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Assessing the influence of sample tagging and library preparation on DNA metabarcoding

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Abstract

Metabarcoding is increasingly used to assess species diversity by high-throughput sequencing where millions of sequences can be generated in parallel and multiple samples can be analysed in one sequencing run. Generating amplified fragments with a unique sequence identifier ('tag') for each sample is crucial, as it allows assigning sequences to the original samples. The tagging through so-called fusion primers is a fast and cheap alternative to commercially produced ligation-based kits. However, little is known about potential bias and inconsistencies introduced by the long nucleotide tail attached to those primers, which could lead to deficient reports of community composition in metabarcoding studies. We therefore tested the consistency and taxa detection efficiency of fusion primers in (1) a one-step and (2) two-step PCR protocol as well as (3) a commercially manufactured Illumina kit using mock communities of known composition. The Illumina kit delivered the most consistent results and detected the highest number of taxa. However, success of the two-step PCR approach was only marginally lower compared to the kit with the additional advantage of a much more competitive price per library. While most taxa were also detected with the one-step PCR approach, the consistency between replicates including read abundance was substantially lower. Our results highlight that method choice depends on the precision needed for analysis as well as on economic considerations and recommend the Illumina kit to obtain most accurate results and the two-step PCR approach as a much cheaper yet very robust alternative.

KEYWORDS
fusion primers, Illumina, index, one-step, tag switching, two-step

1 | INTRODUCTION

Metabarcoding is a powerful molecular tool for biodiversity assessment. The method involves the identification of multiple taxa in environmental samples based on the bulk extraction of DNA and the parallel amplification of short fragments with universal primers (Carew, Pettigrove, Metzeling, & Hoffmann, 2013; Hajibabaei, Shokralla, Zhou, Singer, & Baird, 2011; Taberlet, Coissac, Hajibabaei, & Riesenberg, 2012; Yu et al., 2012). The unique labelling of amplicons allows multiple samples to be sequenced on a single run and ensures the subsequent computational assignment of sequences to their original sample (Binladen et al., 2007; Elbrecht & Leese, 2015; Lundberg, Yourstone, Mieczkowski, Jones, & Dangl, 2013; Schnell, Bohmann, & Gilbert, 2015; Taylor et al., 2008). Following standard library preparation protocols, these tags are typically short nucleotide sequences, which are implemented in commercial and often expensive kits (Bourlat, Haenel, Finnman, & Leray, 2016; Illumina, 2015; Kircher, Sawyer, & Meyer, 2012; Schnell et al., 2015). As an alternative labelling approach, so-called "fusion"—PCR primers have been developed (Elbrecht & Leese, 2015, 2017a; Lundberg et al., 2013). These primers carry a long nucleotide tail consisting of a flow cell binding adapter (P5/P7), a binding site for sequencing primers

