Development and validation of DNA metabarcoding for stream macroinvertebrate community assessment

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Summary

To counteract the worldwide biodiversity decline in freshwater ecosystems due to human impact and ensure the sustainable use of essential ecosystem services (e.g. water and food supply) legally-binding frameworks have been launched, implementing biomonitoring of indicator groups to identify the need for restoration measures as well as to monitor changes and control success of actions taken. Monitoring of groups as taxonomically challenging as benthic macroinvertebrates is slow and cost-intensive requiring high taxonomic expertise but often only supplies an insufficient taxonomic resolution. In scientific approaches, DNA-based metabarcoding is increasingly used for biodiversity assessment especially in taxonomically difficult groups, outperforming morphology-based identification in speed, taxonomic resolution and efficiency. The method is therefore recommended to complement conventional standardised biomonitoring. Despite evident benefits of DNA metabarcoding, methodological limitations in sampling and laboratory processes still exist, requiring further developments while the method is already challenged by newly emerging methods based on whole genome approaches. The presented thesis aims at contributing to the optimization and application of DNA metabarcoding and at developing innovative methods. The main objectives are therefore separated in three parts which comprise I) methodological advancements in DNA metabarcoding, II) the application of DNA metabarcoding with regard to biomonitoring purposes and III) PCR-free methodological innovations to overcome limitations in DNA metabarcoding.

Methodological developments focus on aquatic macroinvertebrate communities with known taxonomic composition (“mock communities”) and reveal DNA extraction from bulk sample fixative and subsequent DNA metabarcoding as a promising tool for diversity assessment of benthic macroinvertebrates, circumventing the yet applied costly step of sample sorting. Its application on field-generated samples successfully detects main species decisive for ecological status assessment but partly fails to detect small and strongly sclerotised taxa clearly indicating the need for further investigations. To understand high inconsistencies in taxa detection through the amplification and tagging of sequencing libraries in a single PCR step (“one-step PCR”) we compare this method with two other tagging methods (“two-step PCR”, Illumina Library Preparation kit). Results indeed reveal the one-step approach as the most inconsistent and inefficient compared to the other two methods. Differences are however

less pronounced then stated in previous studies, rendering the method still applicable for biodiversity assessments due to its low price and contamination risk.

In the second part of my thesis, DNA metabarcoding is applied to field-generated samples of three German river systems (Emscher, Ennepe, Sieg) and within an experimental setup at the Felderbach. The method reliably assesses the diversity of aquatic macroinvertebrate assemblages focusing on the impact of natural and anthropogenic effects on community composition. Influences of diffuse stressors on whole communities and genetic diversity are successfully detected as well as those of defined multiple stressors on the highly diverse dipteran family Chironomidae. Despite prevailing seasonal effects, ecological status class assigned through DNA metabarcoding presence/absence data is congruent with evaluations based on abundance data achieved by morphology-based identification. However, although a successful application of DNA metabarcoding for biodiversity assessment of aquatic macroinvertebrates is recorded, conducted analyses also underline major limitations of the method in the case of incomplete reference databases and the inconsistent assessment of biomass or abundance patterns. Here innovative methods are under development, avoiding PCR and are therefore more promising for biomass assessments (“shotgun metagenomics” or “shotgun mitogenomics”). In the third part of my thesis, we developed a simple centrifugation protocol to enrich mitochondrial genomes before shotgun sequencing of mixed samples. This serves to increase the usability of shotgun mitogenomics, which is currently limited due to the small proportion of informative mitochondrial DNA. Proportion of mitochondrial sequences is successfully increased up to ~ 10 % which enhances the applicability of PCR-free approaches for biodiversity assessment and the potential to achieve biomass data. However, initial mitochondrial copy number per cell and taxonomic group can be highly variable, a fact that needs comprehensive testing before the applicability of this approach for biomass assessment can be finally judged. Further approaches almost completely bypassing the need for taxonomic assignment or biomass values through machine learning algorithms are under development. They open up completely new possibilities for the integration of molecular methods in standardised biomonitoring.

Zusammenfassung

Um den durch den Menschen verursachten weltweiten Biodiversitätsverlust in aquatischen Ökosystemen aufzuhalten und eine nachhaltige Nutzung von lebenswichtigen Ressourcen sicherzustellen, wurden in den letzten Jahrzehnten umfangreiche Gesetzeswerke erarbeitet und in Kraft gesetzt. Diese, wie z.B. die Wasserrahmenrichtlinie (WRRL) der EU, beinhalten die Überwachung der Diversität und Häufigkeit bestimmter Indikatororganismen und bilden die Basis für die Planung von Renaturierungsmaßnahmen sowie deren Erfolgskontrolle. Die Bestimmung der Zusammensetzung von taxonomisch anspruchsvollen Artengemeinschaften (z.B. benthische Makroinvertebraten) ist ein langsamer, kostenintensiver Prozess, der umfassendes taxonomisches Wissen voraussetzt, oftmals aber nur eine unzureichende taxonomische Auflösung erreicht. In wissenschaftlichen Studien wird die DNA-basierte Methode Metabarcoding zunehmend für die Erfassung von Biodiversität genutzt und übertrifft hierbei Morphologie-basierte Verfahren in Geschwindigkeit, Effektivität und der erreichten taxonomischen Genauigkeit. Es besteht daher großer Bedarf, diese Methode zur Komplementierung von herkömmlichen Ansätzen zur Artbestimmung in der angewandten Überwachung von Diversität verstärkt einzusetzen. Trotz der klar ersichtlichen Vorteile von DNA Metabarcoding bestehen immer noch einige Schwachstellen, die zusätzliche methodische Entwicklungen und Optimierungen für die Anwendung notwendig machen. Die hier untersuchten und entwickelten methodischen Innovationen komplementieren die Nutzung von DNA Metabarcoding zur Erfassung von Makroinvertebraten-Artengemeinschaften, gehen zum Teil aber darüber hinaus und erschließen die Analyse von innerartlicher genetischer Diversität. Die vorliegende Arbeit lässt sich in drei wesentliche Bereiche gliedern, dies sind I) Optimierung und Bewertung methodischer Schritte von DNA Metabarcoding im Hinblick auf DNA-Isolation und PCR, II) Anwendung von DNA Metabarcoding zur Bewertung des Einflusses von Stressoren und III) PCR-freie methodische Innovationen zur Erfassung von Biodiversität. Die methodische Entwicklung von DNA Metabarcoding stützt sich hauptsächlich auf Artengemeinschaften mit bekannter Artenzusammensetzung (sogenannte „mock communities“).

Ergebnisse im ersten Teil zeigen, dass die DNA-Extraktion aus dem Fixierungsmittel von Gemischtproben als vielversprechende Methode zur Biodiversitätserfassung anzusehen ist, welche die bis jetzt durchgeführte zeitaufwendige Sortierung der Proben umgeht. Bei der

Anwendung der Methode an Feldproben werden bestimmte Indikatorarten erfolgreich erfasst, während ein geringer Nachweis von kleinen und stark sklerotisierten Arten zu verzeichnen ist. Speziell dazu sind zusätzliche Studien notwendig. Die von vorläufigen Studien aufgezeigten Unstimmigkeiten in der Bestimmung von Artenzusammensetzungen durch die parallele Amplifikation und Indizierung von DNA Metabarcoding-Proben („one-step PCR“) wird überprüft. Hierbei wird die Methode mit zwei weiteren Vorgängen der Indizierung („two-step PCR“ und „Illumina library preparation kit“) verglichen. Dabei erweist sich der Fehler bei der one-step PCR als wesentlich geringer als nach Literaturangaben zu erwarten. Die Methode ist daher, auch auf Grund der geringen Kosten und Anfälligkeit für Kontaminationen für bestimmte Fragestellungen weiter anwendbar.

Im zweiten Teil der vorliegenden Arbeit erfolgt die Anwendung von DNA Metabarcoding zur Erfassung der Makrozoobenthos-Diversität an drei Flüssen in Nordrhein-Westfalen (Emscher, Ennepe, Sieg). Zusätzlich wird die Diversität der Zuckmücken (Chironomidae) im Zuge eines experimentellen Aufbaus am Felderbach untersucht. Die Methode wird erfolgreich für die Erfassung der Diversität von aquatischen Makroinvertebraten eingesetzt. Ein besonderer Fokus liegt auf der Bestimmung und Quantifizierung der Wirkung von natürlichen und anthropogenen Faktoren auf die Artengemeinschaften. Der Einfluss diffuser Stressoren auf komplette Gemeinschaften sowie auf die genetische Diversität wird zuverlässig ermittelt sowie der Einfluss definierter multipler Stressoren auf die artenreiche Familie der Zuckmücken (Chironomidae). Trotz vorherrschender saisonaler Effekte auf Gemeinschaften kann die Gewässergüte basierend auf binären Daten („presence/absence“) zuverlässig bestimmt werden und stimmt mit den Ergebnissen morphologischer Untersuchungen überein. Trotz der erfolgreichen Anwendung von DNA Metabarcoding für die Erfassung von Diversität in aquatischen Ökosystemen unterstreichen die vorgelegten Studien auch weiterhin bestehende Limitationen der Methode wie unvollständige Referenzdatenbanken und die Schwächen bei der Erfassung der Biomasse der untersuchten Taxa. Hierfür befinden sich vielversprechende PCR-freie Methoden in der Entwicklung („shotgun Metagenomik“ oder „shotgun Mitogenomik“).

Um die Brauchbarkeit dieser PCR-freien Methoden, die momentan durch den geringen Anteil an informativer mitochondrialer DNA im methodischen Ablauf limitiert wird, zu verbessern, beschäftigt sich der dritte Teil der vorliegenden Arbeit mit der Entwicklung eines Protokolls zu Anreicherung von Mitochondrien. Hier kann der später erhaltene Anteil an

mitochondrialen Sequenzen durch Zentrifugation auf bis zu ~10 % erhöht werden, was die Möglichkeit der Anwendung der PCR-freien Methode für die Biodiversitätserfassung erhöht. Trotzdem sind noch weitere Untersuchungen bezüglich der variablen Kopienanzahl mitochondrialer DNA pro Zelle und Art notwendig, um die Leistungsfähigkeit der Methode final zu beurteilen. Weitere Ansätze, die über maschinelles Lernen den Aufwand für taxonomischer Zuordnung oder Bestimmung der Biomasse noch weitergehend verringern, befinden sich in der Entwicklung und eröffnen vollkommen neue Möglichkeiten, um molekulare Methoden in standardisiertes Biomonitoring zu integrieren.

Introduction

Anthropogenic impact on nature and the whole earth system has increased tremendously in the last decades ("great acceleration", Steffen et al., 2007). Direct exploitation but also indirect factors such as urbanisation and land use change lead to massive habitat loss and biodiversity decline in ecosystems. The highest species extinction rate today is observed in freshwater systems, which cover less than 1 % of the earth's surface, but comprise an exceptional biodiversity (> 100,000 described species) (Carpenter et al., 1992; Dudgeon et al., 2006; WWF, 2018). Although still difficult to quantify, it is commonly accepted that biodiversity loss results in reduced ecosystem functions and services, thereby negatively affecting our livelihood (Figure 1). The alteration and pollution of freshwater systems and the surrounding landscape by humans led to the destruction and degradation of complete aquatic networks, which are now one of the most endangered habitats on earth. However, freshwater ecosystems supply essential services for humans (and the whole global system) around the world (e.g. water and food supply, transportation, flood and erosion control, power capacity), the sustainability of which is based on functioning ecosystems with intact biodiversity (Falkenmark et al., 2003; Vörösmarty et al., 2010). The restoration and protection of systems and prevailing biodiversity has therefore been a priority in policy goals nationally and internationally in the last decades to sustain ecosystem function of freshwater systems and the linked services essential for humans and biodiversity in general. Legally-binding frameworks have been launched, which require frequent biomonitoring of diversity, depending on efficient and reliable methods to document remaining species and assess biodiversity trends over large scales (Birk et al., 2012; Hering et al., 2010). Currently implemented methods are based on the morphological identification of specimens and therefore cover only a limited spectrum of organismal groups which can be efficiently determined but exclude e.g. a high part of microbial diversity. Even within considered groups, a high taxonomic expertise is needed for specimen identification which is time and money consuming and often leads to an insufficient taxonomic resolution or erroneous determination (Haase et al., 2010). Methodological developments, especially in the field of species detection, have become an essential part of the progress in biodiversity research. Especially with DNA barcoding and High-Throughput Sequencing (HTS) approaches, comprehensive comparative studies became possible with feasible effort outcompeting traditional biodiversity assessment methods in time, costs and accuracy and are even suggested to replace those in legally binding

frameworks. However, considerable weaknesses have still to be overcome and methods optimized to improve especially the significance in the field of species biomass and abundances.

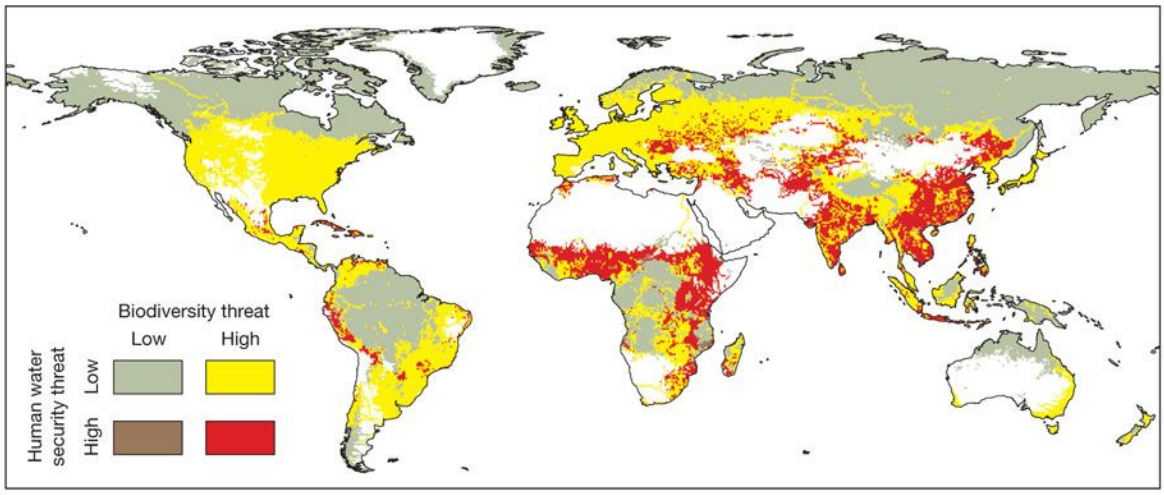

Figure 1: *Human water security threat and biodiversity threat are shown. Especially developing countries face threats to human water security* ***and*** *biodiversity (Vörösmarty et al., 2010).*

Biodiversity assessment in the Water Framework Directive (WFD)

One sophisticated and detailed environmental policy is the European Water Framework Directive (WFD). Inspired by the United States' Clean Water Act (Knopman and Smith, 1993), the WFD 2000/60/EC came into effect in the year 2000 by the European Parliament (European Parliament, 2000). It puts aquatic ecology at the base of water policy and resource management to counteract the unsustainable use of freshwater sources and induce the coexistence of water users and ecological requirements. Managed in collaboration of state and national governments, the WFD claims a 'good' ecological and chemical status of all water bodies (surface, underground, coastal) in member states of the European Union (EU) by 2027 (Hering et al., 2010; Kallis and Butler, 2001). The water body status is determined through biological quality elements (BQEs) as well as hydro-morphological and physio-chemical parameters. Biological quality elements have a descriptive definition of a 'high', 'good', 'moderate', 'poor' or 'bad' status based on the comparison to a water body of similar size, structure and ecology without human impact (Birk et al., 2012; Kallis and Butler, 2001; Logan and Furse, 2002). If the required status conditions of at least 'good' are not fulfilled, restoration measures have to take place to improve the ecosystem status. To assess the prevailing condition and decide upon improvement or deterioration of systems before and after restoration, frequent and reliable biomonitoring of indicator groups is necessary

(England et al., 2008). In stream ecosystems, BQEs refer to the community composition and abundance of macrophytes, phythobenthos, macrozoobenthos (MZB), and fish. Both measurements - inventory and abundance - are typically recorded for ecosystem integrity assessment, including various sensitive taxa as stressor indicators (Birk et al., 2012). As the most widely used assemblage for water and habitat quality, benthic macroinvertebrates mainly include insect larvae, molluscs, worms and crustaceans (Cairns and Pratt, 1993; Purcell et al., 2009). Sampling and identification of this diverse group is rather easy compared to microorganisms, including long-living and sedentary taxa that react strongly and predictable to human influences on aquatic ecosystems. Especially most taxa of the mayflies (**E**phemeroptera), stoneflies (**P**lecoptera) and caddisflies (**T**richoptera) (EPT taxa) are highly sensitive to habitat alterations and form the basis of numerous biological studies investigating aquatic ecosystem quality (e.g. Cairns and Pratt, 1993; Chang et al., 2014; Resh, 2008; Walsh et al., 2001).

Challenges and innovation

Benthic macroinvertebrate communities studied for ecological status assessment are collected in bulk through kick-net sampling and the individuals are traditionally identified to the lowest practical level (species, genus or family) based on morphological characters. The standard protocol in Germany includes the collection of specimens from all available microhabitats according to their relative frequencies in 20 subsamples and the subsequent sorting of samples prior to identification (see Haase et al., 2004 for detailed information). Besides variation due to patchiness (Olsen et al., 2007) or stochasticity of sampling, all steps are potentially affected by human errors, consequently impacting freshwater biomonitoring (Cao et al., 2003; Clarke and Murphy, 2006; Haase et al., 2010). An often-discussed source of error is the identification of specimens based on morphological characters. Due to the high similarity and small body size of many MZB organisms, morphological identification is extremely time consuming and requires a high level of taxonomic expertise, while it still does often not meet species level resolution. This is especially true for larval stages of many of the small insect larvae but also flatworms, leaches or oligochaetes, which are extremely difficult or impossible to identify to the species based on morphology alone. Even if conducted by taxonomic experts, identification is often subjective and impossible to standardize, potentially leading to misidentifications and incomparability (Cao et al., 2003; Haase et al., 2006; Stribling et al., 2003). In addition, cryptic species cannot be determined by morphological features. However, this is necessary, for variable stressor responses have been shown for cryptic species in recent

molecular studies (Feckler et al., 2014; Macher et al., 2016). Long time and high effort needed for morphological identification of MZB taxa led to the designation of a so-called 'Operational Taxalist' for the different countries, where indicator taxa and the respective taxonomic resolution (often only family) is stated. These indicator taxa form the basis for the determination of ecological status class, which however provides restricted ecological information in comparison to holistic and taxonomically highly resolved biodiversity assessments (Yu et al., 2012).

Induced taxonomic error and inaccuracy vary among taxa, laboratories and taxonomists, thus affecting determined diversity and community composition, potentially skewing underlaying data for ecological status assessments (Cao et al., 2003; Haase et al., 2010; Stribling et al., 2008). Inaccuracies and errors can lead to incorrectly conducted or refrained restoration events rendering timely and accurate biodiversity analysis an ongoing challenge for the success of biomonitoring programs. In reaction with the high time and money expenses needed for morphological identification of biomonitoring bulk samples, recent reviews have proposed to complement traditional assessment methods with DNA-based monitoring (Baird and Hajibabaei, 2012; Elbrecht et al., 2017b; Pawlowski et al., 2018; Taberlet et al., 2012; Valentini et al., 2016). However, exemplary morphological studies will remain essential as a basis for reference databases and to calibrate and validate molecular approaches.

DNA metabarcoding

DNA-based monitoring of diversity through analysis of a small segment of the genome has long been applied and accepted in microorganism research (e.g. Nanney, 1982; Pace, 1997; Sogin et al., 2006). The method termed "DNA barcoding" today has been successfully transferred to identify also higher organisms and is an important tool for ecological studies (Ebach and Holdrege, 2005; Janzen et al., 2005; Kress et al., 2015; Sweeney et al., 2011; Valentini et al., 2009). Hebert et al. (2003) proposed a 658 base pair long fragment of the Cytochrome C Oxidase subunit I (COI) gene for the identification of unknown animal specimens because of two main characteristics: i) its variability allows species level discrimination but shows low intraspecific variation and ii) it is haploid, has a high copy number and can be amplified from most animals (Hebert et al., 2003; Ji et al., 2013; Ratnasingham and Hebert, 2007). DNA barcoding implies identification through the extraction, amplification and Sanger-sequencing of a target gene and the subsequent comparison of the query sequence to a barcode reference library built on known species

(Hebert et al., 2003; Marshall, 2005; Ratnasingham and Hebert, 2007; Sanger et al., 1977; Valentini et al., 2009). The most often used databases are the Barcode of Life Data System (BOLD) and the NCBI GenBank database. However, DNA barcoding is restricted to the identification of single specimens since the Sanger sequencing technology is based upon capillary electrophoresis and is limited to sequence a single amplicon of one specimen per run. The method is therefore not practical to determine whole community composition because each individual must be processed separately (Hajibabaei et al., 2007; Ji et al., 2013). Thus it is not applicable for repeated biodiversity assessment as implemented in biomonitoring contexts, where samples include hundreds to thousands of individuals and often high numbers of samples have to be processed (Ji et al., 2013; Taberlet et al., 2012; Yu et al., 2012).

Based on the principle of DNA barcoding coupled with recent advances in High-Throughput Sequencing (HTS) technologies, “DNA metabarcoding” has been developed. The method implements the barcode-based identification of specimens in a massively parallel manner. High-throughput multispecies identification is conducted from bulk samples containing entire organisms (e.g. invertebrate bulk sample) or from environmental samples containing degraded DNA (eDNA; soil, water, faeces etc.) (Hajibabaei et al., 2011; Ji et al., 2013; Taberlet et al., 2012; Valentini et al., 2016). DNA metabarcoding is based on the mass extraction and amplification of the informative barcode of interest using universal primers that are suitable for a specific taxonomic group and require short amplicons and robust PCR conditions to achieve unbiased amplification from a mixture of several DNA templates (Coissac et al., 2012; Hajibabaei et al., 2011; Taberlet et al., 2012; Yu et al., 2012). Through HTS, billions of sequences can be generated in a single sequencing run, which greatly excels the requirements for diversity assessment of one sample. Therefore, many samples including a high number of individuals and taxonomic groups can be multiplexed together on a single flow cell with oligonucleotide index sequences incorporated into the platform-specific sequencing adapter for subsequent assignments (Baird and Hajibabaei, 2012; Goodwin et al., 2016; Hajibabaei et al., 2011; Ji et al., 2013; Taberlet et al., 2012). The MiSeq Illumina sequencing platform is currently dominating metabarcoding methodology and literature due to a high read quality and relatively low purchase costs. However, rapid growth of HTS methods over the past decades produced a variety of techniques and chemistries implementing a progressive throughput and cost reduction (Goodwin et al., 2016; Ji et al., 2013; Piper et al., 2019).

The sequence data finally at hand represent many individuals of different taxa but include also a number of artefacts. Various bioinformatic pipelines exist to proceed and quality filter the huge amount of data. A key issue requiring a difficult trade-off is between sensitivity to detection of rare taxa and excluding the amount of erroneous sequences (e.g. Bolyen et al., 2019; Boyer et al., 2016; Rognes et al., 2016). Software "pipelines" follow similar workflows, initially assigning sequences to original samples (demultiplexing) by the unique oligonucleotide sample indices incorporated into sequencing adapters. In the following, primer sequences are removed and reads are merged into consensus sequences using their overlapping bases. Even if sequencing techniques reach a per base accuracy > 99.9 % out of billions of bases sequenced thousands of sequences still contain errors (Edgar and Flyvbjerg, 2015). A subsequent quality filtering is conducted to remove erroneous or artificial sequences introduced during PCR and sequencing as well as chimeras that can form during PCR from two or more independent templates. However, single substitutions during PCR and length variation is not necessarily associated with low quality scores so the clustering of sequences in Operational Taxonomic Units (OTUs) according to an arbitrary similarity threshold is conducted. The threshold of 97 % is a broadly used compromise between interspecific and intraspecific variation in animals and commonly used to indicate distinct taxa, so that each OTU ideally contains only sequences from one species after clustering (Edgar, 2010; Fu et al., 2012; Ghodsi et al., 2011; Ji et al., 2013). However, actual coalescence depth between species can differ greatly across taxonomic groups. Thus, the application of a single global threshold in analysis of diverse communities can result in splitting of single species across multiple OTUs or lumping of multiple species into the same OTU. This can induce false positive or negative results (Taberlet et al., 2012). Additionally, the aggregation of similar sequences into a single OTU masks all information on intraspecific diversity. Beside alternative clustering methods that do not require fixed similarity thresholds (Mahé et al., 2014) recent studies focused on the analysis of Exact Sequence Variants (ESVs) or haplotypes, which depict information about genetic variability and can be extracted from metabarcoding datasets without any further sampling or laboratory effort (Elbrecht et al., 2018; Laini et al., in review; Tsuji et al., 2019; Turon et al., 2019). The underlying denoising algorithm (Edgar, 2016) uses statistical methods to discern true biological sequences from sequencing noise and correct for single-nucleotide differences without imposing the arbitrary similarity threshold that defines OTUs (Elbrecht et al., 2018; Piper et al., 2019; Turon et al., 2019). Comparisons of OTU representatives or ESV sequences with a reference database in an automated manner results in a list with assigned taxonomy and the concerning number of sequences for this OTU per

sample (Ji et al., 2013). Assignment is mostly conducted with a best-hit classification using alignment based tools such as BLAST (Basic Local Assignment Search Tool (Altschul et al., 1990)) assuming that taxonomy of query sequences will be identical to taxonomy of the most similar sequences in reference databases.

