Environmental DNA time series analysis of a temperate stream reveals distinct seasonal community and functional shifts but no influence of within-stream sampling position

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Abstract

Environmental DNA (eDNA) extracted from water is routinely used in river biodiversity research, and via metabarcoding eDNA can provide comprehensive taxa lists with little effort and cost. However, eDNA-based species detection in streams and rivers may be influenced by sampling season, location, and other key factors such as water temperature and discharge. Research linking these factors and also informing on the potential of eDNA metabarcoding to detect shifts in ecological signatures, such as species phenology and functional feeding groups across seasons, is missing. To address this gap, we collected 102 water samples every two weeks for 15 months at a long-term ecological research (LTER) site and at three different positions in the river's cross section, specifically the water surface, riverbed, and riverbank. We analyzed macroinvertebrate species and molecular Operational Taxonomic Unit (OTU) richness and temporal community turnover across seasons and sampling positions based on COI metabarcoding data. Using Generalized Additive Models, we found a significant influence of sampling season but not sampling position on community composition. Community turnover followed a cyclic pattern, reflecting the continuous change of the macroinvertebrate community throughout the year ('seasonal clock'). Although water temperature had no influence on the inferred community composition, higher discharge reduced the number of Annelida and Ephemeroptera species detectable with eDNA. Most macroinvertebrate taxa showed the highest detection rates in spring, in particular merolimnic species with univoltine life cycles. Further, we detected an increase in proportion of shredders in winter and of parasites in summer. Our results show the usefulness of highly resolved eDNA metabarcoding time series data for ecological research and biodiversity monitoring in streams and rivers.

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Running title

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Abstract

Environmental DNA (eDNA) extracted from water is routinely used in river biodiversity research, and via metabarcoding eDNA can provide comprehensive taxa lists with little effort and cost. However, eDNA-based species detection in streams and rivers may be influenced by sampling season, location, and other key factors such as water temperature and discharge. Research linking these factors and also informing on the potential of eDNA metabarcoding to detect shifts in ecological signatures, such as species phenology and functional feeding groups across seasons, is missing. To address this gap, we collected 102 water samples every two weeks for 15 months at a long-term ecological research (LTER) site and at three different positions in the river's cross section, specifically the water surface, riverbed, and riverbank. We analyzed macroinvertebrate species and molecular Operational Taxonomic Unit (OTU) richness and temporal community turnover across seasons and sampling positions based on COI metabarcoding data. Using Generalized Additive Models, we found a significant influence of sampling season but not sampling position on community composition. Community turnover followed a cyclic pattern, reflecting the continuous change of the macroinvertebrate community throughout the year ('seasonal clock'). Although water temperature had no influence on the inferred community composition, higher discharge reduced the number of Annelida and Ephemeroptera species detectable with eDNA. Most macroinvertebrate taxa showed the highest detection rates in spring, in particular merolimnic species with univoltine life cycles. Further, we detected an increase in proportion of shredders in winter and of parasites in summer. Our results show the usefulness of highly resolved eDNA metabarcoding time series data for ecological research and biodiversity monitoring in streams and rivers.

Keywords

eDNA, time series, bioassessment, bioindication, insects, eLTER, metabarcoding

1. Introduction

Biodiversity in streams and rivers is being impacted by multiple anthropogenic stressors (Jackson et al., 2016). To understand these impacts, their functional consequences, and management effectiveness taxonomically highly resolved information with high spatial and temporal resolution is important. However, such information is difficult to obtain through traditional morphological assessments as many invertebrate species are small or present only in juvenile stages that are difficult to identify. Molecular taxonomic approaches, in particular metabarcoding of environmental DNA (eDNA) collected from water, offer a fast and cost-effective way to assess biodiversity and are routinely used in aquatic bioassessments around the world (Deiner et al., 2017). eDNA metabarcoding is based on extracted DNA shed by organisms via sloughed cells, feces, gametes or other particles into the water and is thus a non-invasive method to assess community composition because assessment is based on water rather than organismal bulk samples (Taberlet et al., 2012; Valentini et al., 2016). DNA metabarcoding uses high-throughput sequencing methods to generate comprehensive taxa lists

(Brantschen et al., 2022; Leese, Sander et al., 2021). However, since the reference databases used to assign taxonomic names to the obtained sequences are still incomplete (Weigand et al., 2019), not all sequences can be assigned to species level. Therefore, molecular Operational Taxonomic Units (OTUs) that are generated according to genetic distance-based similarity thresholds can be used as surrogates for species. Using OTUs in addition to species can reveal further insights into ecological processes (e.g. Beermann et al., 2018).

Despite the obvious advantages, several factors hinder the direct interpretation of eDNA data (Barnes & Turner, 2016; Harrison et al., 2019). First, the possibility to detect a present lotic macroinvertebrate community can be strongly affected by the selected sampling position in the water. Similar to stratified aquatic environments where non-mixed layers need to be considered in the sampling design (Jeunen et al., 2019; Lawson et al., 2019), eDNA molecule distribution may also differ between different positions in lotic environments, such as the water surface versus the riverbed. Accordingly, small scale differences in sampling position both vertically and horizontally may recover different lotic communities (Berger et al., 2020; Macher & Leese, 2017; Thalinger et al., 2021), producing different perspectives of biodiversity change depending on where sampling is conducted. Alternatively, sampling position may have no effect given that eDNA can be transported over long distances of >12 km in streams (Deiner & Altermatt, 2014), which may homogenize eDNA community signals across sampling positions (Macher et al., 2021). Second, several abiotic factors can influence DNA transport and detectability and may thus distort the inferred community (Barnes & Turner, 2016; Harrison et al., 2019), such as discharge and water temperature. Discharge is an important factor influencing eDNA detectability in streams because high discharge could lead to more species being detected by eDNA signals from transported DNA or whole organisms being swept downstream (Fremier et al., 2019; Shogren et al., 2017; Carraro et al., 2018). In contrast, high discharge can also dilute the eDNA signal thus making it more difficult to detect all present species (e.g., Thalinger et al., 2021), which may particularly impede the detection of rare species that are already at low abundance. In addition to discharge, temperature also affects eDNA detectability (Strickler et al., 2015), either negatively if higher temperatures reduce eDNA persistence due to increased enzymatic activity or positively if higher temperatures increase DNA shedding rates (Jo et al., 2019; Kasai et al., 2020; Strickler et al., 2015). These potentially contrasting effects of discharge and temperature make it difficult to predict how they will affect estimates of community composition .