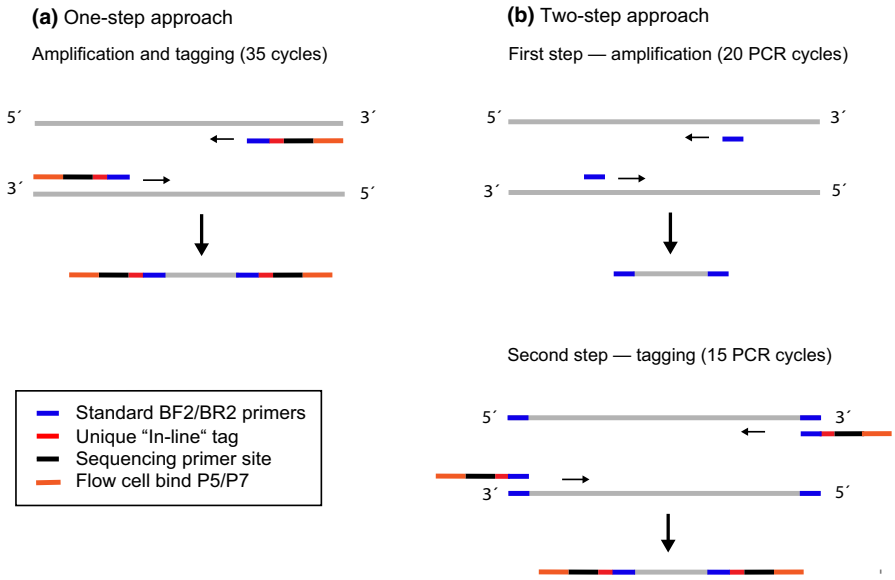

FIGURE 1 Simplified scheme of the one-step and two-step approach implementing fusion primers. (a) Amplification and tagging of the target fragment in a single PCR step. PCR primers carry a long tail with a unique in-line tag, a sequencing primer binding site and the flow cell binding adapters at their 5′-end. After 35 PCR cycles, the amplified fragment is hence provided with the nucleotide tail and prepared for sequencing. (b) Amplification and tagging of the target fragment are separated in two PCR steps. In a first PCR of 20 cycles, the target fragment is amplified using standard primers. In a second PCR of 15 cycles, amplified sequences are tagged using fusion primers as in (a). The amplified fragment is hence provided with the nucleotide tail and prepared for sequencing. For detailed information about the mechanism of the used kit with i5/i/ indexes, see the TruSeq Nano DNA Library Preparation Guide 2015 [Colour figure can be viewed at wileyonlinelibrary.com]

and a label to identify the sample consisting of a short base sequence ("in-line tag") attached to their 5′-end, which ensures the amplification and labelling of samples in one or two PCR steps (Binladen et al., 2007; Elbrecht & Leese, 2015; Lundberg et al., 2013).

In the one-step approach with fusion primers, a target DNA fragment is PCR amplified and simultaneously tagged with the long nucleotide tail attached to the primer's 5′-end (Figure 1a). This one-step method is fast, involves comparatively low costs and laboratory effort and has been successfully used in several metabarcoding studies (Beermann, Zizka, Elbrecht, Baranov, & Leese, 2018; Elbrecht & Leese, 2015, 2017a, 2017b). However, as indicated by former studies (Berry, Mahfoudh, Wagner, & Loy, 2011; O'Donnell, Kelly, Lowell, & Port, 2016), even overhangs of <10 nucleotides in length, so-called tags, on the primer's 5′- end can be associated with substantial PCR bias potentially resulting in extremely different communities inferred depending on the tag combination used. These biases are likely caused by tag-specific mismatches with the PCR template and the subsequent variation of primer binding efficiencies between taxonomic groups (O'Donnell et al., 2016). If the results of metabarcoding studies in fact strongly depend on the tag included, this will also be a problem in approaches implementing fusion primers and would be a major drawback for the use in metabarcoding projects and biodiversity assessment in general. Thus, the topic is a pressing issue in metabarcoding research.

The splitting of the amplification and labelling of a target fragment into two PCR steps (two-step PCR) should reduce the mentioned inefficiencies (Figure 1b). In this method, the target fragment is amplified using standard primers without tags in a first PCR step (but see e.g. Bohmann et al. 2018 for different two-step PCR approach). The labelling and attachment of Illumina adapters occurs in a second PCR, where no interaction with the template is possible, which diminishes the problem of reduced amplification efficiencies caused by mismatches (Berry et al., 2011; Bourlat et al., 2016; Leray & Knowlton, 2017; O'Donnell et al., 2016). Compared to the one-step approach, this method involves one additional PCR step and is therefore more time-consuming, costly and also prone to cross-contamination.

In this study, we tested the consistency and efficiency of taxa detection of fusion primers in a one-step and two-step PCR in comparison with a fabricated TruSeq Nano DNA Library Preparation kit (Illumina, 2015). To test this, we used mock communities consisting of similar tissue amounts from 52, previously identified freshwater taxa and investigated the number of recovered taxa per method. Furthermore, we analysed the consistency of read proportions between technical replicates. Finally, we discuss the implications of the results for future metabarcoding-based freshwater biodiversity assessments.

2 | MATERIAL AND METHODS

2.1 | Sample preparation and sequencing

Experiments were conducted using five mock communities with similar taxa composition (communities A–E) (Elbrecht & Leese, 2015, see Supplementary material 12 therein). Each community consisted of 52 macroinvertebrate individuals belonging to different taxa based on morphological identification (Table S1, Elbrecht & Leese,

2015, see Supplementary material 12 therein). Purified DNA aliquots (25 ng/µl) were processed as described in Elbrecht and Leese (2015). For each community, the three methods (1–3, see below) were applied with three replicates each:

1. *One-step PCR*: The cytochrome c oxidase subunit I (CO1) fragment was amplified and labelled in a single PCR step using uniquely indexed BF2/BR2 fusion primers (Elbrecht & Leese, 2017b, Tables S2 and S4). PCR consisted of 1× PCR buffer (including 2.5 mM Mg^{2+}), 0.2 mM dNTPs, 0.5 µM of both fusion primers, 0.025 U/µl of HotMaster Taq (5 Prime) and 25 ng DNA and was filled up with HPLC H_2O to a total volume of 50 µl. The following PCR program was used: 94°C for 180 s, 35 cycles of 94°C for 30 s, 50°C for 30 s and 65°C for 150 s, followed by a final elongation of 65°C for 5 min in a Thermocycler (Biometra TAdvanced).
2. *Two-step PCR*: In a first PCR step, the CO1 fragment was amplified using untailed BF2/BR2 primers (Elbrecht & Leese, 2017b) with identical PCR conditions to the previous method (one-step), but only 20 PCR cycles. The PCR product was diluted (1:10 with water), and 1 µl of the dilution was used as the template for the second PCR step. Amplified sequences were labelled using uniquely tagged BF2/BR2 fusion primers (Tables S2 and S4) with identical PCR conditions as in the previous step but 15 PCR cycles.
3. *TruSeq Nano DNA Library Prep Kit (Illumina)*: The CO1 fragment was amplified as in the first PCR step described in (2). The PCR product was diluted (1:10 in water) and used as the template for sequence labelling. Sequences were labelled using the TruSeq Nano DNA Library Preparation Kit starting from step 2: "Repair Ends and Select Library Size" to step 5: "Enrich DNA Fragments". The kit implements a ligation-based tagging of amplicons and a selective enrichment of DNA fragments with indexed adapter molecules on both ends through a PCR with eight cycles (Illumina, 2015).

A left-sided size selection of all samples was performed with magnetic beads (SPRIselect BECKMAN COULTER) with a ratio of 0.76×, and the concentration of selected PCR products was measured on a Fragment Analyzer (Advanced Analytical). All samples were equimolarly pooled, and paired-end sequencing was carried out by GATC Biotech AG (Konstanz, Germany) using one MISEQ sequencing run and the 250 bp paired-end v2 kit.

2.2 | Data analysis

Sequences labelled with fusion primers were assigned to their original sample as implemented in JAMP v0.23 (https://github.com/VascoElbrecht/JAMP). Sequences labelled through indexed adapters included in the TruSeq Nano DNA Library Preparation kit were computationally assigned to their original sample by GATC Biotech AG. Subsequent data processing was conducted for all samples as implemented in JAMP v0.23 using standard settings. Paired-end reads were merged (module U_merge_PE), and reverse complements were built where needed (U_revcomp) with USEARCH v10.0.240 (Edgar, 2010). CUTADAPT 1.6 (Martin, 2017) was used to remove primers and to discard sequences of unexpected length so that only reads with a length of 411–431 bp were used for further analyses. The module U_max_ee was used to discard all reads with an expected error >0.5. Sequences were dereplicated, singletons were removed and sequences with ≥97% similarity were clustered into OTUs using UPARSE (U_cluster_otus). OTUs with a minimal read abundance of 0.004% (>1 reads in samples with 30,000 reads, i.e. excluding singletons) in at least one sample were retained for further analysis while other OTUs were discarded. The used script for data analysis is attached in the supplements (Script S1).

Due to differences in read numbers, the data set was rarefied to an equal sequencing depth of 30,000 reads per sample using USEARCH v10.0.240 (*fastx_subsample*) after quality filtering. Taxonomic assignment of obtained sequences was done by comparisons with the reference database of BOLD systems v4 (Ratnasingham & Hebert 2007) as implemented in JAMP v0.23. Different filter thresholds were calculated to determine the minimum read abundance of an OTU in each sample to be included in analyses, ranging from 0.004% (low filtering threshold) to 0.09% (strict value). At low filtering thresholds, a higher number of low abundant OTUs are included in the data set and assigned taxa are displayed in the community composition. Simultaneously, false-positive sequences produced by sequencing mistakes or tag switching also remain in the data set, which can distort the community composition. Increasing filtering thresholds can remove such sequences, but also increase the risk of losing low abundant OTUs.

Detected taxa composition per sample was compared with the reference list of Elbrecht & Leese, 2015. Bray–Curtis and Jaccard dissimilarity indices based on OTUs were calculated between replicates of each sample and the various thresholds using the R (R Development Core Team, 2008) package VEGAN 2.4-2 (Oksanen et al., 2017). Bray–Curtis dissimilarity considers also read numbers per OTU, whereas Jaccard dissimilarity considers only presence or absence of an OTU. No data transformation was conducted for calculations. A one-way analysis of variance (ANOVA) was conducted for the number of detected taxa (dependent variable) and the three different methods (independent variable) at varying thresholds. A post hoc test (Tukey HSD test) was performed to analyse if there were significant differences in taxa detection between the three different methods (threshold 0.004–0.09; Figure 2). The influence of the three methods on Bray–Curtis and Jaccard dissimilarity between replicates was analysed as above, and the data were tested for significance with a post hoc test (Tukey HSD test) at a threshold of 0.004 (Figure 3). Data were visualized using the GGPLOT2 package (Wickham, 2009) in R.

