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ORIGINAL ARTICLE

Seasonal turnover in community composition of stream-associated macroinvertebrates inferred from freshwater environmental DNA metabarcoding

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Abstract

Macroinvertebrate communities are crucial for biodiversity monitoring and assessment of ecological status in stream ecosystems. However, traditional monitoring approaches require intensive sampling and rely on invasive morphological identifications that are time-consuming and dependent on taxonomic expertise. Importantly, sampling is often only carried out once in a year, namely during late winter–spring, where most indicator taxa have larval stages in the streams. Hence, species with divergent phenology might not be detected. Here, we use environmental DNA (eDNA) metabarcoding of filtered water samples collected in both spring and autumn from five streams in Denmark to address seasonal turnover in community composition of stream macroinvertebrates. We find that eDNA read data from the same stream sampling site clearly show different communities in spring and autumn, respectively. For three of the five streams, season even appears to be a more important factor than sampling site for explaining the variation in community composition. Finally, we compare eDNA data with a near-decadal dataset of taxon occurrences in the same five streams based on kick sampling conducted through a national monitoring program. This comparison reveals an overlap in species composition, but also that the two approaches provide complementary rather than identical insights into community composition. Our study demonstrates that aquatic eDNA metabarcoding is useful for species detection across highly diverse taxa and for identifying seasonal patterns in community composition of freshwater macroinvertebrates. Thus, our results have important implications for both fundamental research in aquatic ecology and for applied biomonitoring.

KEYWORDS
arthropods, community structure, environmental DNA, genetic monitoring, metabarcoding, seasonality

1 | INTRODUCTION

Running waters sustain unique biodiversity and provide important ecosystem services (Millennium Ecosystem Assessment, 2005). However, running waters also face multiple anthropogenic threats and are among the most impacted ecosystems on Earth (Strayer & Dudgeon, 2010). Macroinvertebrate communities – particularly the insect orders mayflies (Ephemeroptera), stoneflies (Plecoptera), and caddisflies (Trichoptera), known as the EPT taxa – are central for biomonitoring and for assessing ecological status in lotic ecosystems (Poikane et al., 2020). However, monitoring of stream macroinvertebrates currently requires invasive sampling and relies on time-consuming morphological identifications requiring significant taxonomic expertise. For example, kick sampling is a widely used method in biomonitoring programs for streams and rivers (Feeley et al., 2012), and especially the identification of larval stages of closely related species can be challenging (Pfrender et al., 2010). As a result of these constraints, sampling is often only performed during late winter or spring, coinciding with most indicator taxa having larval stages with sufficient size for identification based on morphological characters. Several important biotic indices such as 'taxon richness' and '% EPT richness' have been identified as relatively unaffected by seasonal variation in macroinvertebrate communities of New Zealand (Stark & Phillips, 2009). However, while the biotic indices used for assessing ecological status may not be strongly influenced by timing of sampling, the macroinvertebrate community composition inevitably changes between seasons. Thus, neglecting temporal sampling may lead to certain species going undetected, because of different phenology or life cycles than those of the indicator species found during spring sampling. Therefore, it is highly relevant to explore supplementary non-invasive and standardized genetic approaches for biomonitoring of stream macroinvertebrates.

Environmental DNA (eDNA) approaches have recently been established as a reliable way of detecting diverse animal taxa in contemporary ecosystems, especially for freshwater systems (Deiner et al., 2017; Taberlet et al., 2018; Thomsen & Willerslev, 2015). Naturally, the first studies focused mostly on single-species detections of vertebrates (Ficetola et al., 2008; Goldberg et al., 2011; Jerde et al., 2011), and rarely macroinvertebrates (Thomsen et al., 2012). However, eDNA metabarcoding – high-throughput sequencing of PCR amplicons produced with generic primers – has provided an efficient approach for analyses of entire communities using eDNA samples (Taberlet et al., 2012, 2018; Thomsen et al., 2012; Zinger et al., 2019), and lately macroinvertebrate community analyses from aquatic eDNA have gained increasing attention (Bista et al., 2017; Elbrecht & Leese, 2017; Fernández et al., 2018; Fernández et al., 2019; Lim et al., 2016; Mächler et al., 2019).

The metabarcoding region of choice for arthropods has generally been the mitochondrial cytochrome C oxidase subunit I (COI) gene, especially because of the vast genetic references available in the Barcode of Life Data System (BOLD) (Ratnasingham & Hebert, 2007). The 16S rRNA gene has, however, been suggested as a better choice due to lower primer bias (Deagle et al., 2014; Elbrecht et al., 2016), but with the caveat of reduced species resolution, mostly due to the lack of reference sequences. While there has been extensive work on developing COI primers with minimal primer bias across different insect groups (Elbrecht & Leese, 2017), most of the suggested primers target longer fragments than what is normally preferred for eDNA studies (Taberlet et al., 2018).

The EU Water Framework Directive (WFD; 2000/60/EC), which requires European nations to obtain a "good" ecological status of their water bodies by 2027, has naturally sparked interest in applying metabarcoding to monitor the presence/absence of ecological indicator taxa (Hering et al., 2018). The first metabarcoding studies primarily focused on using insect bulk samples to monitor the presence of indicator taxa, either as homogenized bulk samples (Elbrecht & Leese, 2015) or using the preservative ethanol used for specimen collection (Hajibabaei et al., 2012). In monitoring programs of Danish streams, the Danish Stream Fauna Index (DSFI) has been the official method for ecological quality assessment in streams for the past two decades (Skriver et al., 2000). In a recent study, Kuntke et al. (2020) applied a metabarcoding approach to bulk samples of macroinvertebrates collected in Danish streams with different DSFI scores and found that the detected taxonomic compositions clearly corroborated the stream quality assessments from classical morphological investigations. A benefit of environmental monitoring using aquatic eDNA samples rather than bulk samples is the simultaneous retrieval of genetic information on indicator species as well as more neglected species, which are not used as bioindicators. eDNA has previously been demonstrated to reflect seasonal turnover in aquatic ecosystems (Bista et al., 2017; Buxton et al., 2018; Sigsgaard et al., 2017), and might, specifically for streams, serve as an indicator of whether and how species composition change between seasons.

Here, we investigate seasonal differences in stream macroinvertebrate communities using eDNA metabarcoding of water samples collected in spring and autumn from five Danish streams. We also evaluate whether such aquatic eDNA metabarcoding data can provide reliable information about the presence of macroinvertebrate taxa, and in particular EPT taxa, by comparing our eDNA data with a near-decadal dataset based on kick sampling carried out at the same sites under a national stream monitoring program.

2 | MATERIALS AND METHODS

2.1 | Study site and sampling

Water sampling for eDNA was performed at five Danish stream sites (Figure 1) on April 24–25 2018 (spring) and again on November 19–20 2018 (autumn). The five sampled sites are named Bøgeskov bæk (Boege), Borre å (Borre), Jeksen bæk (Jeks), Fæbæk (Faebaek), and Tjærbæk (Tjaer). This sampling design was chosen to account for the different developmental stages and seasonal presence of the macroinvertebrates over the year, as these factors are likely to affect shedding rates of eDNA. At each locality, we filtered four water samples per season each of 1 L (2 × 500 ml) using 0.22-μm Sterivex-GP