As a consequence of the phenology of organisms, eDNA detectability in streams might also be influenced by sampling season. Depending on the stream type and region, characteristic abundance patterns can be found for different macroinvertebrate orders, genera and species throughout the year and across years (Cowell et al., 2004, Wagner et al., 2011; Wagner, 2004). In addition, the biology of the different macroinvertebrate taxa has a strong effect on seasonal community composition. One important factor are differences in organismal life cycles. While hololimnic species (species with a fully aquatic life cycle) are presumed to be present in the water the whole year, merolimnic species (species with aquatic larvae and aerial adults) leave the water after hatching and have distinct emergence periods, often lasting up to few months, which can lead to a sudden decline in sampled benthic communities (Fureder et al., 2005; Jackson & Fureder, 2006). Besides life cycle based community composition changes, streams also differ systematically with respect to their functional feeding groups (FFG) in both space and time (Vannote al., 1980). For example, shredders are typically more abundant in autumn, when the amount of allochthonous material in streams is highest (Cummins et al., 1989) and grazers in spring and summer, due to sun exposure supporting the growth of large biofilms. The functional composition of macroinvertebrate communities in the form of different FFG affects ecosystem functioning and is therefore also included in bioassessment approaches (Šporka et al., 2006). Detecting these community dynamics patteres is important in aquatic ecology. There is ample evidence that these types of seasonal differences are reflected in eDNA metabarcoding data (Bista et al., 2017; Dunn et al., 2017; Zizka et al., 2020) and that season or even month of sampling lead to different biological assessment results with eDNA metabarcoding (Jensen et al., 2021; Zizka et al., 2020).

While biodiversity studies addressing larger spatial or temporal scales often suffer from an insufficient resolution (Jackson & Fuereder, 2006; Pilotto et al., 2020), studies using high resolution spatiotemporal data at smaller scales are still scarce. Especially for macroinvertebrates, eDNA metabarcoding data has the po-

tential to assess small temporal and spatial changes in community composition time- and cost-effectively to complement future long-term bioassessment of streams.

Using time series data from a Long-Term Ecological Research (LTER; Mirtl et al., 2018) site, the aim of this study was to test the effect of the sampling position (i.e., different positions in the river's cross section), discharge and temperature, and sampling season on stream macroinvertebrate community composition determined from eDNA. The time series comprises 102 total eDNA samples taken every two weeks for 15 months (from 24.05.2017 to 29.08.2018) at three sampling positions at the same location: (i) the river surface; (ii) the river bottom; and (iii) the river bank. Community composition was determined using high-throughput mitochondrial cytochrome c oxidase subunit I (COI) gene metabarcoding. We tested three hypotheses:

1. Change in community composition will be driven by seasonality but, because of turbulent flow and mixing, not or less the sampling position. Differences in seasons will follow a cyclic pattern throughout the year ('seasonal clock') irrespective of the sampling position.
2. Differences in community composition will reflect the diverging life cycles of different mero- and hololimnic taxa. For example, the species numbers of merolimnic taxa like Ephemeroptera, Plecoptera and Trichoptera (EPT) will decline in summer after emergence, while differences in species numbers will be less pronounced for hololimnic taxa like Annelida and Coleoptera.
3. eDNA metabarcoding will detect seasonal differences in community composition for different functional feeding groups (FFG). In particular, more grazer species will be present in spring and summer, whereas more shredder species will be found in autumn and winter based on seasonal food availability. For parasite species that are dependant on other organisms, overall species occurrence will not follow any seasonal pattern, as it is linked to the presence of different host taxa.

Moreover, we also tested the effects of discharge and water temperature on community composition, although we had no *a priori*expectations of what these effects would be.

2. Materials and methods

2.1 Sampling, filtration and extraction

Sampling, filtration and extraction methods are described in detail in Leese, Sander et al. 2021. In brief, we sampled 102 water samples from the Kinzig river every two weeks within a Long-Term Ecological Research (LTER; Mirtl et al., 2018) site at the Rhine-Main-Observatory [RMO,*https://deims.org/9f9ba137-342d-4813-ae58-a60911c3abc1*] from 24.05.2017 to 29.08.2018. We took three 1-L samples per sampling day at the surface in the middle of the stream, 10 cm above the stream bed in the middle of the stream, and at the riverbank (Fig. 1). After sampling, water samples were put on ice in the field and later stored in a freezer at -20 °C until further processing. Water samples were filtered using a vacuum pump and DNA captured on 0.45 µm Cellulose Nitrate membrane filters (Nalgene, diameter 47 mm). For each day and person who filtered, a negative filter control was included, where only the surrounding air was filtered to control for air and filter contamination. Filters were stored in 1.5 mL Eppendorf tubes filled with 96 % denatured ethanol. DNA extraction was performed following the salt-EtOH-precipitation protocol reported in Weiss & Leese (2016). Subsequent steps included RNA digestion using 1.5 µl RNase A (10 mg/ml, Thermo Fisher Scientific) per sample and clean-up of the samples using a Qiagen MinElute Kit to obtain high-quality DNA.

