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Cooking small and large portions of "biodiversity-soup": Miniaturized DNA metabarcoding PCRs perform as good as large-volume PCRs

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Abstract

DNA metabarcoding is a powerful tool to assess arthropod diversity in environmental bulk samples such as Malaise trap, pitfall trap, or hand net samples. While comparative performance tests for different extraction protocols, primers, and Taq polymerases have been made, the effect of different PCR volumes on bulk sample metabarcoding performance is less explored. Although using small PCR volumes reduces overall costs, they may lead to decreased taxon recovery or higher replicate variability due to increased pipetting imprecision, PCR stochasticity (PCR drift), or inhibition when using high amounts of template community DNA. We here performed a simple DNA metabarcoding experiment to test if species detection and the consistency of technical replicates decrease with decreasing PCR volume in standard reaction tubes. We used a mock community sample consisting of different amounts of DNA from 35 arthropod species, and a Malaise trap sample composed of many thousand insect specimens. PCR volumes tested were 5, 10, 15, 20, 25, and 50 µl. Both samples were replicated 14 times in the first PCR step with two technical replicates each in the second PCR step. Our data show that small PCR volumes did neither have systematically lower species detection or richness values, nor lower consistency between PCR replicates. We therefore recommend low volumes primarily depending on handling constraints. Further, we emphasize the importance of sequencing depth for taxon recovery.

KEYWORDS

amplicon sequencing, biomonitoring, bulk sample, Malaise trap, PCR bias

1 | INTRODUCTION

Biodiversity assessments using DNA metabarcoding are increasingly applied in ecological research and environmental monitoring. In particular for arthropod bioassessments, bulk sample metabarcoding is frequently used for both aquatic and terrestrial environments. The method typically uses homogenized or lysed tissue from specimens as input. Specimens are either collected from traps (Basset et al., 2020; Braukmann et al., 2019; Hardulak et al., 2020; Yu et al., 2012), from manual net catches (Elbrecht et al., 2017; Kuntke et al., 2020; Zizka et al., 2020), or from litter or soil samples (Arribas et al., 2016; Porter et al., 2019). Furthermore, DNA extracted from the sample fixative (Erdozain et al., 2019; Martins et al., 2019; Zizka et al., 2018) or directly from environmental samples (eDNA) (Hajibabaei, Porter, Robinson,

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et al., 2019; Leese et al., 2020; Mächler et al., 2014, 2019) can be used as an input for DNA metabarcoding-based species detection (see e.g., Blackman et al., 2019 for an overview). Different extraction methods have been tested and good practice solutions exist (Majaneva et al., 2018). Likewise, suitable primers have been identified for different purposes (Elbrecht et al., 2019; Elbrecht & Leese, 2017; Hajibabaei et al., 2019; Leray et al., 2013; Tab erlet et al., 2018) and robust metabarcoding Taq polymerases have been suggested (Nichols et al., 2018).

One aspect that has so far received little attention in bulk sample metabarcoding is the role of different PCR volumes of DNA metabarcoding performance. Current studies typically use 10 µl to 50 µl total PCR volumes (Aylagas et al., 2014; Clarke et al., 2017; Elbrecht et al., 2017; Kuntke et al., 2020). Some arthropod metabarcoding studies, however, also successfully used volumes below 10 µl, for example, Braukmann et al., (2019) used 6 µl total reaction volumes for Malaise trap metabarcoding. Volume recommendations for a very reliable PCR mastermix identified for DNA metabarcoding in Nichlos et al. (2018), that is, the Qiagen MasterMix, is even 50 µl. For comparatively high DNA concentrations, which are typically achieved from bulk samples, larger reaction volumes (or smaller template:-mastermix ratios) are sometimes recommended to minimize inhibition (see e.g., Elbrecht, Vamos, et al., 2017). This, however, leads to substantially higher costs, especially for large sample sizes and when considering sample replication (Balint et al., 2018). Even if inhibition does not impact on amplification, there is a risk of increased PCR stochasticity when miniaturizing PCR volumes, for example, due to reagent–tube interactions and because in miniaturized PCR volumes smaller absolute volume deviations have a stronger effect compared to large PCR volumes. For bacterial 16S amplicon sequencing, Minich et al., (2018) systematically tested for this effect. They found that, if input DNA was not too low, the detected number of operational taxonomic units (OTUs) was not lower and PCR stochasticity not higher in small (2.5 and 5 µl) compared to standard 25 µl reaction volumes. For metazoan bulk samples, sometimes referred to as "biodiversity soup" (sensu Yu et al., 2012), no systematic tests have so far been published and recommendations vary among researchers.