Applications

For about a decade now, DNA metabarcoding is comprehensively used for biodiversity assessment of different taxonomic groups covering various habitats and ecosystems (Bienert et al., 2012; Creer et al., 2010; Fahner et al., 2016; Le Bescot et al., 2016; Poisot et al., 2013; Pornon et al., 2016; Ritter et al., 2018; Schmidt et al., 2013; Sogin et al., 2006). Two principal approaches can be discerned in this fast developing field: for higher organisms, extraction from tissue of bulk samples also called "community metabarcoding" is mainly used to investigate local diversity pattern of selected invertebrate groups (Bienert et al., 2012; Ji et al., 2013; Ritter et al., 2018; Yu et al., 2012) or the detection of specified pest species (Piper et al., 2019), while "eDNA metabarcoding" implements the extraction of DNA directly from the environment (e.g. from water, soil, faeces) and aims at catching virtually all biodiversity over a larger taxonomical and geographical scale (Deiner et al., 2016; Stat et al., 2017). eDNA metabarcoding has also proved highly effective in detecting single endangered or invasive species and taxa that are difficult to sample (Andersen et al., 2012; Bohmann et al., 2014; Deiner et al., 2017; Rees et al., 2014; Rodgers et al., 2017). Even diet analyses (Alberdi et al., 2019; De Barba et al., 2014; Pompanon et al., 2012) or the reconstruction of past plant and animal communities (Jørgensen et al., 2012; Willerslev et al., 2007; Willerslev and Cooper, 2005; Yoccoz et al., 2012) can be facilitated by eDNA metabarcoding analysis.

Both metabarcoding approaches have been applied on freshwater ecosystems providing highly effective biodiversity assessment (Deiner et al., 2016, 2017; Elbrecht et al., 2017b; Hajibabaei et al., 2011; Macher et al., 2018; Mächler et al., 2014). However, considering the determination of benthic macroinvertebrate communities as requirement for standardized ecological status assessments, community metabarcoding has been applied and assessed in various studies on 'mock communities' and real environmental samples (e.g. Elbrecht et al., 2017; Hajibabaei et al., 2011; Theissinger et al., 2018) and is thought to be more beneficial compared to eDNA metabarcoding. Especially in freshwater systems, where eDNA is extracted from a water sample, the method potentially assesses biodiversity at a broader

taxonomic and geographical range. This makes it also more prone to miss taxa in a specific, assessment relevant group while in community metabarcoding DNA is extracted from tissue of those specific groups which makes the method more reliable (Hajibabaei et al., 2019a; Macher et al., 2018; Pereira-da-Conceicoa et al., 2019). Community metabarcoding has also proven to be at least comparable to or even more efficient and accurate in species identification and diversity determination of benthic macroinvertebrates than traditional methods based on morphological features (Elbrecht et al., 2017b; Emilson et al., 2017). Various publications therefore encourage the complementary or exclusive use of community metabarcoding as the identification method for benthic macroinvertebrates (Baird and Hajibabaei, 2012; Bush et al., 2019; Taberlet et al., 2012; Valentini et al., 2016). The present study focuses on the development and application of community metabarcoding and methodology, challenges and conclusions refer to this type of the method. eDNA metabarcoding is only briefly investigated and infers separate challenges and applications. In the following the term DNA metabarcoding describes community DNA metabarcoding. The term community metabarcoding is only used in direct comparison to eDNA metabarcoding.

Studies at hand partly focus on the establishing of DNA metabarcoding in the European Water Framework Directive with concrete implementation strategies (Hering et al., 2018; Leese et al., 2018; Pawlowski et al., 2018) which also circumvent the highly discussed and not yet solved problem of retrieving species abundance information from DNA metabarcoding datasets. Next to species diversity, number of individuals or population size (abundance) per identified taxon is part of several current biomonitoring and ecological status assessments, inferring an effect of ecosystem condition on population size of taxa. As stated below in detail, the determination of number of individuals per species is currently not possible with DNA metabarcoding. However, recent studies document that presence-absence data for species lead to highly comparable or even identical status assessments as analyses including additional abundance data (Aylagas et al., 2017; Beentjes et al., 2018; Buchner et al., 2019; Ranasinghe et al., 2012).

Challenges in DNA metabarcoding

Metabarcoding represents a very promising cost-effective tool for reliable biodiversity assessment of freshwater macroinvertebrate communities with fast increasing practical relevance. However, still there remain methodological challenges that potentially reduce the

quality of assessments and defer the transfer into applied biomonitoring. The most concerning challenges and potential solutions are listed in the following.

Sampling

All steps in kick-net sampling of benthic macroinvertebrates before species identification as conducted for standardized biomonitoring are prone to human error. Sources of error range from the choice of sample site and microhabitats over the strength of kicking to the separation of individuals from substrate or subsampling (Haase et al., 2010, 2006; Hering et al., 2010). Most of those error prone steps could be circumvented by the application of eDNA sampling through the filtration of a fixed water volume. As stated above, the method is however adversely to the application of community DNA metabarcoding in the down-stream process (Hajibabaei et al., 2019a; Pereira-da-Conceicoa et al., 2019). For the application of community DNA metabarcoding, established sampling has to be maintained. However, subdividing total samples and the identification of only a subsample of individuals is unnecessary, since extremely high individual numbers can be easily processed. In opposite, the separation of specimens from substrate and debris up to now has to be accomplished using DNA metabarcoding, which is very time consuming and difficult to transfer into biomonitoring protocols that need to be time and money efficient (Haase et al., 2010, 2004). It potentially leads to the overlooking of inconspicuous and in substrate covered specimens especially if only a small time frame is permitted for sample sorting (Haase et al., 2010). Even if a faster procedure and higher taxonomic resolution can be achieved through DNA metabarcoding, specimens would still be neglected if overlooked during separation from substrate. Two main approaches have been investigated to circumvent this separation step. Homogenisation of the whole bulk sample including substrate and following application of DNA metabarcoding with specific primers showed promising results for a fast and comprehensive assessment of benthic macroinvertebrate diversity (Pereira-da-Conceicoa et al., 2019). Another approach includes the extraction of DNA directly from the storage fixative of samples (96 % ethanol most of the times). This approach is based on the assumption that preserved organisms will release DNA into the fixative and represents an intermediate approach between community and eDNA metabarcoding (Figure 2). It has been successfully tested on pre-sorted samples (Hajibabaei et al., 2012; Shokralla et al., 2010) including the evaporation of small volumes of the fixative which is promising for handling bigger sample volumes, e.g. complete bulk samples including debris. A big advantage of the extraction of DNA from the preservative ethanol is, that specimens do not need to be destroyed for

identification. This allows morphological reidentification after sequencing and/or permanent storage of relevant samples. However, many open questions regarding the processing of complete kick samples without the separation of substrate and specimens, the postulated DNA release into the fixative and effects on targeted macroinvertebrate taxa detection exists. A successful application of DNA extraction and subsequent DNA metabarcoding on complete bulk samples would highly increase the efficiency of the method, ensure subsequent morphological reidentification of specimens and increase usability for standardised biomonitoring.

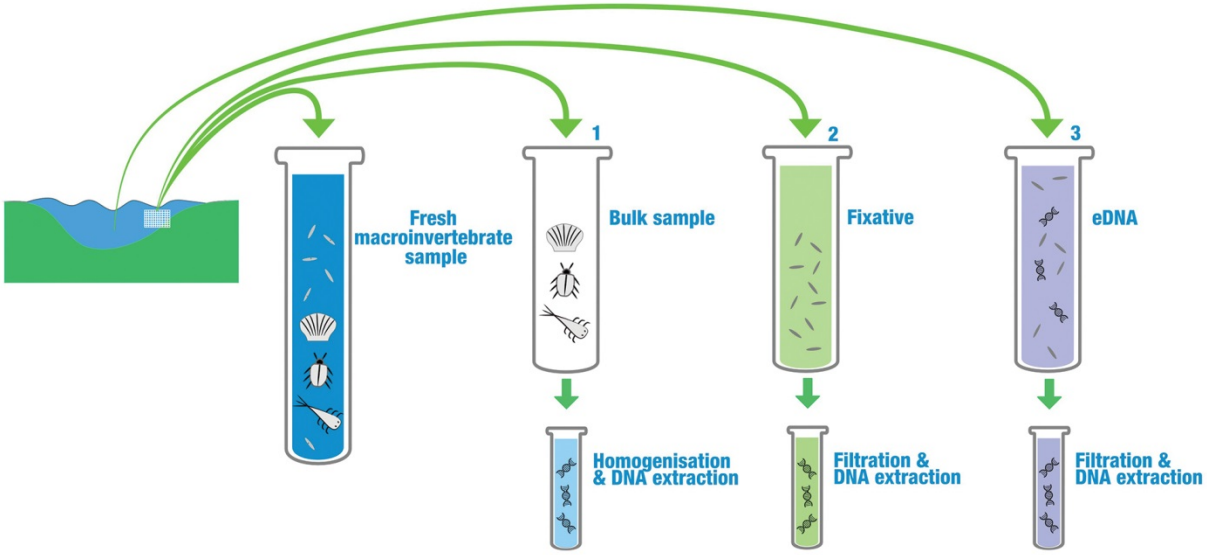

Figure 2: *Three methods to obtain DN for biomonitoring of macroinvertebrates. 1. DNA bulk samples from homogenised specimen samples. 2. DNA extracted from the fixative the sample was preserved in. 3. DNA extracted directly from a water sample (eDNA) (Blackman et al., 2019).*

Laboratory

Primer-template mismatches during PCR, particularly at the 3´ end of the primer where extension takes place, can result in different PCR amplification efficiencies between taxa. Primers do not provide equally good matches to all targeted sequences derived from a bulk sample, introducing amplification bias towards well matched taxa and failing to amplify others. Those disparities generally result in a skewed picture of biodiversity and sequence number per individual and taxon (Deagle et al., 2014; Pawluczyk et al., 2015; Piñol et al., 2019; Taberlet et al., 2012). Primer degeneration can overcome this taxonomic bias to a certain extent but a high level of degeneracy can lead to the off target amplification or the formation of primer dimers (Macher et al., 2018; Mioduchowska et al., 2018). In addition,

template-specific factors can further contribute to PCR bias as the copy number of loci, nucleotide composition and secondary structure, variable amplicon length or specimen biomass (Krehenwinkel et al., 2017). Biases indicate that amplicon sequencing of diverse bulk samples can only be used to achieve presence-absence and not abundance data. This is supported by the fact that many small individuals can constitute the same amount of DNA in a bulk sample as a few big ones resulting in similar read numbers. Good correlations have instead been detected between read number and template biomass of individuals of one species, while the linkage is not possible on a wide taxonomic range due to the above mentioned primer bias (Elbrecht and Leese, 2015; Krehenwinkel et al., 2017).

The individual tagging of sequences according to the sample of origin can be implemented in a single (one-step) or two separated (two-step) PCR steps. So-called “fusion” primers are used with a nucleotide tail attached to the 5’-end consisting of the P5/P7 Illumina adapter, the sequencing primer binding site and a unique base shift (unique tag). In the one-step approach amplification and tagging of sequences occur simultaneously using fusion primers. In opposite, the two-step approach implements the amplification of a target gene with matching primers with no nucleotide tail attached in the first PCR, while individual tagging is ensured through a second PCR with fusion primers (Elbrecht and Leese, 2015; Lundberg et al., 2013). Recent studies reveal a tag-specific intensified primer bias when the one-step method is applied. Inconsistencies are introduced through interactions of the long nucleotide tail with the template for specific tag sequences and taxa, which leads to an inefficient amplification (Berry et al., 2011; O’Donnell et al., 2016). The one-step approach is successfully used for several DNA metabarcoding studies (Elbrecht et al., 2017b, 2017a) and represents a cheap approach for the tagging of sequences with a low susceptibility for contamination. Thus, results of O’Donnell et al. (2016) are surprising and require further investigation also in comparison to the two-step PCR-based approach and manufactured kits.

Bioinformatics

Various bioinformatic pipelines are accessible which allow automated analysis of HTS data with detailed taxa lists as the end product. The strictness of filtering steps and applied thresholds on the dataset is variable and can be selected according to the requirements of research questions (Boyer et al., 2016; Coissac et al., 2012; Edgar, 2010; Edgar and Flyvbjerg, 2015). The already previously discussed step of OTU clustering with a fixed

threshold applied on mixed bulk samples is recently challenged since divergence time and intraspecific variation of taxa can be variable. This can lead to the splitting of one species into several OTUs or the lumping of two different species into one OTU. The extraction of Exact Sequence Variants (ESVs) is therefore an upcoming alternative, investigating single haplotypes and allowing the detection of intraspecific variation (Elbrecht et al., 2018; Tsuji et al., 2019; Turon et al., 2019). Genetic diversity based on the used marker gene can be extracted from DNA metabarcoding datasets without any further sampling or laboratory effort and has been successfully applied in Elbrecht et al. (2018), Tsuji et al. (2019), Turon et al. (2019) and reviewed in Adams et al. (2019). Deciphering sequencing mistakes from rare sequences originating from the investigated sample is the big challenge of this approach which needs further investigations to also evaluate the potential to infer stressor impacts on genetic diversity.

PCR-free molecular methods

To counteract described inconsistencies, PCR free approaches have been investigated recently. ‘Shotgun sequencing’ or ‘shotgun metagenomics’ implement the simple fragmentation of genomic DNA to a fragment length appropriate for sequencing platforms and directly sequence it without any enrichment step (Riesenfeld et al., 2004). This process generates sequence reads comprising a random subsample of mixed community DNA. References are largely incomplete for nuclear genomic data but relatively comprehensive for mitochondrial genes consequently shifting PCR-free methods to “mitochondrial metagenomics” or “mitogenomics”. High copy regions can be assembled into long contigs and even full length mitochondrial genomes for further phylogenetic inference and application to systematics can be retrieved (Papadopoulou et al., 2015). However, the vast majority of reads will be discarded (mainly > 95 %) because they are not from the mitochondrial genome and therefore no reference sequences exist. Deep sequencing is required to reliably detect rare specimens which entails high costs, increases analyzing time and required place for data storage (Arribas et al., 2016; Crampton-Platt et al., 2016; Gómez-Rodríguez et al., 2015; Mamanova et al., 2010). The enrichment of mitochondrial genomes prior to sequencing can increase the informative material in the mixture, shifting the initial ratio of mitochondrial versus nuclear DNA towards a higher mitochondrial proportion. The application of target region enrichment with synthetic probes or baits before shotgun sequencing on a diverse mixed sample has been investigated, showing a successful enrichment of mitochondrial genomes (Liu et al., 2016). However, the use of probes or baits

for target region enrichment can potentially induce biases due to variable capture efficiency (Mamanova et al., 2010). Centrifugation is known to enrich mitochondrial genomes with the most promising results when living tissue is used and organelles are intact (Garber and Yoder, 1983; Tamura and Aotsuka, 1988). A centrifugation protocol for mitochondrial enrichment was applied by Zhou et al. (2013) which showed moderate success and only increased mitochondrial DNA to 0.53 % of the total sequence volume. However, the approach has not been tested on living organisms which indicates a higher enrichment due to the intactness of mitochondria. The successful enrichment of mitochondrial genomes in bulk samples would highly increase the efficiency of mitogenomic approaches for diversity assessments from mixed samples which has been tested in several studies (Choo et al., 2017; Crampton-Platt et al., 2016; Tang et al., 2014). Significant correlations between biomass and sequencing volume of specific taxa in mitogenomic approaches (Bista et al., 2018; Gómez-Rodríguez et al., 2015; Zhou et al., 2013) indicate the usability of this method for biomass or even individual number determination if the enrichment of mitochondrial genomes is successful.

Objectives of this study

DNA metabarcoding has been used in various scientific studies for biodiversity assessment of freshwater macroinvertebrate communities, providing reliable data with high taxonomic resolution superior to traditional identification methods. The approach is therefore proposed to complement or even replace traditional methods in standardized biomonitoring e.g. as part of the Water Framework Directive. However, despite DNA metabarcoding has proven to be highly effective, methodological problems still exist, which need to be solved in order to provide end-users with “best-practice” protocols. Therefore, the first part of this thesis, focussed on method optimisation and validation. In the second part, the methods are applied in a comparative study of river systems, differing in the extent of human impact. The aim of this part is - besides a “proof of methodology” - to identify the effect of environmental stressors to freshwater species biodiversity as well as underlying genetic diversity. As the latter aspect of biodiversity can only be assessed with genetic approaches, it is completely excluded by traditional community assessment approaches but provides important sources also in the context of environmental policies. In the final part of the study a PCR-free mitogenomic approach is applied to enrich mitochondrial genomes for shotgun sequencing which can facilitate taxa detection or mitogenome assemblage of complex samples and also

advances the method for biomass assessment. The objectives of part one focus on optimizing the following crucial steps for DNA metabarcoding:

I) development and evaluation of a protocol to extract DNA of unsorted bulk samples directly from the fixative ethanol and perform metabarcoding.

II) quantification of primer bias induced through the incorporation of Illumina adapters in the PCR primer (fusion primer).

The second part of this thesis uses the established protocols from part one to assess the potential of DNA metabarcoding for stream quality assessment and anthropogenic stressor inference. Next to the Felderbach the model ecosystems chosen are three river systems in Western Germany. The rivers represent (i) a strongly polluted and canalized system that is now part of one of the biggest restoration measures in Europe (Emscher), (ii) a river system prevailing in natural structure but loaded with manifold stressor inflow (Ennepe) and (iii) a river system of natural structure with low stressor inflow (Sieg). All three river systems have been subject to biomonitoring with traditional methods and thus provide excellent basis for an assessment of the performance and reliability of DNA metabarcoding. Principal questions are:

III) does DNA metabarcoding improve detection of stressor responses due to improved taxonomic resolution?

IV) what are seasonal and what are anthropogenic components of community variation in a near-natural and an impacted river and can DNA metabarcoding despite these components be reliably used for ecological status assessment?

V) can intraspecific haplotype diversity be assessed for whole MZB communities and is genetic diversity affected by anthropogenic stressors?

The third part of this thesis is focusing on the PCR-free approach of mitogenomics, which is discussed as a potential molecular method to enable the assessment of species biomass out of mixed samples. Due to the PCR independence the method is not affected by primer bias, but drawn back due to the low proportion of mitochondrial material compared to nuclear material in a mixed sample. The chapter focusses on the following approach:

VI) development of a protocol to enrich mitochondrial genomes for PCR-free methods.

Chapter 1

Methodological advancements in DNA metabarcoding

DNA metabarcoding from sample fixative as a quick and voucher-preserving biodiversity assessment method

Vera MA Zizka, Florian Leese, Bianca Peinert, Matthias F Geiger

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Experimental design and planning: 70 %

Sampling: 60 %

Laboratory work: 60 %

Data analysis: 100 %

Figures: 70 %

Manuscript writing: 80 %

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doctoral candidate supervisor

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ARTICLE

DNA metabarcoding from sample fixative as a quick and voucher-preserving biodiversity assessment method[1]

Vera M.A. Zizka, Florian Leese, Bianca Peinert, and Matthias F. Geiger

Abstract: Metabarcoding is a powerful, increasingly popular tool for biodiversity assessment, but it still suffers from some drawbacks (specimen destruction, separation, and size sorting). In the present study, we tested a non-destructive protocol that excludes any sample sorting, where the ethanol used for sample preserving is filtered and DNA is extracted from the filter for subsequent DNA metabarcoding. When tested on macroinvertebrate mock communities, the method was widely successful but was unable to reliably detect mollusc taxa. Three different protocols (no treatment, shaking, and freezing) were successfully applied to increase DNA release to the fixative. The protocols resulted in similar success in taxa detection (6.8–7 taxa) but differences in read numbers assigned to taxa of interest (33.8%–93.7%). In comparison to conventional bulk sample metabarcoding of environmental samples, taxa with pronounced exoskeleton and small-bodied taxa were especially underrepresented in ethanol samples. For EPT (Ephemeroptera, Plecoptera, Trichoptera) taxa, which are important for determining stream ecological status, the methods detected 46 OTUs in common, with only 4 unique to the ethanol samples and 10 to the bulk samples. These results indicate that fixative-based metabarcoding is a non-destructive, time-saving alternative for biodiversity assessments focussing on taxa used for ecological status determination. However, for a comprehensive assessment on total invertebrate biodiversity, the method may not be sufficient, and conventional bulk sample metabarcoding should be applied.

Key words: non-destructive, environmental DNA, macroinvertebrates, metabarcoding.

Résumé : Le méta-codage à barres est un outil puissant de plus en plus utilisé pour mesurer la biodiversité, mais il présente toujours certains inconvénients (destruction, séparation et triage des spécimens). Dans le présent travail, les auteurs ont exploré un protocole non-destructif qui exclut tout triage des échantillons; l'éthanol employé pour préserver l'échantillon est filtré et l'ADN est extrait du filtre pour être employé subséquemment pour le méta-codage à barres. Lorsque testé sur des communautés simulées de macro-invertébrés, la méthode s'est largement avérée un succès, sans toutefois réussir à détecter de manière fiable les mollusques. Trois protocoles différents (aucun traitement, agitation et congélation) ont été appliqués pour augmenter la libération de l'ADN. Ces protocoles ont entraîné un succès comparable en matière de détection de taxons (6,8 à 7 taxons), mais des nombres variables de séquences assignées aux taxons d'intérêt (33,8 à 93,7 %). Comparativement aux méthodes conventionnelles employées sur des échantillons environnementaux, les taxons avec des exosquelettes prononcés ou encore de petite taille étaient particulièrement sous-représentés au sein des échantillons d'éthanol. Pour les taxons EPT (Ephemeroptera, Plecoptera, Trichoptera), lesquels sont importants pour déterminer le statut écologique des ruisseaux, les méthodes ont permis de détecter 46 OTU en commun, tandis que 4 étaient uniques aux échantillons d'éthanol et 10 aux échantillons traditionnels en composite. Ces résultats indiquent que la méthode de méta-codage à barres faisant appel au fixatif constitue une alternative non-destructive et rapide pour les mesures de la biodiversité portant sur des taxons utilisés pour déterminer l'état des écosystèmes. Cependant, pour une évaluation exhaustive de la biodiversité totale, la méthode n'est peut-être pas suffisante et il faudrait employer des méthodes conventionnelles d'analyse d'échantillons composites. [Traduit par la Rédaction]

Mots-clés : non-destructif, ADN environnemental, macro-invertébrés, méta-codage à barres.

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Introduction

DNA metabarcoding was developed to improve the efficiency, reliability, and resolution of biodiversity assessments. The technique is based on parallel sequencing of standardised DNA barcode markers. Due to the high output of modern sequencing techniques, sequences of thousands of individuals can be generated in one sequencing run and even various samples can be uniquely labelled and pooled. Compared to traditional morphology-based assessments, metabarcoding has the clear advantage of a potentially higher taxonomic resolution, if the right marker is applied and reference databases are sufficient, better comparability, and often also speed (Haase et al. 2010; Hajibabaei et al. 2011, Baird and Hajibabaei 2012; Taberlet et al. 2012; Elbrecht et al. 2017*b*). Even though metabarcoding has been successfully applied for biodiversity assessments (e.g., Shokralla et al. 2012; Yu et al. 2012; Leray and Knowlton 2015; Elbrecht et al. 2017*b*), the method still lacks consistent protocols (Baird and Hajibabaei 2012; Taberlet et al. 2012; Leese et al. 2016, 2018). One drawback of metabarcoding when working with complex environmental samples (e.g., invertebrates from the streambed, so-called bulk metabarcoding) is that organisms have to be separated manually from the by-catch (sediment and other inorganic matter) prior to DNA extraction. This is often time-consuming, substantially limiting the gain in efficiency (Elbrecht et al. 2017*a*). Furthermore, as specimens are usually homogenised and destroyed for DNA extraction they consequently cannot be used for further morphological examination or validation (Zimmermann et al. 2008; Leese et al. 2016). The non-destructive isolation of DNA from the bulk samples' fixative (most often ethanol) has been put forward as a promising alternative (Hajibabaei et al. 2012). However, studies following this idea still included sample sorting (Hajibabaei et al. 2012; Carew et al. 2018) and (or) the usage of lysis buffer (Rohland et al. 2004; Gilbert et al. 2007; Rowley et al. 2007) to soften body structures of specimens for an increased DNA release. This can lead to the complete destruction of smaller specimens or those lacking a strong internal or external skeleton, again prohibiting subsequent morphological identification.

In the present study, we test a fixative-based metabarcoding approach. Our approach differs from that used by Hajibabaei et al. (2012), who obtained template DNA from the fixative after evaporation, in that we extract DNA after a novel filter-based approach including different protocols to increase DNA release into the fixative. We tested the approach on pre-sorted small mock communities of invertebrates and on real environmental samples that included substrate (e.g., sand, stones, litter, etc.) that could potentially also act as PCR inhibitors. First, the general feasibility of ethanol filtration and subsequent extraction of DNA from the filters was evaluated using 15 different mock communities with known taxonomic composition to address whether (*i*) DNA release is high enough to obtain sufficient DNA to correctly recover (with at least one read) the mock community composition, (*ii*) different sample treatment protocols (ultrasonic irradiation, shaking, and freezing) influence DNA release of specimens into the fixative and thereby increase the general success in taxa recovery, (*iii*) the different sample treatment protocols (ultrasonic irradiation, shaking, and freezing) increase the specificity of the method due to increased DNA released from specimens into the fixative and the consequently higher proportion of DNA from target taxa compared to non-target taxa, and (*iv*) the final read abundance is correlated with the biomass or size of specimens. Recognizing that the surface area to volume ratio decreases with increased specimen size, we hypothesized that larger specimens would be less overrepresented in DNA from ethanol compared to bulk sample metabarcoding of homogenized specimens (Elbrecht and Leese 2015).