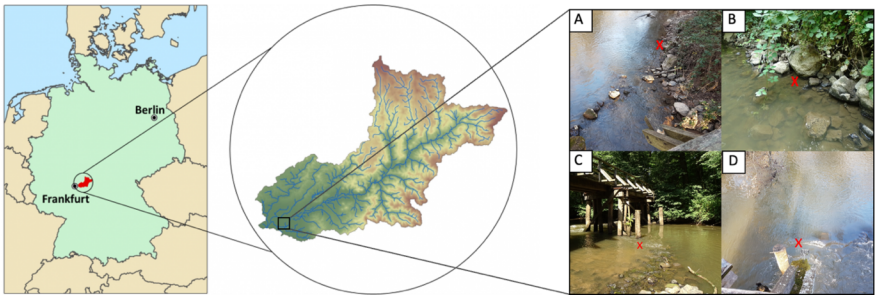

Figure 1: eDNA sampling location within the LTER site RMO in Hesse, Germany. Right: sampling location with the three sampling positions marked with a red cross. A: sampling position: riverbank in winter, B: zoomed in sampling position: riverbank in summer, C: sampling position: surface and riverbed in summer, D: zoomed in sampling position: surface and riverbed in winter.

2.2 DNA metabarcoding

A two-step PCR approach with two replicates per extract was applied (Zizka et al., 2019). For the first step, four length-varying primers for the primer pair fwhF2/EPTDr2n (Leese, Sander et al., 2021; Vamos et al., 2017) were used, with each of them having a universal tail attached. Length variation was due to inline shifts (0–3 Ns) between the universal tail and the primer sequence to maximize diversity between sequences (Elbrecht & Leese, 2015). For the second step, primers matching the universal tail with an i5/i7 index and P5/P7 Illumina adapter attached were used (Buchner et al., 2021a,b). We applied an improved PCR protocol compared to the previous study (Leese, Sander et al., 2021), PCR reactions for both PCR steps contained: 1x Multiplex PCR Master Mix (Qiagen Multiplex PCR Plus Kit), 0.1 µM of each primer, 0.5 x CoralLoad Dye, 1 µL DNA extract/ unpurified PCR product (1^{st} step/ 2^{nd} step) filled up with Rnase-free water to a total volume of 25 µL. Cycling conditions were as follows: first step PCRs consisted of 5 min initial denaturation at 95 °C, followed by a touchdown of 10 cycles of 30 s at 95 °C, 90 s at 64 °C (- 1 °C each cycle) and 30 s at 72 °C and additional 25 cycles of 30 s at 95 °C, 90 s at 54 °C and 30 s at 72 °C, followed by a final elongation for 10 min at 68 °C. Second step PCRs consisted of 5 min initial denaturation at 95 °C followed by 18 cycles of 30 s at 95 °C and 2 min at 72 °C, followed by a final elongation for 10 min at 68 °C. Eight PCR negative controls were added to check for contamination during PCR and tag switching. PCR products were quantified and equimolarly pooled to one library before purification and left-sided size selection with a ratio of 0.76x (Beckman Coulter). The library was sequenced on a single flow cell on an Illumina HiSeq X sequencer using the 300 cycle kit (150 bp paired-end reads) with 5 % Phi-X spike-in added by the sequencing company Macrogen Europe B.V. Ten of the 102 samples of the dataset were amplified and sequenced separately in another library, as they had already been processed in another study (Leese, Sander et al., 2021). For bioinformatic analysis, sequences from the ten previously sequenced samples (Short-Read Archive, Project number: PRJNA664693) and the sequences from the remaining 92 samples (deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB61051) were processed together.

2.3 Bioinformatics

Sequence reads were processed as stated in our previous study in detail (Leese, Sander et al., 2021). Briefly, JAMP v0.67 (https://github.com/VascoElbrecht/JAMP; Elbrecht et al., 2018) was used on default settings to merge paired-end reads and, where needed, to build the reverse complements of the sequences. Primer sequences were removed. To retain only reads of the expected fragment length, sequences with a deviation of >15 bp were excluded from further analyses. Reads with an expected maximum error of >0.5 and singletons were removed before clustering the sequences with a similarity [?]97 % to OTUs. To maximize the number of reads retained, the dereplicated sequences, including singletons, were mapped with a similarity of [?]97 % to the generated OTU dataset. Only OTUs with a minimal read abundance of 0.01 % in at least one sample were retained for further analyses. OTU centroid sequences were compared to the BOLD database for taxonomic annotation using BOLDigger 1.1.4 (Buchner & Leese, 2020). For further analyses, we only considered OTUs with a similarity of [?]90 % to a reference sequence in BOLD. OTUs with a similarity [?]98% were assigned to species, [?]95 % to genus and [?]90% to family level. Replicates were merged with reads summed up and divided by two for each OTU. OTUs for which conflicting taxonomic results were found were checked manually, taking into account if reference specimens were identified by taxonomic experts. Further, the obtained taxa list was compared to the RMO database, which contains detailed information on morphologically identified taxa occurring in this area, and the taxa list was additionally checked by three taxonomic experts to exclude terrestrial taxa and taxa that are impossible or unlikely to occur in the study area.

2.4 Species trait data

We split our dataset into merolimnic and hololimnic species because we expected to see differences in species and OTU numbers over our sampling period based on their different life cycles. Additionally we looked more in detail into species and OTU numbers of the most abundant merolimnic (EPT and Diptera) and hololimnic orders (Annelida and Coleoptera) and their associated FFG. To assign life cycle characteristics and FFG to our taxa, we used trait data from freshwaterecology.info (Schmidt-Kloiber and Hering, 2015) for a given taxon if available. If not available, we used data from a higher taxonomic level, such as genus-level trait values if species-level values were missing. For taxa with no trait values at the species-, genus-, or family-level, we used an averaging procedure whereby values were averaged across species within the genus if species-level values were available and across all taxa within the family if not (Kunz et al., 2022). We used the relative proportion of the different FFG as the proportion of the overall detected feeding types divided by the number of all species found per sampling day.

