Anisus vortex* (NA). Significant relationships (<0.05) are indicated with (*)

	Taxa		Amplicon				Shotgun	
Number	Family	Species	B1FR	FF130R	FFFR	SumAmplicon	pShotgun	MitoNorm
1	Planorbidae	*Anisus vortex*	0.01*	<0.01*	0.06	0.02*	NA	NA
2	Planorbidae	*Bathyomphalus contortus*	<0.01*	0.06	0.07	0.02*	<0.01*	<0.01*
3	Planorbidae	*Planorbis planorbis*	0.03*	0.06	0.09	0.07	0.01*	0.01*
4	Bithyniidae	*Bithynia leachi*	0.11	0.01*	0.04*	0.03*	<0.01*	<0.01*
5	Bithyniidae	*Bithynia tentaculata*	0.58	0.27	0.37	0.46	0.08	0.09
6	Physidae	*Physa fontinalis*	0.25	<0.01*	0.57	0.04*	<0.01*	<0.01*
7	Hydrobiidae	*Potamopyrgus antipodarum*	0.04*	<0.01*	0.02*	0.02*	<0.01*	<0.01*
8	Lymnaeidae	*Radix balthica*	0.03*	0.01*	0.03*	0.01*	<0.01*	0.01*
9	Notonectidae	*Notonecta glauca*	0.02*	0.01*	0.51	0.09	0.01*	0.02*
10	Asellidae	*Asellus aquaticus*	0.05*	0.07	0.15	0.06	0.02*	0.03*
