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Original Articles

Stream water quality assessment by metabarcoding of invertebrates

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ABSTRACT

Stream water quality assessments are conducted by analysing invertebrate communities as a biological quality element (BQE). In Denmark, water quality assessments of streams are often estimated according to the Danish Stream Fauna Index (DSFI). The conventional DSFI method is time consuming and requires highly specialized expertise for species identification of the relevant indicator invertebrates. Furthermore, conventional species identification of relevant indicators may be hampered by differences in, or lack of, developmental stages or due to damages during the sampling process. Metabarcoding has the potential to overcome the challenges associated with conventional morphology-based species identification. Using high-throughput DNA sequencing, metabarcoding of invertebrates collected from stream water provides an alternative to the expertise of taxonomic experts. The present study applies metabarcoding using universal invertebrate primers targeting the mitochondrial cytochrome oxidase I (*COI*) gene to determine stream water qualities. The obtained community profiles were compared to conventional water quality assessments according to the Danish Stream Fauna Index (DSFI). Multivariate data analysis of obtained sequences resulted in distinct clusters of taxonomic units, which reflected the stream water quality as defined by the DSFI. In conclusion, the present study supports the knowledge that invertebrates are efficient as BQE for stream water quality assessment. DNA sequencing by metabarcoding provided a unique fingerprint of the studied communities of invertebrates and was successful in describing the stream water quality.

1. Introduction

Freshwater ecosystems are under pressure due to various anthropogenic influences such as pollution, exploitation, eutrophication, habitat degradation and introduction of invasive species. Standardized assessment methods for biodiversity are essential for maintaining healthy ecosystems (Dudgeon et al., 2006; Elbrecht and Leese, 2017). In 2000, the European commission adopted the Water Framework Directive (WFD) 2000/60/EC, and it was declared that surface-water systems must be in good ecological status. Monitoring the quality of the surface water ecosystems is a prerequisite to fulfil this goal (Birk et al., 2012; Voulvoulis et al., 2017). The WFD demands all European surface waters to reach a so-called “good ecological status”, which is defined by non or minor anthropogenic influences (Stubbington et al., 2017; Voulvoulis et al., 2017). To meet these provisions all EU-member states must implement frequent assessment of surface waters and relevant management strategies to obtain the good ecological status. Assessment methods for surface water differ within the EU, and different physical, chemical and biological quality elements are used in the applied assessment methods (Birk et al., 2012; Stubbington et al., 2017). Chemical quality elements reflects only a momentary snap shot of the water conditions, whereas biological quality elements (BQE) may reflect the long-term water quality (Marchant et al., 2006). Examples of BQE include benthic invertebrates, plants, phytoplankton and fish (Birk et al., 2012). Macroinvertebrates are commonly preferred BQE, as these organisms provide a good indication of ecosystem health in freshwater systems, due to their sensitivity to environmental stressors such as sediment conditions and nutrient levels, which makes them sensitive indicators (Birk et al., 2012; Elbrecht and Leese, 2017; Macher et al., 2016). Assessment methods using invertebrates are based on the collection, sorting and morphological identification of a large number of taxonomic groups. These sorting and identification steps are based on human experience; and results may vary between different laboratories (Haase et al., 2010). Moreover, the conventional procedure is considered to be time consuming, labour intensive and demands skilled taxonomists who are able to identify the relevant invertebrate species

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(Pfrender et al., 2010). Additionally, invertebrates inhabiting streams are mostly in larval stages and some groups do not show sufficient morphological characteristics for identification to species level (Pfrender et al., 2010). Furthermore, specimens which are damaged during the collection process might not be possible to identify (Pfrender et al., 2010). Even though many stream water assessment methods only apply identification of invertebrates to family or genus level, it has been shown that species within the same genus can respond differently to environmental stressors, as shown for e.g. the mayfly genus *Deleatidium* (Macher et al., 2016). Moreover, the decline of taxonomic experts motivates the development of alternative approaches for the quality assessment of stream waters (Elbrecht and Leese, 2017).

In Denmark, stream water quality assessment is often based on macroinvertebrates as BQE. Assessment is conducted according to the Danish Stream Fauna Index (DSFI, Danish: Dansk Vandløb Fauna Indeks – DVFI) (Skriver et al., 2000). The DSFI system divides the stream water quality into seven categories (1–7), with 7 representing the best quality. DSFI categories 5, 6 and 7 are all considered "good ecological status" according to the definition in the WFD. DSFI samples are collected by so-called kick-samples, containing sediments, plant material and benthic invertebrates. The invertebrates are subsequently identified to different taxonomic levels, quantified, and classified into six indicator groups used for calculation of the DSFI categories (for detailed description see Skriver et al., 2000).