In this study, we therefore tested if species detection and richness, as well as the robustness of PCR, decreases with decreasing reaction volume. To test this, we used two different sample types, (i) a mock bulk sample composed of 35 different arthropod species and (ii) a complex arthropod sample composed of thousands of specimens from hundreds of insect species from a German Malaise trap sample. We replicated samples 14 times to obtain robust results and analyze PCR stochasticity.

2 | MATERIAL AND METHODS

2.1 | DNA extraction, PCR, and library preparation

Two different sample types were used in this study: (i) a mock community consisting of 35 distinct macroinvertebrate DNA extracts of known species and concentration and (ii) a size-sorted sample of a Malaise trap (see Figure S1), that had been set up in the Rhine-Main-Observatory for 2 weeks in July 2019, which consisted of several thousand specimens. The Rhine-Main-Observatory is a Long-Term Ecological Research (LTER) site (Haase et al., 2016; Mirtl et al., 2018) east of Frankfurt, Germany (https://deims.org/9f9ba137-342d-4813-ae58-a60911c3abc1). Prior to extraction, the Malaise trap sample was divided into two size classes (small <4 mm; large ≥4 mm), which were pooled in a 5:1 ratio (small:large) prior to extraction to maximize species recovery. While the specimens and species of the small size class contribute most to the diversity of the sample, their biomasses are substantially lower compared to those from the large size class. By increasing the amount of template of the small, species-rich fraction, this fraction gets much better represented and increases species recovery (Elbrecht, Vamos, et al., 2017, Elbrecht et al. 2020). To estimate the detection threshold, DNA extracts with a known copy number were spiked into the Malaise trap sample (50, 500, 5,000, 50,000 mitochondrial copies per PCR assay from *Gammarus pulex*, *Gammarus fossarum*, *Ephemera danica*, and *Oecismus monedula*). Both sample types were extracted with a modified version (Appendix S1) of the NucleoMag Tissue kit (Macherey Nagel, Düren, Germany).

For amplification of both sample types, 6 different assay volumes (5, 10, 15, 20, 25, and 50 µl) with 14 replicates per volume were used in the first PCR. All PCR pipetting steps were conducted on a Biomek FX^{P} liquid handling workstation (Beckmann Coulter, Bread, CA, USA) with custom protocols. Samples were randomly distributed among four 96-well 0.2 ml PCR plates as shown in Figure 1 including one negative control (PCR-grade water) per volume per plate (32 in total) to control for contamination that might happen during robotic pipetting. These sample plates and volumes are typical for arthropod bulk metabarcoding. Samples were amplified using the Qiagen Multiplex PCR Plus Kit (Qiagen, Hilden, Germany) with a final concentration of 1x Multiplex PCR mastermix, 1x CoralLoad Dye, 100 nmol / L of each primer (fwhF2, fwhR2n; Vamos et al., 2017) and 5 ng DNA. Tubes were filled up to the respective final volume with PCR-grade water. For amplification, a touchdown protocol was used: 95°C for 5 min initial denaturation, 10 cycles of 95°C for 30 s denaturation, 68–59°C for 30 s annealing (1°C decrease per cycle), and 72°C for 30 s elongation, followed by 20 cycles with 58°C for annealing, ending with 68°C for 10 min as a final elongation step. For subsequent demultiplexing, all samples from the same plate were tagged with a 4– to 6-bp inline tag and a universal tail attached to the primer (Table S1; Leese et al., 2020). In the second PCR, all samples from the first PCR step were replicated twice. For each plate, each well was indexed individually with primers consisting of the respective matching universal tail sequence and an i5/i7 8-bp index sequence. Samples were amplified with the same concentrations as in the first PCR, with the exception that, depending on the assay volume, 1–10 µl of the first step was used as template (less for the smaller volumes, more for larger ones to compensate for the dilution effect). For amplification, the following protocol was used: 95°C for 5 min initial denaturation, 25 cycles of 95°C for 30 s denaturation, and 72°C for 1 min elongation concluded by 68°C for 10 min final elongation. Amplification success was verified on a 1% agarose gel.

