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Homogenization of insect bulk samples provides more comprehensive yet comparable biodiversity data than non-destructive lysis

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Abstract

The rapid advancement of molecular biodiversity monitoring tools, particularly DNA metabarcoding, has improved specimen identification in bulk samples, such as those from Malaise traps, where traditional morphological identification is impractical. While not being standardized yet, a typical first step in insect bulk sample analysis is the extraction of DNA from homogenized specimens. While this step yields reliable metabarcoding results, it destroys the specimens, preventing further use in monitoring and taxonomic analysis. Non-destructive lysis, which preserves specimen integrity, is still being evaluated for its effectiveness in accurately assessing bulk sample biodiversity. In this study, we assessed the suitability of non-destructive lysis for Malaise trap samples and compared its performance with homogenization using an established metabarcoding workflow. Five bulk samples were collected with Malaise traps. Samples were first incubated in a lysis buffer with Proteinase K (non-destructive lysis) and then homogenized. DNA was extracted from both treatments and metabarcoded to compare OTU richness, accumulation, and beta diversity. On average, homogenized samples yielded 3.8% more OTUs than non-destructive lysis samples. Although homogenization provides a more comprehensive and cost-effective assessment of Malaise trap bulk samples, non-destructive lysis still recovered at least 80% of the OTUs identified through homogenization and revealed similar patterns of community change. While our results show that both methods yield comparable data on insect biodiversity and thus can both be used for monitoring we consider non-destructive lysis as not suitable for integration into automated workflows or large-scale biomonitoring due to the much higher costs. However, undoubtedly the method remains important in cases where morphological integrity must be preserved and additional sampling is not possible.

Introduction

In the last two decades, tools for molecular biodiversity monitoring have rapidly evolved. In particular, DNA metabarcoding has facilitated and accelerated the identification of specimens in bulk samples, also known as “biodiversity soup” (Yu et al. 2012). This acceleration is essential since for samples often contain thousands of specimens and it is therefore impossible to identify specimens within the time frame required for bioassessments and biodiversity monitoring. This limitation of morphological identification becomes prevalent especially for specimen-rich insect samples, e.g. those obtained with Malaise traps that can collect over ten thousand specimens within a single week (Geiger et al. 2016, Karlsson et al. 2020, Lehmann et al. 2021, Remmel et al. 2023). Thus, DNA metabarcoding is increasingly used as a tool to assess species diversity in Malaise trap samples (Steinke et al. 2022, Zizka et al. 2022).

A reliable source material for metabarcoding is DNA extracted from completely homogenized insect bulk samples (Hausmann et al. 2020, Buchner et al. 2023). However, the destruction of specimens makes it impossible to retain vouchers and is therefore not applicable in cases where specimens need to be retained, e.g. for quality control in regulatory monitoring, subsequent phenotypic analysis, or species descriptions. Therefore, two different metabarcoding approaches retaining the morphological integrity of specimens have been proposed: i) analysis of the sample community via the storage ethanol (Erdozain et al. 2019, Kirse et al. 2022) and ii) non-destructive lysis of the bulk samples, during which the samples are incubated in a lysis buffer to obtain insect DNA without homogenization (Batovska 2021, Marquina et al. 2022, Iwaszkiewicz-Eggebrecht et al. 2023). The analysis of preservative ethanol has not proven to be a reliable tool for biodiversity assessment in terrestrial arthropod bulk samples (Marquina et al. 2019, Chimeno et al. 2023), while the non-destructive lysis of insect samples has shown potential to adequately reflect their biodiversity (Carew et al. 2018, Kirse et al. 2022).

Many of these validation studies are based on artificially composed mock communities (Batovska 2021, Marquina et al. 2022, Martoni 2022), which contain fewer species and specimens than a typical Malaise trap sample, making direct transfer of the results questionable. In a study that used the large diversity of Malaise trap samples (Kirse et al. 2022) the treatments of homogenized samples and those treated with non-destructive lysis were not identical, as they differed in lysate volumes and extraction methods, which thus limits direct comparisons of the different methods. However, for method comparison and validation, the treatments should ideally be equal for both methods varying only test parameters - in this case, the input material of either homogenate or lysis buffer obtained via non-destructive lysis. Consequently, there is a clear need to directly compare the performance of bulk sample metabarcoding with specimen-rich

samples using non-destructive lysis and homogenized bulk material to develop method standardization recommendations for insect biodiversity monitoring.