3 | RESULTS

In total, we obtained 10,271,201 read pairs (raw data are available on Short Read Archive, submission number: SRP162847). Of these, 2,121,547 were assigned to the 30 samples (five communities × two methods × three replicates) labelled with fusion primers (Table S4, Figure S1), i.e., 876,035 (8.53%) to samples processed with

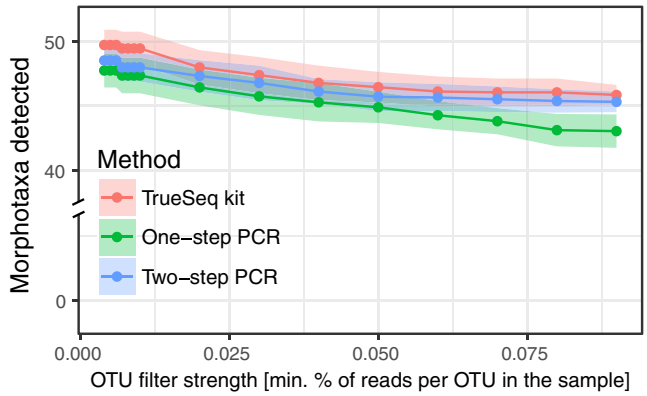

FIGURE 2 Average number of detected known morphotaxa (*y*-axis) for the three compared methods. Filled dots indicate the average proportion of detected taxa across all communities (a–e) and replicates at different filtering thresholds (0.004–0.09). Coloured shading indicates the standard deviation

the one-step and 1,245,512 (12.13%) to the two-step approach. For the 15 samples (5 × 1 × 3) labelled with the TruSeq Nano DNA Library Preparation kit, we obtained 5,282,478 (51.43%) reads. Unused primer combinations produced 111,823 (1.089%) reads, and 105,713 of these were assigned to one primer combination. Other unused combinations comprised a total of 5,749 reads (0.06%) and were thus discarded (Table S4, Figure S1). In total, 2,755,353 reads were rejected as debris after demultiplexing and not used for further analysis (26.83%). Thus, the different samples and methods showed variation in the total read number (average read number one-step approach: 58,402, standard deviation (SD): 23,985; two-step approach: 83,034, SD: 27,024; TruSeq Nano DNA Library Preparation kit: 352,165, SD: 269,251; Table S4, Figure S1). Two samples (C_one_3 [community C, one-step method, replicate three], D_one_2) processed in the one-step PCR had <30,000 reads after paired-end merging and were therefore excluded from further analysis (Table S4, Figure S1).

After taxonomic assignments with BOLD systems v4, the taxa list of each sample was compared with the original taxa list of the mock communities based on morphological identification (Figure 2). Across all samples, the vast majority of reads (on average 99.3% ± 0.9) was assigned to the target taxa included in the mock communities (Table S5), similar to the findings by Elbrecht and Leese (2015) (see Figure S9 therein). At lower filtering thresholds (0.004–0.01), the average number of detected taxa was at minimum 47 (90%) for the three applied methods. Undetected taxa were mainly those individuals with little biomass (e.g. Trombidiformes, *Dugesia*, see Table S3 in Elbrecht and Leese), where detection rate clearly decreased with increasing filtering thresholds. Furthermore, taxa which are known to amplify less successfully with the universal BF2/BR2 primers (Nematoda, Gastropoda, see Elbrecht and Leese (2017b), Weigand & Macher, 2018) were less frequently detected. The highest number of taxa was constantly detected in samples processed with the TruSeq Nano DNA Library Preparation kit (threshold 0.004–0.01: on average 48.9 [±1] taxa, threshold 0.02–0.09:46.4 [±1.2 taxa]). Numbers were significantly higher (Table S3) than those detected in samples processed with the one-step approach for all filtering thresholds (one-step threshold 0.004–0.01: on average 47.3 [±1.3] taxa, threshold 0.02–0.09:44.5 [±1.65] taxa). At low filtering thresholds (0.004–0.01), the number of detected taxa was also significantly higher than in samples processed with the two-step approach (two-step on average 48.1 [±1] taxa). In comparison, at higher filtering thresholds