11	Gammaridae	*Gammarus pulex*	0.52	0.06	0.32	0.46	<0.01*	<0.01*
12	Ephemeridae	*Ephemera danica*	0.04	0.02*	0.06	0.02*	<0.01*	<0.01*
13	Gyrinidae	*Gyrinus marinus*	<0.01*	<0.01*	<0.01*	<0.01*	<0.01*	0.01*
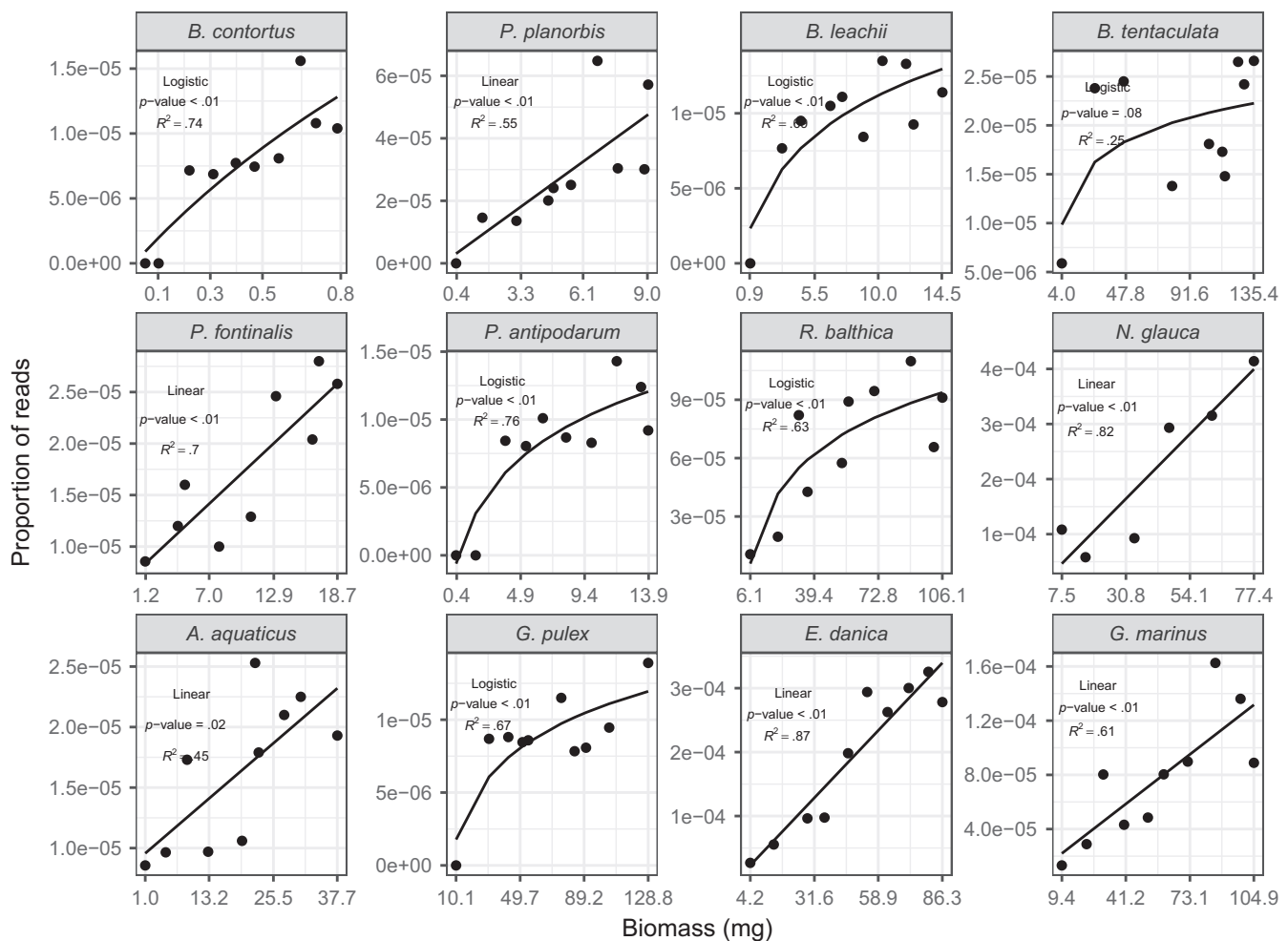
FIGURE 3 Scatter plots and regression analysis of shotgun sequencing results, plotted as sequencing reads vs. biomass (x-axis: biomass, y-axis: proportion of reads). Each box shows data for an individual species and lines show the fit of the model used
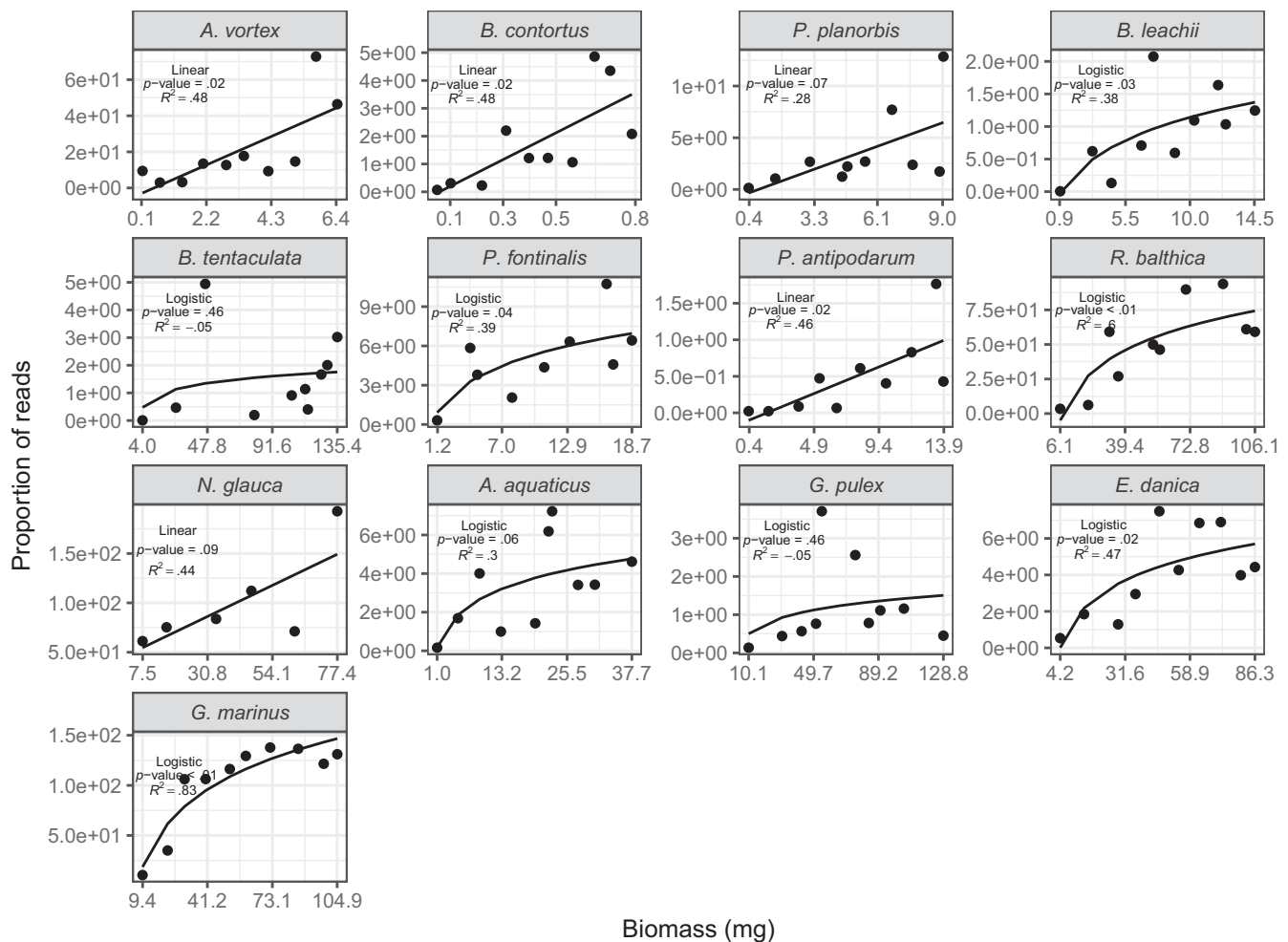
FIGURE 4 Scatter plots and regression analysis of sum of metabarcoding results across amplicons, plotted as sequencing reads vs. biomass (x-axis: biomass, y-axis: proportion of reads). Each box shows data for an individual species and lines show the fit of the model used