The development of next generation sequencing technologies (NGS) has provided a potential alternative to morphology based species identification (Aylagas et al., 2014; Elbrecht and Leese, 2017). High-throughput sequencing enables simultaneous sequencing of a short DNA fragment (barcode) which shows sufficient resolution between species and conserved flanking regions. Sequencing entire communities is termed metabarcoding (Aylagas et al., 2014) and has been widely applied for identifying species composition of eDNA and bulk samples (e.g. Hajibabaei et al., 2011; Valentini et al., 2016; Yamamoto et al., 2017). The usage of DNA-based identifications for ecological quality assessment has been identified as having high potential for implementation within the WFD (Hering et al., 2018; Pawlowski et al., 2018). The mitochondrial gene cytochrome *c* oxidase I (*COI*) has been introduced as a barcode for animals and is widely used in metabarcoding studies (Aylagas et al., 2014; Elbrecht and Leese, 2015; Hebert et al., 2003). It has been shown that metabarcoding using the proposed barcode delivers comparable quality assessment results to morphological based approaches (Aylagas et al., 2016a; Elbrecht et al., 2017b). Nevertheless, these approaches included separation of invertebrates from sediment and plant material in the sample, which does not reduce the time-consuming sorting aspect.

Here, we present a comparative study between conventional DSFI assessed stream water qualities and metabarcoding without introducing a pre-sorting step. The extracted DNA was subjected to amplicon sequencing of the mitochondrial *COI* gene, and analysed using multivariate statistics. Finally, the collected sequences were compared to the conventional morphological approach.

2. Material and methods

2.1. Sampling and conventional quality analysis

Sampling and conventional analysis were conducted in collaboration with the Laboratory of Fish Ecology (Fiskeøkologisk Laboratorium, Helsingør, Denmark) within their surveillance projects according to the DSFI protocol, which is based on a biotic as well as an abiotic assessment (Skriver et al., 2000). A total of 59 samples from 53 different streams in Denmark were sampled (Fig. 1, Table S1) using the kick-sampling method (Bradey and Ormerod, 2002). DSFI samples were decanted using a sieve (mesh size 0.5 mm) and big stones were removed by hand. During the analysis all biotic and abiotic material was kept and recombined once the DSFI category had been determined by an experienced taxonomist. Samples were preserved in 96% ethanol until molecular proceedings were carried out.

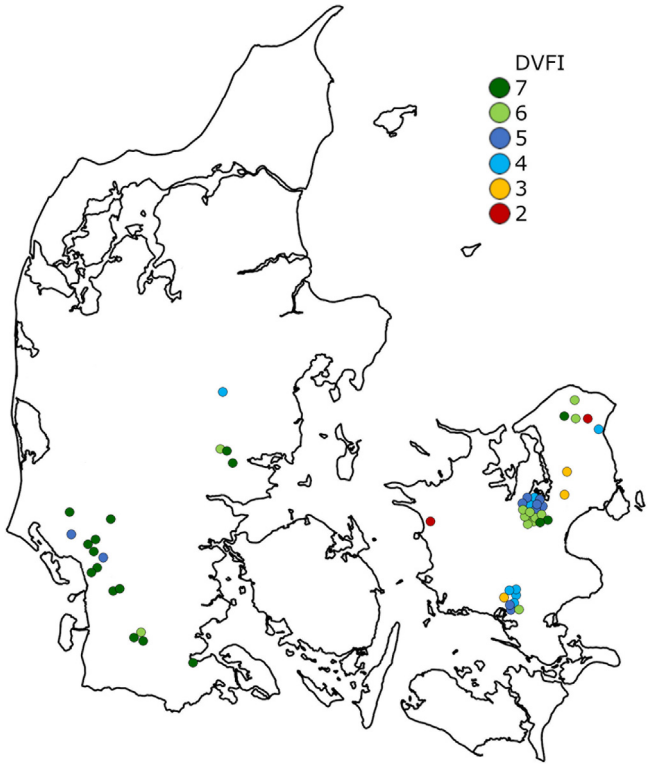

Fig. 1. Locations of the Danish streams included in the study. Samples are coloured by their DSFI category as determined by the conventional method.

2.2. DNA extraction

DSFI samples and sorted biomass were combined and homogenized using a blender (JB 5160 BK, Braun GmbH). To avoid warming of the sample in the homogenizing process, short intervals of 10 s were performed (speed setting 3) until homogenization was completed. Blender and sieve were thoroughly washed between samples and decontaminated by RNase AWAY (Thermo Fisher Scientific). A subsample of each homogenized DSFI sample was transferred to a 50 mL centrifuge tube and immediately stored at −18 °C until DNA extraction. Six subsamples from the 50 mL tube were taken for DNA extraction. DNA was extracted using the QIAamp PowerFecal DNA Kit (Qiagen) according to the manufacturer's protocol. Final elution was conducted in 50 µL elution buffer. DNA concentrations were measured on an Infinite M200 PRO plate reader (TECAN) using Quant-iT BR DNA assay (Thermo Fisher Scientific).

2.3. Amplicon preparation and sequencing

Amplicons of the *COI* gene for each sample were prepared using universal primers mICOIntF (5′-GGWACWGGWTGAACWGTWTAYCCYCC-3′) (Leray et al., 2013) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994) fused with Illumina adapters. Amplicon PCR reactions were carried out in duplicates with a final volume of 25 µL. Each reaction contained 1 µM of each primer, 0.75 mM of $MgSO_4$, 400 nM of each dNTP, 2 mU of Platinum Taq DNA polymerase, 1X Platinum High Fidelity buffer (Thermo Fisher Scientific) and 10 ng of template DNA. Cycling conditions were as follows: initial denaturation at 95 °C for 2 min, 30 cycles of 94 °C for 30 s, 51 °C for 30 s, 68 °C for 1 min and final elongation at 68 °C for 5 min. After pooling of duplicated PCR products, purification was performed using Agencourt AMPure XP beads (Beckmann Coulter) with a bead/sample