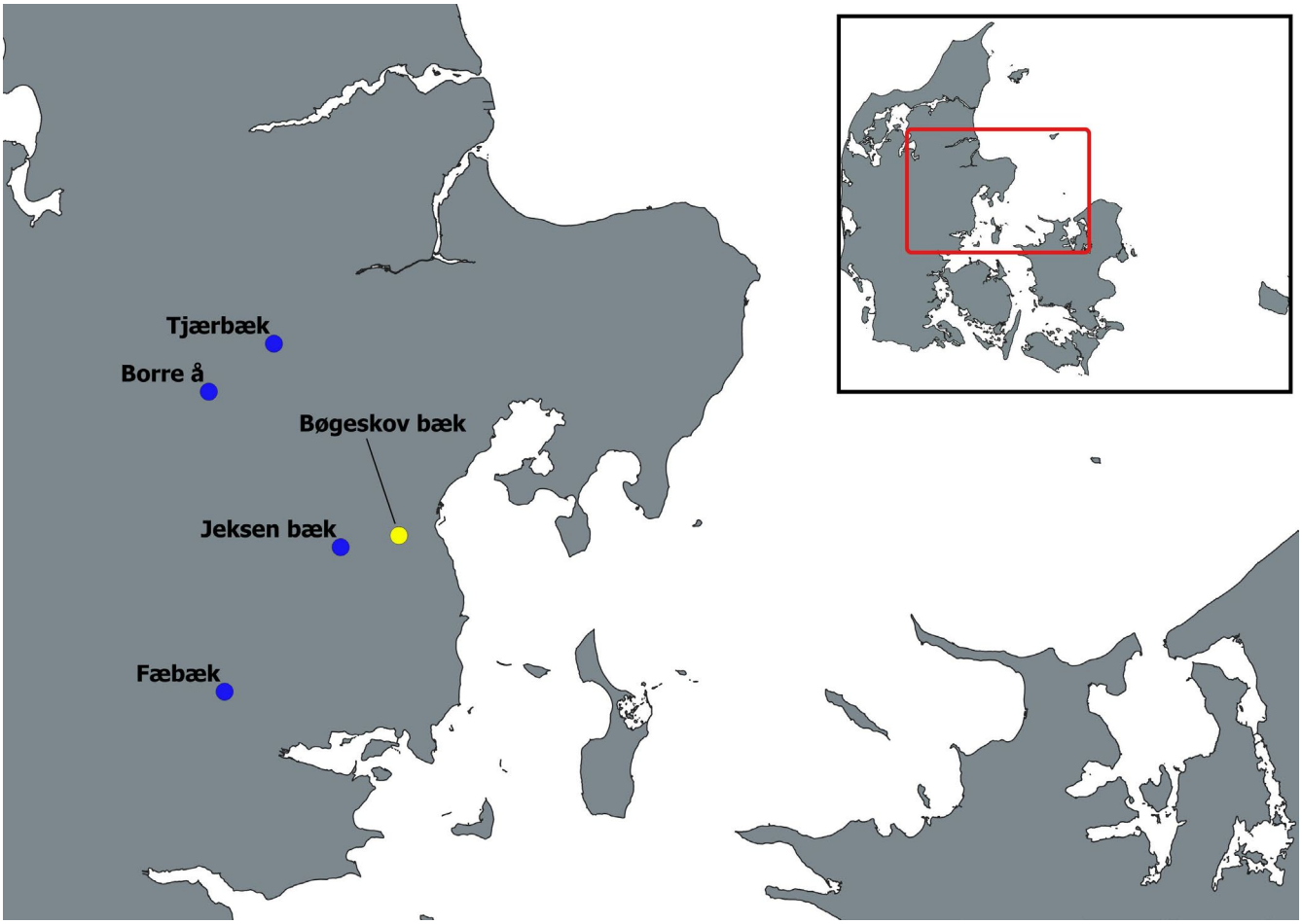

FIGURE 1 Map of sampling sites in Jutland, Denmark. Sampling sites are colored according to their Danish Stream Fauna Index (DSFI) values. Yellow = 4, blue = 7

filters (Merck Life Science). Water was collected from the surface of the stream along a 20-m reach, and we attempted to account for microhabitat differences by sampling above sand, gravel, and rock dominated substrates. We collected one field blank per stream per season by filtering 500 ml of bottled mineral water to keep track of potential DNA contamination, for example from the air. In total, we collected 40 samples of 1 L (five streams, four samples each, and two seasons) as well as 10 field controls. Samples were stored in a box with ice packs immediately after sampling and transferred to a −20°C freezer on the same day after return from the field, where they were kept until DNA extraction.

2.2 | Long-term monitoring data

The national monitoring program for streams in Denmark (NOVANA) includes >4500 sampling sites. Each sampling site represents a stream reach of 100 m. At each site, macrophytes, benthic algae, macroinvertebrates, and fish are surveyed in at least one of the years of each plan cycle in the EU Water Framework Directive (6 years). For a subset of these streams (n = 247), macroinvertebrates have been surveyed annually from 2004 to 2016 and every second year from 2017 to present. Macroinvertebrates are collected using standard kick sampling (mesh size = 500 µm) (for further details, refer Skriver et al., 2000). Taxonomic identification for this subset of streams was to species level for Turbellaria (Tricladida), Hirudinea, Malacostraca, Ephemeroptera, Plecoptera, Trichoptera, Megaloptera, Heteroptera, Gastropoda, Bivalvia, Ptychopteridae, and Simuliidae, genus level for Coleoptera, subfamily level for Chironomidae and Ceratopogonidae, and family level for Oligochaeta. For our study, macroinvertebrate community data obtained through the NOVANA program were extracted from an open source online database at https://oda.dk. The data extracted for the five sites represent spring sampling for nine consecutive years at Tjaer and Jeks (2011–2019), for 2 years at Borre and Boege (2012 and 2018), and for 6 years at Faebaek (2012–2016 and 2018). The five sites where water samples were collected for eDNA analysis were all sampled with kick sampling in the spring of 2018 (within 2 weeks of the spring eDNA sampling).

For each kick sample, the DSFI was calculated according to Skriver et al. (2000). In brief, DSFI is a semi-quantitative indicator developed to detect effects of low oxygen concentrations, and it is based on the presence of positive and negative indicator taxa. The final index score ranges from 1 to 7 with higher scores indicating

higher ecological status. Index scores 5 and 6 characterize good ecological status, and seven characterizes high ecological status.

2.3 | DNA extraction

Extractions of eDNA from filters were performed in the clean laboratory facility at the Department of Biology, Aarhus University, which is a dedicated laboratory for working with samples of low DNA concentration. Regular decontamination routines are in place, including UV light, and only pre-PCR work is carried out in this lab. DNA was extracted using the DNeasy® Blood & Tissue kit (Qiagen), using four times more AL buffer and proteinase K compared to the manufacturers protocol and an incubation time of 3 h. The paired 500-ml samples making up each 1 L water sample were extracted individually, but for subsequent analyses the two extracts of each pair were pooled. An extraction blank was included throughout the extraction process, and final DNA extracts were stored at −20°C.

2.4 | PCR amplification

For eDNA metabarcoding, we used a primer set (BF1 and BR1) targeting a ca. 217 bp fragment of the mitochondrial COI gene and designed for invertebrates (Elbrecht & Leese, 2017). Primers were uniquely tagged. Tags were designed using the OligoTag program (Coissac, 2012) and consisted of six nucleotides with a distance of at least three bases between any two tags. Tags were preceded by two or three random bases; NNN or NN (De Barba et al., 2014) to increase sequence complexity, and identical tags were used on the forward and reverse primers for each sample to avoid tag jumps (Schnell et al., 2015). Four identical PCR runs were set up, each containing: one PCR reaction for each of 20 field samples (five sampling sites, four samples, one season), five field blanks, and five extraction blanks, as well as one PCR reaction with a positive control, and four blank PCR reactions. Each of the 35 reactions in a PCR run had a unique tag (except for the PCR blanks, where a single tag was used for all four reactions), while the same tags were used for PCR replicates of the same sample. This setup was used for samples from both seasons. The preparation of PCR reactions followed Sigsgaard, Olsen, et al. (2020) with 25 μl volumes of 3 μl template DNA, 10 μl HotStarTaq Master Mix (Qiagen), 8 μl ddH2O, 1.5 μl of each primer (10 μM), and 1 μl bovine serum albumin (BSA) (20 mg/ml). Thermocycling parameters were: 95°C for 15 min, 55 cycles of 94°C for 30 s, 46°C for 30 s, 72°C for 1 min, and a final elongation of 72°C for 7 min. Fragment sizes were verified on 2% agarose gel stained with GelRed™.

2.5 | Library building and Illumina sequencing

The PCR products from each PCR run were pooled (excluding the positive control) and the total of eight pools (four PCR replicates per season) were individually purified using Qiagen's MinElute PCR purification kit. The manufacturer's protocol was followed with the exception that samples were incubated with the elution buffer (2*20 μl EB) over two rounds of 37°C for 10 min. A purification blank was included. Library preparation was carried out using the TruSeq DNA PCR-free LT Sample Prep kit (Illumina) with an input of ca. 750 ng of purified PCR product from each pool, as determined with the Qubit HS DNA Kit (Thermofisher Scientific). A library building was also performed on the purification blank. Concentration and fragment size distribution of the libraries were verified using Qubit and an Agilent 4200 TapeStation. The eight libraries were sequenced on an Illumina NovaSeq 6000 by Novogene using 150 PE sequencing and requesting 10 Gb output per library.