Detection of rare taxa proved challenging for both methods, as they failed to detect low biomass species in several cases, while metabarcoding also misrepresented higher biomass species as well. Our results suggest that using shotgun mitogenomic sequencing provides a more consistent and representative estimate of the relationship between reads and biomass from bulk macroinvertebrate samples, compared to amplicon metabarcoding of the COI gene. When considering the single amplicon approach, the metabarcoding data did not provide accurate quantitative information on the biomass of a large proportion of the species in bulk macroinvertebrate samples, although the accuracy of the method slightly improved when results from all three amplicons were combined.

4.1 | Sequencing performance and sample coverage

Sufficient coverage of reference mitogenome sequencing was achieved (Table S6), enabling assembly of almost complete reference mitogenomes. Previous reports suggest that $10\times$ coverage would allow shotgun mitogenome assembly (Zhou et al., 2013), but even at $6.4\times$ coverage for *G. pulex*, we assembled 13,326 bp, at an estimated 83–85% of expected length, by

comparison with other available closely related species [see species *Gammarus duebeni*, 15,651 bp, in Krebes and Bastrop (2012) and *Gammarus roeselli* 15,989 in Macher, Zizka, Weigand, and Leese (2017)]. The low coverage achieved for *G. pulex* could be possibly related to the large nuclear genome for this species (as reported for related species with nuclear genome size 8.5–10.5 pg (Jeffery & Gregory, 2014)), which accounted for the largest proportion of sequencing reads (mito-ratio 0.011%, Table S6). Using existing barcode sequences as baits for mapping, our pipeline allowed lower sequencing coverage to be sufficient compared to de novo assembly (read-based approach) (Crampton-Platt et al., 2016).

For the metabarcoding work, sequencing coverage varied among amplicons, with the shorter amplicon (FF130R, 130 bp) obtaining higher number of reads compared to the other two amplicons (B1FR, 450 bp; FFFR, 658 bp) (Table S4). Such variation in the depth of sequencing could be attributed to Illumina MiSeq sequencing preferentially amplifying shorter reads when sequencing mixed length amplicons or variable efficiency of primer binding (Aird et al., 2011). Additionally, this variation could be attributed to different amplification efficiency when different primer pairs are used across a

range of taxa. Normalizing library contents during sequencing (according to size of molecules included) should therefore be taken into consideration when multiple amplicons are sequenced in the same run.