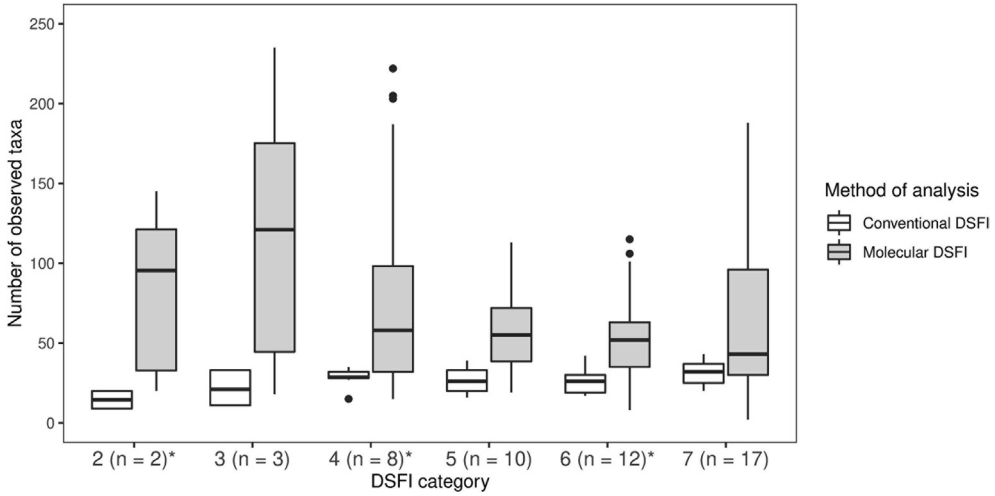

Fig. 2. Observed number of taxa in conventional and molecular DSFI, by category. Only samples where the molecular and conventional data was available were included in the analysis, categories where locations were dropped are marked with an asterisk (*).

ratio of 0.8 according to the manufacturers' protocol. DNA concentrations were evaluated by a Infinite M200 PRO plate reader (TECAN) using Quant-iT HS DNA assay (Thermo Fisher Scientific), while DNA quality was assessed by visualization using the Agilent 2200 TapeStation in combination with D1000 ScreenTape Assay according to the manufacturer's protocol (Agilent Technologies). Subsequently, the obtained amplicons were barcoded with unique Illumina Nextera XT adapters according to manufacturer's protocol (Illumina). Equimolar concentrations of each library were pooled and sequenced on a MiSeq platform using reagent kit v3 (2 × 300 PE, Illumina). An additional positive control of known content was sequenced alongside the sample pool and compared to previous runs to monitor the sequence quality.

2.4. Bioinformatic processing

Raw sequence reads were processed and clustered into ZOTUs using the AmpProc pipeline version 5.1 (https://github.com/eyashiro/AmpProc), without taxonomy assignment. Subsequently, all ZOTUs with at least 10 reads across all samples were taxonomically classified. Taxonomy was initially assigned by BLAST (Altschul et al., 1990), using blastn to extract the best hit, with a minimum e-value set to $1e^{-100}$. Subsequently, manual curation of the obtained taxonomic assignments was performed using additional BLAST searches and comparisons to reference sequences of Danish freshwater invertebrates. Taxonomic assignment limits were applied as described in the "JAMP" pipeline (https://github.com/VascoElbrecht/JAMP). A genus and species classification is given for all ZOTUs with ≥ 98% sequence similarity to the reference sequence, the genus level of the closest relative is given at ≥ 95% sequence similarity, family level for ≥ 90% sequence similarity, and an order level taxonomic assignment is given for ≥ 85% sequence similarity. All ZOTUs with < 85% sequence similarity to reference sequences could not be given a meaningful classification and the reads associated with these were removed from the dataset (n = 43,193, 2.47% of total generated reads).

2.5. Data analysis

Comparative data analysis was performed using R (version 3.5.3) (R Development Core Team, 2015) and Rstudio (version 1.1.463) (RStudio Team, 2015). The R package ampvis2 (Andersen et al., 2018) was used for conducting canonical correspondence analysis (CCA) to visualize dissimilarities between samples based on DSFI classification. A hierarchically clustered heatmap was rendered using gplots (version 3.0.1) (Warnes et al., 2019), using Bray-Curtis distances and ggplot2 (version 2.2.1) (Wickham, 2016) was used to generate all other visualisations.

2.6. Data availability

The raw sequencing data has been made available at the European Nucleotide Archive (ENA) under project accession number PRJEB31952.

3. Results

3.1. Conventional quality analysis

In total 59 sampling points distributed among 53 streams throughout Denmark were examined (Fig. 1). A total of 34 sampling points were classified as good water quality (DSFI category 6 and 7), 19 were assigned medium water quality (DSFI category 4 and 5) and 6 were determined to low water quality (DSFI category 2 and 3) (Table S1).

3.2. Sequence quality

A total of 1,750,431 reads were sequenced across molecular DSFI samples from 59 locations, with an average of 30,180 ± 35,800 sequences per location. Only locations which had yielded 1,300 sequences or more from the collective replicates were included in the analysis. Overall, 335 sample replicates covering 57 sampling points and 52 streams were sequenced to adequate depth, as shown by rarefaction curves (Fig. S1).