2.6 | Data analysis

Raw reads were demultiplexed, trimmed, and filtered using a custom script based on cutadapt (version 1.18) (Martin, 2011), sickle (version 1.33) (Joshi & Fass, 2011), and DADA2 (version 1.6.0) (Callahan et al., 2016) (script available upon request). We used cutadapt to demultiplex sequences (no mismatches to the tags allowed), remove primers, discard untrimmed reads, and specify a minimum read length of 100 bp. We used sickle to trim sequences according to read quality (average required Phred score of 28 in the sliding window). DADA2 was applied to correct for erroneous amplicons as well as merging paired reads (overlap of minimum 5 bp) and removing likely chimeras. The list of final amplicon sequence variants (ASVs) was first searched against a local version of the GenBank nt database (downloaded September 2019) using BLASTn (specifying up to 500 hits, 90% similarity, and 90% query coverage), after which we extracted all ASVs with a metazoan as 'best hit'. We then searched the metazoan ASVs against the BOLD database (Ratnasingham & Hebert, 2007) using the bold_identify function of the 'bold' package (version 0.9.2) in R (version 3.6). Here, we required hits to cover the entire read lengths (query coverage of 100%) and required a minimum of 98% sequence similarity to the best hit. As in Sigsgaard, Olsen, et al. (2020), we determined final taxonomic identifications based on whether there was an overlap in sequence similarity between the sequences of the taxon showing the best hit and other matching taxa. In other words, we looked for barcode gaps (refer e.g., Puillandre et al., 2012), and if there was none, we downgraded the taxonomic identification to the appropriate level (e.g., species to genus, genus to family etc.). If there was a barcode gap, we accepted the best hit as the final identification. The BOLD database contains several inconsistencies in sequence names and descriptions, such as typos, synonyms, and inclusion of identifiers in species names, impeding trustworthy automation of taxonomic identification. To accommodate these issues, all ASVs that were not determined to species level were also manually inspected and updated if appropriate. In a handful of cases, we found examples of sequence similarities ranging from 82% to 100% for the same species, which were caused by a single, mislabeled sequence at low similarity (e.g., 20 hits at 100% and one hit at 82%

similarity). In instances like these, we did a manual BLAST search of the low-similarity hit sequence and disregarded it if it appeared to be a likely result of incorrect identification. Furthermore, we only assigned species level taxonomy in instances with ≥99% similarity to the best hit. In addition to this, we labeled ASVs that could not be determined to species level according to their best hits. Thus, two ASVs with a similar identification outcome would be retained as two separate taxa, if their lists of best hits did not overlap (e.g., two different ASVs both identified as "*Diptera* sp." would become *Diptera* sp.1 and *Diptera* sp.2 if the best hits from each ASV were not overlapping).

After the taxonomic scrutiny, we filtered out all taxa that occurred in higher read counts in a field control, extraction control or PCR control than in any eDNA sample. We furthermore removed all taxa that were only present in one out of four PCR replicates of a sample. Reads were rarified to the median read depth of the samples using the R-package *ROBITools* (version 0.1) to limit the influence of sequencing depth. We performed species accumulation curves on the four PCR replicates per sample and on the four samples taken from the same locality per season. Canonical analysis of principal coordinates (CAP) (refer Anderson & Willis, 2003) based on linear discriminant analysis (LDA) (Park & Park, 2008) of both abundance (based on read counts) and presence/absence data was performed using the CAPdiscrim function of the R-package *BiodiversityR* (version 2.11-3). The CAP analysis was based on uniquely identified taxa, including those not determined to species level (refer previous text). We specified Bray–Curtis as the distance metric and plotted with *ggplot2* (version 3.2.1). Clustered heatmaps were created using the R-package *pheatmap* (version 1.0.12), and Venn diagrams were made using the R-package *eulerr* (version 6.1.0). The most computationally demanding bioinformatic analyses were conducted using the high-performance computing facility GenomeDK, Center for Genome Analysis and Personalized Medicine, Aarhus University.

3 | RESULTS

3.1 | DNA metabarcoding reads

A total of 697 M raw reads corresponding to 348.5 M read pairs were produced. We obtained similar sequencing depth across the eight libraries with 29.29–53.53 M raw read pairs per library (average of 43.56 M reads). After initial sequence quality filtering, trimming, and read merging, we retrieved 0.94–7.54 M reads per sample (average of 4.08 M reads). The final reads yielded a total of 77,923 ASVs for the spring dataset and a total of 62,194 ASVs for the autumn dataset. After filtering for metazoan hits only, a total of 5482–2.1 M reads per sample were retained with an average of 0.38 M (±0.062 M SEM) reads, meaning that >90% of the reads were of non-metazoan origin. We retained 2003 metazoan ASVs from the spring dataset and 1256 metazoan ASVs from the autumn dataset. The majority of the reads found in the controls came from the field controls and were mostly sequences with very poor matches in the NCBI GenBank database (<85% similarity). Still, a few ASVs from the controls survived our criteria and had to be filtered out from the datasets (Data S1).

Rarefaction curves for all four PCR replicates per sample indicated that a plateau in metazoan taxon diversity was reached, and that sequencing depth was thus sufficient, even with the loss of >90% of the data to non-metazoan reads (Figures S1 and S2). Likewise, species accumulation curves for PCR replicates indicated that we included enough PCR replicates to cover the diversity in each sample (Figures S3 and S4). However, species accumulation curves per site per season indicated that four samples were not sufficient to reach saturation of species diversity, and that more samples per site would be advantageous to fully cover the metazoan diversity (Figures S5 and S6). The taxonomic resolution for the spring ASVs was 90.3% identified to genus level and 60.3% to species level, whereas the taxonomic resolution for the autumn ASVs was 84.2% identified to genus level and 52.8% to species level.

3.2 | Traditional kick sample data (NOVANA)

Across the entirety of the near-decadal (2011–2019) kick sample data, specimens from four phyla, eight classes, 19 orders, 54 families, 85 genera, and 86 species were identified in the five streams (Table S1). When only including kick samples collected in spring 2018, specimens from four phyla, seven classes, 13 orders, 39 families, 55 genera, and 53 species were collected and identified. Based on the kick samples from 2018, DSFI scores were four for Boege and seven for Borre, Jeks, Tjaer, and Faebaek.

3.3 | Taxonomic and functional diversity of metazoans

Based on the filtered eDNA dataset, we found a comprehensive diversity of metazoans. Identified taxa were primarily arthropods, but also annelids, mollusks, bryozoans, cnidarians, nematodes, chordates, gastrotrichs, rotiferans, and tardigrades (Table 1). The only phylum found in the NOVANA sampling which was not found with eDNA was Platyhelminthes. For a complete overview of all genera found using eDNA compared with NOVANA sampling, refer Table S1.