In the second part of the study, we used this metabarcoding pipeline to analyse the ethanol fixative from six aquatic multi-habitat environmental samples and compared the results to a conventional tissue-homogenisation-based metabarcoding protocol (e.g., Elbrecht et al. 2017*a*). Specifically we addressed (*i*) whether the two approaches result in the same or similar OTU composition and read distribution, or if a strong effect of the method prevails and therefore different compositions are detected, (*ii*) whether the fixative-based metabarcoding approach might be useful for bioassessment with respect to total number of taxa detected, species overlap between the methods, read distribution, and sequencing effort, and (*iii*) whether the application of the fixative-based metabarcoding on environmental samples reveals similar patterns as observed in the mock community test.

In addition to the assessment of the general performance of fixative-based metabarcoding, another focus was placed on the detection of macroinvertebrate taxa that are important for the calculation of ecological status indices. The groups Ephemeroptera, Plecoptera, Trichoptera (EPT), and Diptera (EPTD) are of particular interest in this respect. Addressing these points in a controlled comparative framework is essential to validate whether using the fixative for metabarcoding can become part of an accelerated bioassessment protocol. The experimental design used in this study enables the validation of fixative-based metabarcoding, which can be used for further method improvements.

Materials and methods

Testing filtration of ethanol and isolation of DNA from filters

Assembly of mock communities

For part one of this study, 15 mock communities were sorted directly in situ at the Deilbach in Velbert, North Rhine Westphalia, Germany, in November 2016. Each

community contained one single specimen from each of the eight morphotaxa *Ancylus*, *Ecdyonurus*, *Ephemera*, *Gammarus*, Gastropoda (*Potamopyrgus* or *Radix*, here not enough specimens of one or the other genera were found during sampling based on morphological identification; individuals were therefore taxonomically merged in Gastropoda), *Hydropsyche*, Leptophlebiidae, and *Polycentropus*. Five different specimen size compositions were used (Figs. 1A–1E; Table S1[2]), and three mock communities of each size composition were assembled (e.g., the following size classes for the eight taxa: A always S[mall] + L[large] + M[edium] + L + M + L + S + M). The three communities of the same size composition were then treated as replicates (e.g., A1–A3) for further analyses. Specimens were collected and directly transferred to a falcon tube containing 50 mL of 96% denatured technical ethanol (410 PharmEur., ethanol phase I). Samples were transported to the laboratory of the University of Duisburg-Essen and stored for 12 h at –20 °C. After 12 h, the ethanol (phase I) was poured over a sieve (mesh size 0.5 mm) to retain animals and was then stored at –20 °C until further processing (protocol 0, see Laboratory protocols). A fresh 50 mL of denatured technical 96% ethanol (410 PharmEur., ethanol phase II) was then added to each of the falcon tubes containing the mock communities. They were then stored at –20 °C until further processing. Specimens were weighed on a scale (Mettler Toledo, XS105 DualRange) and the length was measured on graph paper.

Treatments

After the first ethanol change, the mock communities underwent three different treatments (Fig. 1, protocol 1–3) to potentially increase DNA release into the ethanol. From the five different specimen size compositions, each one of the three replicates was used for a different treatment (protocol 1–3; see Fig. 1, Table S1[2]):

Protocol (1) ultrasonic irradiation: indirect ultrasonic irradiation for 15 min at 35 kHz (Bandalin SONOREX Super rk 510 Hz).

Protocol (2) mechanical shaking: shaking for 2 min at 2000 rpm at room temperature using the Eppendorf ThermoMixer C.

Protocol (3) freezing: samples were kept for 10 min in liquid nitrogen (–196 °C) until ethanol was frozen. Afterwards, samples were left for 15 min at room temperature for thawing. The procedure was repeated three times.

After each treatment, the ethanol (phase II) was filtered through a sieve (mesh size 0.5 mm) to retain animals and was stored at –20 °C until further processing (protocols 1–3, see Laboratory protocols).

Laboratory protocols

Ethanol was filtered through nitrocellulose filters with pore size 0.45 μm (Nalgene Analytical Test Filter Funnel CN, Thermo Scientific) using a vacuum pump (TC–501v, Sparmax). Filtration, as well as all of the following laboratory processes (except treatments), was conducted in a sterile laboratory set up for the treatment of eDNA (environmental DNA) samples, and complete body protection (overalls with hood, mouth protection, and gloves) was worn at all laboratory steps. Filters were dried overnight in petri dishes. Dry filters were ripped into small pieces using sterile plastic tweezers and transferred to 600 μL TNES buffer for subsequent DNA extraction. DNA extraction was carried out using a modified salt extraction protocol (Sunnucks and Hales 1996; adjusted in Weiss and Leese 2016). Extraction success was confirmed on a Fragment Analyzer (Advanced Analytical), and 1 μL of each sample was used for amplicon library preparation in a two-step PCR approach targeting a 421 bp long fragment of the official animal DNA barcode region (5′ end of the mitochondrial cytochrome C oxidase subunit (*CO1*) gene). In the first step, the fragment was amplified using untailed BF2/BR2 primers (Elbrecht and Leese 2017). PCR reactions consisted of 1× PCR buffer (including 2.5 mM Mg^{2+}), 0.2 mM dNTPs, 0.5 μM of each primer, 0.025 U/μL of HotMaster Taq (5 Prime, Gaithersburg, MD, USA), and 1 μL DNA template filled up with HPLC H_2O to a total volume of 50 μL. The following PCR program was used: 94 °C for 180 s; 25 cycles of 94 °C for 30 s, 50 °C for 30 s, and 65 °C for 150 s; followed by a final elongation of 65 °C for 5 min in a Thermocycler (Biometra TAdvanced Thermocycler). For the second PCR step, 1 μL of each PCR product was used with uniquely tagged BF2/BR2 fusion primers. PCR conditions in the second step were identical to the first step, but only 15 cycles were used.

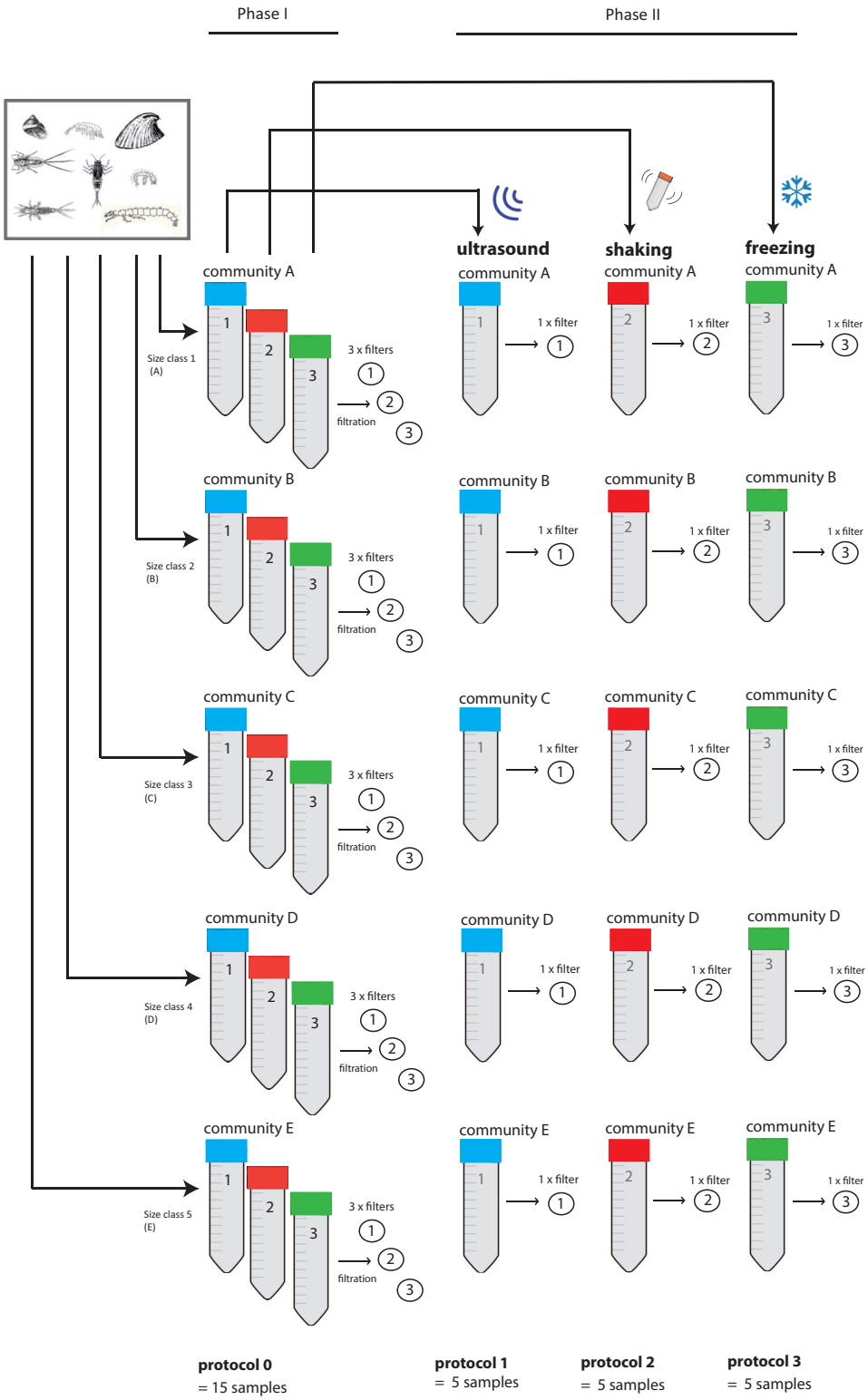
A left-sided size selection of all samples was performed using magnetic beads (SPRIselect BECKMAN COULTER) to remove short fragments (leftover primers and primer dimers). A ratio of 0.76× was applied, and amplicon concentration was measured on a Fragment Analyzer. All samples were equimolarly pooled, and paired-end sequencing was carried out by GATC Biotech AG (Constance, Germany) using one run on the Illumina MiSeq platform with a 250 bp paired-end v2 kit.

Sanger sequencing of specimens

In addition to DNA extraction from filters and subsequent sequencing, full length DNA barcodes of all 120 individuals of the 15 mock communities were obtained through the German Barcode of Life (GBOL) pipeline at ZFMK (Bonn, Germany) to allow direct comparisons of OTUs to the specimens. For single specimen

[2]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/gen-2018-0048.

Fig. 1. In total, 15 different mock communities composed of eight specimens (one individual each from the following taxa: *Ancylus*, *Ecdyonurus*, *Ephemera*, *Gammarus*, Gastropoda (*Potamopyrgus* or *Radix*), *Hydropsyche*, Leptophlebiidae, and *Polycentropus*) were assembled. Individuals of very similar sizes per taxon were compiled for each of the three communities to allow for comparisons of extraction efficiencies among these three replicates (e.g., A1–A3). The ethanol in which specimens were initially preserved after collection was filtered for all 15 communities (protocol 0, phase I). The second ethanol phase was then filtered after one of the following three treatments: ultrasonic irradiation, shaking, or freezing (protocol 1–3).



DNA barcoding, genomic DNA was extracted from subsamples using a BioSprint96 magnetic bead extractor (Qiagen, Hilden, Germany). PCRs were carried out in 20 μL reaction volumes including 2 μL undiluted DNA template, 0.8 μL of each primer (10 pmol/μL; LCO1490-JJ: 5′-CHACWAAYCATAAAGATATYGG-3′ and HCO2198-JJ: 5′-AWACTTCVGGRTGVCCAAARAATCA-3′, Astrin and Stüben 2008), 2 μL Q-Solution, and 10 μL Multiplex PCR Master Mix (Qiagen, Hilden, Germany). We opted for the generation of full length DNA barcodes (658 bp) with the established universal JJ primer set to be able to query the database for reverse identifications. The BF2/BR2 primers used in the metabarcoding tests amplify a 421 bp region within the standard Folmer region with BR2 targeting the same sequence as HCO2198-JJ, but being slightly shorter (5 bp) and having two additional wobble positions. Thermal cycling was performed on GeneAmp PCR System 2700 machines (Applied Biosystems, Foster City, CA, USA) as follows: hot start Taq activation: 15 min at 95 °C; first cycle set (15 repeats): 35 s denaturation at 94 °C, 90 s annealing at 55 °C (–0.2 °C/cycle, touch down), and 90 s extension at 72 °C. Second cycle set (25 repeats): 35 s denaturation at 94 °C, 90 s annealing at 50 °C, and 90 s extension at 72 °C; final elongation 10 min at 72 °C. Purification of PCR products and sequencing in both directions was conducted at BGI (Hongkong, China) using the amplification primers.

Data analysis

For the metabarcoding approach, sequences labelled with fusion primers and indices were assigned to their original sample as implemented in JAMP v0.23 (https://github.com/VascoElbrecht/JAMP). Subsequent data processing was conducted for all samples in JAMP v0.23 using standard settings: paired-end reads were first merged (module U_merge_PE) and reverse complements were built where needed (U_revcomp) with usearch v10.0.240 (Edgar 2010). Cutadapt (Martin 2017) was used to remove primer sequences and to discard sequences of unexpected length so that only reads with a length of 411–431 bp were used for further analyses (module Minmax). The module U_max_ee was used to discard all reads with an expected error > 0.5. Sequences were dereplicated, singletons were removed, and sequences with ≥97% similarity were clustered into OTUs using Uparse (U_cluster_otus). OTUs with a minimal read abundance of 0.01% in at least one sample were retained for further analyses, while other OTUs were discarded. The used script for data analysis can be found in the supplementary data[2]. OTU sequences were compared with the database BOLD using the BOLD ID engine via the module BOLD_webhack in JAMP and taxonomies assigned following rules outlined in Elbrecht et al. (2017*b*): for assignment to species level, a hit with 98% similarity was required; 95% similarity was required for assignment to genus level, 90% for family level, and 85% for order level. To assess the detection success of morphotaxa and the general performance of fixative-based metabarcoding, a comparison of OTU sequences with those generated by single specimen barcoding was conducted. Therefore, the taxa assigned to single specimen sequences via reverse identification on BOLD were searched for in the OTU table, and OTU sequences with matching taxonomy were directly compared to the Sanger sequencing derived barcodes in the software Geneious. For most of the taxa, this comparison was on species level; for the morphotaxon Gastropoda, all OTU sequences assigned to the phylum Mollusca with >85% were compared. Matches between Sanger barcode and OTU sequence ≥97% similarity for an assigned taxon were registered as "recovered". Taxa represented with extremely low read numbers for a certain OTU (i.e., one read) were also counted. In cases where sequences generated through single specimen barcoding showed no similarity to any of the OTU sequences, this comparison was marked with (?) in Tables 1 and S3[2] and is listed as "not recovered".

Comparison of ethanol samples with environmental bulk samples

Sampling

For part two of this study, we compared the performance of fixative-based metabarcoding to tissue-homogenisation-based metabarcoding of six real environmental samples. In spring 2017 (May 2–3), six sites along the Sieg River were sampled (S1, Windeck-Schladern: 50°48′03.7″N, 7°35′17.5″E; S2, Irsenbach-Mündung: 50°46′52.9″N, 7°37′38.5″E; S3, Siegfähre: 50°45′54.7″N, 7°06′25.7″E; S4, Aggermündung: 50°48′02.7″N, 7°10′27.3″E, S5, Bröhl-Mündung: 50°46′55.0″N, 7°18′27.9″E; S6, Bülgenauel: 50°46′29.8″N, 7°21′47.5″E). Multi-habitat sampling was conducted according to the Water Framework Directive (WFD) guidelines (Meier et al. 2006), i.e., by taking 20 subsamples via kick-sampling at each sampling site covering all different microhabitats. Complete samples (containing benthic macroinvertebrates and substrate) were transferred to 96% denatured technical ethanol (permission number for sampling from Amt für Natur- und Landschaftsschutz, Rhein-Sieg-Kreis: 67.1-1.03-19/2016KRO). For each sampling site, two bottles (1 L) were needed to capture the whole bulk sample, which combined the 20 subsamples. The complete sample was equally divided between the two bottles (~25% substrate per bottle). Immediately upon arrival at the laboratory (~3–6 hrs after sampling), the ethanol of all samples was poured off through a sieve (mesh size: 0.5 mm) to retain animals and substrate and stored at –20 °C. New 96% denatured technical ethanol was added to the samples, which were then also stored at –20 °C until further processing.

Processing of ethanol samples

The following laboratory steps were carried out separately for both bottles per sample site to minimize the risk to lose the complete sample due to inhibitors. However, for final data analysis, only OTUs detected in sam-

Table 1. Proportion of total reads (%) assigned to the eight taxa of the mock communities.

Community	Protocol	*Ancylus*	*Ecdyonurus*	*Ephemera*	*Gammarus*	Gastropoda	*Hydropsyche*	Leptophlebiidae	*Polycentropus*	Total (%)
A1	0	0	0.24	0.724	12.156	0.452	17.251	0.578	0.001	31.402
A2	0	0.011	0.037	1.49	0.124	0.017	26.782	0	0.055	28.516
A3	0	0.001	0.156	0.108	0.329	0.091	28.638	0.36	0.043	29.726
B1	0	0.001	0.182	0.375	1.452	?	25.343	?	0.044	27.397
B2	0	0.003	0.114	0.309	1.778	0.046	32.929	3.998	0.185	39.362
B3	0	0	1.304	0.014	0.149	0	38.323	0.023	0.669	40.482
C1	0	0	8.311	8.737	0.762	?	1.084	0.269	0.156	19.319
C2	0	0.009	0.019	1.329	1.801	0.328	0	0	0	3.486
C3	0	0	2.201	0	0.163	1.212	6.205	0.734	0.051	10.566
D1	0	0	0.006	0.298	8.931	?	0.768	0.367	0.216	10.586
D2	0	0.002	0.054	80.586	1.109	0.174	0.692	0.696	0.022	83.335
D3	0	0.011	0.093	0.255	0.358	0.064	77.913	0.088	0.625	79.407
E1	0	0.013	0.869	5.198	2.789	0	0.27	0	0.126	9.265
E2	0	0.007	1.312	33.267	3.994	0.061	9.093	0.269	0.956	48.959
E3	0	0	3.889	0.333	0.115	0.148	1.301	3.932	35.046	44.764
A1	1	0	0	0	0	0	0	0	0	0
B1	1	0	0	0	0	0	0	0	0	0
C1	1	0	0	0	0	0	0	0	0	0
D1	1	0	0	0	0	0	0	0	0	0
E1	1	0	0	0	0	0	0	0	0	0
A2	2	0	49.238	0.838	14.968	0.002	14.968	7.248	0	87.262
B2	2	0.003	0.078	0.703	0.751	0.019	10.579	37.129	0	49.262
C2	2	0.006	0.809	38.681	1.69	0.026	0.034	16.862	0.02	58.128
D2	2	0.003	0.034	47.076	0.398	0.076	1.015	39.326	0	87.928
E2	2	0	0	0	0	0	0	0	0	0
A3	3	0	1.154	53.825	1.508	0.008	10.999	24.392	0.188	93.074
B3	3	0	45.056	12.484	4.494	0	9.52	3.904	16.346	91.804
C3	3	0	0.985	78.12	0.022	0.001	0.436	17.587	0.004	97.155
D3	3	0	2.162	0.06	4.513	0.004	18.509	63.461	0.066	88.775
E3	3	0	0.092	31.456	8.848	0.001	0.092	14.741	42.551	97.781

Note: Proportion of total reads [%] assigned to the eight taxa of the mock communities. Protocols: 0, first ethanol phase in which specimens were preserved after collection; 1, second ethanol phase and treatment with ultrasonic irradiation; 2, second ethanol phase and shaking; 3, second ethanol phase and freezing. Question marks show cases where taxonomic assignments through single-specimen barcoding could not be validated in the metabarcoding dataset.

ples of both bottles were included. This was done to decrease the risk of including sequencing errors and false positives in the analyses and to simplify comparisons with environmental bulk samples. Ethanol filtration of the first phase used for fixation in the field was performed as outlined above for the mock communities and filters were dried overnight in petri dishes. Dry filters were ripped into small pieces using sterile plastic tweezers and transferred to 600 μL TNES buffer for subsequent DNA extraction. For each sample, two PCRs were conducted (2 PCR replicates, $n = 2$), and in subsequent bioinformatic analyses only OTUs were considered, which were present in both PCR replicates to decrease the risk of sequencing errors and false positives. Both PCR steps were conducted with Illustra™ PuRe Taq Ready-To-go™ PCR beads. Per sample, 0.5 μM BF2 and BR2 primer were added to an Illustra bead and made up to a total volume of 25 μL with HPLC H_2O. The following PCR program was used for the first PCR step: 95 °C for 180 s; 25 cycles of 95 °C for 30 s, 48 °C for 30 s, and 72 °C for 60 s; followed by a final elongation of 72 °C for 5 min in a Thermocycler (Biometra TAdvanced Thermocycler). The second PCR step was conducted with similar conditions but with an annealing temperature of 50 °C and 15 cycles. All following steps were carried out as described above (see Laboratory protocols).

Processing of environmental bulk samples

All macroinvertebrates in each sample were separated from the substrate and specimens from one sample were collected in a single tube filled with ethanol. Specimens were counted and categorised according to their size into two classes using standardized reference areas of 5 mm × 2 mm. Individuals fitting into this area were assigned to size class S, individuals exceeding this area were assigned to size class L. All specimens of the respective size class were homogenised to fine powder with an IKA Ultra Turrax Tube Disperser (full speed for 30 min) and DNA was extracted following a modified salt extraction protocol (Sunnucks and Hales 1996; adjusted as in Weiss and Leese 2016). After DNA extraction, the two size categories of the same sample were pooled in proportion to individual numbers (e.g., if 90% of individuals were assigned to size class S and 10% to size class L, for a 20 μL dilution,

18 μL of the extract of size class S was pooled with 2 μL of size class L, see Elbrecht et al. 2017*a* for the detailed description). DNA concentration was quantified using a Qubit 2.0 Fluorometer (Life Technologies, dsDNA BR Assay Kit) and DNA diluted to 25 ng/μL. A two-step PCR (see Laboratory protocols) was conducted for all samples with one PCR replicate each. For each sample, two PCRs were conducted (1 PCR replicate), and in subsequent bioinformatic analyses only OTUs included that were present in both replicates. The PCR products were quantified using a Fragment Analyzer (Advanced Analytical) and all samples were pooled equimolarly. Samples were sent to GATC Biotech (Constance, Germany) and sequenced on an Illumina MiSeq platform with a 250 bp paired-end v2 kit.

Data analysis

Data were analysed using the program JAMP v0.23 (https://github.com/VascoElbrecht/JAMP) as described above. To compare the fixative-based and bulk-sample metabarcoding, the obtained community compositions were visualized using Principal Coordinates analyses (PCoA) implemented PAST v. 3.17 (Hammer et al. 2001) based on Bray–Curtis (read abundance included) and Sørensen (presence-absence) similarities. Further data were visualised using the program R (R Development Core Team 2008) with the package asbio (Aho 2014) and ggplot2 (Wickham 2016).

Results

Testing filtration of ethanol and isolation of DNA from filters on mock communities

All raw data are available on Short Read Archive, project number PRJNA504612. Extraction of DNA from ethanol was successful for all protocols, except protocol 1 (ultrasonic irradiation) where no DNA product was visible on an agarose gel. However, samples were sent for sequencing to confirm that no product was present. A total of 10 659 925 raw reads were obtained from the Illumina MiSeq sequencing run. After demultiplexing, samples obtained from filtering the first ethanol phase (protocol 0, n = 15) comprised on average 325 808 (±170 687) reads. Samples treated with protocol 2 (shaking, n = 5) and protocol 3 (freezing, n = 5) contained on average 347 637 (±236 007) and 456 342 (±279 465) reads. Samples treated with protocol 1 (ultrasonic irradiation, n = 5) contained on average 49 (±28.86) reads and were therefore excluded from further analyses (Table S2[2]). In total 4583 OTUs were detected in this dataset with 950 OTUs assigned to macroinvertebrate taxa. The majority of OTUs were assigned to bacteria and algae.

Recovery and specificity of protocol 0 (no treatment)

The comparison of fixative-based metabarcoding with generated sequences through single-specimen barcoding revealed the recovery of 6.9 (±0.99, n = 15) morphotaxa for protocol 0 (Table S3[2]). On average 33.77% (±23.7) of the total reads of each sample were assigned to OTUs matching the Sanger sequencing-based eight morphotaxa. The taxon that was most often undetected was *Ancylus fluviatilis* (5 of 15 communities). Sequences of *A. fluviatilis* were also rare in the other 10 communities, with on average only 0.006% reads assigned (Tables 1, S3[2]). In 4 of the 15 mock communities, specimens assigned to the morphotaxon Gastropoda could not be detected through fixative-based metabarcoding. In community B1, the gastropod specimen was identified as *Physa* sp. through single specimen barcoding (Table S3[2]), but no similarity (>80%) to any of the OTU sequences was observed. This was similar for the species *Galba truncatula* (community C1). For communities B3, D1, and E1, the gastropod sequences could only be assigned to family level (Succineidae, terrestrial pulmonate gastropod molluscs) through single specimen barcoding. The gastropods in B3 and E1 showed similarities of 96.4% and 97.2% to OTU 1391 (*Succinea putris*) detected in other mock communities, while no reads of B3 and E1 were assigned to this OTU. The gastropod Sanger sequence in D1 showed no similarities to any of the OTU sequences. In the other communities, taxa assigned to the morphotaxon Gastropoda (*Potamopyrgus antipodarum*, *Radix labiata*, *Physella* (*Physa*) *acuta*, and *S. putris*) contributed on average 0.26% to the total number of reads. Only a few arthropods remained undetected in certain mock communities (A2: *Habroleptoides confusa*, C2: *Polycentropus flavomaculatus* and *Hydropsyche saxonica*, C3: *Ephemera danica*, E1: *Ecdyonurus torrentis*). In community B1, the mayfly *Baetis vernus* was identified through single specimen barcoding, but no OTU was assigned. No other Baetidae was highly abundant in this community (Table S5[2]). Further, 19.12% (±9.53) of the reads were assigned to other macroinvertebrate taxa, containing 56 dipteran OTUs. Chironomidae, Simulidae, and Tipulidae were the dominant taxa represented by these OTUs (Table S6[2]).