3.3. Identification of stream macroinvertebrates by conventional and molecular DSFI

A total of 145 unique invertebrate species and taxonomic groups were identified by the conventional method and 435 taxonomic groups by the molecular approach. On average, 27 (± 8) species were detected per DSFI sample by the conventional method, while an average of 67 (± 47) taxonomic groups were identified by the molecular approach (Fig. 2). Overall, the greatest difference in individual species detections was observed in the lowest DSFI categories (2 & 3), and the detected number of species was most similar in the higher DSFI categories, with the least variation between samples seen in categories 5 and 6.

Among the 50 most abundantly detected organisms using the molecular method (Fig. 3a), 25 invertebrate species and taxonomical groups were also identified by the conventional approach, as well as 12

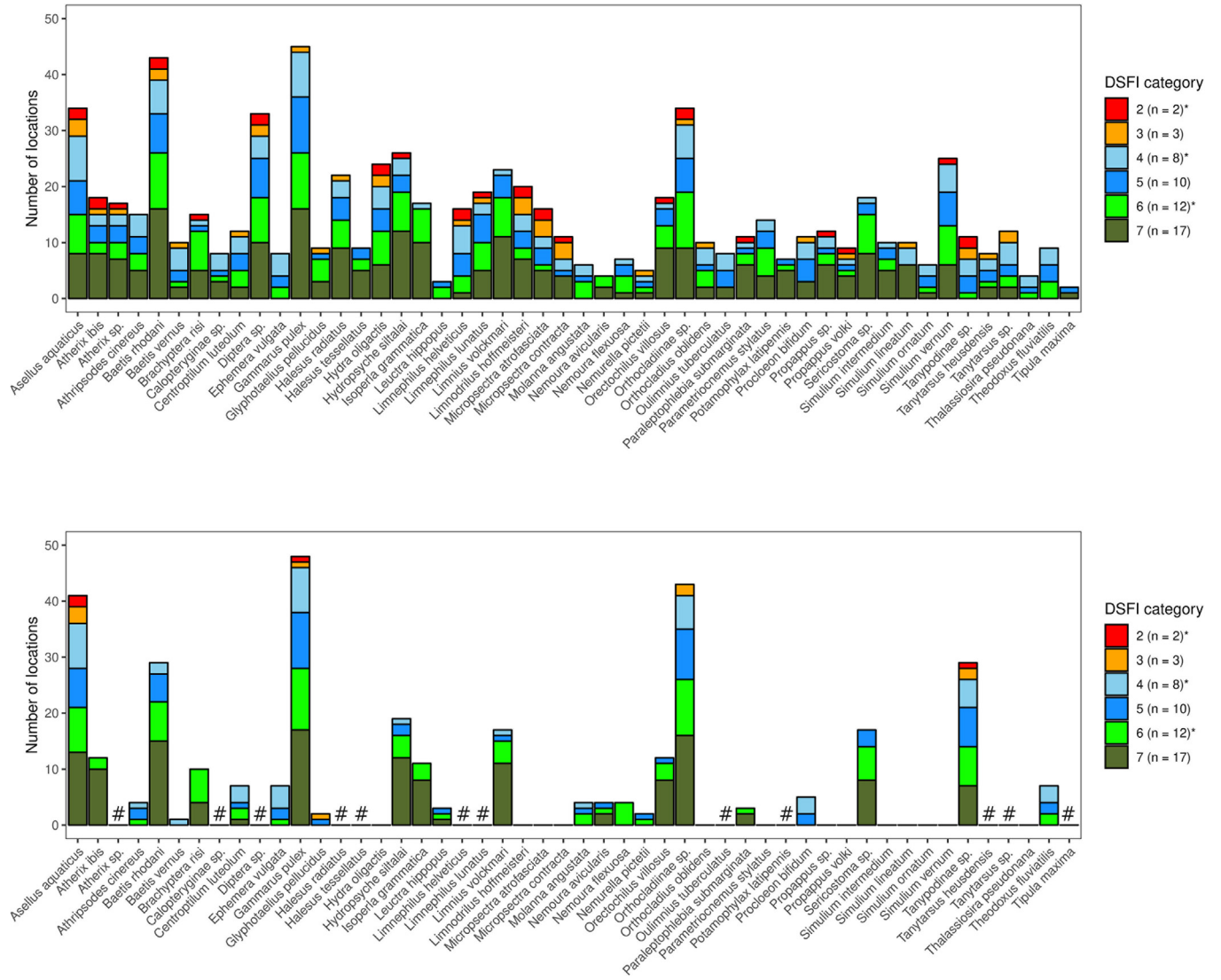

Fig. 3. Occurrence of the 50 most abundantly observed taxa in molecular (a) and conventional (b) DSFI analysis visualised as a stacked bar plot by the number of locations where the organism was observed, coloured by DSFI category as assigned by the conventional analysis. The OTUs in the molecular DSFI were grouped by taxonomic assignment, and organisms where a partial identification was available between the two approaches are marked with a pound sign (#). Only samples where the molecular and conventional data was available were included in the analysis, categories where locations were dropped are marked with an asterisk (*).

partial identifications between both methodologies (Fig. 3b). A notable observation is the presence of the abundantly detected organisms with the molecular method across all sampled DSFI categories, many of which were observed in at least 10 individual locations, but this trend was not apparent in the conventional analysis data. The number of sequences generated for the 50 most abundant organisms using the molecular method did not show a linear relationship with the number of locations the organisms were observed in, nor the DSFI category they were detected in. An overview of the distribution of sequences detected per category per organism is shown in Fig. S2.