4.2 | Sequence read–biomass relationships for both methods

The majority of species presented positive relationships of biomass with the read data for both methods, while only one species showed negative relationship (species *E. danica*, in metabarcoding data). The *E. danica* reverse trend was found for the FFFR (658 bp) amplicon (Figure S7), which was sequenced using the universal Folmer primers (Folmer et al., 1994). The same species also failed to amplify during individual barcoding (Table S1), suggesting that the results are likely to be related to primer incompatibility. In some cases, the use of a logistic model was a better descriptor of the relationship between biomass and read number compared to linear models (Table 4). In most cases (metabrcoding data), the number of reads per amplicon increased logistically with increasing biomass, suggesting a biological link between amplicon read number and species biomass. Both linear and logistic models have been used for the representation of read-to-biomass relationships in published metabarcoding and mitogenomic studies (Doi et al., 2017; Elbrecht & Leese, 2015; Lacoursière-Roussel, Rosabal, & Bernatchez, 2016; Tang et al., 2015; Zhou et al., 2013).

4.3 | Perspective on mitogenomic-based analysis of biodiversity

We performed mitogenome skimming based on custom-generated reference mitochondrial genomes, which allowed use of reduced sequencing depth. In the absence of a reference genome, species *A. vortex* was not included in downstream analysis of shotgun data. Other studies using mitogenomic sequencing to characterize assemblages of leaf beetles (Gómez-Rodríguez et al., 2015) or mass-trapped arthropods (Choo, Crampton-Platt, & Vogler, 2017) have also shown that mitogenome assembly based on reference-sequenced genomes outperforms the de novo approach (without reference library) in accuracy and recovery of diversity. Additionally, availability of reference mitogenomes during analysis allows easier detection and removal of nuclear mitochondrial pseudogenes (NUMTs) (Bensasson, Zhang, Hartl, & Hewitt, 2001) from shotgun sequencing data (Tang et al., 2014).

For the core analyses, the shotgun reads were normalized according to proportion of reads and mito-ratio, which accounted for the variability of mitochondrial sequencing effort compared to the total amount of sequencing reads per community. Our investigation of normalization methods showed similar findings between reads normalized according to mito-ratio and proportion of total reads (Table 4). Normalization of sequencing data is used to account for different DNA concentrations produced by the variability of body size (Gillett et al., 2014), number of individuals and relative abundance in communities. A variety of normalization options is available

(Weiss et al., 2017). Similar normalization strategies as used in the present work have also been used in other studies (e.g., in Tang et al. (2015)).

Mitogenomic sequencing currently uses a very small fraction of the total sequencing data, as the genomic DNA represents the largest amount of total DNA in the sample (Zhou et al., 2013). Depending on the taxon, the genomic to mitochondrial DNA ratio (mito-ratio) might vary, but generally approximately 99% of the reads are attributed to genomic DNA, leaving only 0.5–1% of the data to be used (for insects, the mito-ratio is 0.5% or lower) (Zhou et al., 2013). Attempts to generalize the expected genomic to mitochondrial DNA ratio are difficult as further work on a wider variety of taxa is necessary (Crampton-Platt et al., 2016). In the present work, mitonuclear ratio ranged between 0.011% (*G. pulex*) and 0.692% (*E. danica*), with an average of 0.19% (Table S6), which was lower than the previously described; nevertheless, it did not seem to present a huge challenge for the method, as was demonstrated herein.

To enhance the mtDNA contribution to the data, enrichment via centrifugation (during DNA extraction) (Macher et al., 2017; Zhou et al., 2013) and oligonucleotide capture array (Liu et al., 2016) have been used. In Zhou et al. (2013), only a moderate increase in mtDNA reads was achieved, which accounted for about 0.5% of the total data, while Macher et al. (2017) report a 129- to 140-fold enrichment of mtDNA through centrifugation. Use of capture arrays, designed based on 379 mitochondrial genomes (Liu et al., 2016), increased the mitochondrial ratio by 100-fold compared to previous attempts (mtDNA reads accounted for ca. 42% of the sequencing data after enrichment). Additionally, use of a capture array maintained the original ratio of species biomass in the sample, with a few variations depending on the phylogenetic distance of the test sample species composition compared to the species used for designing the array. The accuracy of capture arrays could be limited by the availability of sequencing information for the target organisms used for designing the probes (Hajibabaei, Singer, Clare, & Hebert, 2007) or due to occasionally picking up nontarget taxa (Liu et al., 2016).