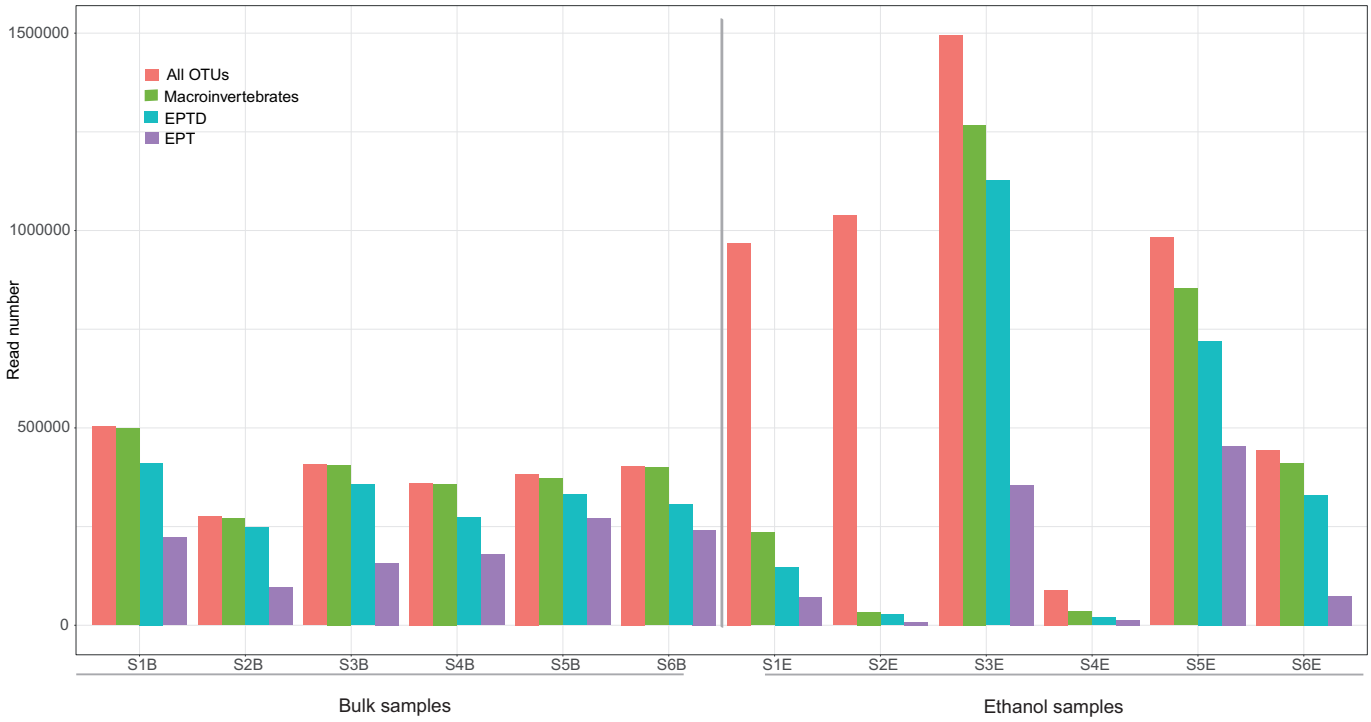
Recovery and specificity of protocol 2 (shaking)

For protocol number 2 (shaking), on average seven (±0.82) of the eight morphotaxa were detected through fixative-based metabarcoding. After bioinformatic analyses, sample E2 showed only 358 reads and was therefore excluded from further analyses. On average, 70.65% (±19.9) of the total reads were assigned to the eight target taxa (Table S5[2]). Additional aquatic macroinvertebrate taxa were detected with 9.35% (±7.2) of the total reads (Table S6[2]). Only *A. fluviatilis* (A2) and *P. flavomaculatus* (A2, B2, and D2) could not be detected (Table S3[2]).

Recovery and specificity of protocol 3 (freezing)

For protocol number 3 (freezing), on average 6.8 (±0.45) morphotaxa were detected with 93.7% (±3.5) of the total number of reads being assigned to the target taxa (Table S3[2]). Only 2.42% (±1.36) were assigned to other macroinvertebrates (Table S6[2]). However, in four of the five samples treated with protocol 3, *A. fluviatilis* was not

Fig. 2. Read numbers per sample site (S1B–S6B corresponding to bulk samples, S1E–S6E corresponding to ethanol samples). Data include all OTUs with a threshold of 0.01% of the total reads in at least one sample. No further filtering threshold was applied. Macroinvertebrates include all OTUs assigned to the taxa Arthropoda, Annelida, Mollusca, and Platyhelminthes with at least 85% similarity to a sequence deposited in BOLD. The category EPTD includes all OTUs assigned to the taxa Ephemeroptera, Plecoptera, Trichoptera, and Diptera with at least 85% similarity compared with the BOLD database. The category EPT includes the same taxa except Diptera.



detected and in sample B3 Succineidae sp. was also not recovered.

Comparison of read numbers and specimen biomass (all protocols)

For protocol 0, a weak correlation between specimen biomass and number of reads was found (Spearman's rho 0.211, $p = 0.02$). Data were strongly scattered and no significant correlation between biomass and number of reads was observed for the other two protocols (Table S4[2]).

Comparison of environmental bulk samples with ethanol samples

Sequencing runs were successfully performed on an Illumina MiSeq platform with a 250 bp paired-end v2 kit. After demultiplexing and quality filtering of the ethanol samples, 5 047 373 (average: 210 307 ± 144 450) reads were retained. DNA extractions from one bottle sampled at sites S4 and S6 yielded less than 400 reads and were therefore excluded from further analyses (Table S7[2]). Sequencing of the second bottle from both sample sites showed a mean number of 90 146 and 443 841 reads, respectively. The bulk sample based DNA extraction resulted in 2 334 707 reads after demultiplexing and quality filtering (average: 194 558 ± 43 135) with no samples containing less than 30 000 reads. For further analyses, only OTUs present in both bottles and both PCR replicates per sample site were kept in the dataset, and read numbers were added up to one OTU table. After this filtering step, ethanol samples included on average 836 027 (±494 981) reads, while bulk samples included on average 388 867 (±74 318) reads (Fig. 2; Table S8[2]).

Comparing OTUs among the two methods revealed a consensus of 357 OTUs, while 640 were unique to the ethanol and 104 to the bulk samples, respectively. Out of the OTUs uniquely found in ethanol samples, the main proportion was assigned to algae (Ochrophyta) (Tables 2, S8[2]). OTUs uniquely found in bulk sample metabarcoding included several beetles (Coleoptera), caddisflies (Trichoptera), as well as a few other aquatic macroinvertebrate taxa (Fig. 3; Table S8[2]).

Looking at OTUs assigned to Arthropoda, Annelida, Mollusca, or Platyhelminthes with at least 85% similarity to records found in BOLD, ethanol samples included on average 472 688 (±494 305, S1E–S6E) reads, while bulk samples contained on average 384 261 (±74 398, S1B–S6B) reads (Fig. 2; Table S9[2]). Comparing this filtered dataset, 262 OTUs were shared between the two methods, while 39 were unique to the ethanol and 97 to the bulk samples. Out of the 42 OTUs assigned to beetle taxa in bulk samples, only 11 were detected in ethanol samples, which showed in addition extremely low number of reads (Fig. 3; Table S9[2]). On the other hand, 12 OTUs

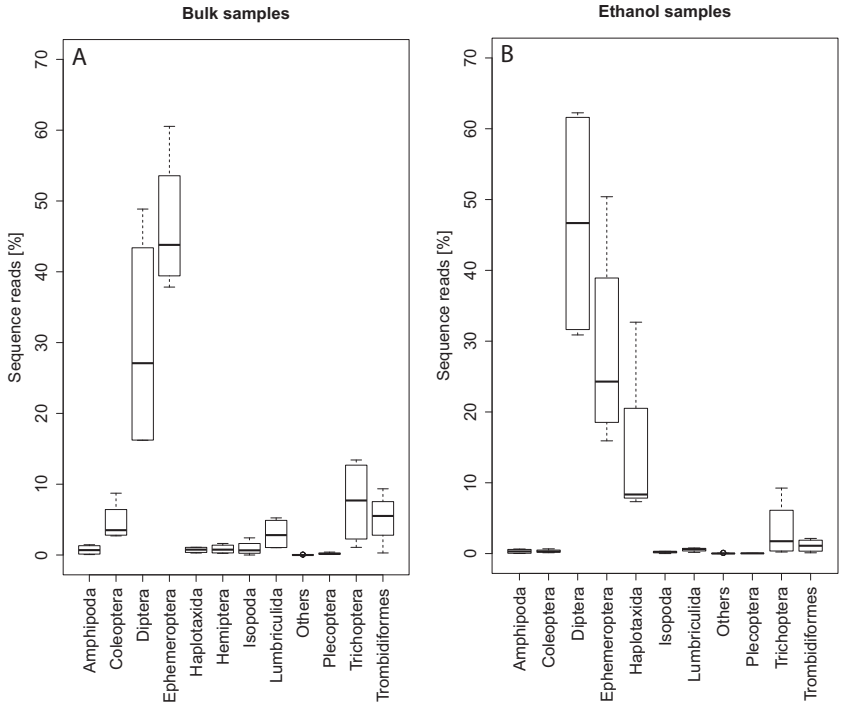
Table 2. Overview of the different datasets used for the analyses.

Dataset	Included samples	OTUs in common	OTUs unique: ethanol	OTUs unique: bulk	Total OTUs
Complete dataset	6	357	640	104	1101
Complete dataset*	6	301	115	98	514
Macroinvertebrates*	6	262	39	97	398
Macroinvertebrates subsample*	4 (excl. S2/S4)	232	39	85	356
EPTD taxa*	4 (excl. S2/S4)	162	16	35	213
EPT taxa*	4 (excl. S2/S4)	46	4	10	60

Note: Overview of the different datasets used for the analyses. Complete dataset includes (*i*) all OTUs above 0.01% of the total reads in at least one sample, and (*ii*) OTUs with at least 85% similarity to a sequence deposited in BOLD. Macroinvertebrates include all OTUs assigned to any taxa of Arthropoda, Annelida, Mollusca, or Platyhelminthes with at least 85% similarity to a sequence deposited in BOLD. The subsample includes only a random 230 000 reads of macroinvertebrates per sample, where S2 and S4 needed to be excluded due to low read numbers. EPTD taxa include all OTUs assigned to Ephemeroptera, Plecoptera, Trichoptera, and Diptera with at least 85% similarity to a sequence deposited in BOLD. Same holds true for the EPT taxa with Diptera excluded.

*OTUs > 85% similarity to a BOLD entry only.

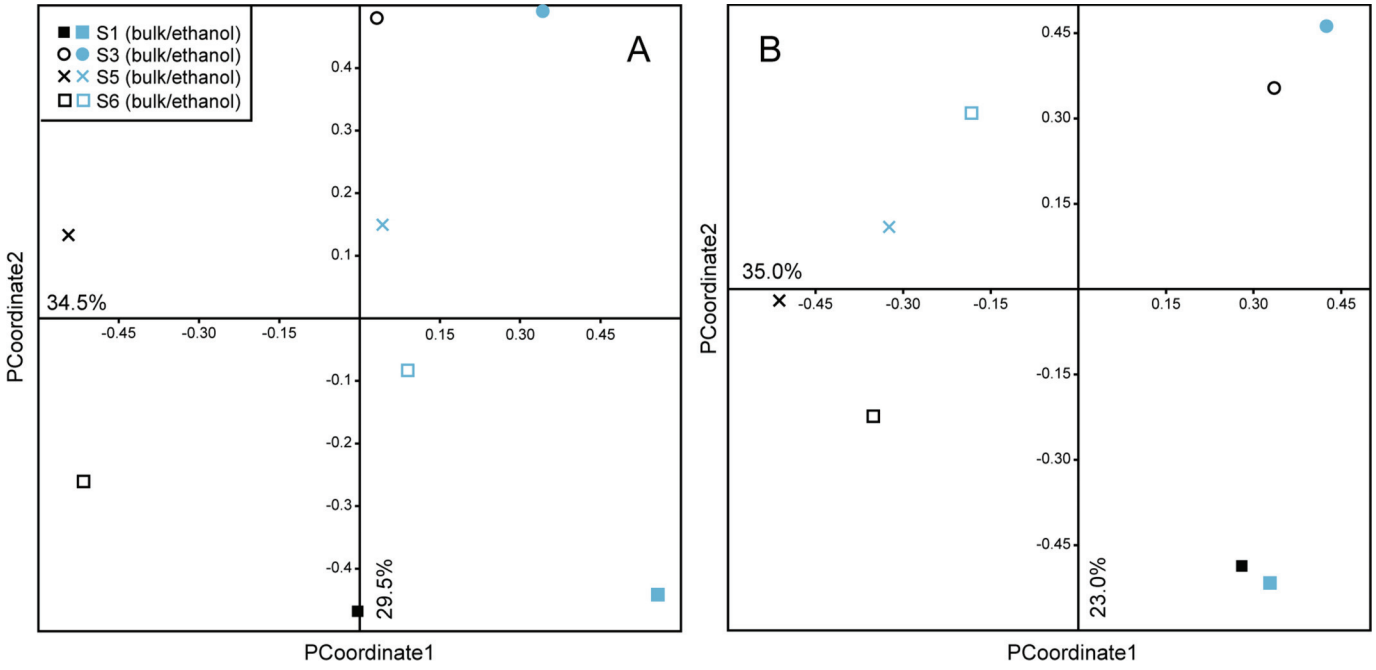
Fig. 3. Proportion of reads per order from all macroinvertebrate taxa in (A) bulk samples and (B) ethanol samples. The category Others includes all orders with read numbers <0.5%.



belonging to the Clitellata (mainly Haplotaxida) were only found in ethanol samples (Table S9[2]). Due to high differences in number of reads per sample (Fig. 2), read subsampling was performed to retain only 230 000 reads identified as macroinvertebrates per sample (Table S10[2]). The samples S2E and S4E showed macroinvertebrate read numbers <50 000 and where therefore excluded from further analysis. Consequently, for the comparisons to bulk samples, also S2B and S4B were excluded. Comparing macroinvertebrate data between the two methods based on the four remaining samples, both datasets had 232 OTUs in common, 39 were unique to the ethanol, and 85 to bulk samples (Table 2).

Reducing the taxonomic focus further (subsampled dataset) to Ephemeroptera (mayflies), Plecoptera (stoneflies), and Trichoptera (caddisflies), the overlap of both methods was 46 OTUs, 4 OTUs were unique to the ethanol samples (Ephemeroptera: *Ephemerella* sp.; Trichoptera: *Goera pilosa*, *Hydropsyche contubernali*, *Anabolia nervosa*) and 10 to the bulk samples (Ephemeroptera: *Habrophlebia lauta*, *Ecdyonurus torrentis*, *Baetis muticus*; Plecoptera: *Leuctra albida*, *Leuctra* sp., *Amphinemura borealis*; Trichoptera: *Oecetis testacea*, *Sericostoma flavicorne* (2×), *Limnephilus lunatus*) (Table S11[2]). Average numbers of reads were higher in bulk samples for all three EPT taxa (Ephemeroptera ethanol: 66 055, bulk: 106 924; Plecoptera: 68.5, 448.5; Trichoptera: 8487.5, 17 190). Including taxa

Fig. 4. Visualization of differences in macroinvertebrate community composition with scatter plots from PCoA for the four retained sample locations S1, S3, S5, and S6 on the 356 OTUs (taxa with >85% similarity to BOLD database entries) based on (A) presence/absence and (B) read abundance data. Each sample has been rarefied to contain only 230 000 reads.



belonging to the Diptera (EPTD) in both methods, 162 identical OTUs were detected, with 16 OTUs unique to the ethanol tests and 35 to the bulk samples. Within those OTUs, 12 uniquely found in bulk samples belonged to the Chironomidae, while eight OTUs assigned to chironomids were only found in ethanol tests (Table 2).

Including all detected macroinvertebrate OTUs > 85% sequence similarity to a BOLD record (356 OTUs after quality filtering and normalization to 230 000 reads/sample), PCoA analyses using similarity measures on presence/absence (Sørensen, Fig. 4A) and abundance (Bray–Curtis, Fig. 4B) data show distinct community compositions for all sample sites. Excluding read numbers (Sørensen, Fig. 4A), 34.5% of the variance is associated with the first axis, along which a clear separation between each sample pair according to the source of DNA (bulk/ethanol) is evident. The second axis depicts 29.5% of the variance (Fig. 4A) and separates the four sample locations, while an effect of the applied method (bulk/ethanol) is almost absent and the sample pairs show nearly identical values. The clear pattern in Fig. 4A is not evident in the PCoA including read number information (Fig. 4B) where an effect of the used method (bulk/ethanol) is not clearly associated to the first or second axis and all sample pairs (but S6) cluster closely together.

When comparing the composition of the 213 OTUs belonging to macroinvertebrate EPTD taxa between the sample sites in the rarefied dataset, the effect of the two different sources of DNA (bulk/ethanol) becomes smaller and the sites cluster closer together in the scatter plots (Fig. 5A) than compared to the results from macroinvertebrate OTU comparisons (Fig. 4A). Including read abundance per OTU information (Bray–Curtis, Fig. 5B), the communities from each sample location appear to become more similar based on the ordination technique, with the exception of S6, where the sample pair is strongly separated along axis two.

Discussion

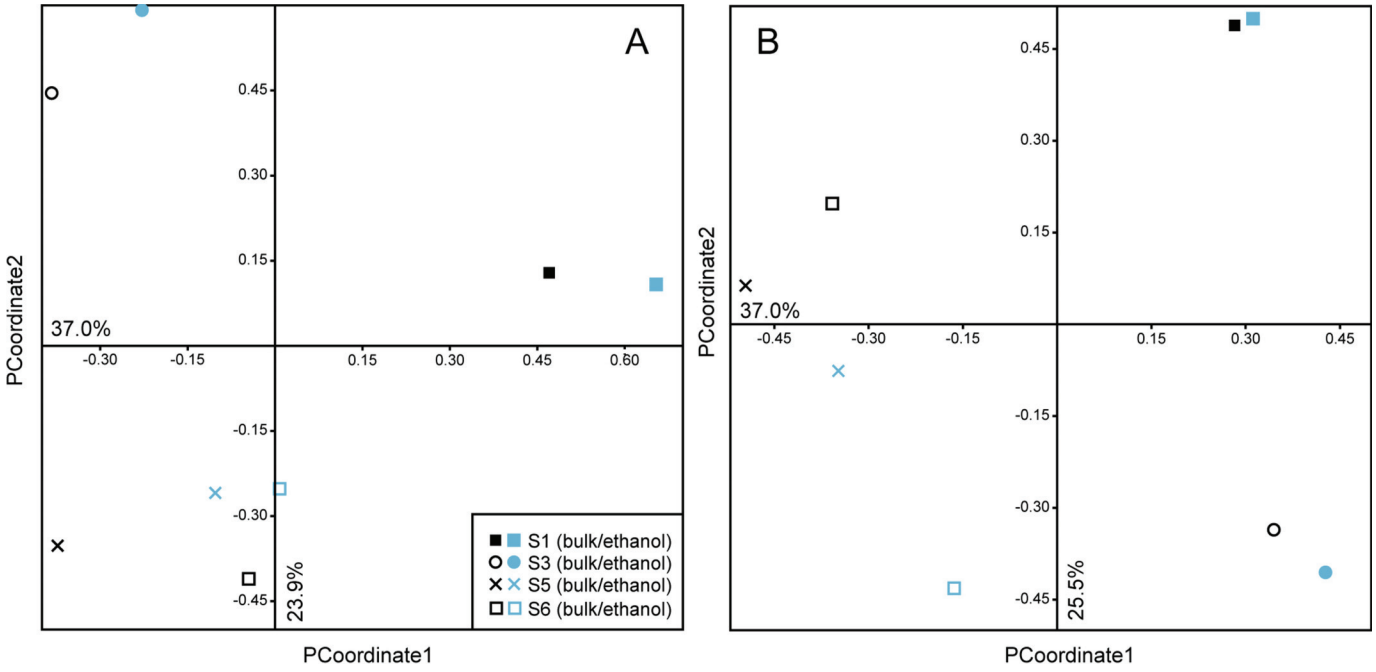
Filtration of ethanol and isolation of DNA from filters — Applicability of the method

As already shown by previous studies (Gilbert et al. 2007; Thomsen et al. 2009; Shokralla et al. 2010; Hajibabaei et al. 2012), it is possible to extract DNA from the fixative, and it successfully worked for three (protocol 0, 2, and 3) of the four filter-based protocols tested in our study. The insufficient sequencing output of samples processed with ultrasonic irradiation (protocol 1) could be due to a low irradiation frequency. The applied ultrasonic water bath (Bandalin SONOREX Super RK 510 H) works at a predetermined frequency of 35 kHz with indirect irradiation only. We chose this approach to exclude potential cross contamination through an ultrasonic probe system with direct contact between the device and sample material. Direct ultrasound irradiation at higher frequencies might induce an increase in cell disruption and thus higher rates of release of DNA to the fixative (e.g., Sinisterra 1992; Rokhina et al. 2009; Kwiatkowska et al. 2011).

Recovery of mock community composition

Taxa detection of arthropod species in samples processed with protocol 0 (first ethanol phase, no treatment), protocol 2 (shaking), and protocol 3 (freezing)

Fig. 5. Visualization of differences in EPTD taxa community composition with scatter plots from PCoA for the four retained sample locations S1, S3, S5, and S6 on the 213 OTUs (taxa with >85% similarity to BOLD database entries) based on (A) presence/absence and (B) read abundance data. Analysis is based on the rarefied dataset (230 000 reads).



was not consistent. With the exception of the genus *Gammarus*, each morphotaxon remained undetected in at least one sample. The unrecovered specimens were mainly categorized to size class S (small) or M (medium), with one unrecovered specimen of *P. flavomaculatus* from size class L (large). This contradicts a relation between specimen size and detection success in fixative-based metabarcoding within taxonomic groups, which is also congruent with findings in Hajibabaei et al. (2012) and correlation results of biomass and read abundance in the present study. It is unlikely that the differences in the recovery of arthropod taxa are due to primer bias since the used primers (BF2/BR2) have been tested and applied in several studies (Elbrecht and Leese 2015; Elbrecht et al. 2017*a*, 2017*b*) on a highly diverse mock community of aquatic invertebrates. The morphology of undetected arthropod species does not indicate systematic differences in DNA release potential due to body structure, which is also supported, because all arthropod taxa, but *Gammarus*, failed to be detected in one sample. A comparative study that quantified the DNA release of the same species to a fixative does not exist, but in Hajibabaei et al. 2012, representatives of same families (Heptageniidae, Leptophlebiidae, and Hydropsychiidae) and largely similar morphological features were successfully detected through fixative-based metabarcoding. Compared to arthropod taxa, mollusc taxa went undetected consistently across all tested protocols. *Ancylus fluviatilis* is of small body size (in the present study on average 4 mm) and dorsally covered by a shell and tissue is only accessible via a small surface area, which can in addition be covered by a layer of mucus (Calow 1974). These morphological characteristics should reduce the DNA release of specimens to the fixative and consequently detection success is decreased. Similar morphological characteristics as *A. fluviatilis* apply to the other mollusc taxa where a reliable recovery was also difficult. Checking for the possibility of primer bias as source for the poor recovery rate for molluscs revealed that while BR2 should give the same good performance as HCO2198-JJ, the BF2 primer sequence showed up to two mismatch positions, for example in *Radix* and *Ancylus* at two neighbouring bases close to the 3′ end of the primer (BF2: -GC- vs. -AG-). We speculate that this is another reason why molluscs were the least reliably detected in our ethanol tests. Taking into account recent findings from Carew et al. (2018) who had also problems in reliably detecting gastropods with operculum, our results indicate that the detection of molluscs through fixative-based metabarcoding might not be reliable at the moment.

Protocol specificity

The different protocols for the mock community treatments should lead to varying proportions between target DNA (directly from the mock community specimens) and DNA from other sources (attached organisms, stomach content, etc.). The specificity and efficiency of the protocols to enrich the target taxa's DNA should thus influence the overall detection success, or at least the proportion of on-target reads. However, as on average seven out of eight taxa were recovered with all three methods, we did not observe an influence on overall de-

tection success. With respect to the proportion of on-target reads, a clear effect was evident: for protocol 0 on average more than 65% of the reads were assigned to OTUs of untargeted taxa, which reduces reads provided for the actual mock community composition. This is in line with the expectations, as samples of protocol 0 are based on the first ethanol in which specimens were preserved. Consequently, a significant amount of DNA from environmental particles attached to the specimens or stomach content had been released into this fraction. Protocol 2 and 3 were applied to ethanol samples into which specimens were transferred after the application of protocol 0 into which loose cellular material was already washed out. Therefore, less DNA from non-target organisms was available in these samples that could influence taxa recovery in these protocols.