The most abundantly detected organisms with both methodologies included representatives of *Crustacea* (*Gammarus pulex*, *Asellus aquaticus*), *Trichoptera* (*Sericostoma* sp., *Hydropsyche siltalai*) as well as members of *Chironomidae* (*Tanypodinae*, *Orthocladiinae*). Organisms exclusively detected by the molecular approach included *Simulium vernum*, *Propappus* sp., *Limnephilus lunatus* and *Limnodrilus hoffmeisteri*.

Canonical correspondence analysis (CCA) using the conventionally assigned water quality category as the constraint revealed clustering of the analysed samples according to their respective categories (Fig. 4a). The samples belonging to locations with DSFI categories 2 and 3 were fully separated into individual clusters, while the samples from DSFI categories 4, 5, 6 and 7 partially overlapped with each other, with the greatest overlap observed in the higher water quality categories.

Generation of CCA models constrained by DSFI category, but subset to the quality categories that were most similar overall (2 & 3, 4 & 5, and 6 & 7, respectively) revealed that separation of the samples within each of the generalised categories was possible. The largest variation difference was observed between categories 2 and 3 (Fig. 4b), while a minor overlap of 1 sampled location (n = 6 replicates) was seen between categories 6 and 7 (Fig. 4d).

3.4. Strong relationship between detected invertebrates and DSFI quality

Hierarchical clustering of the 50 most abundantly observed taxonomic groups revealed relationships between the occurrence and abundance of the individual taxa and the locations they were observed at (Fig. 5). A presence/absence scored heatmap of the same data is displayed in Fig. S3. Similar to what was observed in the ordination analysis (Fig. 4), the samples from the higher water qualities showed overlap in their clustering order, while samples from categories 2 and 3 clustered together separately. Organisms that were observed across most samples in varying abundances included *Gammarus pulex* and

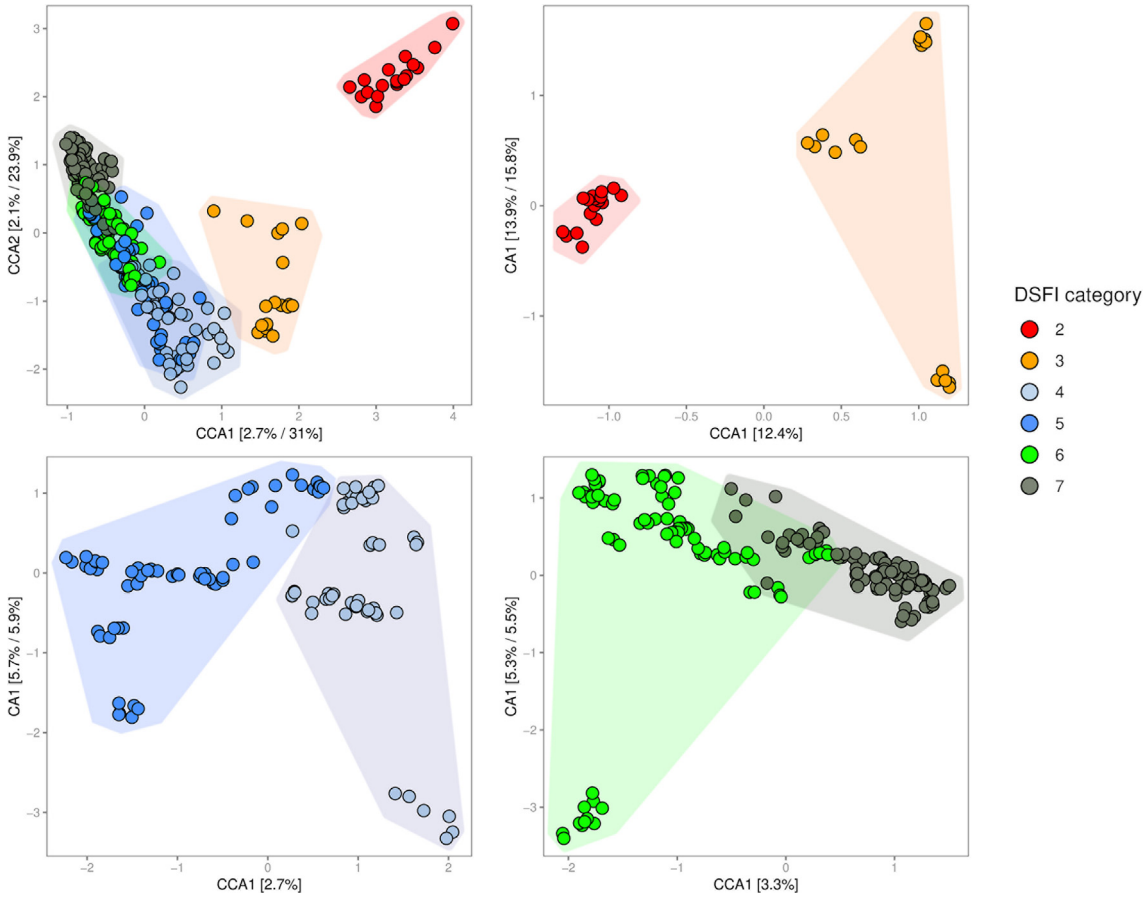

Fig. 4. Molecular DSFI analysis. Canonical correspondence analysis (CCA) of all DSFI samples (a), categories 2 and 3 (b), 4 and 5 (c) and 6 and 7 (d). Samples are coloured by DSFI category as assigned by the conventional DSFI analysis, and a coloured polygon is drawn around samples from the same category.