2.5 Environmental data

Data for discharge was accessible from a gauaging station close to Hanau (www.hlnug.de) <1 km downstream from the sampling site with no tributaries inbetween. Discharge was measured daily with minimum, maximum and average discharge data available. Water temperature data were recorded hourly over the entire sampling period at the sampling site using a temperature logger.

2.6 Statistical analysis

All statistical analyses were performed in R v4.0.5 Venn diagrams were constructed using*http://bioinformatics.psb.ugent.be/webtools/Venn/*. The effect of sampling day, sampling position, and temperature and discharge on overall species/OTU richness was tested using generalized additive models (GAM) with a poisson distribution and a "log" link function implemented by the gam() function in the R package mgcv (Wood, 2015). Where overdispersion was detected, we corrected the standard errors using a quasi-GAM model. Discharge and temperature were respectively quantified as the average discharge and mean hourly temperature of each sampling day (Table S1). To test for the effect of our predictor variable sampling position on our response variable OTU richness/read abundance a Kruskal-Wallis rank sum test was performed, as our data was non-normal and non-linear and we had three group variables. Further we divided our dataset into the two groups merolimnic and hololimnic taxa and subsetted these groups into the most abundant taxa, Diptera and EPT for merolimnic and Annelida and Coleoptera for hololimnic. Then we used GAMs to determine the effect of sampling day, temperature and discharge on species/OTU richness separately for each of the eight groups. To assess the effect of sampling day on the relative proportion for each FFG we used a GAM with a binomial distribution and a "logit" link function. Only the term for sampling day was smoothed in all GAMS via cubic regression splines with a basis dimension $k = 9$. Model diagnostics were checked via plots of residual versus fitted values. The marginal effects, the slopes of the prediction equation, of the significant explanatory variables were plotted at the mean of the covariates to incorporate the possible minimal effects that the insignificant explanatory variables could potentially have on the slope of the significant explanatory variable.

An ANOSIM test was used to test for the effect of sampling position on OTU and species temporal beta-diversity (Jaccard distance) defined as the shift in the identities of named taxa in a specified assemblage over two or more time points (Magurran et al., 2019). Additionally, we partitioned temporal beta-diversity into its components turnover - reflecting replacement of species between samples - and nestedness - occuring when species loss or gain causes species-poor sites to resemble a strict subset of species-rich sites (Baselga, 2010) using the betapart package (Baselga et al., 2018) and visualized their change over the time series as pairwise comparisons in relation to the first timepoint. For comparisons between seasons, we grouped our dataset into spring (March, April, May), summer (June, July, August), autumn (September, October, November) and winter (December, January, February) samples. To visualize differences between sampling positions and seasons for species and OTU community composition, non-metric multidimensional scaling (NMDS) based on Jaccard's distance was conducted using the package vegan (Oksanen et al., 2022).

To identify species that are most strongly associated with a season or only found at a specific season, an indicator species analysis based on presence - absence was performed using the R package indicspecies with the function multipatt() (De Caceres, Legendre, Moretti, 2010). The association of species to seasons was calculated using the indicator value index from Dufrene and Legendre 1997. We used the indicator value index instead of a correlation based index to get results which best match the observed presence of a species rather than preferences of one season over another. Using preferences could lead to the problem of species being reported as indicator for a specific season, although the species was also almost frequently present in other seasons. For biomonitoring aspects it is therefore favourable to use the observed presence of species. The indicator value index ranges between 0 and 1 (with 0 indicating no association) and is calculated as the product of two quantities A, the probability that the surveyed site belongs to the target group (i.e., season) when the species has been found, and B, how frequently the species is found in the different samples from each season. The function determines which season corresponds to the highest association value for each species and then tests the statistical significance of this relationship using a permutation test. We corrected for size differences between groups, as we had different sample sizes between seasons, using the implemented value within the multipatt() function.

3. Results

We obtained 372,431,352 reads and 4,702 OTUs for the 102 eDNA samples after quality filtering and excluding OTUs with read abundance <0.01 % per sample. Reads among samples were unevenly distributed ranging from 88,446 to 4,451,399 with a mean read number of 1,880,966. We excluded two samples that had only 3,282 and 7,928 reads, respectively, to avoid unrepresentative biodiversity data due to too low sequencing depth. These two samples were therefore excluded from the dataset to prevent distorting results due to these outliers. After the database and taxonomist checks, 2,840 OTUs remained which were assigned to 506 species. The uneven read distribution was no concern as neither OTU richness nor species richness were correlated with read numbers (see S1 & S2). Eight controls with no DNA were also included to test for contamination during field or laboratory proceedings. Their read numbers ranged from zero to four, indicating no substantial contamination. The vast majority of reads was assigned to metazoans, especially arthropods and annelids, with <1 % of reads assigned to non-metazoan taxa. In all samples, chironomids had the highest read numbers and constitued 55 to 95 % of every sample.

3.1 Effect of sampling day, sampling position, and environment

Sampling day affected the detection of both species (F = 5.933, p-value = <0.001, ndf = 4.055, ddf = 90.945) and OTU (F = 2.765, p-value = 0.030, ndf = 3.32, ddf = 91.68) richness. In contrast, neither temperature, discharge, nor sampling position had an effect on species or OTU richness. Highest species and OTU richness was detected in spring with lowest numbers in the summer months for all three sampling positions (Figure 2 and Figure S3). Conversely, sampling positions exhibited little difference in read richness, with 99 % of reads shared among the different positions in the stream and only a small fraction of reads being exclusively detected per sampling position ([?]0.1 %) (Figure 2). The sampling positions showed high overlap of reads for shared species (99.14 %) and only a small fraction of reads was assigned to the exclusively detected species per sampling position ([?]0.1 %) (Figure 2). Although some species were exclusively found at one of the three sampling positions (Figure 2), relative occurrence of these species was low in contrast to shared species (Figure S4). We found no significant differences between surface, riverbed or riverbank samples based on OTU richness (Kruskal-Wallis rank sum test: chi-squared = 0.085, p-value = 0.957, ndf = 2, ddf = 97) or read abundance (Kruskal-Wallis rank sum test: chi-squared = 0.774, p-value = 0.679, ndf = 2, ddf = 97).