Applications of mitogenomic sequencing can be used for biodiversity assessment, presenting advantages over traditional approaches similar to those of metabarcoding, such as sample multiplexing. Additionally, mitogenomic sequencing provides increased information content through long mitochondrial contigs which contain multiple protein-coding genes. These long contigs could provide improved phylogenetic resolution and measurement of intraspecific diversity at a more effective rate than single COI barcodes, while reducing the effects of false negatives caused by random dropout of genes due to degradation or variable sequencing coverage (Tang et al., 2014). The number of false positives could also be reduced by the use of longer sequences and selection on stringent criteria for establishing taxon presence. Furthermore, combinations of multiple markers increase delimitation success for closely related species compared to single marker work (Dupuis, Roe, & Sperling, 2012). Nevertheless, as multiple markers derived from mitochondrial reads represent a single linkage group or could

represent mitonuclear discordance as a result of introgression (Weigand et al., 2017), the use of independent nuclear markers should also be considered to resolve the presence of cryptic species (Campos-Soto, Torres-Pérez, & Solari, 2015; Miyamoto, Allard, Adkins, Janecek, & Rodney, 1994).

4.4 | Metabarcoding-based biodiversity analysis

Metabarcoding has been mainly used for the recovery of species richness from community samples, uncovering in many cases extensive diversity which would have been difficult to achieve using traditional methods (Leray & Knowlton, 2015; Sinniger et al., 2016). Additionally, metabarcoding work is increasingly being proposed as an alternative to traditional ecosystem monitoring, where accurate estimations of species abundance in environmental samples are generally desirable (Ji et al., 2013; Shokralla et al., 2015). Sequencing read abundance, where higher proportion of species' biomass would be reflected by a higher proportion of sequencing reads, has been suggested (e.g., Thomas, Deagle, Eveson, Harsch, & Trites, 2016), but these observations do not generally correspond to the majority of findings in the field (e.g., Elbrecht & Leese, 2015). Our results partially support previous findings and mainly reflect the larger uncertainty of assumptions on relative species abundance as derived from metabarcoding workflows. More specifically, the metabarcoding work failed to detect significant relationships between read data and known biomass in the mock communities in several cases (Table 3). The FFFR amplicon data (universal Folmer primers) showed significant read–biomass relationships in only four of 13 species, compared to eight of 13 for the other two amplicons. The discrepancy in efficiency between amplicons could be related to primer specificity or sequencing depth. Because the B1FR and FF130R primers were designed and modified for macroinvertebrate taxa, and second, because the sequencing coverage achieved for the Folmer region (FFFR) compared to amplicon FF130R. Summing of sequencing results from all three amplicons slightly improved the read/biomass relationships, but mainly assisted in removal of false negatives from the metabarcoding data (Table 4).

Multidimensional scaling analysis (nMDS, Bray–Curtis index) of the metabarcoding data revealed similarities in community composition based on the sequencing results for individual amplicons (Figs S11–S12). Such trends indicate that, despite the variations in read–biomass relationships for individual species, the community profiles obtained were still comparable, albeit with some exceptions (communities 9–10, Fig. S11). When assessed against the shotgun data, similar patterns were found across treatments (individual amplicons), but the shotgun data were more condensed across the *y*-axis, resembling more closely results obtained from the B1FR amplicon (Fig. S12).

Selection of the target region can influence metabarcoding results, and the utility of the COI marker has on occasion been questioned with regards to universality of the available priming sites (Deagle et al., 2014). Alternative COI metabarcoding primers have been designed for universal (Gibson et al., 2014; Hajibabaei et al., 2012; Leray et al., 2013) or group-specific detection (e.g., freshwater

invertebrates, Elbrecht & Leese, 2017; Gibson et al., 2014). Additionally, different markers have been proposed for use in characterization of biodiversity through metabarcoding, such as 18S (Zhan et al., 2014) or 16S (Epp et al., 2012), although COI still retains its superior value with some taxonomic groups compared to other markers due to the large repositories of reference sequences already available for a large number of taxa (Clarke, Beard, Swadling, & Deagle, 2017). Increased accuracy in biodiversity detection in community samples could also be achieved through the simultaneous use of multiple amplicons. In Gibson et al. (2014), a set of 11 primer pairs targeting the COI barcoding region was used, showing that combinations of several primers significantly increased the levels of species detection in samples of known content. Our results partially support the idea that the combination of sequencing reads from multiple amplicons can increase the detection rate of species richness of metabarcoding, as improvement of our results varied between the different species (Table 4). Similar findings are also reported by other multimarker studies (Dupuis et al., 2012; Zhan et al., 2014). Furthermore, we should also take into consideration that the use of multiple amplicons or loci also creates additional costs for tagged primers and library preparation as well as handling and data analysis time (Creer et al., 2016).