Therefore, this study aimed to (i) evaluate the suitability of a non-destructive lysis approach applied to Malaise trap samples as well as (ii) to compare the performance of non-destructive lysis and homogenization in identical treatment with a metabarcoding workflow that is already well established and scalable.

We hypothesize that homogenized bulk samples yield higher diversity since DNA will be more accessible after homogenization compared to non-destructive lysis.

Material and methods

Sample collection

Five insect bulk samples were collected using Malaise traps of the LTER type (Soltwedel 2022) from May to June 2022 near the River Boye in Bottrop, Germany. Samples were collected weekly, with insects initially caught in 70% ethanol and subsequently preserved in 96% ethanol. For use in a separate study, all specimens of the family Syrphidae (Diptera) and the orders Ephemeroptera, Plecoptera, and Trichoptera (EPT) were removed from the sample before analysis in this study.

Laboratory processing

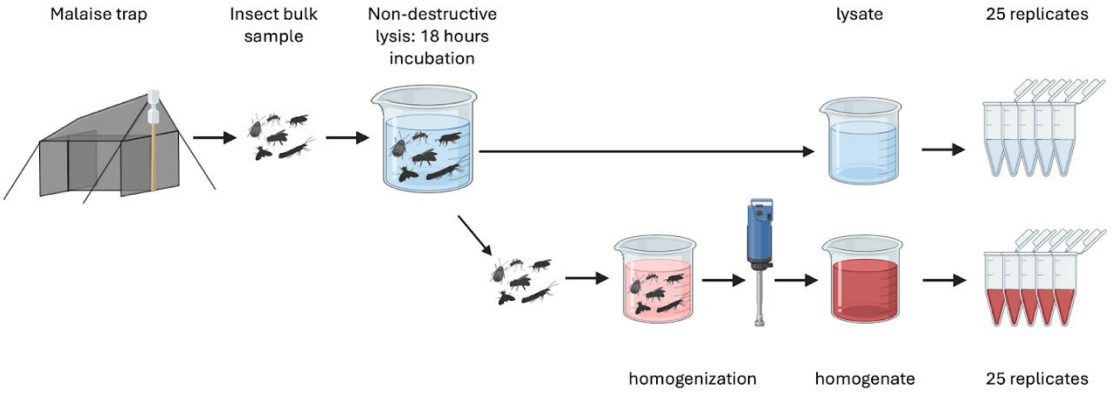

Figure 1: *Laboratory processing of the insect bulk samples. Samples were incubated in lysis buffer for 18 hours. From the lysis buffer 25 replicates were taken for non-destructive lysis processing (top, blue). In parallel, the incubated specimens were homogenized and 25 replicates were taken from the homogenate (bottom row, red).*

For the laboratory workflow (Figure 1), the fixative alcohol was removed from the samples by draining over a sieve (250 µM mesh size) until no more drops leaked from the inverted sample. The samples were

then weighed and subsequently, TNES buffer (Buchner 2022d) was added to the samples until all insects were submerged (140-180 mL, Table S1). For each mL of TNES buffer, 100 µL of Proteinase K stock solution (10 mg/mL) was added to each sample.

The samples were then incubated at 56° C for 18 hours. Afterward, the lysis buffer was drained from the samples with a 250 µM sieve and stored for subsequent analyses. The remaining bulk samples were filled up with 96% ethanol. After short mixing through inversion, the ethanol was immediately exchanged with fresh 96% ethanol to ensure the complete removal of TNES buffer. To ensure equal conditions among the treatments, an identical volume of ethanol was used for each sample as the volume of TNES buffer previously used in the non-destructive lysis. The samples were then homogenized wet with a tissue tearer (BioSpec Products, Bartlesville, USEA) for 2 minutes. The tissue tearer was cleaned in 3% bleach in between samples under a fume hood (Figure 1) (Buchner et al. 2021a).