Across all water samples and seasons, we obtained eDNA from 10 phyla, 18 classes, 37 orders, 105 families, 180 genera, and 212 species (Table 1, Table S2). When species only determined to "genus sp." were included in the species count, we found 268 species in total. This number, however, may be somewhat inflated, as a single species may be counted multiple times in cases where multiple haplotypes for one species occur, and where taxonomic resolution differs for the haplotypes found. Our eDNA findings generally corresponded well with the NOVANA inventory (Table S1). Moreover, the eDNA taxa that were not previously recorded in the NOVANA program for the sampling sites were assessed (expert judgment)

TABLE 1 Overview of sequence abundance (counts and percentages) in both spring and autumn datasets of all metazoan taxa identified. Refer Table S2 for higher taxonomic resolution than provided here. *Note that "sp. included" only counts "genus sp.", and not "family sp." or higher taxonomic levels

Phylum	Class	Order	Families	Genera	Species	"sp."* included	Reads spring	Spring % of total	Reads autumn	Autumn % of total
Annelida	Clitellata	–	0	0	0	0	350	0.01	42	0.00
Annelida	Clitellata	Enchytraeida	1	2	4	4	1042	0.02	2579	0.04
Annelida	Clitellata	Haplotaxida	2	14	21	25	613,519	10.95	1,585,883	27.50
Annelida	Clitellata	Lumbriculida	1	2	2	2	4566	0.08	2355	0.04
Arthropoda	–	–	0	0	0	0	16,765	0.30	0	0.00
Arthropoda	Arachnida	Araneae	4	4	3	4	734	0.01	0	0.00
Arthropoda	Arachnida	Opiliones	1	2	2	2	0	0.00	1279	0.02
Arthropoda	Chilopoda	–	0	0	0	0	0	0.00	114	0.00
Arthropoda	Chilopoda	Geophilomorpha	1	1	1	1	1199	0.02	0	0.00
Arthropoda	Diplopoda	Julida	1	3	2	3	17,891	0.32	55	0.00
Arthropoda	Diplopoda	Polydesmida	1	1	1	1	6475	0.12	902	0.02
Arthropoda	Branchiopoda	Cladocera	2	2	3	3	64	0.00	79,795	1.38
Arthropoda	Hexanauplia	–	0	0	0	0	2182	0.04	0	0.00
Arthropoda	Hexanauplia	Cyclopoida	1	3	3	6	561,729	10.03	388,096	6.73
Arthropoda	Malacostraca	Amphipoda	1	1	1	1	37,993	0.68	5347	0.09
Arthropoda	Malacostraca	Isopoda	4	4	4	4	46,730	0.83	9234	0.16
Arthropoda	Collembola	Entomobryomorpha	3	8	8	11	66,593	1.19	1730	0.03
Arthropoda	Collembola	Neelipleona	1	1	1	1	2362	0,04	0	0.00
Arthropoda	Collembola	Poduromorpha	2	3	3	3	10,194	0.18	0	0.00
Arthropoda	Collembola	Symphypleona	4	5	6	7	8161	0.15	374	0.01
Arthropoda	Insecta	Ephemeroptera	5	7	7	8	225,655	4.03	48,921	0.85
Arthropoda	Insecta	Dermaptera	1	1	1	1	363	0.01	0	0.00
Arthropoda	Insecta	Plecoptera	4	8	10	13	1,123,892	20.07	288,974	5.01
Arthropoda	Insecta	Hemiptera	2	2	2	2	55	0.00	141	0.00
Arthropoda	Insecta	Psocoptera	2	2	2	2	662	0.01	467	0.01
Arthropoda	Insecta	Megaloptera	1	1	1	1	17,975	0.32	0	0.00
Arthropoda	Insecta	Coleoptera	12	24	25	28	166,585	2.97	20,385	0.35
Arthropoda	Insecta	Trichoptera	9	12	9	15	506,892	9.05	144,049	2.50
Arthropoda	Insecta	Lepidoptera	6	7	10	11	7240	0.13	2028	0.04
Arthropoda	Insecta	Diptera	15	41	58	76	996,097	17.78	3,146,972	54.57
Bryozoa	Phylactolaemata	Plumatellida	1	1	1	1	0	0.00	1665	0.03

(Continues)

TABLE 1 (Continued)

Phylum	Class	Order	Families	Genera	Species	"sp."* included	Reads spring	Spring % of total	Reads autumn	Autumn % of total
Chordata	Actinopterygii	Salmoniformes	1	1	0	1	184	0.00	1783	0.03
Cnidaria	Hydrozoa	Anthoathecata	1	1	1	3	6181	0.11	3650	0.06
Cnidaria	Hydrozoa	Limnomedusae	1	1	1	1	0	0.00	4140	0.07
Gastrotricha	Chaetonotoidea	Chaetonotida	1	1	3	4	3075	0.05	2651	0.05
Mollusca	Gastropoda	Littorinimorpha	2	2	2	2	977	0.02	0	0.00
Mollusca	Gastropoda	Pulmonata	2	2	2	2	801	0.01	849	0.01
Mollusca	Gastropoda	Stylommatophora	4	4	7	12	36,229	0.65	1370	0.02
Nematoda	Chromadorea	Rhabditida	1	1	1	1	5605	0.10	0	0.00
Rotifera	Bdelloidea	–	0	0	0	0	8074	0.14	1363	0.02
Rotifera	Monogononta	Ploima	3	3	2	4	796,793	14.23	19,380	0.34
Tardigrada	Eutardigrada	Parachaela	1	2	2	2	299,140	5.34	52	0.00
Total			105	180	212	268	5,601,024	100.00	5,766,625	100.00

to be realistic in terms of habitat and water quality requirements as well as biogeographical constraints. However, we found a total of 12 species unknown to Denmark when compared to the web portal Naturbasen (https://www.naturbasen.dk/), the Danish biodiversity overview project "Allearter" (Skipper, 2017) (www.allearter.dk), and the European species occurrence data project "Fauna Europaea" (de Jong et al., 2014) (https://fauna-eu.org/). These included the sludge worms *Nais alpina* Sperber, 1948 and *Rhyacodrilus subterraneus* Hrabe, 1963 (Annelida: Naididae), the biting midge *Stilobezzia gracilis* Haliday, 1833 (Diptera: Ceratopogonidae), the chironomid midges *Chironomus luridus* Strenzke, 1959, *Chironomus melanescens* Keyl, 1961, *Orthocladius schnelli* Saether, 2004, *Thienemanniella obscura* Brundin, 1947, *Thienemanniella caspersi* Saether, 2003 and *Chaetocladius elisabethae* Makarchenko, 2018 (Diptera: Chironomidae), the gastrotrichs *Chaetonotus borealis* Kolicka, Kotwicki & Dabert, 2018 and *Chaetonotus jaceki* Kolicka, 2017 (Gastrotricha: Chaetonotidae), and the tardigrade *Diphascon higginsi* Binda, 1971 (Tardigrada: Hypsibiidae). The chironomid species (except *T. obscura*) might also be new to Fennoscandia (Lindegaard, 1997). All species were identified with a 100% sequence match. Importantly, BOLD database representation of all con-generic Danish species was checked, which in all cases, except for *Chaetonotus* and *Diphascon*, confirmed that BOLD was complete (*Nais pseudoptusa* not in BOLD but checked in Genbank). As for the gastrotrichs and the tardigrade, there is very limited knowledge on the actual diversity and distribution and we, therefore, regard their presence at these locations as highly uncertain.

Finally, eDNA from several terrestrial taxa were found in the eDNA data, such as species of ground beetles (Carabidae), rove beetles (Staphylinidae), springtails (Collembola), spiders (Araneae), harvestmen (Opiliones), millipedes (Diplopoda) etc. (Table 1, Table S1).

3.4 | Seasonal differentiation in community composition

We found a major difference in the relative dominance of taxonomic orders (based on read abundance) between spring and autumn eDNA samplings (Table 1). While Plecoptera eDNA reads were the most abundant in the spring data (20.07% of metazoan reads), Diptera and Haplotaxida largely dominated the sequencing output from the autumn data, accounting for 54.57% and 27.50% of the metazoan reads, respectively. The CAP analysis on presence/absence data from all taxa also clearly separated the two seasons for all sampling sites. While the sampling sites Boege and Borre formed two somewhat distinct clusters (Figure 2a), the samples from Faebaek, Jeks, and Tjaer overlapped. When only including aquatic taxa, the signal was very similar, with the exception that the autumn samples from Jeks were more similar to the spring samples from Faebaek and Tjaer than they were to the spring samples of Jeks (Figure 2c). Notably, the autumn samples from Boege deviated more from the other samples than when including all taxa, suggesting that this distinctness is primarily explained by the composition of aquatic taxa (Figure 2c). The

abundance data (Figure 2b,d) provided an overall similar distinction of samples based on both seasonality and locality, but with Boege grouping much closer to the other localities than for presence/absence data (Figure 2a,c). The clustered heatmap revealed a grouping similar to that of the CAP analysis, showing that site specificity was a strong factor for Boege and Borre, but in the cluster analysis seasonality divided Tjaer, Faebaek, and Jeks into two different clusters representing spring and autumn data, respectively (Figure 3).