However, most of the reads assigned to untargeted taxa in samples processed with protocol 0 matched various dipterans, but also Clitellata and Ephemeroptera and a few other arthropods (Table S6[2]). In addition to particles or cells attached to the mock community specimens, stomach content of predatory taxa (e.g., *Gammarus*, *Hydropsyche*, and *Polycentropus*) might be the source of this additional diversity. This assumption is supported by the fact that most of the additional OTUs belong to taxa, which frequently serve as prey (mainly dipterans) (Cummins 1973; Klecka and Boukal 2013). Furthermore, it can be observed that some specimens regurgitate their stomach content when being preserved, which consequently should be detected by fixative-based metabarcoding.

To accomplish higher DNA yield and increase taxa detection, a combination of protocol 0 and 3 might be optimal. The inclusion of the stomach contents of specimens would lead to a more comprehensive community assessment. Furthermore, DNA release of taxa remaining undetected through protocol 0 could be increased with protocol 3. This is supported by the fact that all arthropod taxa, which could not be detected in samples treated with protocol 0, were recovered in samples processed with protocol 3. The combination of protocols could include an ethanol change in between, as was conducted for the present study, or simply an additional freezing step to the ethanol phase of protocol 0. The second possibility would also decrease workload, because it excludes the ethanol change step and only includes the transfer of samples to liquid nitrogen or an ultra-low temperature freezer (–150 °C) after sampling for a determined time (e.g., one hour).

Correlation between final read number and biomass

A weak correlation between biomass (weight of specimens in g) and number of reads was found for protocol 0. The strong scattering of the underlying data points and the fact that no significant correlation between biomass and read numbers was evident in the two other treatments indicate that biomass of individuals has a limited effect on the final read number, especially within taxonomic groups. However, the present results show that individuals release different amounts of DNA, which in turn biases the generated read numbers. For fixative-based metabarcoding, these biases seem to be more dependent on the morphology of the specimens or on the random dispersal of cells or even body structures on the filter. To help circumvent the problem of capturing complete cells on the 0.45 μm filter, additional filtering of the ethanol with a finer mesh size than applied in the present study (0.5 mm) could be applied before processing. This has also recently been proposed in a comparison of different filtration techniques for eDNA from water samples (Majaneva et al. 2018).

Comparison of environmental bulk samples with ethanol samples

A comparison between bulk and ethanol samples based on all detected taxa revealed completely different communities (Fig. 4). Specific untargeted groups (Bacillariophyta, Ochrophyta, Proteobacteria, etc.) are mainly present in ethanol samples, because whole kick net bulk samples include algae, parts of wood, gravel, and other substrate and consequently released DNA into the fixative, or function as substrate for other groups (bacteria, etc.). The degeneracy of the BF2/BR2 primers used in this study, lead to the amplification of taxa not even closely related to the targeted macroinvertebrates and might explain the high abundance of untargeted taxa in the dataset (Elbrecht and Leese 2017; Macher et al. 2018). This phenomenon is also known from eDNA studies where, in addition to the taxa of interest, many other groups were present in final sequencing results (Deiner et al. 2016; Macher et al. 2018). While for the sample points S3, S5, and S6, the riverbed substrate was dominated by gravel and stones, for sample points S1 and S2, the substrate was overgrown with algae. Here, a brief separation of organisms from larger plant material or a preceding filtering step would presumably decrease the number of reads assigned to untargeted taxa. Furthermore, if a particular taxonomic group is of interest, the usage of more specific primers would increase amplification accuracy (Elbrecht and Leese 2017).

For rarefied samples (230 000 reads) including only macroinvertebrates, the orders Diptera (mainly chironomids) and Haplotaxida were more strongly represented in read numbers in the ethanol samples (Fig. 3) and for Haplotaxida OTU richness was also higher. Both orders comprise of taxa with soft body structures and surfaces, where only the head is partly sclerotized. These taxa can therefore have an increased DNA release compared to other organisms with more pronounced exoskeletons. On the other hand, many chironomids, as well as oligochaetes, serve as prey for other macroinvertebrates (Hildrew and Townsend 1982; Krisp and Maier 2005; Klecka and Boukal 2013) and the additional OTUs or reads could result from DNA of regurgitated prey. This is

also concordant with results of fixative-based metabarcoding of mock communities and observations during specimen preservation (see above). The detection of prey organisms could be a positive by-product of this protocol, as it provides a more detailed insight into the present community. For bulk sample metabarcoding approaches, we speculate that the detection of prey organisms remains difficult unless a much higher sequencing depth is applied. This is due to the lower proportion of prey DNA in stomach compared to the actual specimen tissue.

The high number of detected chironomid OTUs (>80) and assigned species names to them (62 with >98% sequence similarity) is especially promising, as these non-biting midges are ecologically highly diverse and dominant in many aquatic ecosystems (Ferrington 2007). Chironomids have therefore been proposed as indicator group for freshwater ecosystem quality, but as their morphological identification is extremely difficult they are not used for WFD status assessment in Germany (cf. Operationelle Taxaliste). These difficulties and the lack of taxonomic experts for chironomids are also the reason for the low coverage of species with DNA barcodes available (~25% of 700 species in Germany; www.bolgermany.de). Our non-destructive approach for metabarcoding benthic communities might provide a promising additional source for reference chironomid species, which can either be studied morphologically after having been detected and taxonomically assigned, or can indicate specific sample locations for emergence trapping (as the adult males are usually needed for an unambiguous morphological identification). The potential of this approach is illustrated by the fact that 29 of the 62 via BOLD species-level-identified chironomids are still missing in the German Barcode of Life reference database (as of 21 March 2018) and were therefore identified by DNA sequence comparisons to material from other countries. Theissinger et al. (2018) recently showed how valuable species level knowledge on chironomids would be, as several species are indicators for good water quality (low saprobic values), but they are most often lumped together as *Chironomus* sp. with a high saprobic value indicative for bad water quality. Further evidence for the great potential of chironomids to identify stressor responses in freshwater ecosystems with metabarcoding has been highlighted by Beermann et al. (2018), where over 100 OTUs with distinct responses to stressors were found.

Bulk sample metabarcoding revealed a higher OTU diversity assigned to macroinvertebrates (317), and read numbers for Amphipoda, Coleoptera, Isopoda, Lumbriculida, Trichoptera, and Trombidiformes were also higher than in the ethanol samples (Fig. 3). We speculate that this is due to a lower relative number of template DNA fragments for the PCR for the aforementioned taxa in the DNA mixture from the filters. Representatives of these taxa often have a pronounced exoskeleton or are surrounded by a case of stable material (trichopterans), which can lead to the restraint of DNA. Additionally, Trombidiformes are extremely small, which further reduces total DNA release. The decreased number of reads available for those taxa might further be explained by the competition for sequencing coverage, especially when high fractions are "used up" for other taxonomic groups (algae, etc., see above). For other taxa, which do not fulfil these features, it is difficult to conclude whether those were rare species, as was also stated as a reason for lower recovery in Hajibabaei et al. 2012, or if they belonged to extremely small specimens, as we did not inspect the bulk samples in detail or count specimens.

Regarding EPT taxa, the two methods provided 46 overlapping OTUs with an additional 4 OTUs unique to the ethanol samples and 10 OTUs unique to bulk samples. Within the EU WFD, these taxonomic groups are essential biological quality elements (BQEs) for the assessment of the ecological status of rivers and lakes (Meier et al. 2006). These results indicate the usefulness of this method for biodiversity assessment of certain taxa or for biomonitoring approaches without the time-consuming step of specimen sorting and the consequent decrease in costs compared to bulk sample metabarcoding or morphological identifications. In particular, metabarcoding of the fixative could become an especially viable alternative in cases where legal frameworks do not allow homogenizing the organismal samples.

The comparison of our findings from the fixative-based metabarcoding of mock communities and its application to real environmental samples exemplifies the general feasibility of this method. The biggest advantages over destructive metabarcoding approaches are the detection of additional taxa (from stomach content, etc.) and the possibility for morphological a posteriori studies. On the downside are the poor detection rate of mollusc taxa and a higher proportion of non-target reads, which lowers the cost-efficiency per sequencing run. Both issues could be improved by the development of either better primers, or the addition of another set of more specific primers. Adopting the findings of the mock community experiment to environmental samples, a combination of protocol 0 (first fixative phase) and 3 (freezing), could further enhance our understanding of real community compositions.

Conclusions

We showed that metabarcoding of DNA extracted from fixative (ethanol) is in general possible, although several false negative cases are to be expected (especially mollusc taxa). Mock community and field sample analyses showed that the detection of the biological quality elements (EPTD taxa) relevant for ecological status assessment was highly successful and comparable to taxa

lists generated by bulk sample metabarcoding. The extraction of DNA from ethanol and the subsequent metabarcoding is thus a promising less-invasive and time efficient alternative to standard specimen-based metabarcoding approaches.

Conflict of interest statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Assessing the influence of sample tagging and library preparation on DNA metabarcoding

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RESOURCE ARTICLE

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Assessing the influence of sample tagging and library preparation on DNA metabarcoding

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Abstract

Metabarcoding is increasingly used to assess species diversity by high-throughput sequencing where millions of sequences can be generated in parallel and multiple samples can be analysed in one sequencing run. Generating amplified fragments with a unique sequence identifier ('tag') for each sample is crucial, as it allows assigning sequences to the original samples. The tagging through so-called fusion primers is a fast and cheap alternative to commercially produced ligation-based kits. However, little is known about potential bias and inconsistencies introduced by the long nucleotide tail attached to those primers, which could lead to deficient reports of community composition in metabarcoding studies. We therefore tested the consistency and taxa detection efficiency of fusion primers in (1) a one-step and (2) two-step PCR protocol as well as (3) a commercially manufactured Illumina kit using mock communities of known composition. The Illumina kit delivered the most consistent results and detected the highest number of taxa. However, success of the two-step PCR approach was only marginally lower compared to the kit with the additional advantage of a much more competitive price per library. While most taxa were also detected with the one-step PCR approach, the consistency between replicates including read abundance was substantially lower. Our results highlight that method choice depends on the precision needed for analysis as well as on economic considerations and recommend the Illumina kit to obtain most accurate results and the two-step PCR approach as a much cheaper yet very robust alternative.

KEYWORDS
fusion primers, Illumina, index, one-step, tag switching, two-step

1 | INTRODUCTION

Metabarcoding is a powerful molecular tool for biodiversity assessment. The method involves the identification of multiple taxa in environmental samples based on the bulk extraction of DNA and the parallel amplification of short fragments with universal primers (Carew, Pettigrove, Metzeling, & Hoffmann, 2013; Hajibabaei, Shokralla, Zhou, Singer, & Baird, 2011; Taberlet, Coissac, Hajibabaei, & Riesenberg, 2012; Yu et al., 2012). The unique labelling of amplicons allows multiple samples to be sequenced on a single run and ensures the subsequent computational assignment of sequences to their original sample (Binladen et al., 2007; Elbrecht & Leese, 2015; Lundberg, Yourstone, Mieczkowski, Jones, & Dangl, 2013; Schnell, Bohmann, & Gilbert, 2015; Taylor et al., 2008). Following standard library preparation protocols, these tags are typically short nucleotide sequences, which are implemented in commercial and often expensive kits (Bourlat, Haenel, Finnman, & Leray, 2016; Illumina, 2015; Kircher, Sawyer, & Meyer, 2012; Schnell et al., 2015). As an alternative labelling approach, so-called "fusion"—PCR primers have been developed (Elbrecht & Leese, 2015, 2017a; Lundberg et al., 2013). These primers carry a long nucleotide tail consisting of a flow cell binding adapter (P5/P7), a binding site for sequencing primers

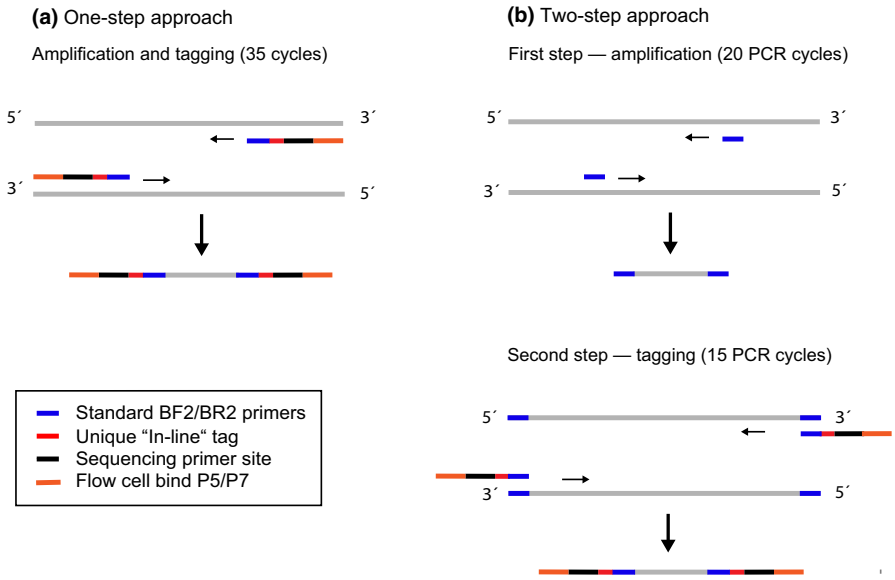

FIGURE 1 Simplified scheme of the one-step and two-step approach implementing fusion primers. (a) Amplification and tagging of the target fragment in a single PCR step. PCR primers carry a long tail with a unique in-line tag, a sequencing primer binding site and the flow cell binding adapters at their 5′-end. After 35 PCR cycles, the amplified fragment is hence provided with the nucleotide tail and prepared for sequencing. (b) Amplification and tagging of the target fragment are separated in two PCR steps. In a first PCR of 20 cycles, the target fragment is amplified using standard primers. In a second PCR of 15 cycles, amplified sequences are tagged using fusion primers as in (a). The amplified fragment is hence provided with the nucleotide tail and prepared for sequencing. For detailed information about the mechanism of the used kit with i5/i/ indexes, see the TruSeq Nano DNA Library Preparation Guide 2015 [Colour figure can be viewed at wileyonlinelibrary.com]

and a label to identify the sample consisting of a short base sequence ("in-line tag") attached to their 5′-end, which ensures the amplification and labelling of samples in one or two PCR steps (Binladen et al., 2007; Elbrecht & Leese, 2015; Lundberg et al., 2013).

In the one-step approach with fusion primers, a target DNA fragment is PCR amplified and simultaneously tagged with the long nucleotide tail attached to the primer's 5′-end (Figure 1a). This one-step method is fast, involves comparatively low costs and laboratory effort and has been successfully used in several metabarcoding studies (Beermann, Zizka, Elbrecht, Baranov, & Leese, 2018; Elbrecht & Leese, 2015, 2017a, 2017b). However, as indicated by former studies (Berry, Mahfoudh, Wagner, & Loy, 2011; O'Donnell, Kelly, Lowell, & Port, 2016), even overhangs of <10 nucleotides in length, so-called tags, on the primer's 5′- end can be associated with substantial PCR bias potentially resulting in extremely different communities inferred depending on the tag combination used. These biases are likely caused by tag-specific mismatches with the PCR template and the subsequent variation of primer binding efficiencies between taxonomic groups (O'Donnell et al., 2016). If the results of metabarcoding studies in fact strongly depend on the tag included, this will also be a problem in approaches implementing fusion primers and would be a major drawback for the use in metabarcoding projects and biodiversity assessment in general. Thus, the topic is a pressing issue in metabarcoding research.

The splitting of the amplification and labelling of a target fragment into two PCR steps (two-step PCR) should reduce the mentioned inefficiencies (Figure 1b). In this method, the target fragment is amplified using standard primers without tags in a first PCR step (but see e.g. Bohmann et al. 2018 for different two-step PCR approach). The labelling and attachment of Illumina adapters occurs in a second PCR, where no interaction with the template is possible, which diminishes the problem of reduced amplification efficiencies caused by mismatches (Berry et al., 2011; Bourlat et al., 2016; Leray & Knowlton, 2017; O'Donnell et al., 2016). Compared to the one-step approach, this method involves one additional PCR step and is therefore more time-consuming, costly and also prone to cross-contamination.

In this study, we tested the consistency and efficiency of taxa detection of fusion primers in a one-step and two-step PCR in comparison with a fabricated TruSeq Nano DNA Library Preparation kit (Illumina, 2015). To test this, we used mock communities consisting of similar tissue amounts from 52, previously identified freshwater taxa and investigated the number of recovered taxa per method. Furthermore, we analysed the consistency of read proportions between technical replicates. Finally, we discuss the implications of the results for future metabarcoding-based freshwater biodiversity assessments.

2 | MATERIAL AND METHODS

2.1 | Sample preparation and sequencing

Experiments were conducted using five mock communities with similar taxa composition (communities A–E) (Elbrecht & Leese, 2015, see Supplementary material 12 therein). Each community consisted of 52 macroinvertebrate individuals belonging to different taxa based on morphological identification (Table S1, Elbrecht & Leese,

2015, see Supplementary material 12 therein). Purified DNA aliquots (25 ng/µl) were processed as described in Elbrecht and Leese (2015). For each community, the three methods (1–3, see below) were applied with three replicates each:

1. *One-step PCR*: The cytochrome c oxidase subunit I (CO1) fragment was amplified and labelled in a single PCR step using uniquely indexed BF2/BR2 fusion primers (Elbrecht & Leese, 2017b, Tables S2 and S4). PCR consisted of 1× PCR buffer (including 2.5 mM Mg^{2+}), 0.2 mM dNTPs, 0.5 µM of both fusion primers, 0.025 U/µl of HotMaster Taq (5 Prime) and 25 ng DNA and was filled up with HPLC H_2O to a total volume of 50 µl. The following PCR program was used: 94°C for 180 s, 35 cycles of 94°C for 30 s, 50°C for 30 s and 65°C for 150 s, followed by a final elongation of 65°C for 5 min in a Thermocycler (Biometra TAdvanced).
2. *Two-step PCR*: In a first PCR step, the CO1 fragment was amplified using untailed BF2/BR2 primers (Elbrecht & Leese, 2017b) with identical PCR conditions to the previous method (one-step), but only 20 PCR cycles. The PCR product was diluted (1:10 with water), and 1 µl of the dilution was used as the template for the second PCR step. Amplified sequences were labelled using uniquely tagged BF2/BR2 fusion primers (Tables S2 and S4) with identical PCR conditions as in the previous step but 15 PCR cycles.
3. *TruSeq Nano DNA Library Prep Kit (Illumina)*: The CO1 fragment was amplified as in the first PCR step described in (2). The PCR product was diluted (1:10 in water) and used as the template for sequence labelling. Sequences were labelled using the TruSeq Nano DNA Library Preparation Kit starting from step 2: "Repair Ends and Select Library Size" to step 5: "Enrich DNA Fragments". The kit implements a ligation-based tagging of amplicons and a selective enrichment of DNA fragments with indexed adapter molecules on both ends through a PCR with eight cycles (Illumina, 2015).

A left-sided size selection of all samples was performed with magnetic beads (SPRIselect BECKMAN COULTER) with a ratio of 0.76×, and the concentration of selected PCR products was measured on a Fragment Analyzer (Advanced Analytical). All samples were equimolarly pooled, and paired-end sequencing was carried out by GATC Biotech AG (Konstanz, Germany) using one MISEQ sequencing run and the 250 bp paired-end v2 kit.

2.2 | Data analysis

Sequences labelled with fusion primers were assigned to their original sample as implemented in JAMP v0.23 (https://github.com/VascoElbrecht/JAMP). Sequences labelled through indexed adapters included in the TruSeq Nano DNA Library Preparation kit were computationally assigned to their original sample by GATC Biotech AG. Subsequent data processing was conducted for all samples as implemented in JAMP v0.23 using standard settings. Paired-end reads were merged (module U_merge_PE), and reverse complements were built where needed (U_revcomp) with USEARCH v10.0.240 (Edgar, 2010). CUTADAPT 1.6 (Martin, 2017) was used to remove primers and to discard sequences of unexpected length so that only reads with a length of 411–431 bp were used for further analyses. The module U_max_ee was used to discard all reads with an expected error >0.5. Sequences were dereplicated, singletons were removed and sequences with ≥97% similarity were clustered into OTUs using UPARSE (U_cluster_otus). OTUs with a minimal read abundance of 0.004% (>1 reads in samples with 30,000 reads, i.e. excluding singletons) in at least one sample were retained for further analysis while other OTUs were discarded. The used script for data analysis is attached in the supplements (Script S1).

Due to differences in read numbers, the data set was rarefied to an equal sequencing depth of 30,000 reads per sample using USEARCH v10.0.240 (*fastx_subsample*) after quality filtering. Taxonomic assignment of obtained sequences was done by comparisons with the reference database of BOLD systems v4 (Ratnasingham & Hebert 2007) as implemented in JAMP v0.23. Different filter thresholds were calculated to determine the minimum read abundance of an OTU in each sample to be included in analyses, ranging from 0.004% (low filtering threshold) to 0.09% (strict value). At low filtering thresholds, a higher number of low abundant OTUs are included in the data set and assigned taxa are displayed in the community composition. Simultaneously, false-positive sequences produced by sequencing mistakes or tag switching also remain in the data set, which can distort the community composition. Increasing filtering thresholds can remove such sequences, but also increase the risk of losing low abundant OTUs.

Detected taxa composition per sample was compared with the reference list of Elbrecht & Leese, 2015. Bray–Curtis and Jaccard dissimilarity indices based on OTUs were calculated between replicates of each sample and the various thresholds using the R (R Development Core Team, 2008) package VEGAN 2.4-2 (Oksanen et al., 2017). Bray–Curtis dissimilarity considers also read numbers per OTU, whereas Jaccard dissimilarity considers only presence or absence of an OTU. No data transformation was conducted for calculations. A one-way analysis of variance (ANOVA) was conducted for the number of detected taxa (dependent variable) and the three different methods (independent variable) at varying thresholds. A post hoc test (Tukey HSD test) was performed to analyse if there were significant differences in taxa detection between the three different methods (threshold 0.004–0.09; Figure 2). The influence of the three methods on Bray–Curtis and Jaccard dissimilarity between replicates was analysed as above, and the data were tested for significance with a post hoc test (Tukey HSD test) at a threshold of 0.004 (Figure 3). Data were visualized using the GGPLOT2 package (Wickham, 2009) in R.

3 | RESULTS

In total, we obtained 10,271,201 read pairs (raw data are available on Short Read Archive, submission number: SRP162847). Of these, 2,121,547 were assigned to the 30 samples (five communities × two methods × three replicates) labelled with fusion primers (Table S4, Figure S1), i.e., 876,035 (8.53%) to samples processed with

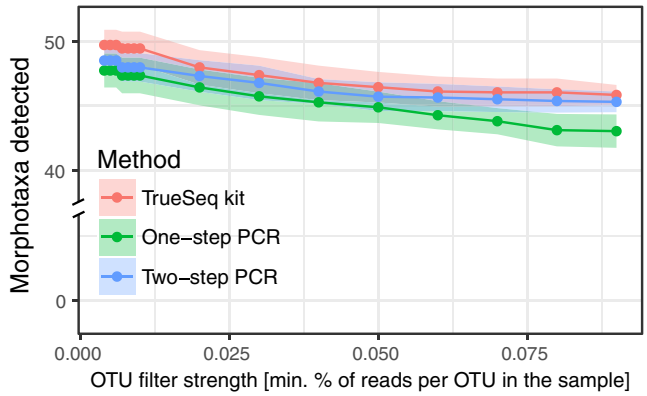

FIGURE 2 Average number of detected known morphotaxa (*y*-axis) for the three compared methods. Filled dots indicate the average proportion of detected taxa across all communities (a–e) and replicates at different filtering thresholds (0.004–0.09). Coloured shading indicates the standard deviation

the one-step and 1,245,512 (12.13%) to the two-step approach. For the 15 samples (5 × 1 × 3) labelled with the TruSeq Nano DNA Library Preparation kit, we obtained 5,282,478 (51.43%) reads. Unused primer combinations produced 111,823 (1.089%) reads, and 105,713 of these were assigned to one primer combination. Other unused combinations comprised a total of 5,749 reads (0.06%) and were thus discarded (Table S4, Figure S1). In total, 2,755,353 reads were rejected as debris after demultiplexing and not used for further analysis (26.83%). Thus, the different samples and methods showed variation in the total read number (average read number one-step approach: 58,402, standard deviation (SD): 23,985; two-step approach: 83,034, SD: 27,024; TruSeq Nano DNA Library Preparation kit: 352,165, SD: 269,251; Table S4, Figure S1). Two samples (C_one_3 [community C, one-step method, replicate three], D_one_2) processed in the one-step PCR had <30,000 reads after paired-end merging and were therefore excluded from further analysis (Table S4, Figure S1).