From the TNES buffer removed from each sample, 25 subsamples with a volume of 1 mL each were transferred into 2 mL microcentrifuge tubes. All subsequent steps were performed on an automated liquid handling robot (Hamilton Vantage, Hamilton Bonaduz AG, Switzerland). Identical to the non-destructive lysis procedure, 25 subsamples of 1 mL homogenate were taken from each of the five bulk samples. The tissue was pelleted via centrifugation at 4.000 x g for 3 minutes. The remaining ethanol was removed and lysis was conducted through bead-beating in 1 mL lysis buffer with the same concentration as in the non-destructive lysis protocol (Buchner 2022d). The lysis of homogenate was performed at 56 °C for 20 min at 1400 rpm. For all samples, regardless of treatment, remaining debris in the samples was pelleted via centrifugation at 4.000 x g for 3 minutes. The samples were then transferred into 96-well plates in 2 technical replicates with 300 µL of lysate each according to the workflow proposed in (Buchner et al. 2021b) with 12 negative controls per plate.

DNA was extracted with a silica spin column-based protocol (Buchner 2022a) via a vacuum manifold. Extraction success was checked on a 1% agarose gel. Samples were amplified in a first PCR with the Qiagen Multiplex Plus Kit (Qiagen, Hilden, Germany) with a final concentration of 1x Multiplex Mastermix, 200 nM of each primer (fwh2F, fwhR2n, Vamos et al. (2017)) filled up to a total volume of 10 uL with PCR-grade water. Cycling conditions were as follows: Initial denaturation for 5 min at 95° C, 20 cycles of denaturation (95° C, 0:30 min) annealing (58° C, 1:30 min) and extension (72° C, 0:30 min) finished with a final elongation step at 68° C for 10:00 min. Each of the PCR plates used in the first PCR was tagged with a unique combination of inline tags. Additionally, the primer contained a universal binding site for the primer used in the second PCR step to anneal (Table S2). A bead-based cleanup was conducted in between the two PCR steps (Buchner 2022b). For the second PCR samples were amplified with a final concentration of 1x Multiplex Mastermix, 1x CoralLoad loading dye and 100 µM of each primer. Each of the wells was

individually tagged with Illumina indices with the following cycling conditions: Initial denaturation for 5:00 min at 95° C, 25 cycles of denaturation (95° C, 0:30 min) annealing (61° C, 1:30 min) and extension (72° C, 0:30 min) and a final elongation at 68° C for 10:00 min. PCR success was checked on a 1% agarose gel. PCR products were normalized with a bead-based protocol (Buchner 2022c). To remove overamplified products and remaining primer dimers, a reconditioning PCR (Buchner 2024) was performed. Finally, library quality was checked with a Fragment analyzer (High sensitivity NGS Fragment Analysis Kit; Advanced analytical, Ankeny, USA).

Sequencing was performed on an Illumina NovaSeq 6000 instrument with 300 cycles (paired-end 2x150 bp) at GENEWIZ, Leipzig, Germany.

Bioinformatic analysis

Raw data of the sequencing runs were delivered demultiplexed by index read. Additional demultiplexing of the inline tags was achieved with the Python package “demultiplexer” (v1.2.0) Sequencing data were then processed with the APSCALE pipeline (Buchner et al. 2022), including paired-end merging, primer trimming, quality filtering, dereplication, OTU clustering and lulu filtering according to default settings. Sequences were clustered into operational taxonomic units (OTUs) based on a 97% similarity threshold. The subsequent taxonomic assignment was performed with the Barcode of Life database via BOLDigger (Ratnasingham and Hebert 2007, Buchner and Leese 2020). The analyses of richness and similarity were based on the identified OTUs, irrespective of their species matches in the Barcode of life Database. As an additional filtering step, only reads detected in both technical replicates were accepted as true signals and these reads were subsequently summed, leading to a final number of 25 replicates per sample used in the subsequent analysis. Reads were rarefied per sample to achieve equal sequencing depth for all replicates of a sample in the final OTU table (Table S3). The effect of the treatment (non-destructive lysis or homogenization) on OTU richness was estimated with a linear model in Python with the statsmodels module. Richness per treatment boxplots were created in Python seaborn and richness differences per sample were estimated using a Mann-Whitney-U test. An OTU accumulation curve was created with a custom Python script (Script S1) to simulate subsequent addition of replicates and the resulting increase in OTU richness per added replicate. For this purpose, the respective combination replicates was drawn from the pool of all possible combinations 1000 times at maximum and all possible combinations were drawn in low replicate numbers in which the maximum of possible combinations was below 1000. For a principal component analysis (PCA), Jaccard beta diversity distances were calculated as a distance measure between samples. The full Python code for this analysis is provided in Script S1.