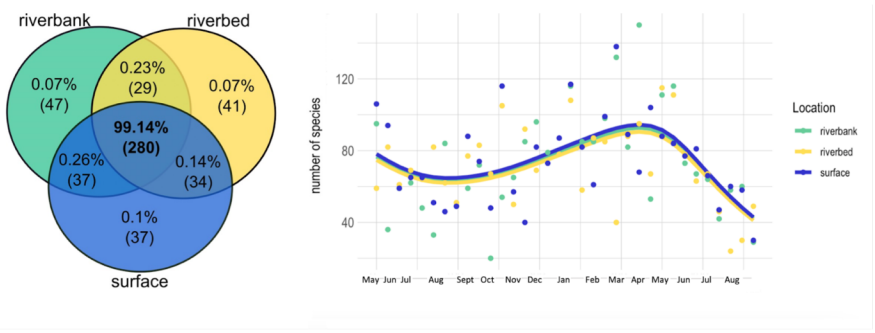

Figure 2: Comparison of the three sampling positions. Left: Venn diagram for the different combinations of sampling positions. For each combination read proportion in relation to overall macroinvertebrate reads is given in percent, numbers of species shown in brackets. Right: species richness over time for each sampling position. Points represent pure species numbers, solid lines represent the fitted values of the GAM.

Additionally, the NMDS analysis showed no differences in species or OTU beta-diversity between sampling positions based on ANOSIM tests (p-value: 0.7371/0.7079) (Figure S5 & S6). Based on these results, samples from the different sampling positions were merged for each sampling day and subsequent analyses focused on sampling time and seasonal differences. NMDS analysis for the merged samples revealed a strong impact of sampling season on community structure (Figure 3 & S7). Summer and winter samples were separated on the first axis, spring and autumn samples on the second axis, producing a cyclic temporal beta-diversity pattern resembling a "seasonal clock". When temporal beta-diversity was partitioned into turnover and nestedness, turnover was the driving factor for change in beta-diversity while nestedness was of less importance (Figure S8). Over the year, turnover increased over the winter samples and decreased again in summer with nestedness showing the opposite trend, reaching its highest value in summer 2018 (Figure S8).

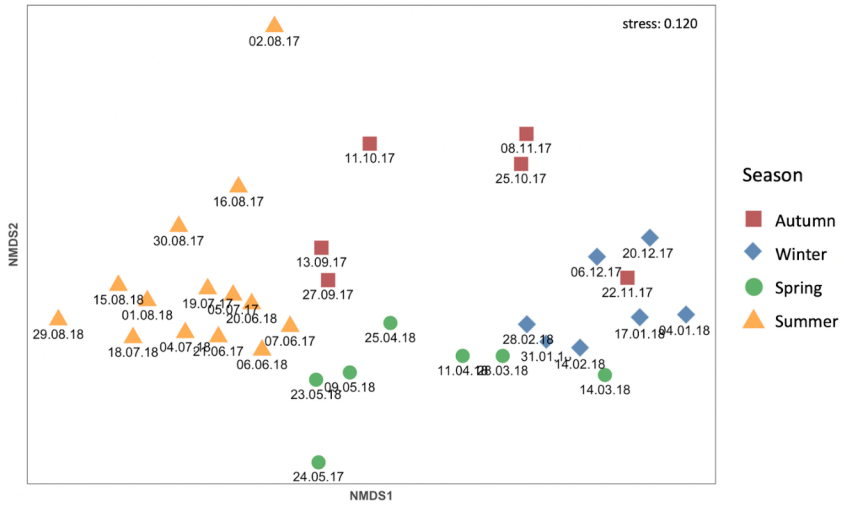

Figure 3: NMDS based on Jaccard's distance for MZB species for the 34 samples after merging sampling position samples for each time point. Autumn samples: September, October, November; Winter samples: December, January, February; Spring samples: March, April, May; Summer samples: June, July, August.

3.2 Mero- vs hololimnic

Sampling day affected the detection of overall merolimnic species richness (F = 4.419, p-value = 0.005, ndf = 3.59, ddf = 27.41), but not OTU richness, which was also true for the most species-rich merolimnic taxon Diptera (species richness: F = 3.231, p-value = 0.021, ndf = 3.649, ddf = 27.351). For merolimnic, most species were detected in spring (115),) and lowest richness was detected in summer (60) (Figure S13), as well as for the most species-rich merolimnic subgroup Diptera (spring: 95 species; summer: 50 species) (Figure S14). While the detection of merolimnic subgroups Ephemeroptera, Plecoptera and Trichoptera species richness was unaffected by sampling day, it had an effect on the detection of OTU richness (E: Chi-squared = 39.11, p-value = <0.001, ndf = 7.27, ddf = 23.73; P: Chi-squared = 21.01, p-value = 0.005, ndf = 6.156, ddf = 24.844; T: Chi-squared = 20.11, p-value = 0.002, ndf = 4.636, ddf = 26.364). For Ephemeroptera, most OTUs were detected in autumn (15) and in late spring (20). In contrast, the detected Plecoptera and Trichoptera OTU richness was highest from December to March (10) and January to March (20) respectively (Figure S15). Lowest numbers for all EPT were detected in summer (E: 6, P: 1, T: 6).