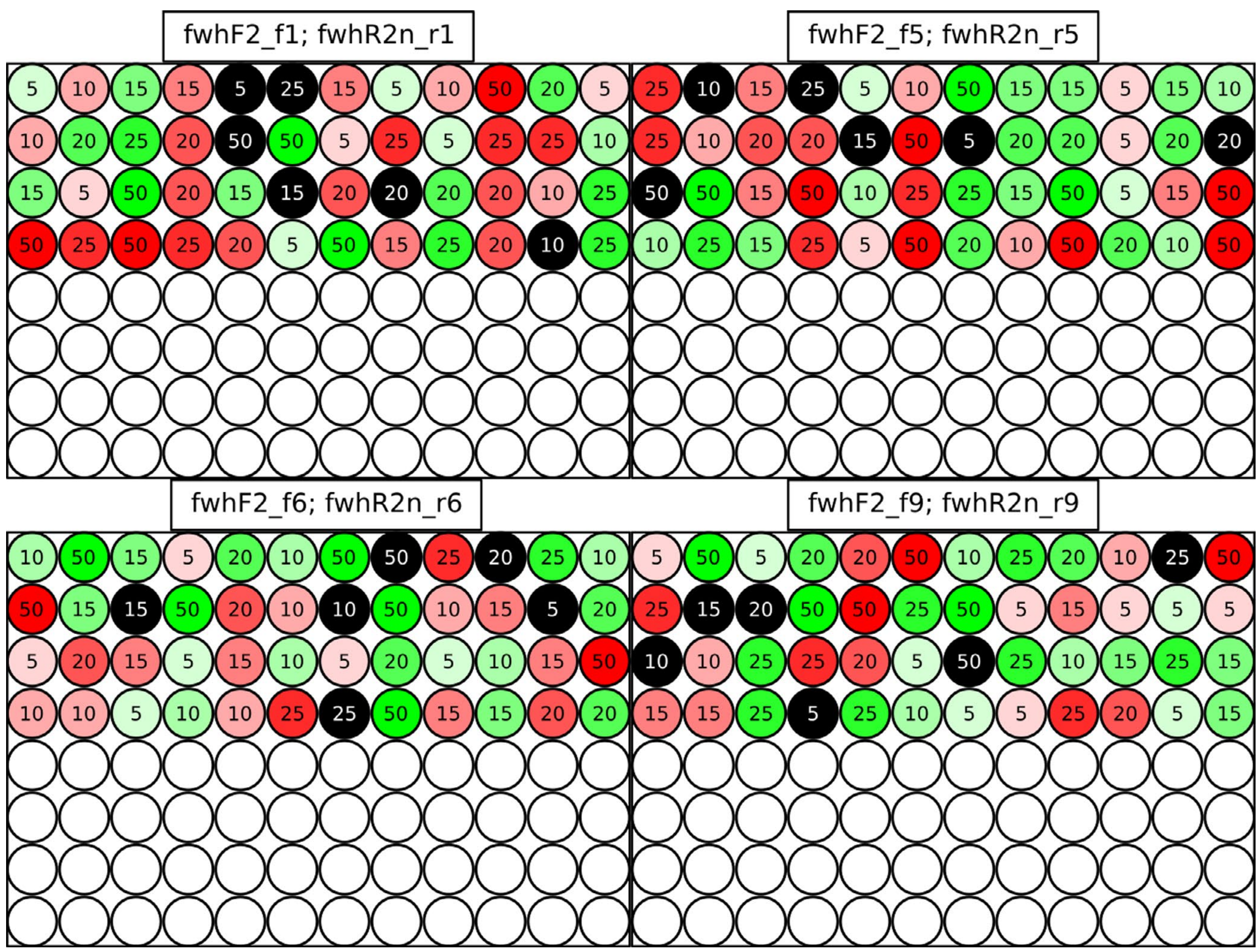

FIGURE 1 Plate design for the 1st step PCR. Numbers indicate the PCR assay volume. Red: Mock community, green: Malaise trap, black: no template control (NTC)

Assay volume was normalized to 50 µl with PCR-grade water and DNA concentration was measured using a Qubit 2.0 (High Sensitivity Kit, Thermo Fisher Scientific, Beverly, USA). All uniquely indexed samples were pooled with equimolar concentrations into one library. The library was concentrated in a vacuum centrifuge (Eppendorf Concentrator Plus, Eppendorf, Hamburg, Germany) and cleaned up with a double-sided size selection with a ratio of 0.75x/0.6x using the NucleoMag Kit (Macherey Nagel, Düren, Germany) to remove any nonspecific products. The library was sequenced on an Illumina MiSeq (V3 kit, 2x300 bp) at CeGat GmbH (Tübingen, Germany).