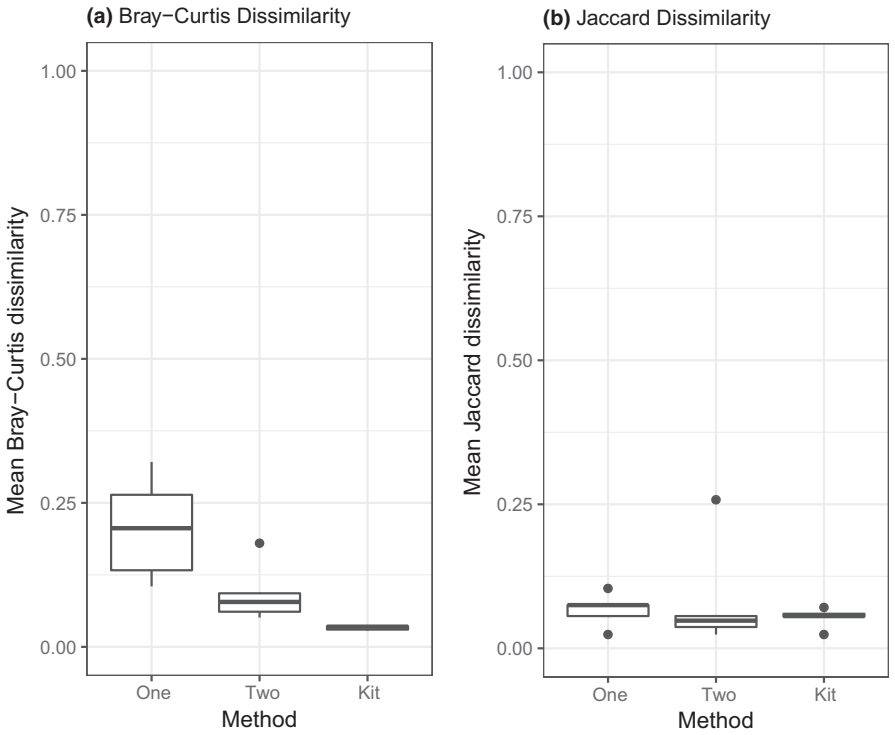

FIGURE 3 Boxplots for Bray–Curtis (a) and Jaccard (b) OTU dissimilarity indices among replicates of the different amplicon labelling methods: "one" = one-step PCR, "two" = two-step PCR, "kit" = TruSeq Nano DNA Library Preparation kit. The figure displays analyses with a filtering threshold of 0.004, which was used for previous metabarcoding studies. Increased thresholds showed no changes in dissimilarity values (see Figures S1 and S2 for further information)

results showed no significant differences (two-step threshold 0.02–0.09:45.7 [±1.2] taxa, Table S3). Up to a threshold of 0.05, on average one taxon more was detected with the two-step (47.7) than with the one-step (46.7) approach (Figure 2), although this difference was not significant. However, with higher thresholds (>0.05), differences became significant (Table S3) and on average two taxa more were detected with the two-step method. From threshold 0.01 on, the average detection clearly decreased for all three methods but more slowly for samples processed with the kit and the two-step than with the one-step approach (Figure 2). Thereby, 45.8 and 45.3 taxa were still detected at a strict threshold of 0.09 with the kit and the two-step method, respectively. With the one-step method, taxa detection was 43 at a threshold of 0.09. Samples processed with this method showed also the highest standard deviation. Detailed information of number of detected taxa per method without a subsampling after quality filtering is given in Figure S4. Results are largely congruent with those of the subsampled data set.

No distinct differences could be observed for calculated Bray–Curtis and Jaccard dissimilarity indices with varying filtering thresholds (Figures S2 and S3). Therefore, the threshold of 0.004 was selected for Figure 3a,b. The Bray–Curtis dissimilarity index among replicates was low for samples processed with the TruSeq Nano DNA Library Preparation kit (<0.1). Dissimilarity values were higher for replicates processed with fusion primers in a two-step PCR but also remained below 0.15 except for one outlier. Replicates of samples processed with fusion primers in a one-step PCR showed the highest Bray–Curtis dissimilarity (Figure 3a) and the highest variance, which was, however, below 0.3 (Figure 3a). Differences between replicates processed with this method were significantly higher than differences between replicates processed with the two-step method ($p = 0.0278$) or the kit ($p = 0.0017$). Differences between the two-step method and the commercial kit approach were not significant ($p = 0.2962$, ANOVA $F = 10.76$). The Jaccard dissimilarity index of samples processed with the kit and fusion primers in a two-step PCR was below 0.15. Again, in case of the two-step approach, one outlier arose with a dissimilarity value of 0.25. Replicates of samples processed with fusion primers in a one-step PCR showed the highest Jaccard dissimilarity and the highest variance (Figure 3b). However, all dissimilarity values were below 0.15. No significant difference could be observed between the methods at a threshold of 0.004 (Figure 3b; ANOVA $F = 0.347$, $p = 0.714$).