Sampling day had an effect on the detection of overall hololimnic species richness (Chi-squared = 32.28, p-value = <0.001, ndf = 5.498, ddf = 25.502) but not OTU richness. Most hololimnic species were detected between autumn and spring with relatively stable numbers inbetween (30) (Figure S16). The detection of the most abundant hololimnic taxon, Annelida, was influenced by sampling day for both species (Chi-squared = 20.56, p-value = 0.004, ndf = 5.392, ddf = 25.608) and OTU richness (Chi-squared = 2.534, p-value = 0.043, ndf = 5.243, ddf = 25.757) with the highest richness detected in autumn (species: 20/OTUs: 50) and a constant decline afterwards (summer: 5/10) (Figure S17 and S18). The number of detected species and OTUs for the second most abundant hololimnic taxon, Coleoptera, was not affected by sampling day.

Temperature had no effect on the detection of species nor OTU richness for any of the tested groups. Higher discharge negatively affected the detection of Ephemeroptera and overall hololimnic and Annelida species (E: Chi-squared = 4.213, p-value = 0.04, ndf = 1, ddf = 28.292; hololimnic: Chi-squared = 6.639, p-value = 0.01, ndf = 1, ddf = 25.502; Annelida: Chi-squared = 6.182, p-value = 0.013, ndf = 1, ddf = 25.608) (see Figures S11, S10 and S12). Per 10 m^3/s increase in discharge Ephemeroptera lost 1 species, hololimnic taxa 5 species and Annelida 3 species.

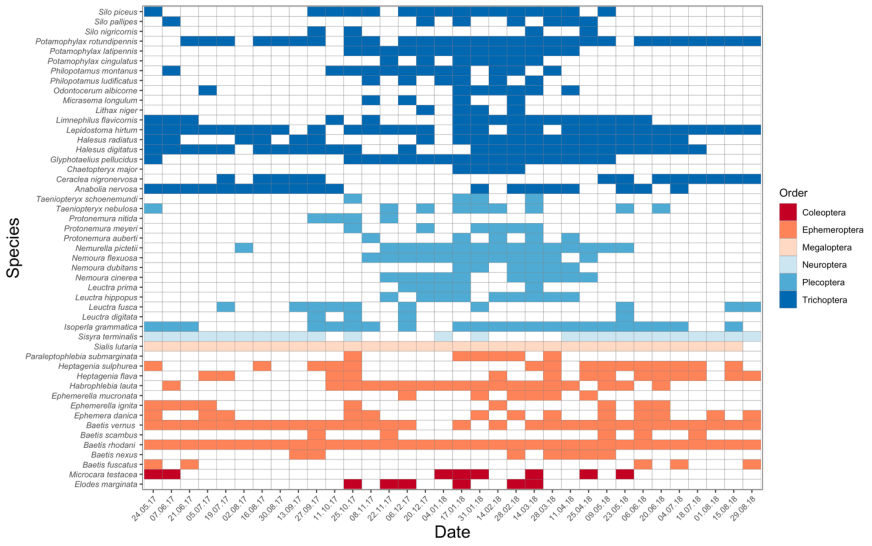

Figure 4: Co-occurence plot of the most common merolimnic species that occur at least in 10 % of the samples. Diptera are excluded because species numbers were too high to visualize clearly.

The indicator species analysis identified most species indicative for a certain season or seasons when either winter or spring samples were included in the combinations: autumn + spring + winter: 35 indicator species;

spring + winter: 30; winter: 24; spring: 20. Groups including the summer samples showed the lowest number of indicator species: autumn + summer: 2 indicator species; summer: 1; autumn + summer + winter: 1; spring + summer + winter: 1, indicating the presence of few species characteristic only for summer. Some species were exclusively found in one season, like the Plecoptera *Protonemura nitida* in autumn and the Trichoptera *Chaetopteryx major* in winter (see also Figure 5 & S18). Species not found in summer, but quite frequently in all other seasons, were Trichoptera *Silo piceus, Potamophylax latipennis,*Plecoptera *Nemoura flexuosa,* Diptera *Tipula paludosa, Rheocricotopus atripes,* Coleoptera *Platambus maculatus* and Annelida *Tubifex tubifex* . Species that were almost exclusively (high A value) and often (high B value) found in spring and winter included Trichoptera *Glyphotaelius pellucidus, Limnephilus flavicornis, Halesus radiatus and* Plecoptera *Nemurella pictetii, Isoperla grammatica* . Here, results were congruent with the NMDS and co-occurrence plots showing a clear distinction between summer and winter samples. In some cases, more than one OTU was assigned to the same species. Here, most OTUs showed similar association values. However, for *Baetis vernus* the different OTUs showed different associations even though it had no significant association to any season(s) when all OTUs were combined. One OTU was significantly associated with autumn, one with autumn + spring and two with autumn + spring + summer.

3.3 Functional feeding groups

Of the 505 detected species, we were able to assign trait values to 477 (94%), with 323 assigned at the species-level, 106 at the genus-level, and 48 assigned via gap-filling. The resulting trait data encompassed ten total FFG of: active filter feeders (aff: 66 species), gatherers (gat: 345), grazers (gra: 228), miners (min: 9), others (25), parasites (par: 7), passive filter feeders (pff: 31), predators (pre: 149), shredders (shr: 120) and xylophagous feeders (xyl: 8). Sampling day affected the relative proportion of shredders (Chi-squared = 38.111, p-value = <0.001, ndf = 3.78, ddf = 308.152) and parasites (Chi-squared = 14.2, p-value = 0.007, ndf = 3.187, ddf = 308.152), with no effects on the other FFGs. The relative proportion of shredder species increased from summer 2017 to winter from 6 % to 15 % and declined then again towards summer, reaching <5 % in summer 2018 (Figure 5A). Parasites showed a drop in relative proportion from 2 % in summer 2017 to <1 % in autumn and winter with many timepoints having no parasites detected at all. After winter relative proportion increased again to 3 % in summer 2018 (Figure 5B).