Results

After the 18-hour non-destructive lysis treatment in TNES and Proteinase K, the specimens were already very soft and their suitability for individual handling for morphological determination was extremely limited.

The non-destructive lysis treatment recovered significantly fewer OTUs in four out of five samples (Mann-Whitney-U test, $p<0.001$ in all samples, Fig. 2 a-e). In comparison, 3.8 % more OTUs were recovered through homogenization than by non-destructive lysis of the same samples.

The analysis of the OTU accumulation curve with increased replication showed that in three out of five samples the number of detected OTUs was higher in homogenate samples even with a low number of replicates. Furthermore, additional replicates did not increase the richness recovered from non-destructive lysis samples to a level comparable to that of the homogenate samples.

The ordination analysis based on Jaccard diversity showed that both methods recovered systemically different communities separated through axis 1 and that variation between replicates among technical replicates (axis 2) was very low (Fig. 2 k-o). Additionally, in three samples, the homogenate showed higher within-treatment similarity than the non-destructive lysis samples, i.e. a higher variability in communities recovered from identical samples by non-destructive lysis.

The number of OTUs exclusively detected in homogenized samples was on average 75% higher compared to those detected in non-destructive lysis samples (Fig. 2 p-t). Still, the majority of OTUs were detected in both treatments, i.e. 80-84% of shared OTUs across all samples (Fig. 2 p-t).