2.2 | Bioinformatic analysis

Raw data of the sequencing run were delivered demultiplexed by index reads. Further demultiplexing of the inline tag was done with the python script “demultiplexer” (https://github.com/DominikBuchner/demultiplexer). Reads were then further processed with the JAMP pipeline (v0.67; https://github.com/VascoElbrecht/JAMP). First, paired-end reads were merged using Usearch (v11.0.667, Edgar, 2010) through the command U_merge (fastq_pctid=75). Primer sequences were trimmed using Cutadapt (v2.5, (Martin, 2011), and only reads with a length of 205 bp (±10) were retained. Prior to OTU clustering using a similarity threshold of 97%, reads were dereplicated and singletons excluded. Only clusters with at least 0.01% abundance in one sample were used in further analysis. Taxonomic assignment was carried out using BOLDigger (v1.2.1; https://github.com/DominikBuchner/BOLDigger; Buchner & Leese, 2020). The best hit was determined with the BOLDigger method, which selects the most common hit above a 98% similarity threshold. For all subsequent analysis steps, the resulting read table (Table S2) combined with the taxonomic assignment was used.

2.3 | Data filtering and statistical analysis

All subsequent filtering and analysis steps were done using a custom python script (Appendix S2). To reduce noise introduced by incomplete entries in the BOLD database, all OTUs without species-level

assignment were discarded as well as species names that contained numbers or special characters (i.e., "sp." or falsely entered epithet information). To account for tag-switching and cross-contamination, the maximum reads for every species in the 32 negative controls were subtracted from the respective species' read numbers. After computing sequencing depth and the number of shared species for the technical replicates in the second PCR, only species that were present in both of these replicates were retained for further analysis. To further account for possible low-level cross-contamination only those species were retained, that were found in at least 2 of the 14 replicates in the first PCR.

Datasets were checked for normality using the Shapiro–Wilk test. Since only a small fraction of the datasets were normally distributed, nonparametric tests were used in the downstream analysis. To check for correlation of assay volume and species richness or mean overlap between replicates, the Spearman rank-order correlation was used. Possible systematic deviations in the means for groups were checked using the Kruskal–Wallis test. In case of significant differences between groups, the Dunn's test was applied to compute all pairwise comparisons and significance levels were Bonferroni corrected to account for multiple testing. All statistical tests were conducted using the python packages "scipy" and "scikit_posthocs" (Terpilowski, 2019; Virtanen et al., 2020) and all plots were created with the python package "seaborn" (Waskom et al. 2020).

3 | RESULTS

Sequencing yielded 12,234,306 read pairs. After demultiplexing and filtering, we retained on average 20,935 read pairs (55,91–32,906) per sample. PCR volume had a significant effect on the obtained read count per sample for both the mock community (Kruskal–Wallis test, $p < 0.001$; Table S3; Figures S2, S3) and the Malaise sample ($p = 0.004$). For mock community samples, read counts of 5 and 50 μl volumes were significantly lower than for all other tested volumes (Dunn's test, $p < 0.001$–$p = 0.044$, Table S4; Figures S2, S3), but did not significantly differ from each other. In comparison, for the Malaise trap samples, read counts of the 50 μl volume samples also differed significantly from all other volumes (Dunn's test, $p < 0.005$–$p = 0.041$, Table S4; Figures S2, S3) except for 5 μl, but the latter did not significantly differ from any other volume.

Despite these differences in read counts per assay volume, there was no correlation of tested PCR volumes and recovered species richness for mock community (Spearman's ρ, $p = 0.825$; Table S5, Figure 2) or Malaise trap samples ($p = 0.534$). Additionally, species richness was not systematically different in any assay volume (mock community: Kruskal–Wallis test, $p = 0.722$; Malaise trap: $p = 0.073$; Table S3, Figure 2 and Figure S4), further supporting the former result.