4 | DISCUSSION

Our results show that all three tested methods could reliably detect the taxa composition of a diverse macrozoobenthic community. This was supported by Jaccard dissimilarity calculations that showed no significant differences between the three methods. The average number of detected taxa was constantly highest in samples processed with the TruSeq Nano Library Preparation kit; yet, differences to the two-step approach were minor and insignificant for most filtering thresholds, with only one or two more taxa detected by the Illumina kit. More than 90% of the taxa were still detected in samples processed with the one-step approach at lower filtering thresholds. When considering read abundance for individual OTUs in the comparison via Bray–Curtis dissimilarity, the Illumina kit showed the highest consistency. Again, dissimilarity estimates for the two-step approach were only insignificantly higher. For the one-step method, Bray–Curtis dissimilarity values between replicates were the highest and also significantly different to the other two methods indicating a lower consistency and usability for quantitative analysis of this approach. However, in comparison with previous studies (Berry et al., 2011; O'Donnell et al., 2016) our findings indicate low primer-specific bias caused by the nucleotide overhang of the primers 5′-end. This is also congruent with findings by Leray and Knowlton (2017), who did a similar study on a mock community consisting of 34 marine macroinvertebrate taxa.

As indicated by Schnell et al. (2015), tag switching ('tag jumps') for an Illumina kit-based analysis was higher (2.5%–2.7%) than the rate observed for the one-step fusion primer approach (0.01%, Elbrecht and Leese (2015, 2017a)), which leads to a higher number of deficient sequences in the data set. For the present study, tag switching, which is determined by the numbers of reads assigned to unused primer combinations, is difficult to define. This is due to one primer combination (BF20/BR20), which was not used during laboratory processes but consisted of approximately 100,000 reads after demultiplexing. The emergence of these sequences is unclear. Cross-contamination could be a source for the incorrect tagging (Schnell et al., 2015); however, this is not consistent with the other results. The inclusion of these 100,000 sequences for calculations of tag switching indicates a proportion of 1.062%, whereas the proportion of tag switching is reduced to 0.058% when this combination is excluded.

Samples processed with the three different methods showed extremely different read numbers. In particular, the samples processed with the TruSeq Nano DNA Library Prep kit showed constantly the highest read numbers, with up to 1,122,685 for one sample (Figure S1, Table S4). All samples, independent from the used tagging method, were equimolarly pooled before sequencing, with a foregone concentration measurement of DNA fragments (Elbrecht & Leese, 2015; Illumina, 2016). However, the sequencing primer binding site of fusion primers used in the present study differs from the ones used in the Illumina kit (see Elbrecht and Leese (2015), Illumina, 2015, 2016). Differences can be due to recent changes of sequencing primer used on the Illumina platform or a mistake during primer design. Since reads from both indexing methods were sequenced in one run, a greater affinity of the sequencing primer to sequences labelled with the Illumina kit is likely and might have led to the observed differences in read numbers. Furthermore, the protocol implemented in the kit used different PCR ingredients (e.g. Taq polymerase in PCR cocktail) and a lower number of PCR cycles were performed (28 vs. 35 cycles).

The two methods implementing fusion primers with tags have some advantages over commercial kits due to their lower cost and required handling time. For the labelling of the 15 samples of the present study, two sets of the TruSeq Nano DNA Library Preparation kit were needed for 800 € each (set A/B—12 index combinations, 24 reactions each), resulting in a price of 106 € for each sample (Illumina,

2015). In comparison, the two-step approach for all 15 samples cost approximately 314.40 € in total and 20.96 € per sample, with the one-step approach costing slightly less. In detail, the ordered fusion primers (100 µM) cost approx. 36 € per primer (80–160 reactions) and 288 € in total, as four primer pairs were needed to achieve the number of primer combinations required for the individual tagging of the 15 samples. Other consumables needed for the two-step approach for all 15 samples cost approximately 26.40 € (and slightly less for the one-step method). The tagging process with fusion primers implements only one or two PCR steps (depending on the method) and a final size selection, while a successful library preparation with the TruSeq Nano Library Preparation kit is based on several preceding steps (end repair, creating an A-overhang for ligation). The kit approach also requires various clean-up steps, rendering the method more time-consuming and prone to cross-contamination (Schnell et al., 2015).