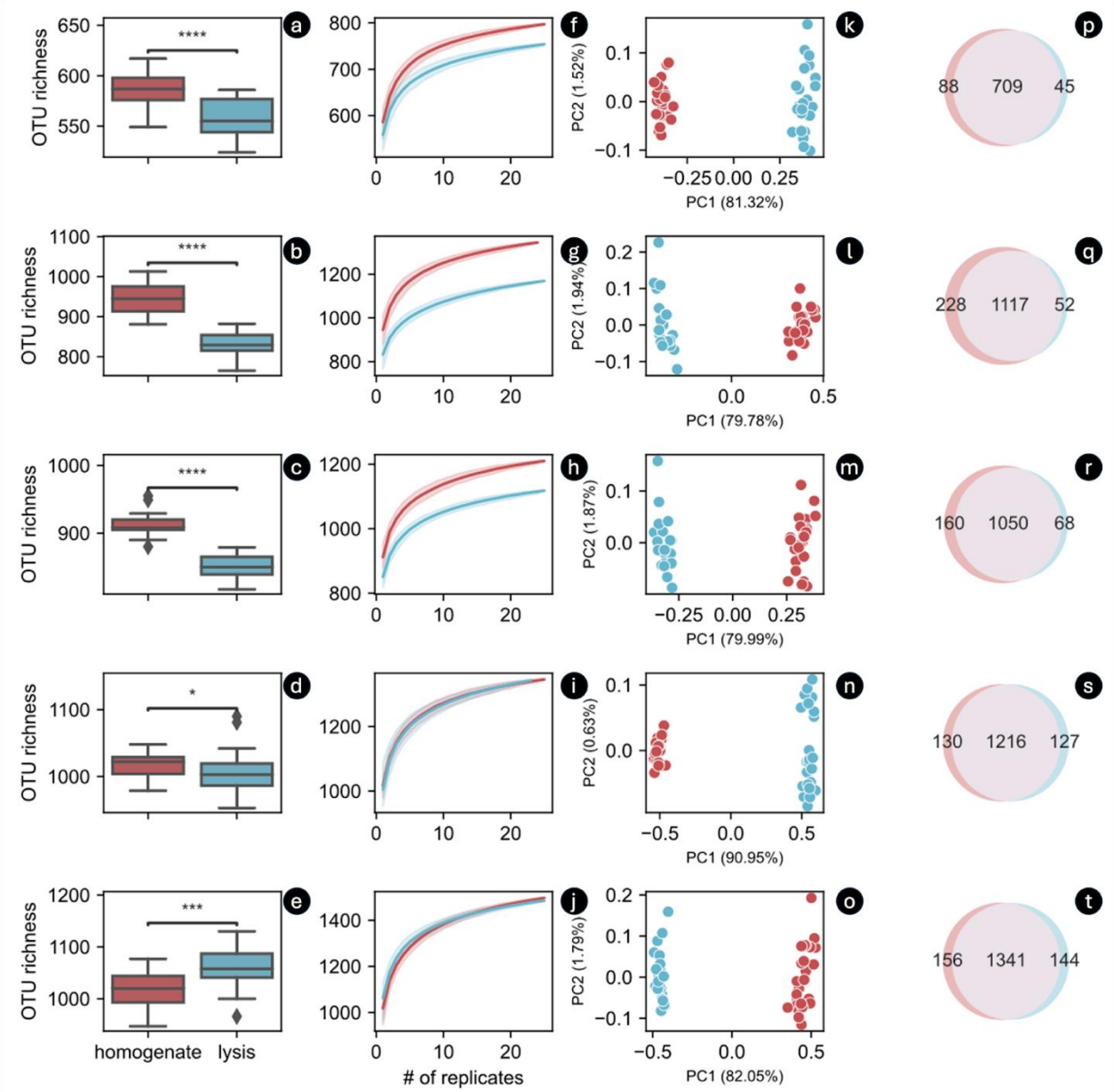

Figure 2: *Comparison of different metrics for Malaise traps analyzed based on homogenized bulk samples (red) and via non-destructive lysis (blue).* ***a-e*** *OTU richness per sample, asterisks indicate significant differences between methods (Mann-Whitney-U test, p-value < 0.05 = *, p-value < 0.01 = **, p-value < 0.001 = ***, p-value < 0.0001 = ****).* ***f-j*** *OTU accumulation curve based on the 25 technical replicates per sample and method. The curve shows the median value of all possible or max. 1000 random replicate draws for homogenate (red) and non-destructive lysis samples (blue).* ***k-o*** *PCA plots indicating community dissimilarity (Jaccard distance) of the 25 replicates per method.* ***p-t*** *Venn diagrams indicating OTU overlap as well as OTUs exclusively detected by each method.*

A comparison of the community similarity across all samples collected in 5 consecutive weeks in the same area reveals that both methods detect similar patterns in beta diversity irrespective of the treatment (Fig. 3).