After taxonomic assignments with BOLD systems v4, the taxa list of each sample was compared with the original taxa list of the mock communities based on morphological identification (Figure 2). Across all samples, the vast majority of reads (on average 99.3% ± 0.9) was assigned to the target taxa included in the mock communities (Table S5), similar to the findings by Elbrecht and Leese (2015) (see Figure S9 therein). At lower filtering thresholds (0.004–0.01), the average number of detected taxa was at minimum 47 (90%) for the three applied methods. Undetected taxa were mainly those individuals with little biomass (e.g. Trombidiformes, *Dugesia*, see Table S3 in Elbrecht and Leese), where detection rate clearly decreased with increasing filtering thresholds. Furthermore, taxa which are known to amplify less successfully with the universal BF2/BR2 primers (Nematoda, Gastropoda, see Elbrecht and Leese (2017b), Weigand & Macher, 2018) were less frequently detected. The highest number of taxa was constantly detected in samples processed with the TruSeq Nano DNA Library Preparation kit (threshold 0.004–0.01: on average 48.9 [±1] taxa, threshold 0.02–0.09:46.4 [±1.2 taxa]). Numbers were significantly higher (Table S3) than those detected in samples processed with the one-step approach for all filtering thresholds (one-step threshold 0.004–0.01: on average 47.3 [±1.3] taxa, threshold 0.02–0.09:44.5 [±1.65] taxa). At low filtering thresholds (0.004–0.01), the number of detected taxa was also significantly higher than in samples processed with the two-step approach (two-step on average 48.1 [±1] taxa). In comparison, at higher filtering thresholds

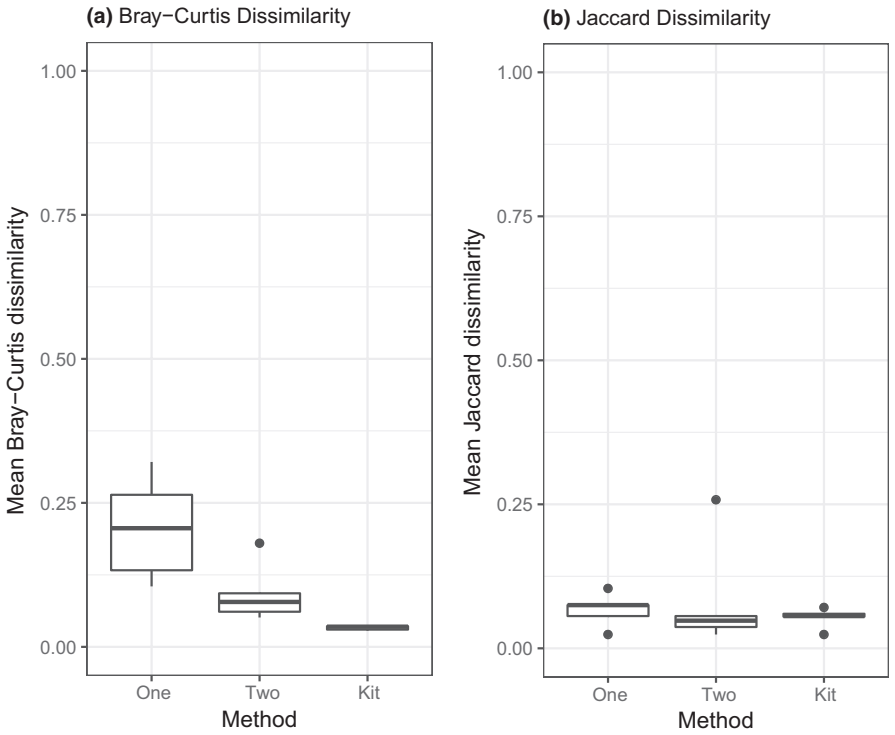

FIGURE 3 Boxplots for Bray–Curtis (a) and Jaccard (b) OTU dissimilarity indices among replicates of the different amplicon labelling methods: "one" = one-step PCR, "two" = two-step PCR, "kit" = TruSeq Nano DNA Library Preparation kit. The figure displays analyses with a filtering threshold of 0.004, which was used for previous metabarcoding studies. Increased thresholds showed no changes in dissimilarity values (see Figures S1 and S2 for further information)

results showed no significant differences (two-step threshold 0.02–0.09:45.7 [±1.2] taxa, Table S3). Up to a threshold of 0.05, on average one taxon more was detected with the two-step (47.7) than with the one-step (46.7) approach (Figure 2), although this difference was not significant. However, with higher thresholds (>0.05), differences became significant (Table S3) and on average two taxa more were detected with the two-step method. From threshold 0.01 on, the average detection clearly decreased for all three methods but more slowly for samples processed with the kit and the two-step than with the one-step approach (Figure 2). Thereby, 45.8 and 45.3 taxa were still detected at a strict threshold of 0.09 with the kit and the two-step method, respectively. With the one-step method, taxa detection was 43 at a threshold of 0.09. Samples processed with this method showed also the highest standard deviation. Detailed information of number of detected taxa per method without a subsampling after quality filtering is given in Figure S4. Results are largely congruent with those of the subsampled data set.

No distinct differences could be observed for calculated Bray–Curtis and Jaccard dissimilarity indices with varying filtering thresholds (Figures S2 and S3). Therefore, the threshold of 0.004 was selected for Figure 3a,b. The Bray–Curtis dissimilarity index among replicates was low for samples processed with the TruSeq Nano DNA Library Preparation kit (<0.1). Dissimilarity values were higher for replicates processed with fusion primers in a two-step PCR but also remained below 0.15 except for one outlier. Replicates of samples processed with fusion primers in a one-step PCR showed the highest Bray–Curtis dissimilarity (Figure 3a) and the highest variance, which was, however, below 0.3 (Figure 3a). Differences between replicates processed with this method were significantly higher than differences between replicates processed with the two-step method ($p = 0.0278$) or the kit ($p = 0.0017$). Differences between the two-step method and the commercial kit approach were not significant ($p = 0.2962$, ANOVA $F = 10.76$). The Jaccard dissimilarity index of samples processed with the kit and fusion primers in a two-step PCR was below 0.15. Again, in case of the two-step approach, one outlier arose with a dissimilarity value of 0.25. Replicates of samples processed with fusion primers in a one-step PCR showed the highest Jaccard dissimilarity and the highest variance (Figure 3b). However, all dissimilarity values were below 0.15. No significant difference could be observed between the methods at a threshold of 0.004 (Figure 3b; ANOVA $F = 0.347$, $p = 0.714$).

4 | DISCUSSION

Our results show that all three tested methods could reliably detect the taxa composition of a diverse macrozoobenthic community. This was supported by Jaccard dissimilarity calculations that showed no significant differences between the three methods. The average number of detected taxa was constantly highest in samples processed with the TruSeq Nano Library Preparation kit; yet, differences to the two-step approach were minor and insignificant for most filtering thresholds, with only one or two more taxa detected by the Illumina kit. More than 90% of the taxa were still detected in samples processed with the one-step approach at lower filtering thresholds. When considering read abundance for individual OTUs in the comparison via Bray–Curtis dissimilarity, the Illumina kit showed the highest consistency. Again, dissimilarity estimates for the two-step approach were only insignificantly higher. For the one-step method, Bray–Curtis dissimilarity values between replicates were the highest and also significantly different to the other two methods indicating a lower consistency and usability for quantitative analysis of this approach. However, in comparison with previous studies (Berry et al., 2011; O'Donnell et al., 2016) our findings indicate low primer-specific bias caused by the nucleotide overhang of the primers 5′-end. This is also congruent with findings by Leray and Knowlton (2017), who did a similar study on a mock community consisting of 34 marine macroinvertebrate taxa.

As indicated by Schnell et al. (2015), tag switching ('tag jumps') for an Illumina kit-based analysis was higher (2.5%–2.7%) than the rate observed for the one-step fusion primer approach (0.01%, Elbrecht and Leese (2015, 2017a)), which leads to a higher number of deficient sequences in the data set. For the present study, tag switching, which is determined by the numbers of reads assigned to unused primer combinations, is difficult to define. This is due to one primer combination (BF20/BR20), which was not used during laboratory processes but consisted of approximately 100,000 reads after demultiplexing. The emergence of these sequences is unclear. Cross-contamination could be a source for the incorrect tagging (Schnell et al., 2015); however, this is not consistent with the other results. The inclusion of these 100,000 sequences for calculations of tag switching indicates a proportion of 1.062%, whereas the proportion of tag switching is reduced to 0.058% when this combination is excluded.

Samples processed with the three different methods showed extremely different read numbers. In particular, the samples processed with the TruSeq Nano DNA Library Prep kit showed constantly the highest read numbers, with up to 1,122,685 for one sample (Figure S1, Table S4). All samples, independent from the used tagging method, were equimolarly pooled before sequencing, with a foregone concentration measurement of DNA fragments (Elbrecht & Leese, 2015; Illumina, 2016). However, the sequencing primer binding site of fusion primers used in the present study differs from the ones used in the Illumina kit (see Elbrecht and Leese (2015), Illumina, 2015, 2016). Differences can be due to recent changes of sequencing primer used on the Illumina platform or a mistake during primer design. Since reads from both indexing methods were sequenced in one run, a greater affinity of the sequencing primer to sequences labelled with the Illumina kit is likely and might have led to the observed differences in read numbers. Furthermore, the protocol implemented in the kit used different PCR ingredients (e.g. Taq polymerase in PCR cocktail) and a lower number of PCR cycles were performed (28 vs. 35 cycles).

The two methods implementing fusion primers with tags have some advantages over commercial kits due to their lower cost and required handling time. For the labelling of the 15 samples of the present study, two sets of the TruSeq Nano DNA Library Preparation kit were needed for 800 € each (set A/B—12 index combinations, 24 reactions each), resulting in a price of 106 € for each sample (Illumina,

2015). In comparison, the two-step approach for all 15 samples cost approximately 314.40 € in total and 20.96 € per sample, with the one-step approach costing slightly less. In detail, the ordered fusion primers (100 µM) cost approx. 36 € per primer (80–160 reactions) and 288 € in total, as four primer pairs were needed to achieve the number of primer combinations required for the individual tagging of the 15 samples. Other consumables needed for the two-step approach for all 15 samples cost approximately 26.40 € (and slightly less for the one-step method). The tagging process with fusion primers implements only one or two PCR steps (depending on the method) and a final size selection, while a successful library preparation with the TruSeq Nano Library Preparation kit is based on several preceding steps (end repair, creating an A-overhang for ligation). The kit approach also requires various clean-up steps, rendering the method more time-consuming and prone to cross-contamination (Schnell et al., 2015).

Comparing the one-step and two-step methods that add tags via fusion primers (with identical Illumina 5′-tails), the two-step approach showed a higher consistency and taxa detection efficiency. However, differences between the two approaches were not as strong as described in previous studies (Berry et al., 2011; O'Donnell et al., 2016). An additional advantage of the two-step method is presumed to lie in its decreased susceptibility to inhibitors. While this hypothesis was not specifically tested, samples including inhibiting substances (humic acids, tannins, flavonoids contained in alder foliage on which many freshwater species feed) could not be amplified in a single PCR step, but showed clear products when the two-step method was applied (using similar numbers of PCR cycles). These observations indicate that the untailed, shorter primers used in the first step of the two-step PCR method increase amplification success. The dilution of the product of the first PCR step also dilutes any associated inhibiting substances, subsequently resulting in a lower influence of the inhibitors on the fusion primers in the second PCR step where the tagging occurs.

In contrast, the advantage of the one-step approach is that it requires less time and is less prone to cross-contamination than the two-step approach because it omits the second PCR step. This makes it applicable for metabarcoding approaches using clean samples that are known to contain low amounts of inhibiting substances and in cases where detecting the maximum number of species in a sample is not important. For detailed, larger-scale biodiversity assessments, the choice of method is coupled with financial possibilities. Here, the more expensive Illumina kit may deliver the most consistent results, but is by far the most expensive and time-consuming method. With limited time and budget, the two-step PCR approach, which showed in addition no significantly lower consistency despite a greater number of PCR cycles applied, is a very good alternative with almost similar performance in consistency and taxa detection efficiency.

5 | CONCLUSION

Our results show that the commercial kit shows the most consistent and promising results for detailed biodiversity assessment of macroinvertebrates. However, the two-step PCR approach implementing fusion primers had almost comparable benchmarks in terms of taxa detection and consistency between replicates. Both the two-step PCR approach and the kit performed significantly better than the one-step PCR approach in terms of consistency and PCR efficiency, as well as showing a reduced sensitivity to inhibitors. Due to these advantages and the comparatively low costs, we recommend the two-step PCR method for cost-efficient large-scale metabarcoding approaches. However, the commercial TruSeq Nano DNA Library Prep kit detected most taxa on average and showed highest consistency between replicates and is therefore the best option for elaborate studies where a detailed and comprehensive diversity assessment is necessary.

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CONFLICT OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the study.

AUTHOR CONTRIBUTIONS

F.L., V.E., J.N.M. and V.Z. conceived the ideas and designed the methodology; V.Z. carried out the laboratory work and performed bioinformatic analyses; V.Z. and F.L. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA ACCESSIBILITY

DNA sequences: raw data are accessible on GenBank (Short Read Archive; submission number SRP162847); processed data are available in the online Supporting Information.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Chapter 2

Application of DNA metabarcoding

DNA metabarcoding reveals the complex and hidden responses of chironomids to multiple stressors

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RESEARCH

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DNA metabarcoding reveals the complex and hidden responses of chironomids to multiple stressors

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Abstract

Background: Chironomids, or non-biting midges, often dominate stream invertebrate communities in terms of biomass, abundance, and species richness and play an important role in riverine food webs. Despite these clear facts, the insect family Chironomidae is often treated as a single family in ecological studies or bioassessments given the difficulty to determine specimens further. We investigated stressor responses of single chironomid operational taxonomic units (OTUs) to three globally important stressors (increased salinity, fine sediment and reduced water flow velocity) in a highly replicated mesocosm experiment using a full-factorial design (eight treatment combinations with eight replicates each).

Results: In total, 183 chironomid OTUs (97% similarity) were obtained by applying a quantitative DNA metabarcoding approach. Whereas on the typically applied family level, chironomids responded positively to added fine sediment and reduced water velocity in the streambed and negatively to reduced velocity in the leaf litter, an OTU-level analysis revealed a total of 15 different response patterns among the 35 most common OTUs only. The response patterns ranged from (a) insensitivity to any experimental manipulation over (b) highly specific sensitivities to only one stressor to (c) additive multiple-stressor effects and even (d) complex interactions.

Conclusion: Even though most OTUs (> 85%) could not be assigned to a formally described species due to a lack of accurate reference data bases at present, the results indicate increased explanatory power with higher taxonomic resolution. Thus, our results highlight the potential of DNA-based approaches when studying environmental impacts, especially for this ecologically important taxon and in the context of multiple stressors.

Keywords: Taxonomic resolution, Chironomidae, OTU, Stressor responses, Field experiment

Background

Chironomidae ('non-biting midges') is a highly diverse family of insects with an estimated global richness of up to 20,000 species [11]. They occur in every zoogeographic region including Antarctica [4], and their larvae inhabit limnic, marine, terrestrial, and even subterranean environments [1, 13]. Chironomids occur over a wide range of environmental gradients, including gradients of pH, salinity, dissolved oxygen, water level, and temperature (reviewed in: [3, 56]). In streams and lakes, they are frequently the most abundant group of insects [56] often accounting for at least 50% of the total macroinvertebrate species [3]. Because of their species richness and high abundance, chironomids play an important role in aquatic and terrestrial food webs (e.g., [75]). Chironomidae larvae are found in all functional feeding groups (gatherers, filterers, scrapers, shredders, and predators) and many species are able to exhibit different feeding modes [6]. Chironomid larvae themselves are prey to fish [58] and many species of invertebrates (reviewed in [56]).

Despite their species richness, diversity, abundance, and ecological importance, the taxon Chironomidae has not experienced the same autecological in-depth research as other freshwater taxa [56]. Thus, despite

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detailed investigation of single species such as *Chironomus riparius* [3, 53] or *Chironomus plumosus* [30, 32], many ecological studies only treat Chironomidae at family or subfamily level. This divergence is partly attributed to their small body size and difficulties in the morphological identification of their larvae and adults, which hinders studying the autecology of species, especially in field studies. Beyond that, the taxonomic impediment combined with the high abundance of Chironomidae makes it often not feasible to use the taxon in ecological studies or for morphology-based routine biomonitoring below family or subfamily level. Even though for Chironomidae an increased taxonomic resolution can be achieved by studying pupal exuviae rather than larvae [24], exuviae drift downstream on the water and thus do not provide the exact same information on time and space as benthic sampling.

Identifying communities with many species using classical taxonomy demands increased time, money, and expertise (e.g., [33, 34]). Therefore, the concept of taxonomic sufficiency [21] was developed, which suggests using higher taxonomic levels than species as taxonomic surrogates. Consequently, many studies addressing the concept of taxonomic sufficiency revolve around the question if there is a loss of information when higher taxonomic levels are used (e.g., [37, 46, 68]). However, a precise alternative to morphological species identification is nowadays possible with molecular methods such as DNA barcoding [29] or its fast and cost-efficient extension DNA metabarcoding [66], which allows for simultaneous analysis of hundreds of specimens and species. Molecular methods are already widely used for species identification (e.g., [8, 28, 73, 76]) and have been shown to be beneficial for bioassessments [19, 22, 25, 64]. These new DNA-based techniques now also make it possible to test and even circumvent the concept of taxonomic sufficiency. They hold the potential to provide highly resolved data on community change under stressor conditions that is typically not assessed. The importance of this additional layer of resolution for ecosystem process understanding and subsequently management measures has been rarely explored so far. Key targets for such studies should be highly abundant, ecologically relevant and species-rich taxa like Chironomidae.

In this study, we selected Chironomidae as an ecological key taxon and investigated specific stressor responses of single Chironomidae operational taxonomic units (OTUs)—likely representing individual species—obtained by DNA metabarcoding. The chironomids were obtained from a former mesocosm field experiment [5] that studied effects of three globally important stressors on stream communities. The tested stressors were increased salinity, added fine sediment, and reduced water flow velocity, alone and in all possible combinations. Among the macroinvertebrate taxa in the experiment, the Chironomidae were the most abundant group (59,325 specimens, 59.6% of all specimens), and due to their sheer abundance, they were identified morphologically only to family level. In the mesocosm experiment, two microhabitats were distinguished and analyzed separately: channel substratum (here referred to as 'streambed') and leaf litter. On family level, chironomid abundances of the streambed increased with added fine sediment and reduced water flow velocity. In the microhabitat leaf litter, chironomid abundance decreased upon flow velocity reduction, which combined with the streambed results could be interpreted as behavioral shift from the leaf litter to the streambed. Changes in salinity did not affect the Chironomidae abundances in any microhabitat [5].

In view of recent studies [9, 69], we expected to find a large number of different chironomid species/OTUs in the experiment. However, following the concept of taxonomic sufficiency, we predicted similar responses to the different stressors for the vast majority of chironomid species/OTUs as on family level (see above).

Methods

Mesocosm experiment

A multiple stressor mesocosm field experiment (original experiment design by [54]; see [5] for full experiment description) was conducted from 8 March to 22 April 2014 at the Felderbach (Germany, North Rhine-Westphalia, 51°20′59.09″N, 7°10′14.03″E, 136 m a.s.l). Stream water was continuously pumped into the experiment to maintain a constant water flow into each of 64 mesocosms (25 cm diameter, volume 3.5 L; Microwave Ring Moulds, Interworld, Auckland, New Zealand), which were arranged in four blocks of 16 mesocosms each. The mesocosms contained the two compartments channel substratum (300 mL fine sediment [< 2 mm], 900 g gravel [2–30 mm] and seven stones [> 30 mm]) and leaf litter bags (12.5 × 6.5 cm, 5 mm mesh size, 2.5 g leafs) of dried alder leafs, which resemble the two microhabitats streambed and leaf litter in the study. Colonization of mesocosms occurred via drift (water intake pump mesh size 4 mm) and was complemented with macroinvertebrates from multi-habitat kick-net sampling (96 kick samples in total, 12 benthic kick samples per 8 channels, area of benthic habitat provided per individual channel: 0.163 m^2). The experiment ran for 46 days (24-day colonization, 22-day manipulative period). Responses of macroinvertebrates to stressors were tested in a 2 × 2 × 2 full-factorial design with two levels of each factor and 8 replicates per treatment combination (see [5] for details on the chosen factor levels): salinity (ambient [18.2 mg/L, SD ± 4.1]

versus increased [312.2 mg/L, SD ± 78.5] chloride concentration), fine sediment (ambient [300 mL < 2 mm] versus added [300 mL < 2 mm plus 450 mL < 0.5 mm]), and water flow velocity (normal [16.5 cm/s, SD ± 0.1] versus reduced [9.6 cm/s, SD ± 0.1]). Macroinvertebrates were sampled at the last day of the experiment by first taking out the leaf litter bags followed by sieving the channel substratum for macroinvertebrates. Macroinvertebrates from both microhabitats were stored separately for every single mesocosm in 96% ethanol (which was replaced with fresh 96% ethanol the same day) at − 20 °C until further processing. All macroinvertebrates were identified morphologically and counted. For the present study only Chironomidae, which were not identified further below family level, were used for further molecular analysis.

DNA extraction, DNA metabarcoding, and bioinformatics

For the molecular analysis, chironomids were obtained from both microhabitats (streambed, leaf litter) from all 64 mesocosms. The 128 samples were dried separately in 1.5 mL Eppendorf tubes on a heating block at 50 °C for 16–24 h, before weighing their respective dry mass (Additional file 1). Specimens were grinded in their respective tube by five zirconia beads (2.0 mm Zirconia Beads, BioSpec Products, Bartlesville, USA) at 6 m/s for 3 × 45 s on a FastPrep®-24 tissue grinder (MP Biomedicals, Eschwege, Germany). DNA was extracted from the grinded tissue using a modified salt extraction protocol [65]; modified by [74], followed by an RNA digestion step using 1 µL RNase A on 50 µL sample for 30 min at 37 °C and a clean-up step using the NucleoSpin® Gel and PCR Clean-up kit (Macherey–Nagel, Düren, Germany) to minimize the concentration of possible inhibitors prior to PCR. DNA concentrations of the extracted and cleaned-up samples were measured using the Qubit 2.0 (Broad Range Kit, Thermo Fisher Scientific, Beverly, USA) and diluted to 25 ng/µL. DNA was amplified using a two-step PCR protocol. In the first step, DNA was amplified using illustra puretaq ready-to-go PCR beads (GE Healthcare UK Limited, Little Chalfont, UK) with 12.5 ng DNA and 0.5 µM of each primer (BF2, BR2; [17]) filled up to 25 µL with sterile H_2O at: 94 °C for 3 min initial denaturation, followed by 25 cycles of 94 °C for 30 s denaturation, 50 °C for 30 s annealing, 72 °C for 2 min elongation, and final elongation at 72 °C for 5 min. PCR success was validated by gel electrophoresis before the PCR product was cleaned-up using the NucleoSpin® Gel and PCR Clean-up kit (Macherey–Nagel, Düren, Germany) to remove any bovine serum albumin (BSA) from the samples. In the second step, DNA was amplified using 1 × Buffer, 0.2 mM dNTPs, 0.5 µM of each primer, 0.025 U/µL 5Prime HotMaster Taq DNA Polymerase (Quantabio, Beverly, USA), 1 µL DNA template from the first PCR step, filled up to 50 µL with sterile H_2O. The above-described PCR protocol was applied, with 15 instead of 25 cycles, in the second step. Samples were individually tagged in the second PCR step using fusion primers ([17]; all primer combinations can be found in Additional file 2). DNA concentrations were measured after the second PCR step (Additional file 1) using the Fragment Analyzer (Standard Sensitivity NGS Fragment Analysis Kit; Advanced Analytical, Ankeny, USA). Two libraries were pooled including specimen abundance as a factor (Additional file 1). The first library consisted of all samples from the experimental blocks 1 and 4 and the second library of all samples from experimental blocks 2 and 3. Pooled this way, both libraries contained an equal amount of samples from both microhabitats (32 streambed and 32 leaf litter samples per library), an equal amount of samples per treatment (four replicates of each treatment combination) and nearly the same number of chironomid specimens (29,915 specimens [block 1 and 4] versus 29,410 specimens [block 2 and 3]). A left-sided size selection was performed for both libraries using 0.76× SPRI select (Beckman Coulter, Krefeld, Germany). Each library was sequenced using the MiSeq platform with a paired-end v2 kit (read length 2 × 250 bp) at GATC, Germany (5% PhiX spike into increase sequence diversity). Raw data were processed with R JAMP v023 (https://github.com/VascoElbrecht/JAMP). Sequences were assigned to their original sample using the module Demultiplexing_shifted, followed by paired-end merging using Usearch (v10.0.240, [14]) through the JAMP module U_merge_PE. Reverse complement sequences were generated where necessary and quality filtering was conducted (maxee = 1; [14]). Pre-processing of sequences included primer removal (Cutadapt v1.9; [43]) discarding of reads ± 10 bp of the expected length and dereplication with removing singletons. Operational taxonomic unit (OTU) clustering was conducted (97% similarity) and clusters with at least 0.01% abundance in one sample (after mapping of the reads including singletons) were included for further analyses. After remapping the remaining reads of OTUs that were discarded in the first filtering step, a second filtering step was conducted for each sample separately, in which only OTUs with an abundance of at least 0.01% in the respective sample were retained. OTUs were assigned to a taxonomic group by comparison with the Barcode of Life Database (BOLD, http://www.boldsystems.org/; accessed 02.08.2017) as implemented in JAMP. Since a 4–5% threshold has been suggested to be appropriate to delineate species of the chironomid genus *Tanytarsus* [39], we carried out a second analysis using a

combination of Usearch (v10.0.240; [14]) and Vsearch (v2.8.1; [60]) with a 5% clustering threshold to obtain a more conservative estimation of the actual number of species.