Comparing the one-step and two-step methods that add tags via fusion primers (with identical Illumina 5′-tails), the two-step approach showed a higher consistency and taxa detection efficiency. However, differences between the two approaches were not as strong as described in previous studies (Berry et al., 2011; O'Donnell et al., 2016). An additional advantage of the two-step method is presumed to lie in its decreased susceptibility to inhibitors. While this hypothesis was not specifically tested, samples including inhibiting substances (humic acids, tannins, flavonoids contained in alder foliage on which many freshwater species feed) could not be amplified in a single PCR step, but showed clear products when the two-step method was applied (using similar numbers of PCR cycles). These observations indicate that the untailed, shorter primers used in the first step of the two-step PCR method increase amplification success. The dilution of the product of the first PCR step also dilutes any associated inhibiting substances, subsequently resulting in a lower influence of the inhibitors on the fusion primers in the second PCR step where the tagging occurs.

In contrast, the advantage of the one-step approach is that it requires less time and is less prone to cross-contamination than the two-step approach because it omits the second PCR step. This makes it applicable for metabarcoding approaches using clean samples that are known to contain low amounts of inhibiting substances and in cases where detecting the maximum number of species in a sample is not important. For detailed, larger-scale biodiversity assessments, the choice of method is coupled with financial possibilities. Here, the more expensive Illumina kit may deliver the most consistent results, but is by far the most expensive and time-consuming method. With limited time and budget, the two-step PCR approach, which showed in addition no significantly lower consistency despite a greater number of PCR cycles applied, is a very good alternative with almost similar performance in consistency and taxa detection efficiency.

5 | CONCLUSION

Our results show that the commercial kit shows the most consistent and promising results for detailed biodiversity assessment of macroinvertebrates. However, the two-step PCR approach implementing fusion primers had almost comparable benchmarks in terms of taxa detection and consistency between replicates. Both the two-step PCR approach and the kit performed significantly better than the one-step PCR approach in terms of consistency and PCR efficiency, as well as showing a reduced sensitivity to inhibitors. Due to these advantages and the comparatively low costs, we recommend the two-step PCR method for cost-efficient large-scale metabarcoding approaches. However, the commercial TruSeq Nano DNA Library Prep kit detected most taxa on average and showed highest consistency between replicates and is therefore the best option for elaborate studies where a detailed and comprehensive diversity assessment is necessary.

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CONFLICT OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the study.

AUTHOR CONTRIBUTIONS

F.L., V.E., J.N.M. and V.Z. conceived the ideas and designed the methodology; V.Z. carried out the laboratory work and performed bioinformatic analyses; V.Z. and F.L. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA ACCESSIBILITY

DNA sequences: raw data are accessible on GenBank (Short Read Archive; submission number SRP162847); processed data are available in the online Supporting Information.

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REFERENCES

Beermann, A. J., Zizka, V. M. A., Elbrecht, V., Baranov, V., & Leese, F. (2018). DNA metabarcoding reveals the complex and hidden responses of

chironomids to multiple stressors. *Environmental Science Europe*, *30*, 26. https://doi.org/10.1186/s12302-018-0157-x

Berry, D., Mahfoudh, K. B., Wagner, M., & Loy, A. (2011). Barcoded primers used in multiplex amplicon pyrosequencing bias amplification. *Applied and Environmental Microbiology*, *77*(21), 7846–7849. https://doi.org/10.1128/AEM.05220-11

Bohmann, K., Gopalakrishnan, S., Nielsen, M., dos Santos Bay Nielsen, L., Jones, G., Streicker, D. G., & Gilbert, M. T. P. (2018). Using DNA metabarcoding for simultaneous inference of common vampire bat diet and population structure. *Molecular Ecology Resources*, *18*(5), 1050–1063. https://doi.org/10.1111/1755-0998.12891.

Bourlat, S. J., Haenel, Q., Finnman, J., & Leray, M. (2016). Preparation of amplicon libraries for metabarcoding of marine eukaryotes using Illumina MISEQ: The dual-PCR method. In S. Bourlat (Eds.), *Marine genomics. Methods in molecular biology* (*1452*, pp. 197-207) . New York, NY: Humana Press.

Binladen, J., Gilbert, M. T. P., Bollback, J. P., Panitz, F., Benedixen, C., Nielsen, R., & Willerslev, E. (2007). The use of coded PCR primers enables high-throughput sequencing of multiple homolog amplification products by 454 parallel sequencing. *PLoS One*, *2*, e197. https://doi.org/10.1371/journal.pone.0000197

Carew, M. E., Pettigrove, V. J., Metzeling, L., & Hoffmann, A. A. (2013). Environmental monitoring using next generation sequencing: Rapid identification of macroinvertebrate bioindicator species. *Frontiers in Zoology*, *8*, 1–15. https://doi.org/10.1186/1742-9994-10-45

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*(19), 2460–2461. https://doi.org/10.1093/bioinformatics/btq461