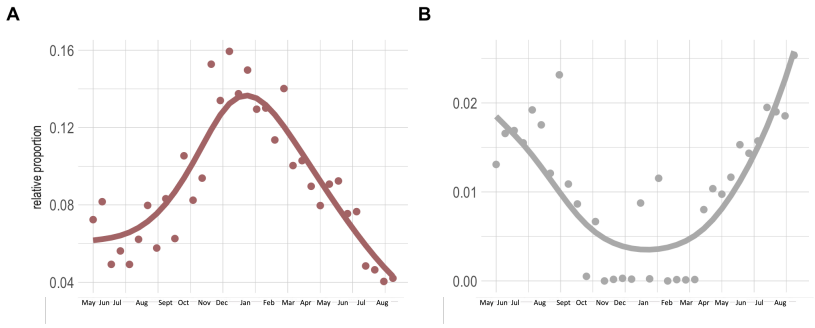

Figure 5: Relative proportion of species belonging to a disctinct functional feeding group over time. A: shr (shredders); B: par (parasites). Points represent pure values, solid lines represent the fitted values of the GAM.

4. Discussion

4.1 Influence of sampling time and position on community composition

Our results indicated that differences in community composition were caused by seasonality but not sampling position, which matched our expectations despite positional compositional differences occurring in other studies examining fish and macroinvertebrates (e.g. Berger et al., 2020; Macher & Leese, 2017; Thalinger et

al., 2021). High similarity among sampling positions likely occurred due to the complete mixing of eDNA in the water body, with smaller compositional differences occurring by replication, the three water samples taken within the sampling site, and not by systematic differences between positions (Macher et al., 2021). The stream sampled in our study had a water depth <1 m and sampling positions were less then 10 m apart with a mostly turbulent flow, all of which can reduce community heterogeneity (see Shogren et al., 2017; Fremier et al., 2019 for further discussions). As we sampled only one location in a single temperate stream, it is not possible to derive general predictions from our results regarding eDNA detection probability of macroinvertebrates between different sampling positions in other streams and regions. As a next step, it would be of interest to systematically compare sampling position differences among divergent stream types.

Temporal beta-diversity shifted in a seasonally cyclical and gradual pattern, which was primarily driven by turnover, with turnover and nestedness both returning to the same level after one year. The potential of eDNA in detecting seasonal shifts in community composition has been already shown for marine environments (Jensen et al., 2022; Liu et al., 2022) but not for freshwater.

4.2 Influence of different life cycles on species and OTU detection

We hypothesized that community composition will differ depending on life cycle differences between mero- and hololimnic taxa. Accordingly, we found a strong effect of sampling season on all tested groups, except Coleoptera. Species richness of merolimnic and hololimnic taxa were both affected by sampling day but in a different way. In contrast to merolimnic taxa, hololimnic taxa showed a more stable number of species between autumn 2017 and spring 2018 likely due to the absence of emergence events, as hypothesized. For the most species-rich hololimnic group, Annelida, most species were detected in autumn with the most species-rich taxa being assigned to the family Naididae. The high diversity of Naididae probably comes from a high number of asexually produced individuals, or through shedding of the sexually produced individuals from autumn or dying of the adults after cocoon laying (Learner et al., 1978). Contrary to our second hypothesis, we did detect lower species numbers in summer also for the hololimnic group Annelida, but the decrease was more steady instead of an abrupt drop in species numbers after peaking as for merolimnic species.

In general, merolimnic species detection in the water depends on several factors, for example time and duration of the flight period and emergence, abundance of the species in the water, number of generations per year, larval development and occurrence of dormancy/diapause. Diptera were the most species-rich merolimnic order with most taxa belonging to family Chironomidae. We detected a high diversity of Diptera in spring and early summer which is consistent with the results of other studies, implying a reduced diversity in winter and the highest diversity in early summer before emergence, as emergence of chironomids is correlated with high temperatures (Armitage et al., 2012; Bista et al., 2017). The high number of species in spring is likely due to a high growth rate preparing the larvae for emergence in summer, also explaining the drop in detected species number for the later summer month. For the species-rich mero- and hololimnic groups, except Annelida, no effect of sampling day on OTU richness was detected, possibly due the high number of OTUs per group which can show distinct responses to seasonal changes even within the same species. Contrary, for Ephemeroptera, Plecoptera and Trichoptera, only OTU richness was affected by sampling day, likely due to the lower numbers of species and therefore smaller shifts in species richness over time. Plecoptera and Trichoptera OTU richness showed one peak each, in winter and early spring, which is likely linked to an increase in biomass that will probably reach its peak right before emergence coinciding with our second hypothesis expecting a low richness in summer. Plecoptera OTU richness peaked a bit earlier than Trichoptera OTU richness which is congruent with an on average earlier emergence of many Plecoptera species compared to most Trichoptera species (Graf et al., 2008; Graf et al., 2009; Graf et al., 2022a,b; Schmidt-Kloiber and Hering, 2015). By comparison, Ephemeroptera OTU richness had two peaks, one in autumn and one in late spring. Species details revealed that from 21 Ephemeroptera species, ten have a bivoltine (mainly genus *Baetis*), one a flexible (*Caenis beskidensis*) and two a semivoltine (genus*Ephemera*) lifecycle (Buffagni et al., 2009; Buffagni et al., 2022; Schmidt-Kloiber and Hering, 2015). In contrast, the detected Plecoptera and Trichoptera were mostly univoltine species.