3.5 | Complementarity of eDNA metabarcoding data and traditional kick sampling

Venn diagrams of all aquatic taxa from both eDNA and NOVANA kick samples indicated a general overlap between the two methods (Figure 4). When only including aquatic taxa, we found a total of 118 species using eDNA (27 of which are also found in NOVANA kick sampling), and an additional 26 species found only in NOVANA, giving a total of 144 taxa (Figure 4). For aquatic taxa, between 5 and 16 species per site were shared between eDNA spring data and NOVANA data (collected within two weeks of each other) (Figure 5). eDNA from the spring sampling consistently yielded higher species richness than NOVANA data did. Autumn eDNA sampling yielded fewer taxa, but several were not detected in the spring by either NOVANA or eDNA sampling. Thus, the autumn eDNA sampling provided between 4 and 17 additional species across all sampling sites. In general, taxa lists based on eDNA and kick samples became increasingly dissimilar with increasing taxonomic resolution (from phylum to species level), most likely partly explained by the different taxonomic resolution reached with the two methods. Similarly, Venn diagrams of only EPT taxa showed some overlap between methods, but also an increasing dissimilarity between the two methods as taxonomic resolution increased (Figure 6). At two of five sampling sites (Jeks and Faebaek), no additional EPT taxa were found by including the autumn eDNA sampling compared to only using the spring sampling, while at Tjaer, Boege and Borre, one, two and three additional EPT taxa were found in the autumn eDNA sampling, respectively (Figure 6).

4 | DISCUSSION

Environmental DNA analyses of freshwater communities are increasingly being developed and implemented in aquatic research – especially for macroinvertebrates in lotic ecosystems. The current study demonstrates that eDNA metabarcoding provides additional insight of true macroinvertebrate diversity in streams. Furthermore, an eDNA sampling covering both spring and autumn was found to add considerably to the number of detected taxa compared to a single season of sampling (Figure 4), as well as elucidating an important seasonal difference in community composition. Comparison with a monitoring dataset based on traditional kick sampling revealed important complementarity of the two approaches.

4.1 | Seasonally differentiated communities

Stream macroinvertebrate species vary in phenology and thus seasonal abundance (Füreder et al., 2005; Soulsby et al., 2001; Šporka et al., 2006). Lending further support to existing studies, our results clearly showed that the community composition of stream macroinvertebrates inferred using eDNA strongly differed between spring and autumn (Figure 2). Spring sampling consistently detected more taxa compared to autumn sampling at both family and species level (Figures S7 and S8). Cluster analysis indicated that season might be a more important factor for determining community composition than sampling site for three of the five localities (Figure 3). However, for the sampling sites Borre and Boege, samples clustered together first and foremost according to their respective sampling site. The distinctness of the Boege samples aligns well with the fact that Boege has a lower fauna class than the remaining localities (DSFI score of 4 vs. 7). While Borre had the same DSFI score as Tjaer, Faebaek, and Jeks, it appeared to have a different species composition compared to the latter three localities, as site specificity outweighed seasonality in the clustering of this stream. The clear separation from other sampling sites can be explained by, among others, the presence of species such as the mayfly *Heptagenia sulphurea* Muller, 1776 and the chironomid *Tanytarsus brundini* Lindeberg, 1963, which are only found in this locality. It has been documented previously in other ecosystems that seasonality in community composition can be studied using eDNA (Buxton et al., 2018; Sigsgaard et al., 2017), but this has not, to our knowledge, been addressed for running waters so far.

The eDNA presence/absence data appeared to better distinguish sampling sites according to site specific DSFI scores compared to read abundance data (Figure 2). Overall, both presence/absence and abundance-based data provided a clear signal from both the sampling site and the seasonality. This supports Buchner et al. (2019), who found that incorporating eDNA abundance data performed highly similar to presence/absence data with regards to assigning ecological status classes, although they used bulk samples and not water samples. However, the abundance data from this study did show an interesting transition from a dominance of Plecoptera (primarily Nemouridae) in the spring sampling (20.07% of reads) to a complete dominance of Diptera (primarily *Simulium* spp. and Diptera spp.) and Haplotaxida (primarily Naididae sp.) in the autumn (54.57% and 27.50%, respectively (Table 1, Table S2). This could suggest that there is (a) an increase in dipteran and haplotaxid abundance or biomass in the autumn or (b) plecopterans and other spring-abundant taxa are less abundant or comprise less biomass in the autumn or (c) an effect of both (a) and (b). Šporka et al. (2006) similarly found a much higher abundance of Diptera during October compared to spring. However, they also found an increase in Trichoptera and Ephemeroptera abundance when comparing October sampling to spring sampling – a trend not visible in our data. These are interesting findings that warrant additional investigation of relative eDNA read abundances and which, if supported by collections of physical specimens during autumn sampling, have their merits for furthering

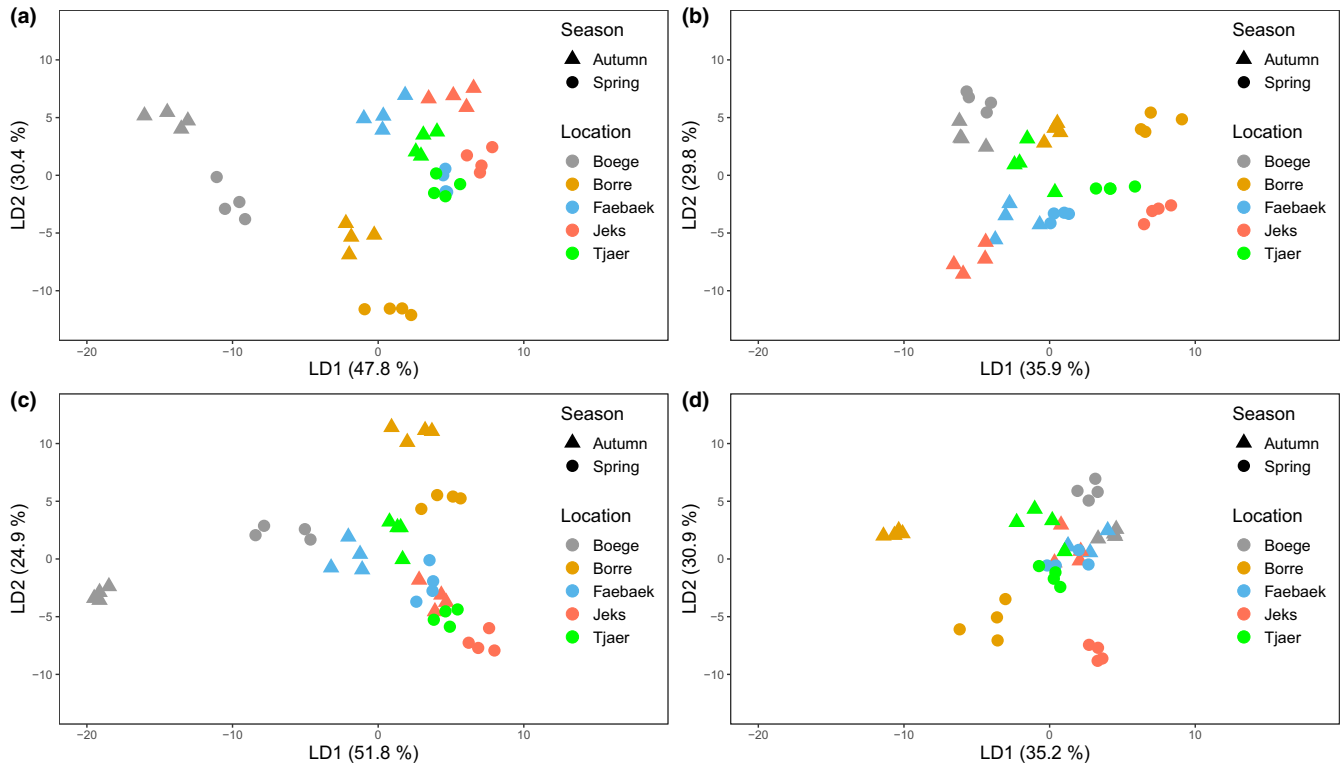

FIGURE 2 Canonical analysis of principal coordinates (CAP) based on linear discriminant analysis (LDA) of all eDNA samples across localities and seasons sampled. LD1 and LD2 represent the two eigenvalues explaining the most variance in the data. (a) All data included, presence/absence data, (b) all data included, abundance data, (c) only aquatic taxa, presence/absence data, and (d) only aquatic taxa, abundance data