Diptera sp. The samples from locations with DSFI category 2 showed a cluster of organisms that were almost exclusively found abundantly there, including *Micropsectra atrofasciata*, *Limnodrilus hoffmeisteri* and *Hydra oligactis*. Organisms such as *Baetis rhodani*, *Halesus radiatus*, *Hydropsyche siltalai* and several *Simulium* species were primarily observed in the samples stemming the locations with the highest water qualities, or at least in greater abundance compared to locations of a lower water quality. Clusters limited to a small number of samples were also observed, such as abundant presence of *Oulimnius tuberculatus*, *Ephemera vulgara*, *Theodoxus fluviatilis* and *Procloeon bifidum* in a smaller number of samples from DSFI categories 4 and 5.

In order to investigate if the identified invertebrates could be resolved to specific categories, and thus be of potential importance for the molecular water quality assessment, Venn diagrams were generated for the occurrence of abundant organisms (> 0.1% of total abundance in a sample) for samples from adjoining DSFI categories (data not shown). ZOTUs that occurred in at least 51% of all replicates representing a single category were extracted and summarised in Fig. S4. A total of 38 unique ZOTUs with a strong association to a single or more DSFI categories were identified. The figure identifies potential organisms of interest as indicators for the 6 DSFI categories included in the present study. In line with the observed canonical correspondence model, the largest number of organisms associated to a specific DSFI category was observed for the lower water qualities (2 & 3), including *Aulodrilus pluriseta,* two species of *Micropsectra* and *Limnodrilus hoffmeisteri*. The highest water quality categories were associated by taxonomic groups including *Baetis rhodani* and *Hydropsyche siltalai,* which occurred with greater frequency as water quality increased. *Simulium vernum* was found associated most abundantly to categories 4, 5 and 6.

4. Discussion

4.1. Development of molecular methods for species identification

Species identification using molecular techniques such as next generation sequencing has the potential to reduce processing time by high sample throughput. Furthermore, processing several samples at once reduces labour intensity and time spent on sample analysis. Moreover, metabarcoding enables reliable and objective identification to species level, independent of the specimens' life stage (Aylagas et al., 2014). Establishing alternative methods for freshwater assessments is of high interest due to decreasing numbers of experienced taxonomic experts (Elbrecht and Leese, 2017). Molecular approaches have the potential to become an alternative method, while also reducing the processing time and costs for stream water assessment (Hering et al., 2018; Pawlowski et al., 2018). Although the cost for NGS analysis and morphological-based assessments have been assessed to be comparable (Elbrecht et al., 2017b; Stein et al., 2014), this is likely to be different in the near future due to the steadily declining cost for DNA sequencing.

Assignment of proper phylogenetic affiliations of the generated barcodes is dependent on the currently available databases but may be improved by establishing a sequence database of relevant stream macroinvertebrates. The content of invertebrates in the present study covers 59 samples from 53 different Danish streams. All 53 streams were analysed by traditional morphology-based identification and indexed into quality assessment groups according to Danish standards (DSFI). A metabarcoding approach using amplicon sequencing of the mitochondrial *COI* gene on the same samples revealed the presence of a total of 1228 ZOTUs of which 657 could be identified at the species level, and collectively represented 435 taxonomic groups at different