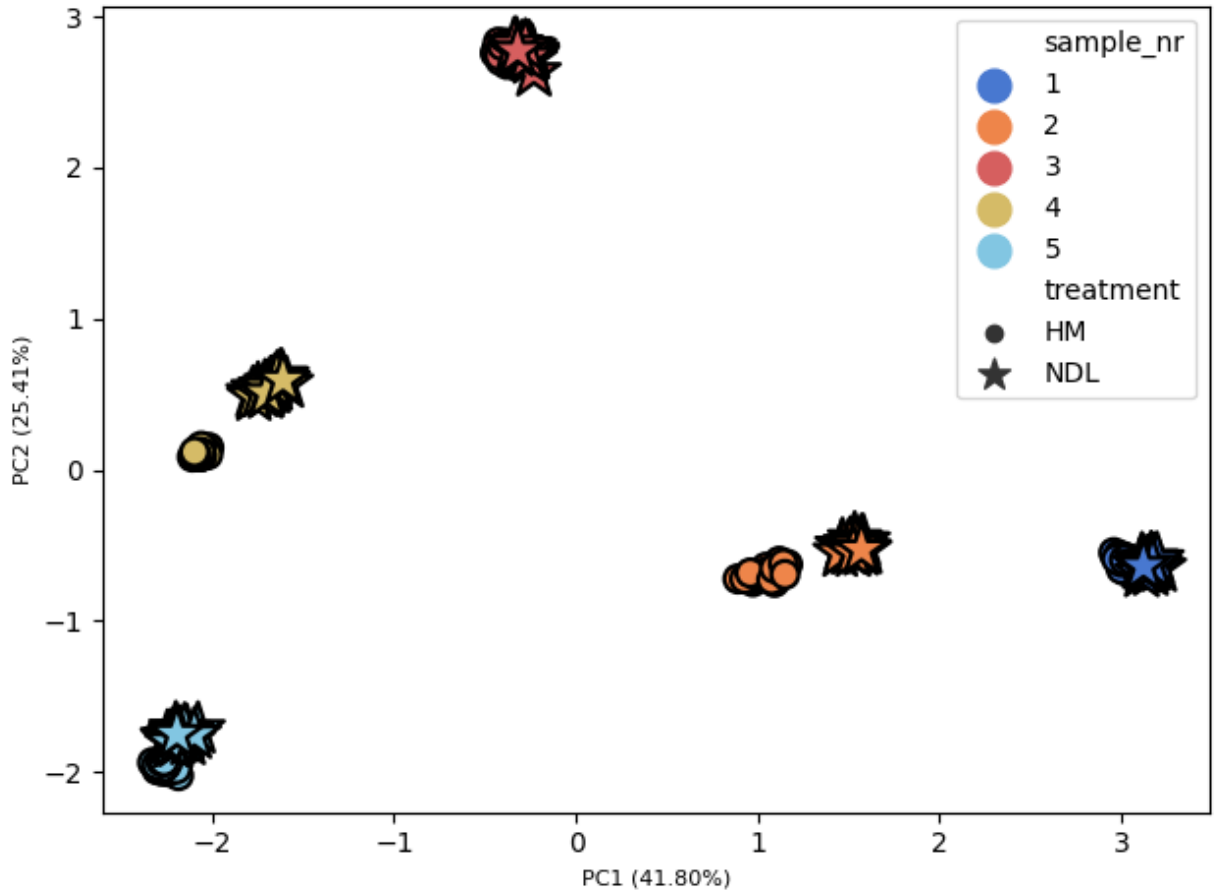

Figure 3: *PCA plot indicating community dissimilarity (Jaccard distance) based on all five Malaise trap samples (different colors), the two different methods (circle = homogenate, HM, asterisk = non-destructive lysis, NDL) and the 25 individual replicates per sample and method.*

Discussion

Our study revealed that in most cases metabarcoding of arthropod samples from non-destructive lysis detected a smaller fraction of the community compared to homogenized samples of the same communities. This was true for the recovered OTU richness as well as for the number of OTUs exclusively detected by the two methods. Homogenization accesses DNA from strongly sclerotized or small specimens that may not release significant amounts of DNA into the lysis buffer during non-destructive lysis (Zizka et al. 2019, Martins et al. 2021, Martoni 2022). In addition, the number of cells in contact with the lysis buffer is higher in homogenate compared to non-destructive lysis, logically resulting in a higher DNA yield when treated with equal protocols. This likely results in lower OTU recovery with non-destructive lysis due to fewer DNA molecules obtained compared to those extracted from homogenized tissue. Other studies (Kirse et al. 2022) found a lower yield of DNA in homogenate compared to non-destructive lysis samples.