Elbrecht, V., & Leese, F. (2015). Can DNA-based ecosystem assessments quantify species abundance? Testing primer bias and biomass-sequence relationships with an innovative metabarcoding protocol. *PLoS One*, *10*(7), 1–16. https://doi.org/10.1371/journal.pone.0130324

Elbrecht, V., & Leese, F. (2017a). Development and validation of DNA metabarcoding COI primers for aquatic invertebrates using the R package "PRIMERMINER". *Methods in Ecology and Evolution*, *8*, 622–626. https://doi.org/10.1111/2041-210X.12687

Elbrecht, V., & Leese, F. (2017b). Validation and development of freshwater invertebrate metabarcoding COI primers for environmental impact assessment. *Frontiers in Environmental Science*, *5*, 11. https://doi.org/10.3389/fenvs.2017.00011

Hajibabaei, M., Shokralla, S., Zhou, X., Singer, G. A. C., & Baird, D. J. (2011). Environmental barcoding: A next-generation sequencing approach for biomonitoring applications using river benthos. *PLoS One*, *6*(4), e17497. https://doi.org/10.1371/journal.pone.0017497

Illumina (2015). *TruSeq® Nano DNA library prep reference guide*.

Illumina (2016). *Illumina adapter sequences*.

Kircher, M., Sawyer, S., & Meyer, M. (2012). Double indexing overcomes inaccuracies in multiplex sequencing on the Illumina platform. *Nucleic Acids Research*, *40*(1), 1–8. https://doi.org/10.1093/nar/gkr771

Leray, M., & Knowlton, N. (2017). Random sampling causes the low reproducibility of rare eukaryotic OTUs in Illumina COI metabarcoding. *PeerJ*, *5*, e3006. https://doi.org/10.7717/peerj.3006

Lundberg, D. S., Yourstone, S., Mieczkowski, P., Jones, C. D., & Dangl, J. L. (2013). Practical innovations for high-throughput amplicon sequencing. *Nature Methods*, *10*(10), 999–1002. https://doi.org/10.1038/nmeth.2634

Martin, M. (2017). CUTADAPT removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal*, *17*(1), 17–19.

O'Donnell, J. L., Kelly, R. P., Lowell, N. C., & Port, J. A. (2016). Indexed PCR primers induce template-specific bioas in large-scale DNA sequencing studies. *PLoS One*, *11*(3), e0148698. https://doi.org/10.1371/journal.pone.0148698

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., … Wagner, H. (2017). *VEGAN: Community ecology package*. R package version 2.4-3. Retrieved from https://CRAN.R-project.org/package=vegan

R Development Core Team (2008). *R: A language and environment for statistical computing*. Vienna, Austria: The R Foundation for Statistical Computing.

Ratnasingham, S., & Hebert, P. D. N. (2007). BOLD: The Barcode of Life Data System. *Molecular Ecology Resources*, *7*(3), 355–364. https://doi.org/10.1111/j.1471-8286.2007.01678.x

Schnell, I. B., Bohmann, K., & Gilbert, T. B. (2015). Tag jumps illuminated – Reducing sequence-to-sample misidentification in metabarcoding studies. *Molecular Ecology Resources*, *15*, 1289–1303. https://doi.org/10.1111/1755-0998.12402

Taberlet, P., Coissac, E., Hajibabaei, M., & Riesenberg, L. H. (2012). Environmental DNA. *Molecular Ecology*, *21*(8), 1789–1793. https://doi.org/10.1111/j.1365-294X.2012.05542.x

Taylor, D. L., Booth, M. G., McFarland, J. W., Herriott, I. C., Lennon, N. J., Nusbaum, C., & Marr, T. G. (2008). Increasing ecological inference from high throughput sequencing of fungi in the environment through a tagging approach. *Molecular Ecology Resources*, *8*, 742–752. https://doi.org/10.1111/j.1755-0998.2008.02094x

Weigand, A. M., & Macher, J.-N. (2018). A DNA metabarcoding protocol for hyporheic freshwater meiofauna: Evaluating highly degenerate COI primers and replication strategy. *Metabarcoding and Metagenomics*, *2*, e26869. https://doi.org/10.3897/mbmg.2.26869

Wickham, H. (2009). *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer Verlag.

Yu, D. W., Ji, Y., Emerson, B. C., Wang, X., Ye, C., Yang, C., & Ding, Z. (2012). Biodiversity soup: Metabarcoding of arthropods for rapid biodiversity assessment and biomonitoring. *Methods in Ecology and Evolution*, *3*(4), 613–623. https://doi.org/10.1111/j.2041-210X.2012.00198.x

SUPPORTING INFORMATION

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

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