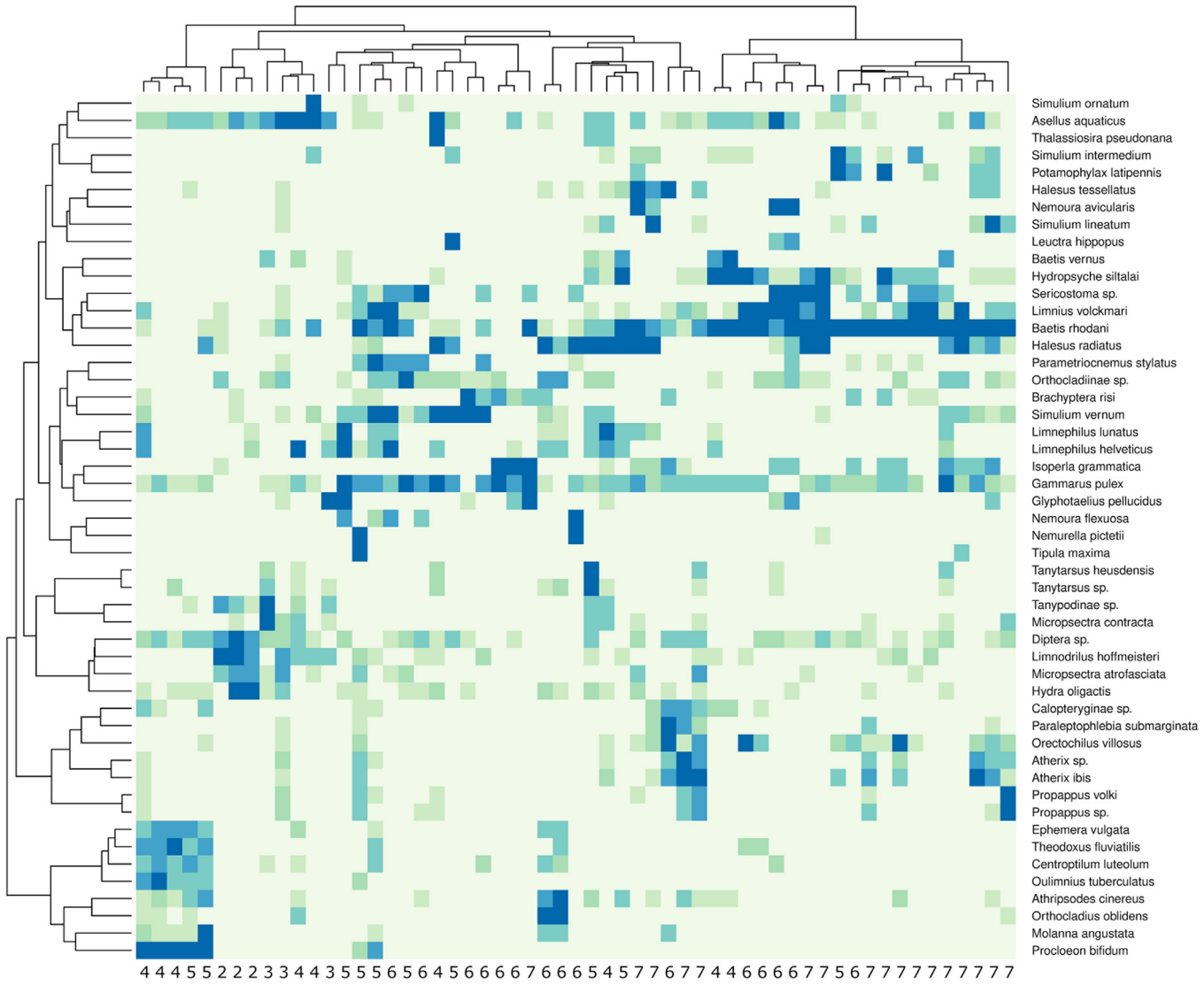

Fig. 5. Hierarchically clustered heatmap of the 50 most abundant taxa detected by molecular DSFI, clustered by Bray-Curtis distances for detected species and locations. The highest possible taxonomic classification of each group is displayed.

phylogenetic levels.

4.2. Stream water quality assessment comparison of a conventional and molecular approach

The present study showed that by sequencing subsamples of homogenized DSFI samples ranging from low to high stream water qualities, distinct clusters representing similar invertebrate compositions were obtained. The low stream-water quality samples were more distinct from the other samples, while better stream-water quality showed relatively high resemblances. The relative differences between water-quality samples formed clusters that resembled the DSFI categories. Due to the greater differentiation seen in the water samples with low quality estimates compared to those of higher quality categories, it was not possible to separate the higher water quality samples in the same ordination analysis due to their greater relative similarity. However, further restricting the analysis to two neighbouring DSFI categories enabled clustering of identified ZOTUs into their respective DSFI category. A clear separation of the clusters within low stream-water quality was observed, while a minor overlap was seen within the samples representing higher stream-water qualities. These molecular results suggest that the metabarcoding of invertebrates collected from stream-water systems can be used to evaluate the state of stream-water bodies. The resolution and accuracy may be lower between two neighbouring DSFI categories with the highest stream-water quality (6 & 7). However, this overlapping tendency may not only reflect lack of resolution in the metabarcoding approach, but also mirror a lack of precision in the traditional stream-water quality assessment. Such limitations are supported by observations by the traditional approach particularly in distinguishing between the higher DSFI categories.

4.3. Challenges in conventional stream water quality assessment

Conventional assessment methods for stream water quality determination differ between countries and classification of different stream assessment categories unified quality categories would realize comparability between studies. Moreover, in accordance with the WFD, water quality needs to achieve a “good ecological status”. In the Danish implementation of WFD, “good ecological status” covers DSFI category 5, 6 and 7. Detailed distinction within high quality categories is not always necessary. More conserved separation of DSFI categories within the same quality category by the conventional assessment method may be caused by considering abundances of some species. Sequencing data of eukaryotic species are not exchangeable to absolute abundances of

species as it is based on relative abundance of sequencing reads (Yu et al., 2012). Moreover, different biases related to DNA extraction, primer efficiencies and specificities complicate quantifications by NGS (Aylagas et al., 2016a). Furthermore, different sizes of specimens, and even life stages, may result in different read abundance of sequences e.g. one large organism might result in the same read abundance as many small organisms of the same species (Elbrecht and Leese, 2015). Pre-sorting of specimens according to their size would reduce this effect and also decrease the risk of overlooking small specimens (Elbrecht et al., 2017a). However, pre-sorting is a time-consuming process and causes also the risk of overlooking specimens (Haase et al., 2010). Developing indices based on absence/presence data would increase the value of metabarcoding data for assessing biodiversity (Aylagas et al., 2014; Elbrecht and Leese, 2015). This approach has been applied for assessing marine benthic biodiversity and achieved comparable results as morphological analysis (Aylagas et al., 2016a). However, metabarcoding studies of mock communities showed positive correlations between biomass and read abundance which could provide an alternative to approach absolute species numbers (Elbrecht and Leese, 2015).