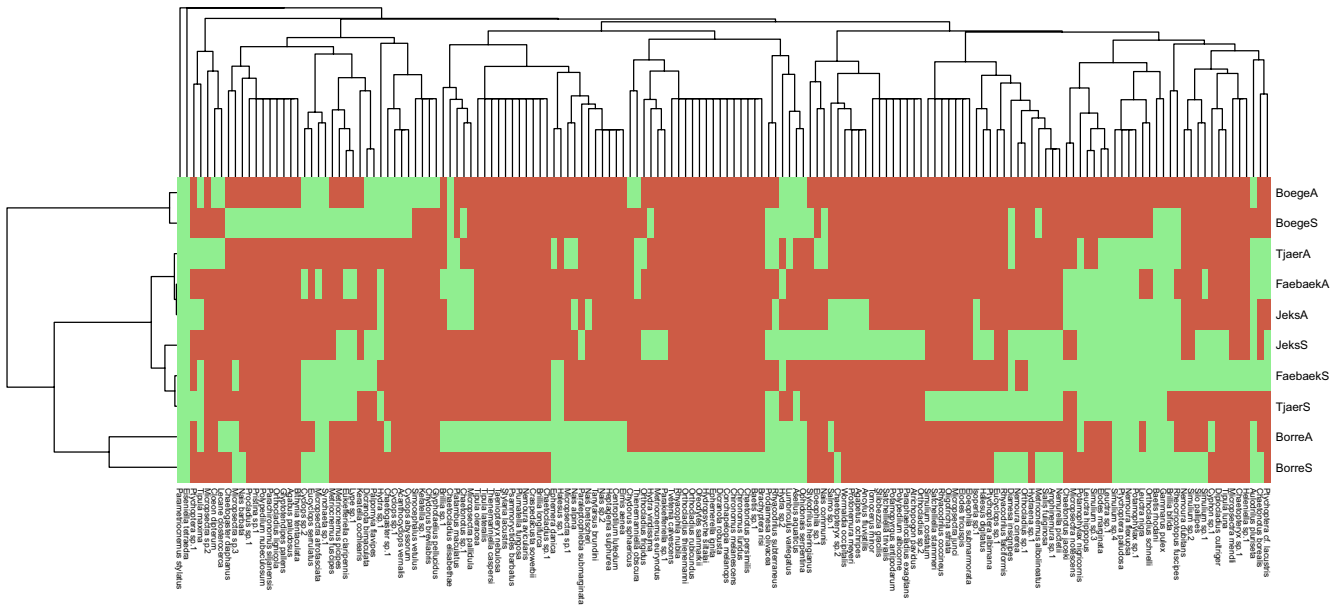

FIGURE 3 Clustered heatmap of all aquatic taxa found (green = present, red = absent) at the five sampling sites across the two seasons using eDNA metabarcoding. The locality suffixes "S" and "A" denote spring and autumn data, respectively

our understanding of stream macroinvertebrate ecology through eDNA methods.

While previous studies have shown reliable correlations between biomass and read abundances from bulk metabarcoding (Elbrecht & Leese, 2015), there is little knowledge on how eDNA samples perform in representing abundance signals of stream macroinvertebrates. Furthermore, aquatic eDNA is affected by discharge and morphological structures (e.g., substrate composition

and aquatic plant dominance), complicating interpretations of DNA origin in the stream systems (Mächler et al., 2019; Stewart, 2019; Wacker et al., 2019). However, Carraro et al. (2020) recently showed that eDNA data coupled with knowledge of hydrology patterns and kick sampling can be used to model and upscale spatial biodiversity inference for both presence/absence and relative abundance of EPT taxa. Integrating knowledge of eDNA data, stream hydrology and modeling tools could have an immense impact on where to place future conservation efforts and in identifying biodiversity hotspots in these freshwater systems. With both existing literature and our results indicating a shift in macroinvertebrate community composition over the year, it seems essential to implement seasonality data into such modelling approaches as well.

In Denmark, stream water assessments are routinely carried out once a year in the late winter or spring. While there is accumulating evidence that at least some of the metrics used for ecological assessment of the streams may not be overly affected by seasonality (Šporka et al., 2006; Stark & Phillips, 2009), we argue that seasonal changes in the community composition of macroinvertebrates should be carefully considered when making high-resolution biodiversity assessments.

4.2 | Comparison of traditional kick sampling and eDNA

Generally, we found good concordance between results from eDNA metabarcoding and traditional kick sampling (Figures 4–6, Table S1). For example, the mayfly *H. sulphurea* occurred only at Borre, and was detected with both eDNA and kick sampling. Similarly, the mayfly *Ephemera danica* Muller, 1764 was detected in the same three sampling sites for kick sampling and eDNA spring sampling, and at two of the same localities for the autumn sampling. However, the methods were more concordant at higher taxonomic levels (from phylum to family level), whereas for genera and species level important differences emerged. For instance, the stonefly *Perlodes microcephalus* Pictet, 1833 and the caddisfly *Tinodes pallidulus* McLachlan, 1878, which were detected with kick sampling at two and one sampling sites, respectively, were not detected by eDNA at all. We also found several examples of important indicator taxa that were detected only with eDNA and not with kick sampling, such as the mayflies *Ephemerella ignata* Poda, 1761 and *Cloeon dipterum* Linnaeus, 1761, the stonefly *Taeniopteryx nebulosi* Linnaeus, 1758, and the caddisflies *Wormaldia occipitalis* Pictet, 1834 and *Oligotricha striata* Linnaeus, 1758. However, some of these species undoubtedly represent eDNA input from upstream habitats (springs and ponds) and, thus, not local presence. For aquatic invertebrates in general, more species were consistently obtained using eDNA (Figures 4 and 5). This is not surprising given the limited selection of species included in taxonomic lists of interest for kick sampling surveys (including NOVANA). Non-indicator taxa are consistently neglected in traditional sampling, where the purpose is to monitor ecological status. Thus, several aquatic taxa including seven genera of crustaceans (of which kick sampling only detected *Gammarus*), five genera in Lumbricidae (of which kick sampling only detected *Eiseniella*), eight genera in Naididae (of which kick sampling detected none), as well as the phyla Cnidaria, Bryozoa, Nematoda, Rotifera and Tardigrada were identified using eDNA but not kick sampling (Table S1). Another notable difference between eDNA and kick sampling was the input from terrestrial species to the eDNA samples (Table S1). These are species that live in the surrounding habitat of the streams and their DNA traces most likely originate from specimens that have fallen into the water and thus have excreted eDNA into the stream similarly to the aquatic insects.

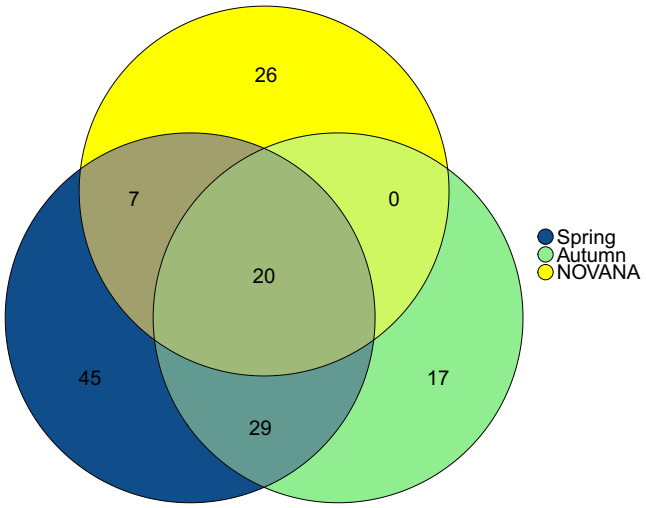

FIGURE 4 Venn diagram showing overall species overlap of aquatic taxa from eDNA spring sampling (Spring), eDNA autumn sampling (Autumn), and NOVANA kick sample data across all the sites sampled in 2018. Note that numbers only include taxa that were identified to species level

Our results altogether indicate that the eDNA and traditional methods are complementary, and that more comprehensive analyses of stream macroinvertebrate communities can be performed using a combination of several approaches across seasons. Gleason et al. (2020) recently found that eDNA water sampling is less congruent than bulk metabarcoding with conventional kick sampling. This is likely connected to the fact that aquatic eDNA detects organisms from a wider area compared to kick/bulk samples (Macher et al., 2018). While this complicates comparisons between the methods, the wider spatial coverage of aquatic eDNA can be useful for upscaling spatial biodiversity inferences, as mentioned in the section above (Carraro et al., 2020).