Occurrence even between species within a merolimnic order differed between seasons. The indicator species

analysis revealed that *Glyphotaelius pellucidus* is detectable in all winter and spring months and absent in summer which coincides with the species having a long flight period after emergence and a known dormancy (Graf et al., 2008; Graf et al., 2022; Schmidt-Kloiber, A. and Hering D., 2015). In contrast *Philopotamus ludificatus*has also a long flight period and dormancy but was detected less frequently but had similar read numbers as *G. pellucidus* (Graf et al., 2008; Graf et al., 2022; Schmidt-Kloiber and Hering, 2015). As neither differences between life cycle characteristics, nor read numbers were present, the less frequent detection of *P. ludificatus* was likely because the species was rarer at the sampling location or because of other unknown factors influencing the detectability of the species. Other species like the Ephemeroptera *Baetis rhodani* and*Baetis vernus* are detectable throughout the year with *B. vernus* being less frequently detectable than *B. rhodani* . The more frequent detection of *B. rhodani* can be explained due to the species being also sometimes trivoltine and *B. vernus* being only known for a bivoltine life cycle (Buffagni et al., 2009; Buffagni et al., 2022; Schmidt-Kloiber and Hering, 2015) and therefore, larvae of*B. rhodani* are probably more frequent. Additionally the OTUs detected belonging to species *B. vernus* differed in their seasonal occurrence with one OTU only occurring in autumn. It is known that different OTUs can show distinct responses to environmental changes (Beermann et al., 2018), and for the *B. vernus* group cryptic diversity has been recorded (Stahls & Savolainen, 2008). This strengthens the assumption that the differences in detection between the OTUs is based on different responses to environmental changes and therefore an underestimation of the diversity within *B. vernus* . A limitation in using eDNA to assess patterns of seasonality is the persistence of eDNA in the environment for up to several days or weeks, and apart from that, the uncertainty of capturing living or dead cells (Dejean et al., 2011; Pilliod et al., 2014; Thomsen et al., 2012a,b). Nonetheless, our results demonstrate that the patterns we found are consistent with the phenology of the different taxa thus further encouraging that the DNA we detected mostly originated from living organisms.

4.3 Influence of season on the distribution of feeding types

For two of the functionl feeding groups (FFG), we observed an influence of sampling time on the relative proportion of FFG, partly confirming our third hypothesis. Shredders showed a large increase from summer to winter and a decrease afterwards. As shredders feed on allochthonous material such as decomposed leaf litter, the increase of the relative proportion of shredders in winter is likely due to an increase in their food after fallen leaves accumulate in the stream (Cummins, 1974; Cummins et al., 1989). Although we expected to detect a higher number of grazers in the warmer months, we did not detect an effect of sampling day on the relative proportion of grazers. As the number of detected species was in general extremely low in the summer samples, the lack of seasonal effect on the proportion of grazers detected is likely the result of the extremely dry and warm summer in 2018 reducing the abundance of temperature sensitive taxa. Since we only looked at presence/absence data, it is possible that abundance data would have shown higher proportions of grazers in summer. Proportion of parasites was highest in summer and their occurrence was likely linked to their hosts. The exclusively parasitc larvae of *Sisyra terminalis* , which was frequently found in the spring and summer but not winter samples, is known to parasite on sponges and the species *Spongilla lacustris* and *Eunapius fragilis* , which were also present in our dataset, are known to form colonies during warm seasons and produce gemmules over the cold seasons (Gugel, 2001; Weißmair, 1994). Additionally, also the Trichoptera *Ceraclea nigronervosa* , which feeds on Spongillidae (Graf et al., 2002), was detected in the summer and spring month but not in the winter months, further encouraging the influence of the abundance of sponges on the detection of their hosts/predators. These results are promising for future eDNA based biomonitoring, as we could detect shifts in the relative proportion of different FFG and therefore changes in functional composition of the macroinvertebrate community over time, which could help to determine changes in ecosystem functioning (Minshall et al., 1992).

4.4 Influence of water temperature and discharge on species and OUT richness

We neither detected an influence of temperature on overall species nor on OTU richness, thus indicating that other factors like sampling time or season had a greater effect on eDNA community composition. In contrast, we found a negative effect of increasing discharge on hololimnic taxa, Annelida, and Ephemeroptera

species richness. For Ephemeroptera with a p-value close to 0.05 this should be interpreted as a trend and future studies are needed to verify the effect of discharge. For hololimnic species, where Annelida were the most species-rich group, a possible explanation could be that the possibility of many Annelida specimens being swept away with high discharge is low as most annelids are embedded within the sediment and are potentially moving even deeper into the sediment with high discharge. This could lead to a higher dilution effect and therefore lower annelid DNA concentrations as specimens are not washed away.

In the summer months, an extremely low species and OTU richness was detected for all groups with August 2018 being the month with the lowest richness. The summer 2018 was outstandingly warm with low rainfall and high sun exposure. The exceptionally warm temperatures probably affected abundance and growth rates of the more sensitive species and therefore causing a decrease in detection rates but also increased DNAse activity or microbial break DNA down leading to faster DNA degradation rates could be an influencing factor.

Conclusion Our data show how eDNA-based time-series data on a stream macroinvertebrate community can support ecological research and biomonitoring of rivers, as we detected seasonal differences and small temporal shifts in community composition and key functional feeding groups. Based on our results it is difficult to predict eDNA detection probability of different sampling positions in general, as we only sampled three positions in one location of one stream for 15 months. As a clear advantage of eDNA metabrcoding, our study detected many species of often morphologically neglected taxa such as annelids and chironomids that are often underrepresented in other studies. By including such taxa in the analysis, eDNA metabarcoding could function as a complementary tool for long-term monitoring when assessing differences in macroinvertebrate community composition on a highly resolved temporal scale. This could help to disentangle variations in community composition due to recent and extreme changes from gradual changes, as seen with our "seasonal clock" data which showed gradual shifts in temporal beta-diversity, and help deriving guidelines for future eDNA-based monitoring of rivers and streams.

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Data availability statement

All raw reads will be made accessible via the European Nucleotide Archive (ENA) prior to publication under accession number: PRJEB61051.

Hosted file

`Supplement_timeseries_EPTDr2n.docx` available at https://authorea.com/users/602937/articles/633564-environmental-dna-time-series-analysis-of-a-temperate-stream-reveals-distinct-seasonal-community-and-functional-shifts-but-no-influence-of-within-stream-sampling-position