4.4. Challenges in metabarcoding of freshwater ecosystems

Metabarcoding of stream water samples has several technical challenges (Pawlowski et al., 2018), starting with DNA extractions. Sediment samples contain, besides invertebrates, plant material and various inorganic matters (clay, silt, sand, stones) and organic matters (e.g. humic substances), which are known inhibitors for PCR reactions (Malcolm, 1990; Wilson, 1997). Therefore, commercial DNA extraction kits or specified protocols which remove these inhibitors efficiently should be used. The choice of target gene and suitable primers also presents a challenge. The barcode region of the cytochrome *c* oxidase I (*COI*) gene, as used in this study, has been widely applied for species identification and biodiversity studies. However, it has also been shown that universal primers for this barcode are not efficient for all eukaryotes (Geller et al., 2013). Therefore, group specific primers were developed, including universal invertebrate primers (Folmer et al., 1994; Leray et al., 2013). Nevertheless, within the group of invertebrates, amplification efficiencies using these universal primers differ and multiple primers are needed for identifying benthic invertebrate fauna (Pfrender et al., 2010). Alternative target genes such as the nuclear *18S rRNA* gene have been suggested for marine nematodes (Dell'Anno et al., 2015). For freshwater macroinvertebrates a combination of the mitochondrial genes *COI* and *cyt b* achieved higher detection rates than the solely use of one gene (Carew et al., 2013). Other studies have suggested the use of a combination of the gene coding for nuclear *18S rRNA* and *COI* for invertebrate communities (Cowart et al., 2015). However, the use of *COI* has been successfully applied for species identification of mixed stream invertebrate communities using newly developed primers optimized for stream invertebrates (Elbrecht et al., 2017b; Elbrecht and Leese, 2017). In comparison to morphology based identification of mixed invertebrate samples, metabarcoding achieved similar results, and even improved species detection for invertebrate families which are known to be challenging for morphological identification (Elbrecht et al., 2017b). Further studies could focus on using the developed approach for analysing eDNA samples of streams as a non-invasive method to not only assess stream water quality but also to detect biodiversity affiliated to river ecosystems (Deiner et al., 2016). The present study showed that it was possible to identify organisms associated to a single or a range of DSFI categories, without filtering or otherwise weighting of the obtained sequencing data (Fig. S4). However, due the disparity between samples from different DSFI categories, and the lower number of sampling locations in general, this analysis is of low strength, and only represents a tendency between the occurrences of different taxonomic groups in streams of diverging water quality categories. It is expected that continued application of metabarcoding will improve the empiric ecological knowledge regarding the role and importance of the individual invertebrate species, as well as a large increase in the number of sampled locations, will improve this correlation in future studies and will be able to pinpoint new taxa as indicators for stream quality assessment.

4.5. Potential of metabarcoding as a stream water quality assessment tool

The present study shows the potential of metabarcoding for assessing stream water quality in practice and was able to classify samples based on their obtained DNA sequences into quality categories without prior sorting procedure which decreases processing time per sample. Further validation and optimization of the suggested approach could be carried out by amending with standard mock communities including sediment material to evaluate effects of DNA extractions, primer efficiencies and PCR conditions (Elbrecht et al., 2017b). Moreover, for identification of sequences, databases need to be improved by barcoding single specimens of interest (Aylagas et al., 2014; Elbrecht and Leese, 2017). However, as different primers are currently used for assessing invertebrate communities (e.g. Aylagas et al., 2016b; Elbrecht and Leese, 2017), reference libraries containing complete mitochondrial genomes would be optimal to facilitate metabarcoding studies.

In perspective, the developed approach could already be used to evaluate an unknown sample based on its placement within the obtained clusters of DSFI categorised samples in this study and forms the basis for establishing a molecular method for predicting stream water quality. The applied methodology revealed a greater diversity of detected species compared to the conventional method (Fig. 2), as well as broader detection of these species across sampled locations (Fig. 3). While this greater yield in detected species broadens the number of potential indicators upon which to base the quality assessment, it is important to note that the conventional analysis is based on carefully selected organisms as indicators for stream-ecosystem quality. Furthermore, it was also shown that the distribution of reads obtained from the individual species differ greatly between locations (Fig. S2), as expected with the distribution of species and individuals based on ecological quality. These results highlight the need for refinement of the data analysis in order to improve accuracy and robustness in future applications on a larger scale. Further acquisition of sequencing data from additional locations will make it possible to begin the development of robust models for the prediction of stream water quality. In conclusion, the obtained results show great promise for the development of a molecular method for the assessment of stream water ecosystems based on macroinvertebrates as BQE.

Author contributions

MH and JLN conceived the ideas, designed the work and achieved the funding; FK did the sampling and laboratory work; NdJ and FK analysed the data with guidance from JLN; NdJ and JLN led the writing of the manuscript with contributions from FK and MH.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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