4.3 | Primer use and database coverage

The large proportion of non-arthropod ASVs found in this study suggests that the applied primers amplify several non-target taxa. More than 90% of the data generated are of non-metazoan origin, a similar amount to that found by Gleason et al. (2020), who used the equally degenerate BF2 and BR2 primers (Elbrecht & Leese, 2017). This is

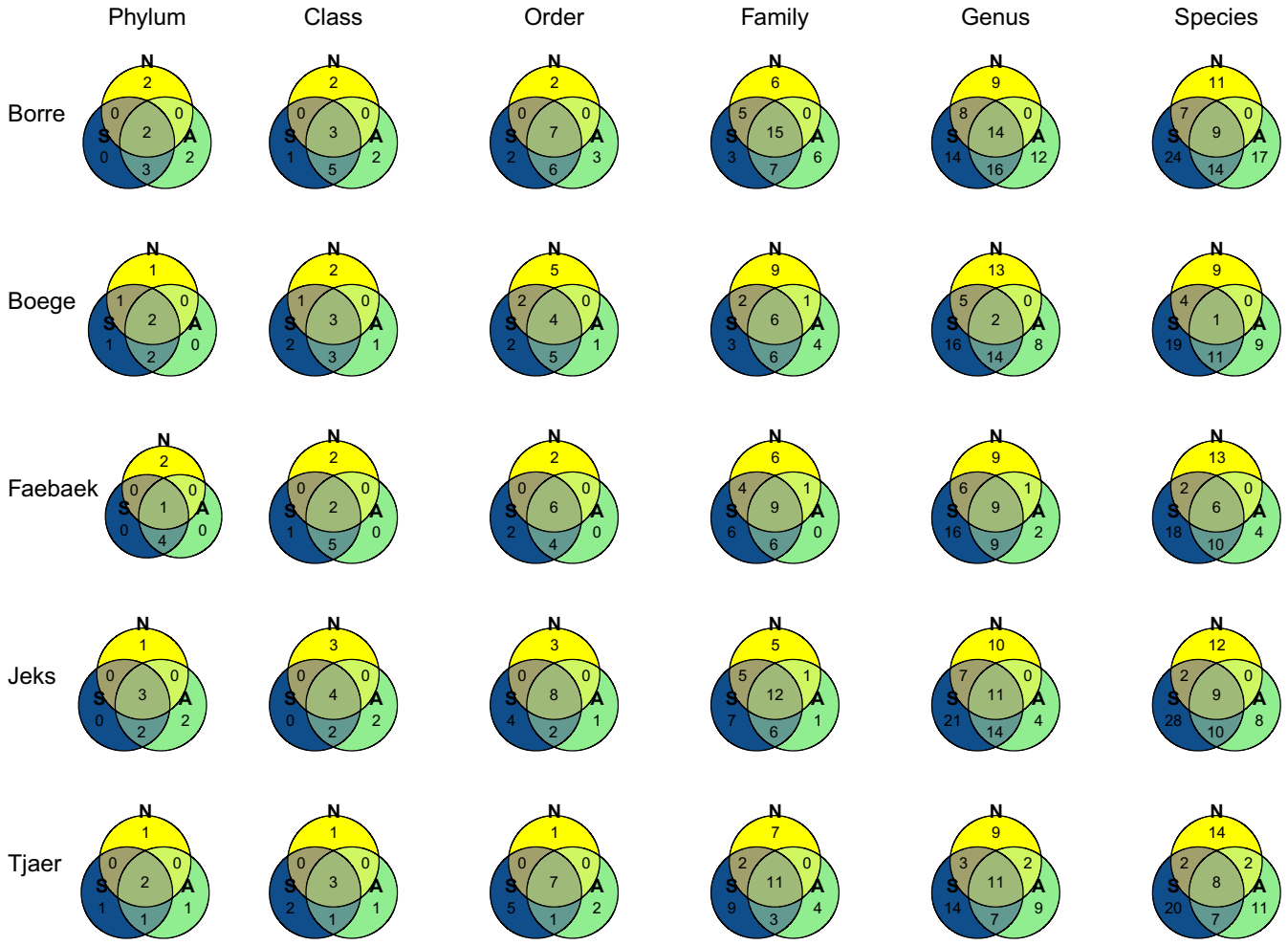

FIGURE 5 Venn diagrams showing overlap between aquatic taxa from eDNA spring (S), eDNA autumn (A), and NOVANA kick sample data for 2018 (*N*) for each of the five sampling sites. Overlap is here presented at phylum, class, order, family, genus and species level, respectively. Note that numbers only include taxa that were at minimum identified to the respective taxonomic levels of each plot

not surprising given that these primer pairs were developed to ensure broad and equal amplification of arthropods across many taxa, with the intent to apply them to bulk arthropod samples (Elbrecht & Leese, 2017). Target specificity of the applied primer set is thus relatively low, but the potential for quantification is good due to very little primer bias (Piñol et al., 2019), owing in part to the primers' highly degenerate design. The presence of 83 families of arthropods across 23 orders in this dataset demonstrates the taxonomic breadth of the primers. With cost-efficiency of high-throughput DNA sequencing rapidly increasing, the low amplification specificity is unlikely to be a major issue for the eDNA approach in the future. Finally, the additional aquatic and terrestrial non-arthropod species found with eDNA might be beneficial in representing a more comprehensive view of stream biodiversity. Indeed, it is continuously becoming more common practice in eDNA studies to target all eukaryotes simultaneously (Guardiola et al., 2015; Leray et al., 2013; Wangensteen et al., 2018) for community composition inferences.

While 16S seems to be a promising alternative to COI metabarcoding for inferences on benthic invertebrates (Ficetola et al., 2020), especially regarding primer bias, the amount of missing reference sequences is problematic. The lack of database coverage is a serious impediment for biodiversity studies using eDNA metabarcoding, but the issue is continuously alleviated as more reference sequences are generated. Imperfect taxonomic identification is due to the fact that not all described species have a DNA reference sequence for the particular target fragments, and/or that the specific primers cannot positively discriminate all species. As an example from this study, *Isoperla grammatica* Poda, 1761 can be identified to species level with morphology, whereas eDNA can only identify it to genus level, due to several *Isoperla* species having the same DNA barcode. Furthermore, only an estimated 10% of the species on Earth have been scientifically described, which is important to consider, especially when working in tropical ecosystems with high species richness.

Related to reference database coverage is the challenge of properly identifying the origin of eDNA metabarcoding reads. In the current study, most of the ASVs (60.3% and 52.8%, for the spring and autumn ASVs, respectively) are identified to species level, and we chose a conservative approach of naming the remaining taxa sp.1, sp.2 etc. when an unambiguous identification could not be made.