4.5 | False negatives, detection of rare diversity and closely related species

The percentage of false-negative detections for the shotgun work reached 5.6%, while false-negative detections for metabarcoding were either comparable or somewhat lower per amplicon at 2.2% (B1FR), 4.5% (FF130R) and 5.6% (FFFR). However, metabarcoding was more unpredictable due to false negatives also occurring for species with higher biomass in the communities (e.g., 10 specimens of *E. danica*, FFFR amplicon). The shotgun method only missed rare species at the lowest end of biomass presence, which could be indicative of a need for higher sequencing depth for detection of rare species, as well as small-bodied species with limited contribution to overall biomass. Primer binding-related bias could have caused false negatives or abnormal biomass representation in metabarcoding data, through primer incompatibility (as was likely the case for species *E. danica*) or low sequencing depth, while variation in sequencing depth could also influence the quantitative relationships between reads and species biomass (Hajibabaei et al., 2011). Increased sequencing depth or use of multiple primers has been suggested to enhance the detection of species of smaller biomass or lower relative abundance in the samples through metabarcoding (Hajibabaei et al., 2012). Generally, the inability of either method to detect rare or low biomass species could have significant implications for conservation surveys, as is the case for many endangered or invasive species (Zhan & MacIsaac, 2015).

Finally, the ability to discriminate between closely related species in community samples should also be taken into consideration. We analysed two closely related congener species of gastropods,

Bithynia leachii and *Bithynia tentaculata*. Shotgun sequencing was able to effectively differentiate between the two species. Annotation of the reads for the two species by mapping onto the previously generated reference mitogenomes provided more confidence in the results. Similarly, identification of metabarcoding reads for the two species was achieved by BLAST identification of OTUs against individual barcode reference sequences. Using phylogenetic (NJ) analysis further supported the correct annotation of the sequences.

4.6 | Future perspectives

Overall, the mitogenomic approach could present more effective and accurate detection of biomass and shifts in biomass in mixed bulk samples taken from the wild, compared to the more widely used to date COI metabarcoding. Nevertheless, the cost of metabarcoding currently remains lower than shotgun sequencing (in both cases, multiplexing is reducing the analytical cost) and would allow metabarcoding to be used for applied ecosystem monitoring, especially in cases where accurate biomass information is not required, and good reference databases of the target groups exist. Additionally, current metabarcoding costs are comparable to morphology-based identification while providing comparable assessment results (e.g., stream bioassessment Elbrecht et al. (2017)).

The importance of optimizing and standardizing such sequencing approaches is linked to generating more informative estimates of ecological interactions across taxa and trophic levels, as well as ecosystem functioning (Darling et al., 2017). Traditional monitoring is applied on individual or a limited number of species, which may not necessarily capture subtle responses to ecological change. Simultaneous monitoring across trophic levels, especially where the nature and dynamics of trophic interactions as well as other biotic interactions linked to competition and predation, might reveal more meaningful ecological signals relevant to biomonitoring (Woodward, Gray, & Baird, 2013). High-throughput sequencing (HTS) data of species richness and relative abundance could be enhanced in the future through automated pipelines utilizing automated samplers and machine learning methods to reconstruct ecological networks and investigate ecological interactions at an unprecedented scale (Bohan et al., 2017). Such advances in technologies underpin the utility of HTS applications, both in generating comparable data sets across large spatial and temporal scales, as well as capturing increasingly subtle, but ecologically informative, signals of response to environmental change.

DATA ACCESSIBILITY

The data for this work can be accessed at the European Nucleotide Archive (ENA) database (Study accession PRJEB23036).

AUTHOR CONTRIBUTIONS

The experiments were designed by I.B., S.C. and G.R.C.; contribution to experimental design was made by K.W., M.H., X.Z. and M.C.; morphometric measurements and analysis were performed by I.B.; DNA extractions and metabarcoding library prep were optimized and performed by I.B.; bioinformatics analysis of metabarcoding data was performed by I.B.; statistical analysis was performed by M.S. and I.B.; metabarcoding sequencing was run by S.S. and M.H.; sequencing and bioinformatics analysis of mitogenomics was performed by M.T., S.L. and X.Z.; invertebrate specimens were taxonomically identified by D.B.; first version of the manuscript was written by I.B.; the manuscript was edited by S.C. and G.R.C.; and comments were provided by all authors.

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