When comparing consistency of technical PCR replicates (all 14 "assay volume replicates" were run in PCR replicate pairs), we observed no significant differences in shared species richness between replicates for the different assay volumes (mock community: Kruskal–Wallis test, $p = 0.15$; Malaise trap: $p = 0.057$; Table S3, Figures S5, S6). In comparison, with on average 92% (range: 80%–100%; Figure 3) shared species, the relative number of shared species across all 14 samples per assay volume was higher for the less diverse mock community than for the species-rich Malaise trap samples with an average of 73% (range: 56% to 86%). When comparing the consistency of species richness across all tested assay volumes, we detected a weak correlation of shared species between replicates and assay volumes in both the mock community (Spearman's ρ,

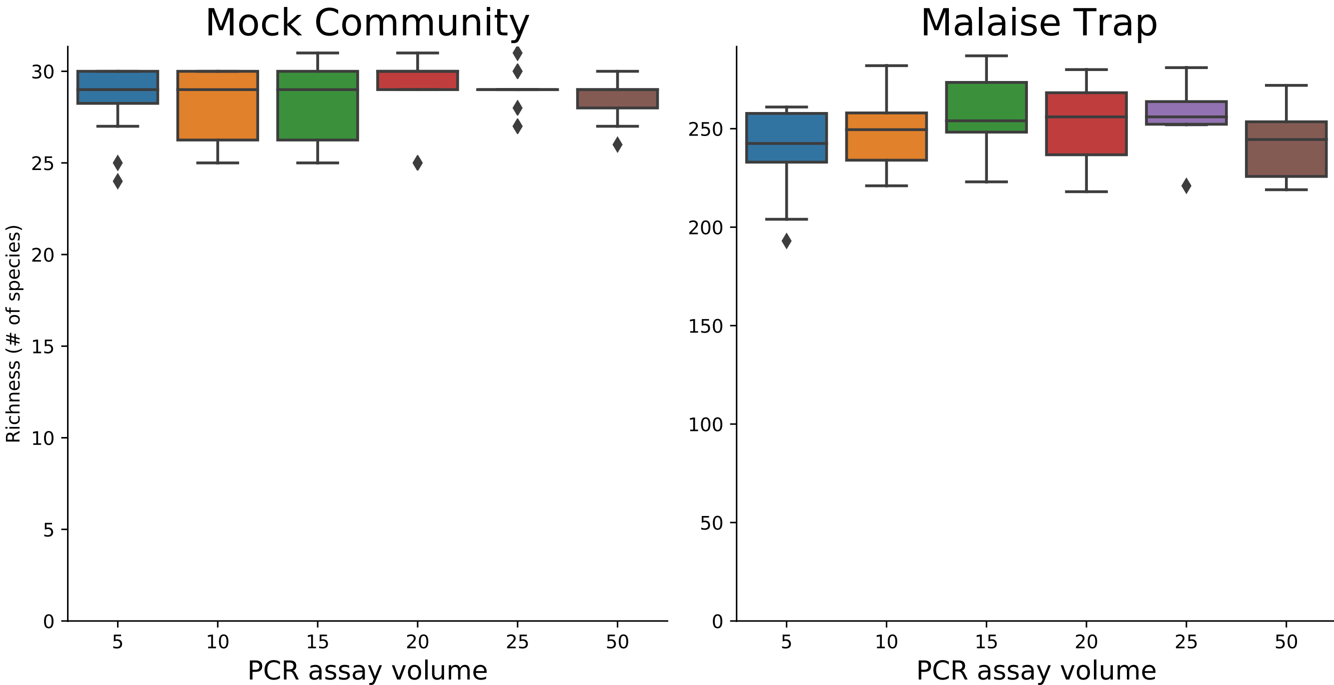

FIGURE 2 Species richness per PCR assay volume of mock community (left) and Malaise trap samples (right). The box indicates the 25th–75th percentile, with the whiskers showing 1.5 times the interquartile range. The middle line shows the median of the data