Conceptual model, read abundances, and statistics

It is assumed that the initial chironomid communities in the experiment by Beermann et al. [5] were similar or at least did not vary systematically between the treatments. Differences in specimen abundances at the end of the experiment are considered to be the effect of the applied stressors and a shift of organisms from one microhabitat to the other to represent a behavioral shift. DNA metabarcoding fails at picking up the exact specimen abundances [15, 57], which are needed for arithmetic-mean-based statistics that are used for analyzing stressor effects. Species and specimens differ in their biomass, and, therefore, also in their amount of DNA [18]. These differences are further amplified in a PCR, a pattern which is also modified by differences in primer binding efficiency [15], ultimately distorting the use of read abundances as a proxy for specimen abundances. In this study, we used highly degenerated primers [16, 17] and focused on a single family. To test if the species, potentially being found in this study, can be recovered with the used primers, we clustered (97% similarity, PrimerMiner v0.18; [16]) and aligned all publically available chironomid COI sequences from specimens sampled in Germany from BOLD (downloaded 01.06.2017, Additional file 3). We then tested the match of our used forward primer (BF2) sequence against all the available OTUs showing that the primer matched to > 98.5% of the OTUs without a single mismatch, supporting a generally good match. The reverse primer could not be tested as it is located in the HCO2198 binding region. Even though a primer bias between species cannot be excluded (i.e., certain species might be over- or underrepresented in their read abundance as a result of different primer binding efficiencies), this bias is expected to not systematically vary between the same OTUs in different treatments. A bias due to unequal biomass of specimens [15, 18] cannot be excluded, but is expected to be comparably small here, because chironomid specimens show much less variation in biomass among different species than other taxa such as Ephemeroptera, Plecoptera, and Trichoptera. Furthermore, when analyzing stressor effects, the number of reads of a single OTU is compared to the number of reads of the same OTU and not to those of other OTUs (with the exception of community variables, see below). Unless the different treatments of the experiment significantly impacted on growth rates during the experimental manipulation and thereby on organism size and biomass, the same OTUs under different treatments should contribute a comparable amount of DNA per specimen. We argue that read abundances can be used in our case as a proxy for real abundances. Even though our data do not allow for quantifying the mean number of individuals affected by a stressor treatment, we argue that the analysis of read abundances is sufficient to infer stressor responses of the respective OTUs. In addition, although a high variation in the number of reads per OTU and sample may be expected, this study is backed by a high number of replicates (8) per treatment combination, which makes the results robust against random variation. Hence, read abundances of OTUs were used as an input for statistical tests. Since the sequencing depth per sample can vary for technical reasons the number of reads was standardized (see Additional file 4) prior to statistical analyses. Data were analyzed using SPSS 23 (IBM SPSS Statistics; IBM Company, Chicago, IL, U.S.A.) and R v3.3.3 [67]. We examined the responses of three community metrics for both microhabitats: OTU richness, Simpson's index of diversity, and Pielou's evenness. For each metric, we performed an ANOVA with salinity, fine sediment and flow velocity as fixed factors. The ANOVA model was intercept (d.f. 1) + salinity (1) + fine sediment (1) + velocity (1) + salinity × fine sediment (1) + salinity × velocity (1) + fine sediment × salinity (1) + salinity × fine sediment × velocity (1) + error (56, $n = 64$). Since null hypothesis significance testing does not provide any estimates of the magnitude of an effect of interest [47], we calculated standardized effect sizes for all results with $p \leq 0.10$ to allow evaluating the biological relevance of our findings (partial η^2 values, range 0–1). To assess treatment effects on invertebrate community composition, we performed a MANOVA with the multivariate equivalent of the model above for all OTUs with a read abundance of at least 50,000 reads (0.39%) for the total read abundance and at least 30,000 reads (0.23%) for the respective microhabitat (35 OTUs in total, 24 OTUs for the streambed, and 25 OTUs for the leaf litter). Moreover, we examined the between-subjects effects in the MANOVA for each common taxon to determine their individual responses. After exploratory analysis, community-level and taxon data were log-transformed (log + 1) to improve normality and homoscedasticity. OTU sequences of the analyzed 35 OTUs where compared to the Barcode of Life Database to explore the taxonomy of the OTUs found in this study. The criteria to accept a species name were at least 95% similarity to a reference sequence, ≥ 5 published reference sequences, adult reference specimens

and ≥ 1 visible project with a visible specimen identifier (see Additional file 5 for details).

Results

A total of 20,598,800 reads were generated in both sequencing runs combined (Library 1: 10,277,200; Library 2: 10,321,600). After bioinformatic processing (13,012,030 reads remaining) and taxon assignment 12,975,968 reads were identified as belonging to 183 Chironomidae operational taxonomic units (OTUs, 97% similarity; see Additional file 6 for reads belonging to other taxonomic groups). The alternative approach of using a 5% clustering threshold resulted in 142 OTUs, but will not be referred to from here on unless specifically mentioned. The average sequencing depth was 259 (±67 SD) and 199 (±102 SD) reads/specimen for the streambed and leaf litter, respectively, before standardizing the reads for further analysis. The number of reads per sample correlated with the number of specimens per sample (Spearman's rho, $r_s = 0.88$, $p < 0.001$, $n = 128$). 31.3% of these reads are from streambed and 68.7% from leaf litter samples. A total of 35 OTUs (i.e., the most common OTUs) were analyzed for individual stressor responses (Tables 1 and 2), of which 14 OTUs were well represented (i.e., within the chosen read abundance threshold) in both microhabitats, while 10 OTUs were more exclusive (i.e., the chosen threshold was only met for one microhabitat) in the streambed and 11 OTUs in the leaf litter. Eight OTUs of the streambed (33%) and 9 of the leaf litter (36%) did not respond to the experimental manipulations. For the remaining OTUs, we observed 14 different response patterns (Tables 1 and 2) across both microhabitats. When comparing OTUs analyzed for both microhabitats, 3 OTUs (OTU 3, 7, 11) did not respond to any treatment combination in either microhabitat, 5 OTUs (OTUs 5, 16, 18, 262, and 466) responded to the experimental manipulation in one microhabitat but not the other and the remaining 6 OTUs (OTUs 1, 2, 4, 9, 17, and 21) showed different response patterns in the respective microhabitat. OTUs 1 (29.6%), 2 (17.1%), 3 (6%), 4 (5.8%), and 466 (5.1%) were the most abundant OTUs across both microhabitats accounting for > 63% of the total reads.

Streambed

The community metrics OTU richness and Simpson's diversity increased upon fine sediment addition (Table 1, Fig. 1). The MANOVA revealed that streambed community composition (24 OTUs) was affected by added fine sediment and reduced flow velocity. Concordant with that result, fine sediment had an effect on 12 OTUs (50%) and flow reduction on 9 OTUs (37.5%), of which 5 OTUs responded to both factors in a double positive manner (additive effects; e.g., OTU 1, Table 1, Fig. 2). The effect of added fine sediment was positive for all affected OTUs except for OTU 16, which decreased in abundance upon fine sediment addition. All 9 OTUs affected by the manipulation in flow velocity increased in abundance when the flow velocity was reduced. It is notable that 7 out of 9 OTUs that responded to the change in flow velocity were OTUs being more exclusive to the streambed than the leaf litter. Only one OTU (OTU 4, Fig. 2) responded to salinity manipulation by increasing with higher salinity. Positive synergistic interactions between salinity and sediment (OTU 6), salinity and velocity (OTU 6, OTU 25) and fine sediment and velocity (OTU 4) were found, i.e., the respective OTUs showed a higher increase in abundance than expected based on the single stressor effects. A complex three-way interaction affected OTU evenness. While reduced flow and fine sediment addition affected OTU evenness negatively at ambient salinity and positively at increased salinity, all three stressors combined had a positive antagonistic effect (i.e., less positive than predicted additively; following the terminology by [55]).

Leaf litter

The OTU richness decreased upon flow reduction in the leaf litter (Table 2, Fig. 1). Manipulation of flow velocity had an effect on 13 OTUs (52%), which all decreased upon flow reduction, and concordantly, the MANOVA revealed that flow velocity had an effect on the community composition (25 OTUs). The abundance of two OTUs (OTU 4 and 9) increased with higher salinity, while the abundance of one OTU (OTU 5) decreased. Fine sediment affected one OTU (OTU 30) resulting in a higher abundance upon fine sediment addition. A single two-way interaction was observed for OTU 9. OTU 9 decreased in abundance upon flow reduction and fine sediment addition, but responded in a negative antagonistic (i.e., less negative than predicted additively; [55]) way when both stressors where combined. Two complex three-way interactions were observed for OTU 21 and OTU 17. At ambient salinity, OTU 21 decreased in abundance with reduced flow and added fine sediment, but increased when both stressors where combined. At increased salinity, OTU 21 still decreased upon flow reduction, but increased with added fine sediment and most notably decreased when all three stressors where combined. OTU 17 decreased with reduced flow and added fine sediment, but increased when both stressors where combined at ambient salinity. These effects were reversed at increased salinity, i.e., OTU 17 increased upon flow reduction and fine sediment addition and decreased when all three stressors were present.

Table 1 Summary of (M)ANOVA results for the habitat "streambed"

Streambed Response variable	% reads	% habitat reads	*N* *p* value	*S* *p* value	*V* *p* value	*N* × *S* *p* value	*C*	*N* × *V* *p* value	*C*	*S* × *TV* *p* value	*C*	*N* × *S* × *V* *p* value	*p*
Total read abundance			0.730	**< 0.001 (0.32)**↑	**0.001 (0.18)**↑	0.349		0.726		0.369		0.520	
Simpson's diversity			0.193	**0.007 (0.12)**↑	0.161	0.401		0.159		0.108		0.732	
Pielou's evenness			0.970	0.105	0.227	0.571		0.163		0.437		**0.029 (0.08)**	
OTU richness			0.832	**< 0.001 (0.38)**↑	0.261	0.833		0.473		0.660		0.968	
Community composition (MANOVA, 24 OTUs)	27.9	89.1	0.276	**< 0.001 (0.87)**	**0.000 (0.73)**	0.796		0.618		0.504		0.552	
OTU_1	6.17	19.7	0.453	**< 0.001 (0.29)**↑	**0.034 (0.08)**↑	0.641		0.927		0.421		0.859	A
OTU_3	4.51	14.4	0.775	0.908	0.933	0.183		0.309		0.924		0.367	B
OTU_4	2.03	6.5	**< 0.001 (0.20)** ↑	**< 0.001 (0.65)**↑	0.111	0.606		0.967		**0.033 (0.08)**	+ *S*	0.370	C
OTU_9	1.17	3.7	0.078 (0.05) ↑	**< 0.001 (0.54)**↑	**0.004 (0.14)**↑	0.279		0.816		0.317		0.634	A
OTU_2	0.90	2.9	0.934	0.947	0.092 (0.05) ↓	0.222		0.740		0.977		0.479	B
OTU_5	0.75	2.4	0.624	0.463	0.079 (0.05) ↑	0.650		0.518		0.496		0.826	B
OTU_7	0.46	1.5	0.306	0.081 (0.05)↑	0.820	0.997		0.685		0.603		0.248	B
OTU_18	0.40	1.3	0.758	**< 0.001 (0.33)**↑	0.563	0.372		0.532		0.413		0.218	D
OTU_466	0.33	1.1	0.730	0.097	0.252	0.726		0.752		0.129		0.864	B
OTU_262	0.30	1.0	0.428	**< 0.001 (0.36)**↑	0.095 (0.05)↑	0.999		0.188		0.066 (0.06)	+ *S*	0.149	D
OTU_17	0.29	0.9	0.394	**0.001 (0.18)**↑	0.096 (0.05)↑	0.407		0.770		0.954		0.814	D
OTU_11	0.28	0.9	0.248	0.840	0.842	0.285		0.691		0.070 (0.06)	− *S*	0.397	B
OTU_16	0.27	0.9	0.066 (0.06) ↓	**0.002 (0.16)**↓	0.330	0.671		0.181		0.479		0.285	E
OTU_21	0.26	0.8	0.107	**0.005 (0.13)**↑	0.223	0.211		0.409		0.440		0.209	D
OTU_6	2.18	7.0	0.252	0.065 (0.06)↑	**0.003 (0.14)**↑	**0.014 (0.10)**	+ *S*	**0.021 (0.09)**	+ *S*	0.653		0.285	F
OTU_15	1.39	4.5	0.998	0.765	**0.005 (0.13)**↑	0.285		0.589		0.303		0.412	G
OTU_12	1.15	3.7	0.211	0.142	**< 0.001 (0.29)**↑	0.921		0.957		0.234		0.489	G
OTU_8	0.97	3.1	0.483	0.976	**0.002 (0.16)**↑	0.627		0.569		0.705		0.533	G
OTU_14	0.96	3.1	0.953	0.065 (0.06) ↑	0.068 (0.06)↑	0.606		0.499		0.531		0.525	B
OTU_23	0.77	2.5	0.391	**0.028 (0.08)**↑	**< 0.001 (0.21)**↑	0.534		0.795		0.261		0.472	A
OTU_28	0.87	2.8	0.727	**< 0.001 (0.51)**↑	**< 0.000 (0.25)**↑	0.860		0.961		0.819		0.176	A
OTU_31	0.57	1.8	0.686	0.987	0.195	0.543		0.672		0.601		0.727	B
OTU_19	0.47	1.5	0.770	**0.003 (0.15)**↑	0.126	0.931		0.746		0.485		0.235	D
OTU_25	0.44	1.4	0.493	**0.005 (0.13)**↑	**0.027 (0.08)**↑	0.302		**0.045 (0.07)**	+ *S*	0.320		0.982	H

Significant *p* values (< 0.05) are printed in bold. Effect sizes (partial η^2, range 0–1) are presented in brackets for *p* values < 0.1. Arrows indicate increase (↑) and decrease (↓) of abundance under the stressor condition compared to the non-stressor condition. Multivariate *p* values are for the Pillai's Trace statistic. Interactions of combined stressors (*C*) were positive synergistic (+ *S*) or negative synergistic (− *S*). OTUs analyzed for both microhabitats are indicated by a bold underline, while OTUs being more exclusive to the streambed are indicated by a underline. *N* salinity, *S* fine sediment, *V* velocity, *P* response pattern

Table 2 Summary of (M)ANOVA results for the habitat "leaf litter"

Leaf litter Response variable	% reads	% habitat reads	*N* *p* value	*S* *p* value	*V* *p* value	*N* × *S* *p* value	*C*	*N* × *V* *p* value	*C*	*S* × *V* *p* value	*C*	*N* × *S* × *V* *p* value	*P*
Total read abundance			0.098 (0.05)↑	0.943	**< 0.001 (0.48)**↓	0.100		0.745		0.557		0.167	
Simpson's diversity			0.356	0.807	0.095	0.371		0.693		0.866		0.799	
Pielou's evenness			0.439	0.612	0.226	0.538		0.667		0.365		0.941	
OTU Richness			0.902	0.658	**0.002 (0.15)**↓	0.162		0.891		0.938		0.213	
Community composition (MANOVA, 25 OTUs)	65.01	94.65	0.131	0.529	**< 0.001 (0.76)**	0.193		0.626		0.507		0.396	
OTU_1	23.38	34.04	0.197	0.967	**< 0.001 (0.22)**↓	0.080 (0.05)	+ *A*	0.967		0.542		0.264	I
OTU_2	16.23	23.63	0.804	0.535	**0.001 (0.17)**↓	0.568		0.355		0.468		0.625	I
OTU_466	4.72	6.86	0.343	0.770	**< 0.001 (0.27)**↓	0.219		0.866		0.796		0.778	I
OTU_4	3.73	5.42	**0.016 (0.10)**↑	0.083 (0.05)↑	**0.005 (0.13)**↓	0.567		0.628		0.683		0.262	J
OTU_7	2.43	3.53	0.369	0.842	0.150	0.545		0.392		0.483		0.665	B
OTU_5	1.91	2.78	**0.030 (0.08)**↓	0.982	0.882	0.308		0.212		0.542		0.286	K
OTU_3	1.48	2.15	0.619	0.681	0.481	0.742		0.449		0.282		0.658	B
OTU_21	1.06	1.55	0.545	0.819	**0.000 (0.41)**↓	0.553		0.787		0.384		**0.016 (0.10)**	L
OTU_11	0.61	0.88	0.320	0.715	0.249	0.678		0.076 (0.06)	− *S*	0.677		0.953	B
OTU_17	0.41	0.59	0.052 (0.07)↑	0.198	0.938	0.304		0.507		0.474		**0.041 (0.07)**	M
OTU_9	0.39	0.56	**0.041 (0.07)**↑	0.926	0.082 (0.05)↓	0.186		0.300		**0.029 (0.08)**	− *A*	0.368	N
OTU_262	0.34	0.50	0.224	0.390	0.217	0.230		0.998		0.415		0.714	B
OTU_18	0.33	0.47	0.787	0.293	0.605	0.314		0.501		0.104		0.798	B
OTU_16	0.24	0.35	0.521	0.091 (0.05)↑	0.862	0.349		0.243		0.249		0.947	B
OTU_138	1.49	2.18	0.949	0.994	**0.014 (0.10)**↓	0.927		0.556		0.242		0.573	I
OTU_29	0.93	1.36	0.340	0.948	0.322	0.061	+ *A*	0.441		0.359		0.264	B
OTU_316	0.97	1.41	0.665	0.765	**0.042 (0.07)**↓	0.692		0.092 (0.05)	+ *A*	0.463		0.119	I
OTU_10	0.78	1.13	0.110	0.444	**0.011 (0.11)**↓	0.745		0.410		0.596		0.141	I
OTU_20	0.80	1.17	0.297	0.946	**0.014 (0.10)**↓	0.190		0.164		0.971		0.534	I
OTU_116	0.69	1.01	0.656	0.717	**0.035 (0.08)**↓	0.604		0.194		0.313		0.216	I
OTU_13	0.57	0.84	0.660	0.690	**< 0.001 (0.26)**↓	0.300		0.940		0.134		0.574	I
OTU_30	0.46	0.67	0.943	**0.025 (0.09)**↑	**0.002 (0.16)**↓	0.263		0.673		0.296		0.111	O
OTU_293	0.36	0.53	0.708	0.400	0.197	0.967		0.764		0.456		0.127	B
OTU_27	0.37	0.54	0.300	0.128	**< 0.001 (0.22)**↓	0.944		0.768		0.981		0.591	I
OTU_26	0.36	0.52	0.843	0.197	0.212	0.170		0.529		0.914		0.560	B

Significant *p* values (< 0.05) are printed in bold. Effect sizes (partial η^2, range 0–1) are presented in brackets for *p* values < 0.1. Arrows indicate increase (↑) and decrease (↓) of abundance under the stressor condition compared to the non-stressor condition. Multivariate *p* values are for the Pillai's Trace statistic. Interactions of combined stressors (*C*) were positive antagonistic (+ *A*), negative antagonistic (− *A*) or negative synergistic (− *S*). OTUs analyzed for both microhabitats are indicated by a bold underline, while OTUs being more exclusive to the leaf litter are indicated by a underline. *N* salinity, *S* fine sediment, *V* velocity, *P* response pattern

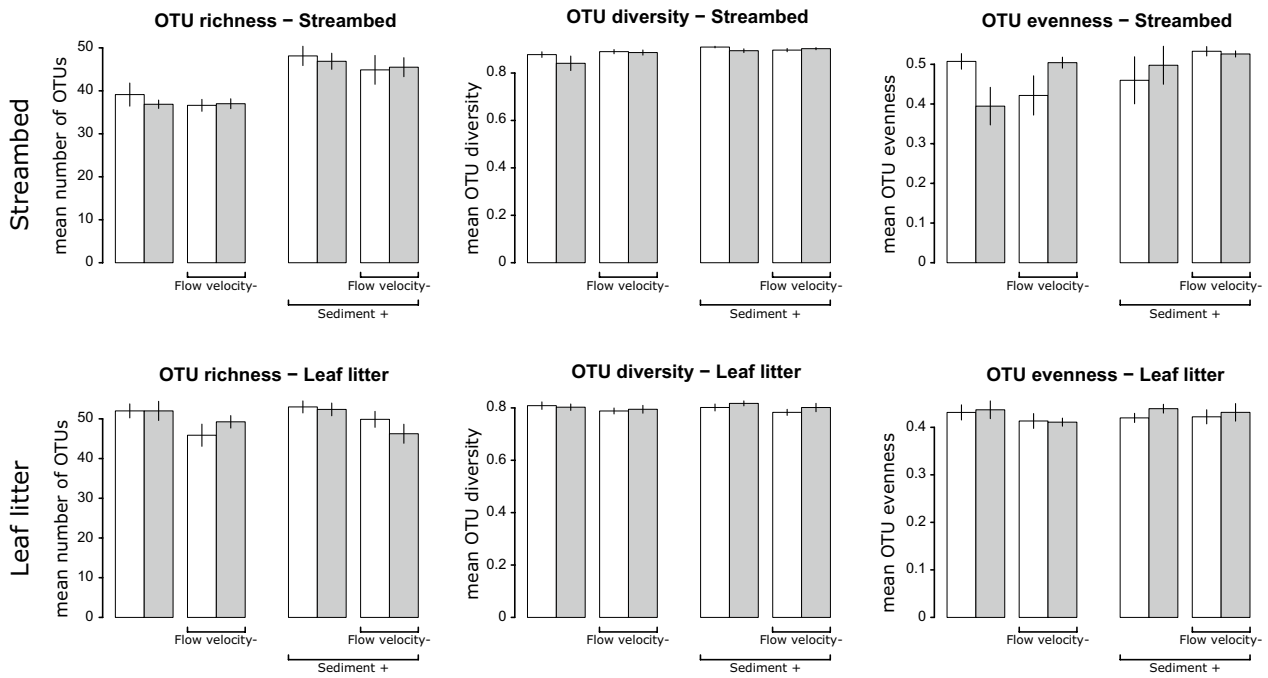

Fig. 1 Community metrics OTU richness, diversity, and evenness of chironomid OTUs in both microhabitats: streambed and leaf litter. White bars represent ambient and grey bars increased salinity. Error bars represent standard errors. Sample size for each treatment combination is $n = 8$

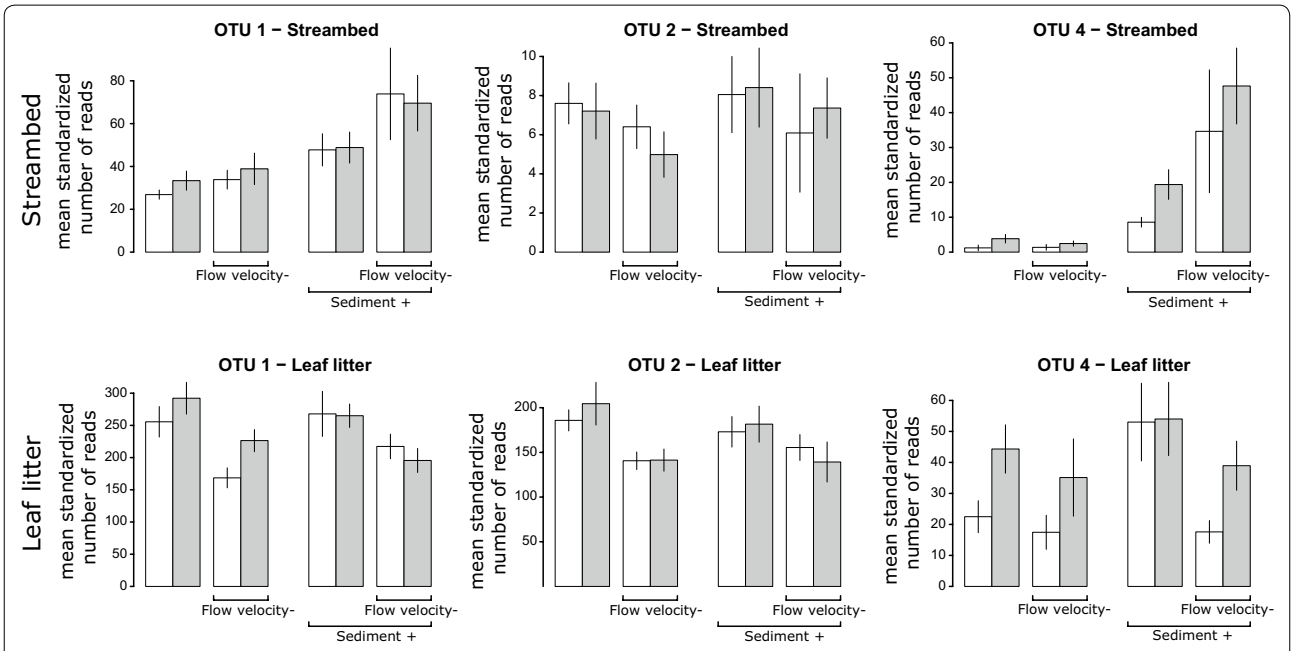

Fig. 2 Read abundances of OTU 1, OTU 2, and OTU 4 in their respective microhabitats: streambed and leaf litter. White bars represent ambient and grey bars increased salinity. Error bars represent standard errors. Sample size for each treatment combination is $n = 8$

Taxon name assignment

Based on the available reference sequences on the Barcode of Life Database (BOLD), it was impossible to assign the majority of OTUs to formally described species with binominal nomenclature with our assignment criteria. For our 35 most common OTUs, 7 OTUs

matched to reference sequences only at family, 14 OTUs to genus, and 14 OTUs to species level (see Additional file 5 for all details). Furthermore, it is notable that the reference sequences with the best matches for OTU 2 (99.52%), OTU 138 (97.36%), OTU 466 (98.06%), and OTU 316 (98.32%) all bear the same species name *Brillia bifida*. The same case was observed for OTU 20 and 29, which best match to reference sequences belonging to *Polypedilum convictum* with a match of 99.52 and 100%, respectively. Whereas only one OTU bearing the name *B. bifida* was obtained when clustering was based on a 5% threshold, still, two separate OTUs were found for *P. convictum* (99.52% and 100% similarity to reference sequences, respectively). When statistically reanalyzed the one *B. bifidia,* OTU responded the same way as the individual separate OTUs, i.e., being insensitive to manipulation in the streambed and decreasing in the leaf litter with reduced flow velocity (ANOVA, flow velocity, $p < 0.01$; partial $\eta^2 = 0.251$). In addition, the individual response patterns of both *P. convictum* OTUs were unaffected by the different clustering thresholds.