However, since different DNA extraction methods and lysate volumes were used in the study, the comparability of the two methods is limited due to these methodological shortcomings. This presents a concern for formal method validation and standardization. The results of our study indicate that the amount of DNA is higher in homogenate than in non-destructive lysis samples since they recover a more extensive OTU community. Furthermore, a lower concentration of DNA molecules resulting from non-destructive lysis may also lead to a dilution effect that decreases the likelihood of detection, especially for OTUs close to the detection limit of the method. Earlier studies on the effect of lysate volume (Nielsen et al. 2019) did not reveal differences in OTU recovery with a fivefold increase in lysate volume, this study however only used samples with a low number of specimen with the largest sample being 10 mL. Additionally, this study was based on phenol-chloroform extraction, which is much more suitable for small amounts of DNA and, therefore, more likely to avoid a dilution effect. In addition, we assume that in non-destructive lysis, a certain amount of DNA is leached from the sample that would otherwise be available in the homogenate. Therefore, we expect that homogenization alone recovers an even more extensive community than when performed after non-destructive lysis as done in this study. However, this expectation cannot be validated in this study, but must be considered, especially in experimental setups where DNA degradation is a concern. Furthermore, in non-destructive lysis a certain number of small specimens fully disintegrated, leaving their recovery solely possible in non-destructive lysis but not in homogenization.

Even though non-destructive lysis avoids homogenization, the morphological integrity of the samples was strongly degraded after 18 hours of incubation. For this study, a long incubation time was chosen for maximum DNA yield and consequently the highest possible recovery of OTUs (Marquina et al. 2022). However, in applications targeted at maintaining morphological integrity, shorter incubation times may be advisable despite the lower expected DNA yield. This, however, may decrease the number of detected species and thus a trade-off remains. An alternative could be to replicate sampling or split samples and use one half with a protocol optimized for preservation for morphological inspection and use the other half with a protocol optimized for DNA extraction. This however leads to the effect that all species with only a single individual cannot be found in both fractions, reducing the recovered richness.

The direct comparison of both methods reveals similar ecological patterns, which is encouraging as it allows for comparability. However, non-destructive lysis has several important downsides to consider: The higher variability between communities recovered with non-destructive lysis as well as the relatively lower richness recovered indicate that study designs relying on non-destructive lysis should consider a higher number of replicates or the extraction of a larger lysate volume to achieve a comparable species recovery. Ideally, all of the lysate would be extracted to maximize species recovery. This however makes automated

sample analysis very complicated or even impossible. Additionally, the high amounts of Proteinase K necessary for non-destructive lysis make this protocol rather expensive with a cost of approximately 40-60 € per sample depending on the sample biomass solely for the lysis of samples. This is already as expensive as the entire sample analysis in our regular workflow (Buchner et al. 2024). Lower concentrations of Proteinase K have also been applied (Porco et al. 2010), but this is expected to reduce the effectiveness of the non-destructive lysis and deviates from recommended working concentrations for commercially available kits. Furthermore, non-destructive lysis requires more manual labor and much longer handling times compared to homogenization. Therefore, we conclude that non-destructive lysis is not suitable for integration into (automated) standardized monitoring protocols soon. Moreover, the benefits of non-destructive lysis for biodiversity monitoring and integrative taxonomy are not obvious: DNA metabarcoding is most useful in cases where morphological identification is impractical, i.e., for analysis of large numbers of hard to identify specimens. But due to the inability to link the species / OTU list generated via metabarcoding to individual specimens, this key problem persists after mild lysis. Even if species / OTUs of interest are detected in the final lists, it will be very difficult and time-consuming to screen the samples for them, and often the morphology may also suffer from the treatment. However, in specific applications where preserving specimens' morphological integrity is crucial and the budget is sufficient, non-destructive lysis can still be of high value. In conclusion, homogenization offers a more comprehensive and cost-effective assessment of Malaise trap insect communities compared to non-destructive lysis. Despite the higher costs, non-destructive lysis recovers at least 80% of the species found with homogenization, indicating that both methods can be intercalibrated and used in standardized biodiversity assessments depending on the specific objectives of a study. While non-destructive lysis has several limitations, including higher costs, greater manual labor, and longer handling times, making it less suitable for integration into automated standardized monitoring protocols, it can be invaluable for applications where preserving the morphological integrity of samples is crucial.

Data accessibility statement

Demultiplexed raw read data for this publication will be uploaded to the ENA under the accession number PRJEB76617.

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Author contributions

LW, DB, and FL drafted the study design. JE conducted the sampling in the field as well as the sample sorting. DB and LW conducted the laboratory processing of the sampling and the bioinformatic analysis of the data. LW wrote the first draft of the manuscript, which was edited by all coauthors.

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