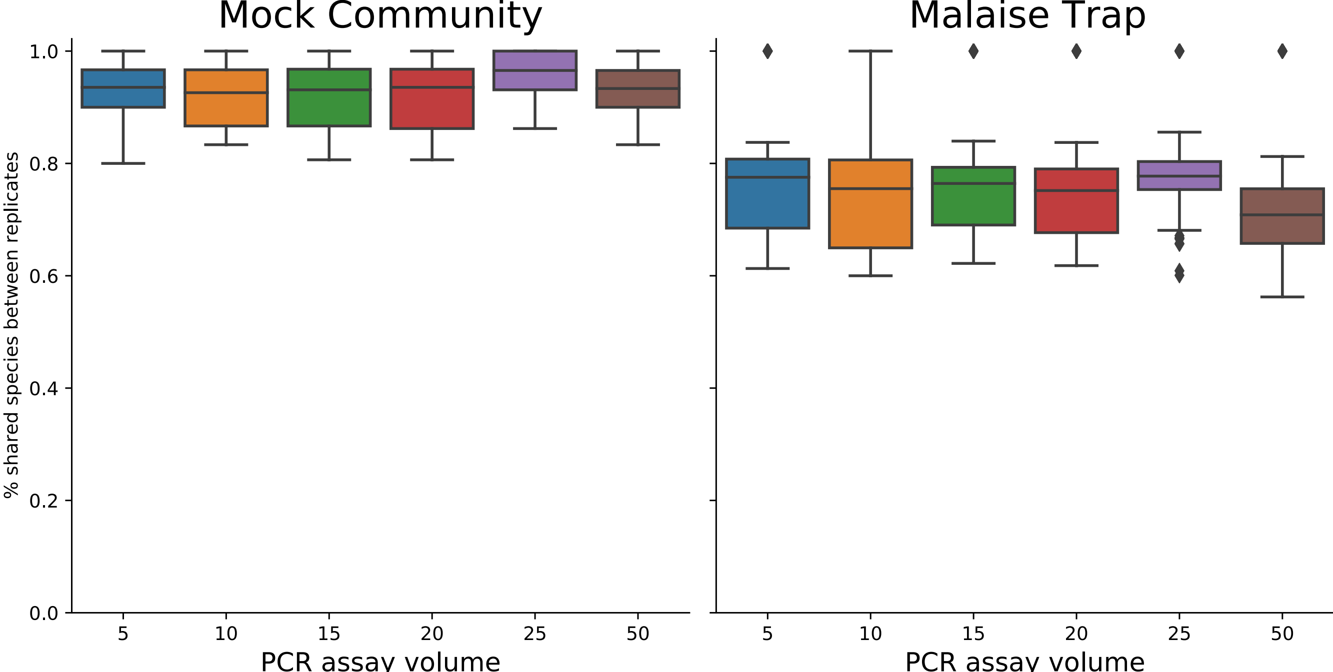

FIGURE 3 Proportion of shared species between replicates of mock community (left) and Malaise trap samples (right). The box indicates the 25th–75th percentile, with the whiskers showing 1.5 times the interquartile range. The middle line shows the median of the data

p =0.037, ρ = 0.089; Figure 3, Figures S7, S8; Table S5) and Malaise trap samples (p <0.001, ρ = −0.145).

As a quantitative measure for the two different sample types, mock community samples were checked for detection success of mock community species and Malaise trap samples were spiked with a known number of mitochondrial copies of four different species not expected to be present in a Malaise trap. Of all 35 mock community species, 23 species were detected in all replicates of all assay volumes, while four species were not detected a single time and the detection success of the remaining eight species averaged 70% (27%–99%) across all tested assay volumes (Table S6). Results of the spike-in controls indicate that sequencing depth was too low to recover rare templates (i.e., rare or small species). We did not find any reads of *Gammarus pulex* or *Ephemera danica* in the samples (50 and 5,000 mitochondrial copies per PCR replicate and assay volume, respectively). For *Gammarus fossarum* (500 mitochondrial copies per PCR replicate and assay), we found on average 5.5 reads (range: 2 to 11) per replicate and assay, and a detection rate between 0.21 (15 and 25 µl) and 0.43 (10 µl), and for *Oecismus monedula* (50,000 copies per replicate and assay) between 13 (93%) and 14 (100%) with an average of 131.7 reads (range: 7 to 310) per replicate and assay (Table S7). In both cases, species or spike-in detection rate was not correlated with the assay volume (Spearman's ρ, p =0.67/0.92, ρ = 0.03/−0.02; Table S5).