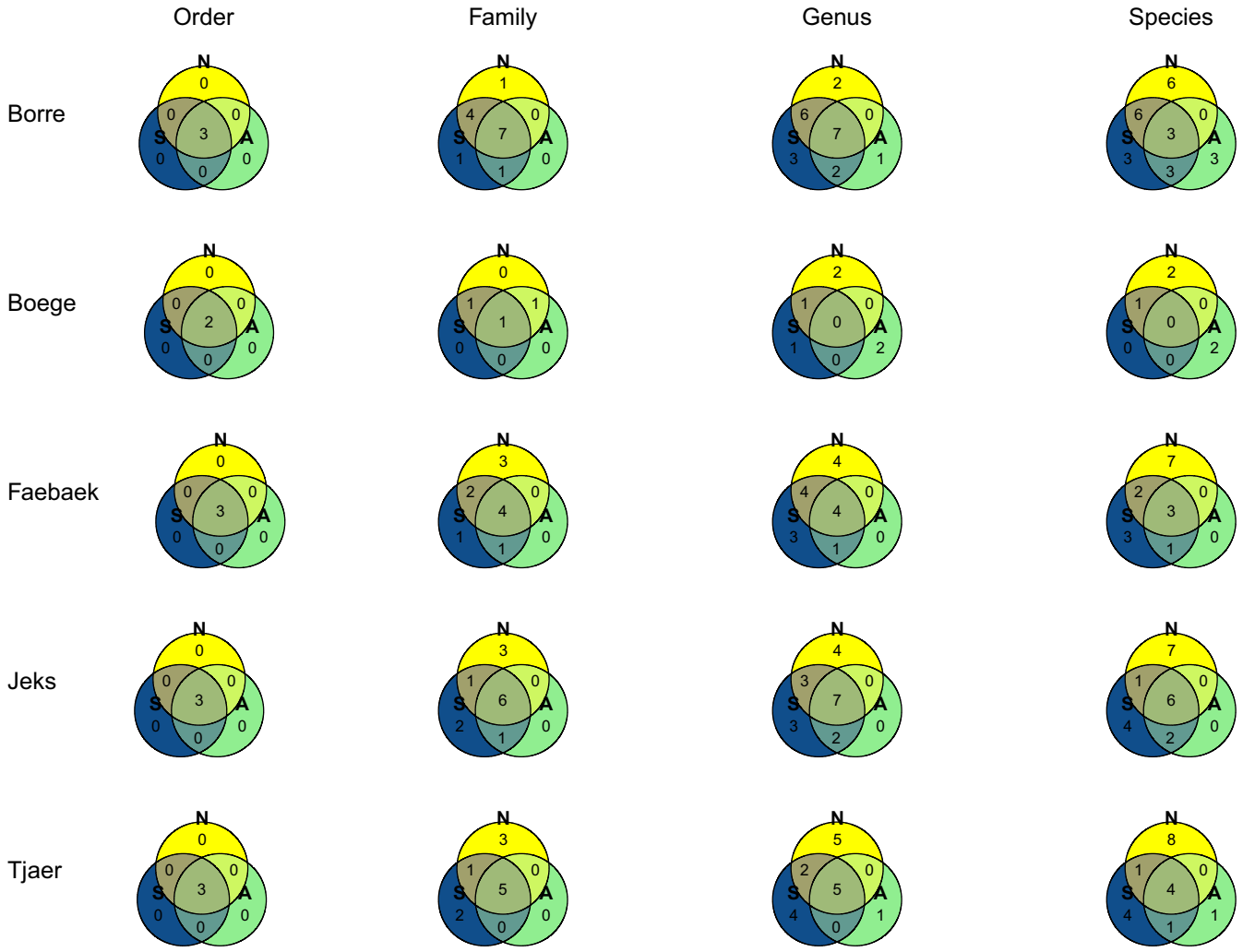

FIGURE 6 Venn diagrams showing overlap between EPT taxa from eDNA spring (S), eDNA autumn (A), and NOVANA kick sample data (*N*) for 2018 for each of the five sampling sites. Overlap is here presented at order, family, genus and species level, respectively. Note that numbers only include taxa that were at minimum identified to the respective taxonomic levels of each plot

While the BOLD database covers an impressive diversity of animal taxa, fully automated taxonomic assignment is hampered by issues relating to synonymy, typos, and identifiers included in species names. Furthermore, single cases of wrongly identified species present in the database may prevent taxonomic assignment due to falsely missing barcode gaps. We believe that taxonomic assignment cannot be fully automated as long as these issues persist, and that blindly trusting database hits should be avoided. However, manual labor can be reduced by focusing on likely problematic ASVs, such as those with no apparent barcode gap between the top matching taxa.

4.4 | Detection of potentially new species to the Danish fauna

We found eDNA traces from 12 species that are 'new' to Denmark according to existing databases of national faunistic records. All the new species found represent species known to occur in neighboring countries (e.g., Germany, Sweden), and represent animal groups where limited taxonomic expertise exists in Denmark (e.g., relatively little work has been done on adult Chironomidae, representing most of the new species, at least in recent time). It is, therefore, certainly possible that these species could occur unnoticed in Denmark. Indeed, most of the new species are represented in low read abundance and in few samples, suggesting that they may not be very abundant. Especially the detected genus *Chironomus* includes many species that have yet to be sequenced for the COI barcode, meaning that current species-level identifications may turn out to be ambiguous (if other species in the genus have the same barcode sequence). Importantly, the BOLD database contains public barcodes for all the species found in the NOVANA data except one (*Epoicocladius ephemerae* Kieffer, 1924, Diptera: Chironomidae). Presence of all Danish con-generic species was confirmed in BOLD for the 12 'new' species, and the database was complete for all con-generic species except for *Chaetonotus* (Gastrotricha) and *Diphascon* (Tardigrada). Altogether, this strongly indicates that database completeness is a

negligible issue in interpreting comparisons between the two methods and for detection of unexpected taxa (Figures 4–6). Nonetheless, our findings warrant further investigation and we stress the need for physical confirmation of specimens, before the presence of these new Danish species can be finally confirmed.

4.5 | Perspectives for freshwater ecology research and biomonitoring

In order to halt the alarming rates of freshwater biodiversity declines (Reid et al., 2019; Vörösmarty et al., 2010), efficient biomonitoring tools are pivotal. Sampling aquatic eDNA can provide non-invasive complimentary insights into the species composition of macroinvertebrates in streams. eDNA methods can also add an additional layer of information from the sampled streams, as they detect DNA from species occurring in upstream parts of the stream system, as well as terrestrial species in the surrounding areas. Importantly, eDNA sampling can simultaneously retrieve biodiversity information on indicator species and on more neglected aquatic species that are not used for ecological indices, and which may otherwise be completely overlooked.

While eDNA sampling is less congruent than bulk metabarcoding with conventional kick sampling (Gleason et al., 2020), water sampling can still be used as an easily standardized method for temporal and spatial inference on important ecological metrics used for streams. This can be done in connection with conventional kick sampling, but perhaps also as a standalone index, as has been suggested for marine macroinvertebrate monitoring using bulk metabarcoding (Aylagas et al., 2014). There are still many avenues to be explored for macroinvertebrate eDNA detection, such as taxonomy-free approaches with machine learning as has been done with diatom eDNA metabarcoding (Apothéloz-Perret-Gentil et al., 2020), and eDNA sampling could even help alleviate the need for multiple different sampling events, as the same sample can be used to infer multiple stream indices across different taxa (Maitland et al., 2020).

Seasonal changes in macroinvertebrate community composition related to species phenology is a well-known phenomenon (Füreder et al., 2005; Šporka et al., 2006), yet poorly addressed due to the monetary resources and methodological standardization needed for conducting extensive time series surveys. We here show that community composition detected with aquatic eDNA in streams vary with seasonality, and demonstrate changes in taxon dominance over the year by using relative read counts. If applied in connection with knowledge of local hydrology and modeling approaches, eDNA approaches can help provide an informed baseline for future conservation efforts in lotic ecosystems. Seasonal sampling may furthermore be important for understanding climate-mediated phenological shifts (Füreder et al., 2005; Thomsen et al., 2016) and could potentially be coupled with automated sampling throughout the year (Hansen et al., 2020) in more standardized monitoring programs. In addition, intraspecific variation can be detected using eDNA (Elbrecht et al., 2018; Jensen et al., 2021; reviewed by Sigsgaard, Jensen, et al., 2020), enabling a better understanding of the impact of environmental stressors, as demonstrated by Zizka et al. (2020) using bulk sampling of macroinvertebrates. This would be a major advantage of the eDNA method compared with conventional kick sampling and morphological identification.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

MRJ, AB-P, JJR and PFT conceived the ideas for the project. MRJ, EES and PFT carried out sampling. MRJ analyzed the eDNA data with input from EES, SA and PFT. MRJ analyzed the monitoring data with input from AB-P, JJR, PW-L and PFT. MRJ and PFT led the manuscript writing with input from all authors. All authors contributed to the manuscript drafts and approved the final version.

DATA AVAILABILITY STATEMENT

The raw sequencing data along with files for demultiplexing are available on Dryad. The data can be accessed via https://doi.org/10.5061/dryad.1zcrjdfrc. Any enquiries should be directed at the corresponding author.

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SUPPORTING INFORMATION

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

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