4 | DISCUSSION

In this DNA metabarcoding study, we tested if species detection or richness from arthropod bulk sample DNA (5 ng total DNA) is positively correlated with the used PCR reaction volume for a short amplicon (205 bp) and the Qiagen Multiplex PCR Plus Kit in 0.2 ml standard reaction tubes with 100 nmol/L primer concentration. All samples were successfully amplified at first attempt regardless of the volume used. Further, we found that reaction volume is uncorrelated with species or spike-in detection rate or species richness regardless of sample type. This confirms findings made for 16S bacterial communities (Minich et al., 2018), where OTU richness was also similar between 2.5, 5, and 25 µl reactions. When comparing the two different sample types used, we found the overlap among the 14 PCR replicates to be higher in the less complex mock community sample than in the Malaise trap sample, but also here, consistency between replicates did not increase with reaction volume, but was similarly high for all volumes. To obtain a similarly high consistency among replicates for the extremely species-rich Malaise trap sample, a greater sequencing depth would be needed, or pooling would need to be adjusted to better reflect the expected species diversity in the different sample types.

For 50 µl volumes of both sample types, we obtained a significantly lower number of reads after filtering, while for the mock community sample read numbers were also significantly lower for the 5 µl reaction. The lower sequencing depth for 50 µl reactions was probably a result of the used laboratory workflow. Because unspecific amplification (e.g., primer dimer) was generally low, we decided to directly measure concentrations with Qubit Fluorometer and pooled equimolarly depending on the concentrations measured. However, for the 50 µl reactions, a higher primer:template ratio was used (as input template amount was standardized), which likely led to a systematically higher unspecific amplification, overestimating the target template concentration when using Qubit for quantification. Therefore, probably less target template was

pooled in comparison to the other reaction volumes. For some 5 μl reactions, measured concentrations were slightly lower than needed for equimolar pooling, which might have influenced sequencing depth here for the mock community samples. In both cases, the issue could be overcome by using any clean-up method prior to pooling or using a quantification method that accounts for amplicon size (e.g., Bioanalyzer, Fragment Analyzer). However, the difference in read numbers is not directly correlated with the reaction volume.

For the mock community, sufficient sequencing depth can easily be tested by analyzing the detection rates for the different species. This is not possible for samples with unknown species composition. However, these samples can either be controlled by checking replicate consistency or detection rate of spike-ins with known DNA template copy numbers. While the first method only allows distinguishing between sufficient or insufficient sequencing depth, the latter additionally allows estimating a threshold for the amount of input DNA needed for species detection. This method is already applied in PCR-free study designs (Chen et al. 2016, Yu et al. 2020), and we here tried to adapt it to our metabarcoding workflow. Even though this method had worked in principle, primer bias has to be better considered when choosing species as we could detect the 500 copy spike-in in some replicates, but not the 5.000 copy spike-in probably due to primer bias. This issue could be overcome, for example, by using a synthetic DNA template (Lutzmayer et al., 2017) that is not affected by primer bias.

We conclude that the outcome of metabarcoding studies, especially for arthropod bulk samples with little inhibition, is not systematically influenced by the PCR volume used. Thus, studies using different PCR volumes should lead to comparable results. Even though all PCRs amplified successfully at first attempt, inhibition might be a concern when scaling down PCR assay volumes as a result of either high template or inhibitor concentration introduced by different sample types (e.g., soil or litter samples, Schrader et al. 2012). While both issues can be solved by dilution of template DNA, it might be beneficial to increase PCR volume for such samples with high inhibitor concentrations, or to use inhibitor removal protocols prior to PCR (Schrader et al. 2012, Hu et al. 2015).

While further exploration is needed for individual sample types and the practicability in laboratory handling has to be considered, our results encourage the use of small PCR volumes in metabarcoding studies. The cost savings up to an order of magnitude open up possibilities to increase the number of biological replicates.

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CONFLICT OF INTEREST

The authors have no competing interests.

AUTHOR CONTRIBUTION

Dominik Buchner: Conceptualization (equal); Formal analysis (lead); Writing-original draft (equal); Writing-review & editing (equal). **Florian Leese:** Conceptualization (equal); Project administration (equal); Writing-original draft (equal); Writing-review & editing (equal). **Arne Beermann:** Conceptualization (equal); Writing-original draft (equal); Writing-review & editing (equal). **Martina Weiss:** Conceptualization (equal); Project administration (equal); Writing-original draft (equal); Writing-review & editing (equal).

DATA AVAILABILITY STATEMENT

Demultiplexed raw read data for this publication has been uploaded to Zenodo.org and can be accessed via 10.5281/zenodo.4769118.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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