Discussion

Chironomidae richness

Our experiment revealed that the Chironomidae specimens collected from the Felderbach comprised a total of 183 operational taxonomic units (OTUs). It is not straightforward to assign species to OTUs given incomplete reference databases, because using a fixed threshold of 3% as used in our study is prone to over split genetically diverse species into more than one entity. However, it needs to be emphasized that the high number of OTUs found in this study originates from only one site in one stream and one season. Moreover, even when using a much more conservative OTU clustering threshold of 5% that has been suggested to be more appropriate for Chironomidae species delimitation [39], still, 142 OTUs are found to co-exist in this stream. According to Cranston [13], a richness of more than 100 chironomid species is not uncommon, especially in lotic environments, unclear if the scale of the lotic environment refers to a stream, a stream and its tributaries, a catchment or just one site of the stream. Concordantly, a high number of Chironomidae species are also documented in the long-term data from the first-order stream Breitenbach in Germany, where over 100 species were recorded for different stream sites [72]. A comparison to the number of only 745 Chironomidae species recorded for Germany [62] suggests either a high number of species that are widely distributed ecological generalists or, more likely, that very many chironomid species are still undescribed.

Comparison of stressor effects on family and OTU level—taxonomic (in)sufficiency?

On family level, Chironomidae abundance increased with added fine sediment and reduced flow velocity in the streambed but decreased upon flow reduction in the leaf litter (see [5]). In case the concept of taxonomic sufficiency can be applied for this study, the same response patterns should be detected for the majority of OTUs. In sharp contrast, analyzing stressor responses at OTU level revealed not 2 but 15 different stressor response patterns among only the 35 most common (i.e., with the highest overall read abundances) OTUs, ranging from (a) not responding to any experimental manipulation to (b) being affected by one factor to (c) additive effects, and (d) even complex interactions. This clearly shows that the diversity of possible responses to environmental disturbance is not reflected on family level and that the concept of taxonomic sufficiency is insufficient for this study on Chironomidae. However, despite many insensitive OTUs, it is notable that from the sensitive OTUs of the streambed (45.8% for fine sediment, 37.5% for flow velocity), almost all responded in the same direction as indicated by the family level response (i.e., increased upon fine sediment addition or flow reduction, respectively). The same pattern was observed for the leaf litter, where 52% of the OTUs responded negatively to flow reduction, as indicated by a decrease in abundance on family level. In this particular case, analyzing abundances on family level was an indicator that many species or at least high abundant species within the family were affected by the respective stressor. However, as this is a case study, it needs to be validated if this is a general trend or an isolated case. In this regard, OTU 16 represents a counterexample to the overall picture as it decreased and not increased upon fine sediment addition in the streambed. In addition, OTU-level-based analysis revealed that salinity had an effect on single OTUs (one in the streambed and three in the leaf litter) and fine sediment on one OTU in the leaf litter (OTU 30). Furthermore, analyzing stressor effects on single chironomid OTUs showed that stressor effects can be complex and far beyond observable for a pooled family metric. All these single response patterns go unnoticed when the stressor response is analyzed on coarse taxonomic level. Similar results were observed for the mayfly genus *Deleatidium* in New Zealand, where analyzing single OTUs revealed different stressor response patterns for different OTUs, whereas no effect was observed on genus level [41]. It needs to be highlighted that the 15 different response patterns in this study were observed for the 35 common OTUs manipulating only three factors with two factor levels each. Stream ecosystems, however, can be impacted by a multitude of stressors with a wide range of respective intensities. Even if profound

data on how single species respond to different stressors would be available, which is clearly not the case for many taxa, multiple stressors can act in surprising ways that cannot always be predicted based on their single stressor effects [31, 50]. In this regard, an increase in taxonomic resolution means a significant increase in information of how single OTUs respond to environmental changes.

The overall migration from leaf litter to streambed upon water flow reduction as observed on family level [5] could not be confirmed on OTU level. Except for OTU 1, there is no overlap of OTUs decreasing in the leaf litter and increasing in the streambed upon flow reduction. It cannot be ultimately excluded that the analysis was insufficient in detecting a migration of OTUs, but the results strongly suggest that the stressor pattern on family level does not reflect the responses of single OTUs.

The concept of taxonomic sufficiency is currently regarded as a pragmatic trade-off that maximizes the speed and comparability of taxonomic lists for as little money as possible at the cost of losing taxonomic resolution. As an example, identifying chironomids to species rather than family level can more than double the identification time when based on morphology [27] and is associated with a large error. Yet, balancing this trade-off between speed, cost, and precision becomes obsolete when applying DNA-based identification methods. Hence, with the application of DNA-based methods, the concept of taxonomic sufficiency that came largely as a pragmatic compromise has no real application in many cases anymore if determination can be done with DNA barcoding. The approach allows for maximum taxonomic resolution, comes with no or only marginally higher additional costs [19], and sample processing for metabarcoding at species level is fast.

OTU ecology

The comparison of OTU sequences with the Barcode of life database (BOLD) suggests that only a very small fraction of OTUs can be assigned reliably to formally described species with binominal nomenclature at this time. Even though this situation will improve in the future, when more reference sequences are released, assigning species names to chironomid OTUs will be an ongoing problem as comparisons with sequence databases might provide contradicting and puzzling results (as observed for Chironomidae, [7]. It needs to be considered if studying autecology is sufficient on OTU level in case a species name cannot be assigned. The potential of studying ecology on OTU level is maybe best being demonstrated not by the case, where no species can be assigned to an OTU, but by the reverse cases of several OTUs being assigned to the same species. In this study, the sequences of four OTUs (2, 138, 316, and 466) match best to reference sequences on BOLD that all bear the name *Brillia bifida*. For other Chironomidae species, a comparably high species delineation threshold (e.g., for the genus Tanytarsus; [39]) has been suggested and all four OTUs here might very well represent *B. bifida* from a morphological point of view. Thus, one could treat these four OTUs as one response variable in the stressor analysis. While two of the four OTUs (2 and 466) were only analyzed in the streambed and were both insensitive to the experimental manipulation, all four OTUs in the leaf litter responded negatively to flow reduction. In this case, pooling the four OTUs prior to stressor analysis would have delivered the same result, which was confirmed by a second statistical analysis based on the 5% clustering data. In contrast, OTU 20 and OTU 29, which both correspond to *Polypedilum convictum*, show a different response to flow reduction in the leaf litter and pooling these according to a different threshold would have changed the biological interpretation. While our study cannot clarify species status of these OTUs, the data can reveal differences between closely related OTUs.

It might be of insignificance if two molecular entities represent (a) distinct species, (b) different 'ecotypes' or 'evolutionary significant units' or (c) separated populations of which members are still capable of reproducing with members of the other population. Diversity and responding to changing environmental conditions are not limited to distinct species, as also single individuals with a different genetic setup (intraspecific variation) can be capable of coping differently with different environmental conditions. Hence, OTU-based analysis ideally even down to unique-sequence-based haplo- or genotypes hold great potential to extend the traditional morphospecies-based view and should be favored. This is because the data are compatible with morphospecies or higher level (genus, family) data but (i) capture also morphologically cryptic species [41] and (ii) also allow detecting changes inside species [20]. Based on OTU, haplo- or genotype data, different fitness values of individual ecotypes, sub-species, or populations can be identified [36, 51], which substantially extends our view of biotic responses to environmental factors. While it is possible to use OTUs as entities in ecological studies, it is of indisputably greater strengths if these OTUs can be assigned to formally described species. Only by this, the full available species knowledge can be connected to the OTU-based analyses. Thus, this should be a clear target for future integrative molecular ecological studies.

OTU 1—*Rheocricotopus fuscipes* (Kieffer, 1909)

Most reads were assigned to OTU 1, which represents *Rheocricotopus fuscipes*. *R. fuscipes* is widely distributed in Europe [62] and can occur in densities of more than

8.000 larvae/m^2 [40]. The high read abundance (29.6% of all reads) suggests that *R. fuscipes* was also highly abundant in this study. OTU 1 was well represented in both of the study's microhabitats, streambed, and leaf litter, which is in agreement with the described broad range of microhabitats for *R. fuscipes* (stones, gravels, plants, coarse detritus, sandy bottoms; reviewed in [44]). In contrast to other studies, where *R. fuscipes* is commonly found in fast-flowing streams [38, 59], an increase of OTU 1 was observed upon flow reduction in the streambed. Despite being common in fast-flowing waters in Central Europe, the species occurs in numerous habitats including the littoral zone of lakes, suggesting a preference for high oxygen levels rather than high flow rates [38, 40]. The increase in read abundance for this species in the streambed might be explained by immigration of specimens from the leaf litter, where a notable decline was observed. Possibly, reduced flow velocity resulted in a decrease in oxygen in the leaf litter forcing specimens to leave the unfavorable microhabitat.

OTU 2—*Brillia bifida* (Kieffer, 1909)

OTU 2 corresponds to *Brillia bifida*. Larvae of *B. bifida* are mainly found on decaying leaves [70, 71], which they are suspected to feed on [12]. This is in agreement with the high read abundance of OTU 2 found in the leaf litter microhabitat compared to the streambed. Interestingly enough, *B. bifida* is never found in high densities [44], but contributes the second most reads in this study. This can either be explained by a comparably high biomass of *B. bifida* and a high primer binding efficiency compared to other chironomid species in our experiment (i.e., they overproportionally contribute to the number of total reads) or poses a contrasting ecological information to the known literature. The species has been noticed to be reasonably independent to current velocity [44], which is only partly in agreement with our results as the abundance decreased in the leaf litter when flow velocity was reduced but was unaffected in the streambed. While OTU 2 did not respond to fine sediment or salinity manipulation in this experiment, *B. bifida* is a species to be found in no or only little polluted water [45], which highlights the importance of studying the effects of different stressors, as even species sensitive to one stressor can be insensitive to another and vice versa.

OTU 13—*Tvetenia calvescens* (Edwards, 1929)

OTU 13 is *Tvetenia calvescens* with certainty. In our study, this species was almost exclusively found in the leaf litter. This stands in contrast to the literature that describes *T. calvescens* as being abundant on gravel, stones, plants, and mosses (reviewed by [44]). These findings indicate that the streambed might have been already unsuitable as a habitat even without fine sediment addition. *T. calvescens* is usually the most common species of the genus in streams with flow velocities of 0.5–1.0 m/s, can be found in stretches with slower currents, and is absent in typical lowland brooks with slower currents (reviewed by [44] *T. calvescens* is suspected to be sensitive to low oxygen content [44]. The overall low read number assigned to *T. calvescens* in this study indicates that this species was rare. The low abundance might be explained by the general low flow velocity (16.5 cm/s) which is supported by even lower read abundances for this species for the reduced flow velocity (9.6 cm/s) treatment.

OTU 28—*Prodiamesa olivacea* (Meigen, 1818)

OTU 28 corresponds to *Prodiamesa olivacea*, which can be easily described in all stages [44], resulting in more extensive ecological information being available on this species. Larvae of *P. olivacea* are bottom inhabitants and are rarely found on stones or among vegetation [71]. They can be numerous in organic silt and over-represented in sand with coarse and fine detritus [70]. The results of this study are in broad agreement with the aforementioned findings, as *P. olivacea* was almost exclusively found in the streambed and increased further with added fine sediment. In addition, *P. olivacea* responded positively to reduced flow velocity in this study. Even though the larvae are scarce in stagnant water, they are known to thrive well in stretches with a slow flow when enough decomposing material and silt is available [44]. Despite rarely being found in brackish waters and being classified as haloxenic ("salinity tolerance class 1"; [44, 49, 63]), the species was not affected by an increase in salinity in this study, suggesting that it can tolerate a certain increase in salinity.

OTU 3—*Micropsectra pallidula* (Meigen, 1830)

OTU 3 corresponds to the *M. pallidula*, which is difficult or impossible to identify at larval stage [52]. No extensive ecological information is available on this species. However, it is known to inhabit oligotrophic lakes and to be common in silt of rivers in Central Europe [26, 52]. Many *Micropsectra* larvae are oxygen-demanding, rheophilous species, but some species, i.e., *M. atrofasciata* (Kieffer, 1911) can tolerate a wide range of substrates, current velocities, and even some shifts in salinity [26, 49], which is probably also true for *M. pallidula* that did not respond to any experimental manipulations in this study. In this case, DNA-based identification made it possible to gather these autecological information, which would have otherwise not been accessible.

Evidence for indirect effects

It was beyond the scope of this study to discuss the stressor response of every single OTU. It would also not have been possible, because only few OTUs can be reliably assigned to species and partly because there is a lack of ecological information. However, it is very likely that the applied stressors had an indirect effect on most chironomid OTUs rather than a direct physiological effect. The results of Beermann et al. [5] showed that many Ephemeroptera, Plecoptera, and Trichoptera taxa decrease in abundance upon added fine sediment and reduced flow velocity. Since many chironomids have a higher tolerance for more extreme conditions and the competition decreases with the applied stressors, they are likely increasing in abundance. A further indirect ecological effect might be due to the experimental manipulation affecting the microbial community. Microbes, i.e., bacteria and fungi, are the nutritional basis for many invertebrates either directly as food or indirectly as many invertebrates rely on microbial processes like decomposition of leaf litter. This highlights the need to extend multiple stressor analyses as done here to microbial taxa to understand community responses to multiple stressors and identify the relevance of indirect ecological effects.

Limitations and outlook

One of the biggest limitations of using DNA metabarcoding for multiple stressor research is that read abundances do not reflect species abundance, but are influenced by species biomass, mitochondrial copy numbers, and primer efficiency. Exact abundances are not being picked up especially for multicellular organisms [15]. Therefore, stressor analyses, as conducted here, do not allow for quantifying changes in species abundance upon respective experimental manipulations or anthropogenic stressors. Hence, additional data on species and life-stage biomass would be beneficial for metabarcoding studies to approximate species abundance. Furthermore, the options of PCR-free methods such as mitochondrial metagenomics [10] might greatly improve biomass and abundance estimations, but need to be studied further as there is scarce information on factors that influence the number of mitochondria per unit of biomass (season, life stage, active versus inactive species). The application of specific strategies to enrich mitochondria in metagenomic studies [42] can be helpful in principle, yet challenges may arise, because specimens have to be processed immediately and cannot be stored prior to laboratory processing. In any case, it needs to be considered if the benefit of taxonomic resolution and being able to analyze single OTUs outweighs the disadvantage of not having exact abundances, which will be greatest for taxa that are small, highly abundant, have a difficult morphology or for which there is a lack of experts such as Chironomidae, Acari, Nematoda and Oligochaeta.

Another limitation of using DNA metabarcoding for analyzing the effects of environmental disturbances to species is that the molecular method does not provide any information on the life stage of the detected species. However, different responses to stressors for the same OTUs might be observed, when different life stages are investigated (e.g., Kefford et al.[35]), that might be visually recognized when identification is based on morphology. Furthermore, different life stages are always encountered when field experiments are conducted in different seasons, but are rarely distinguished. For biomonitoring on the other hand this should be a minor problem, as sampling is conducted at the same time of the year annually in many biomonitoring programs (e.g., the Water Framework Directive, WFD [23], Europe).

Pinder [56] stated for Chironomidae that "specific identification may not [..] be justified for routine monitoring purposes, at least within the limitations imposed by the present state of knowledge regarding the ecological preferences and tolerances of most species." However, chironomids have already been shown to be of value for freshwater biomonitoring [48, 61]. Data as generated here and elsewhere [2] have the potential to greatly improve studying autecology of species, as it is not hindered by taxonomic impediment. With a more profound understanding of single species ecology, taxa that are widely neglected or only used on a superficial taxonomic level for biomonitoring might be utilized as bioindicators. With the variety of stressor response patterns found here, this study adds further support that Chironomidae specimens should not be treated as one taxon in biomonitoring programs and stressor research.

Conclusion

With more than 180 OTUs, we revealed a very high chironomid diversity with DNA metabarcoding in a multiple stressor experiment conducted at a single low mountain stream in Germany. The multiple stressor responses at OTU-level, and not only at family level as traditionally done, demonstrate the many and very different response patterns for individual chironomid OTUs (15 response patterns for the 35 most abundant OTUs). Thus, our study highlights the power of increased taxonomic resolution and adds ecological information made accessible by metabarcoding.

Additional files

Additional file 1. Chironomid specimen abundance, dry mass, DNA concentrations, and library details.

Additional file 2. Primer combinations.

Additional file 3. Publically available chironomid COI sequences from specimens sampled in Germany from BOLD(downloaded 01.06.2017).

Additional file 4. Read abundances and standardization of read numbers.

Additional file 5. Taxonomic assignment of the 35 most common chironomid OTUs.

Additional file 6. Sequencing reads assigned to other taxonomic groups.

Authors' contributions

Study design: AB, FL, VE, and VZ; lab work: AB and VZ; bioinformatics: VE and VZ; data analysis: AB and FL; writing the manuscript: AB, FL, VB, and VZ. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All sequences generated in this study are deposited at NCBI (SRA accession SRP154194).

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DNA metabarcoding of stream invertebrates reveals spatio-temporal variation but consistent status class assessments in a natural and urban river

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Sampling: 70 %

Laboratory work: 100 %

Data analysis: 100 %

Figures: 100 %

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DNA metabarcoding of stream invertebrates reveals spatio-temporal variation but consistent status class assessments in a natural and urban river

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Highlights:

- DNA metabarcoding reveals strong seasonal variation of stream invertebrate communities
- DNA metabarcoding detects site-specific impacts in a stressed urban stream
- Presence-absence and read-abundance data yield similar results
- Ecological status assessments are very similar among seasons
- The robustness of the method encourages the wider application for biomonitoring

Abstract

DNA metabarcoding of macroinvertebrates is increasingly used for aquatic bioassessment and -monitoring. A major strength of metabarcoding is the high taxonomic resolution provided while the inability to deliver reliable abundance data is regarded a main drawback. Data on the potential of metabarcoding to disentangle site-specific and seasonal variation with presence-absence data have been poorly explored. In addition, the robustness of ecological status assessments compliant with the European Water Framework Directive (Directive 2000/60/EG) have only been compared in few studies, not one of which studied changes across seasons. Therefore, we here investigated seasonal as well as site-specific effects on macroinvertebrate communities at six stream sites in a near-natural (Sieg) and urban (Emscher) German stream through metabarcoding. Furthermore, we evaluated the usability of the method as a tool for ecological status assessments. Our data showed distinct seasonal effects on OTU composition at the near-natural river Sieg. However, ecological status assessment was constant through seasons and comparable to assessments based on morphological identification. In contrast to the river Sieg, we found strong site and stressor-specific impacts in the heterogeneous urban stream Emscher. Here, ecological status assessment varied between sampling sites ranging from good to bad status but were largely consistent between seasons. Our study demonstrates the ability of presence-absence metabarcoding data to reliably assess invertebrate community composition in streams and infer environmental (natural or anthropogenic) impacts. Together with the technical robustness, our study encourages the wider adoption of the technique in stream bioassessment and -monitoring.

1. Introduction

Streams are dynamic ecosystems harbouring a high biodiversity in their natural state, but are under immense threat due to human alterations (Dobson et al., 2006; Elmqvist et al., 2003; Hooper et al., 2012; Vörösmarty et al., 2010). Invertebrates, macrozoobenthos in particular, constitute the most diverse organismal group in streams and are critical for stream ecosystem function and integrity (Wallace and Webster, 1996). Specific macroinvertebrate taxa (mainly Ephemeroptera, Plecoptera, Trichoptera, so-called ‘EPT taxa’) are highly sensitive to alterations of habitat or abiotic factors and their diversity and abundance is therefore the most commonly used biological quality element for stream bioassessment and -monitoring world-wide (Hering et al., 2004a, 2004b; Meier et al., 2004; Reynoldson et al., 1997; Rosenberg and

Resh, 1993; Usseglio-Polatera et al., 2000; Wallace and Webster, 1996). Many invertebrates, especially the EPT taxa, have evolved adaptations to the heterogenous conditions of stream ecosystems, which are reflected in their life cycle characteristics. E.g. in temperate stream ecosystems the majority of univoltine stream insects inhabit stream habitats most of the year and typically hatch in spring/summer in order to reproduce. The varying size of different life stages leads to strong temporal fluctuations in abundance and especially biomass for these groups in an ecosystem (Einarsson et al., 2002; Jackson and Füreder, 2006a; Šporka et al., 2006) and may lead to changes in status assessments when performed at different time points (but see Stark and Phillips (2009)). Standardised assessments that are based on morphological identification of organisms usually take also abundances into account, and take place at defined, similar seasoned points in time to allow for comparisons between years.

In recent years, new genetic high-throughput approaches, especially DNA metabarcoding, were proposed for bioassessments of macroinvertebrates with potential advantages over traditional approaches, such as costs, time, and higher taxonomic resolution (see Elbrecht et al., 2017b; Haase et al., 2010; Hering et al., 2018b; Leese et al., 2018; Taberlet et al., 2012 for further information). Up to now it is not possible to reliably determine specimen abundances with metabarcoding due to methodological biases restricting analyses mostly to presence/absence data (Krehenwinkel et al., 2017a; Piñol et al., 2015; Thomas et al., 2016). However, various studies show a strong correlation between individual abundance and read number per sample (Bista et al., 2018; Elbrecht et al., 2017b; Elbrecht and Leese, 2015). The insufficient determination of individual abundance limits the assessment of detailed numerical changes in community dynamics through metabarcoding. However, presence/absence data with high taxonomic resolution can be reliably assessed including taxa of low biomass and different life stages. Assessment results based on those data therefore might be less susceptible to the actual abundance of the target species in an ecosystem except for very rare ones and therefore should be less influenced by factors affecting individual abundances, e.g seasonality (Singer et al., 2019). While the impact of seasonal changes on aquatic macroinvertebrate community composition has been investigated in several studies using morphological data (e.g. Dolédec, 1989; Jackson and Füreder, 2006b; Šporka et al., 2006; Stark and Phillips, 2009; Wagner et al., 2000) studies based on molecular identification are rare, mainly focusing on microorganisms (Alam et al., 2001; Bock et al., 2018; Chain et al., 2016; Guardiola et al., 2016; Moreira and López-García, 2002; Stoeckle et al., 2017), or vertebrates (de Souza et al., 2016; Stoeckle et al., 2017). The insights suggest that (e)DNA

metabarcoding holds strong potential to reveal shifts in community dynamics on a high taxonomic level either based on relative read abundance or site occupancy data. To the best of our knowledge, only the study by Bista et al. (2017) analysed patterns of aquatic macroinvertebrates (non-biting midges from a Lake in UK) over the course of one year in lakes based on eDNA and bulk sample (community) data. For chironomids, seasonal changes in community composition was reflected in both data sets, yet the taxonomic focus was much narrower than for typical stream assessments.

In this study, we aimed to broadly assess stream macroinvertebrate taxon diversity and community composition in two seasons (spring and autumn) for two consecutive years in order to disentangle temporal from spatial patterns and the influence on ecological status determination. We selected two streams for the comparison, an intermediate-size, near-natural river (Sieg) as well as a heavily impacted, intermediate-size urban river (Emscher). The Sieg contains various sensitive freshwater taxa such as EPT and mainly shows a good ecological status (Beckmann et al., 2005; Gellert, 1987; Neumann, 2002; van Dijk et al., 1995). In contrast, the Emscher river is located in the densely populated industrial Ruhr area and was used as a sewage channel for decades. It is now part of the largest and among the most expensive restoration events in Europe. Small parts of the main channel and several tributaries are completely restored, other are still highly degraded and used as sewage channels (Bonada et al., 2005; Gerner et al., 2018; Winking et al., 2014). Therefore, the Emscher constitutes a network of differently-impacted and stressed stream sections in contrast to the Sieg. We sampled benthic macroinvertebrates in autumn 2016, spring 2017, autumn 2017, and spring 2018 and compared diversity of inferred operational taxonomic units (OTUs) assigned to macroinvertebrates to test the following hypotheses.

(I) Metabarcoding-based assessment of community composition of macroinvertebrates is largely constant between seasons, especially when using presence-absence data.

(II) Seasonal variation is more pronounced in the near-natural stream while site-specific spatial differences predominate in the heavily stressed urban river.

(III) Given expectations from hypothesis I, we expect ecological status assessment to yield largely similar results between seasons.

2. Material and Methods

Six sites each of the rivers Sieg and Emscher in North Rhine-Westphalia (Western Germany) were sampled two times in autumn/winter (Sieg: September 2016/2017, Emscher: November/December 2016/2017) and spring (Sieg and Emscher: April/May 2017/2018) for benthic macroinvertebrates. The autumn sampling of river Emscher had to be shifted to end of November 2016 due to logistic issues. To keep data comparable within streams, the sampling period of autumn 2017 was also shifted to this time of the year (beginning of December). Sample locations at river Sieg are in similar largely unstressed conditions and a good ecological status class was assessed for all locations as part of the ongoing federal bioassessment based on morphological data (https://www.elwasweb.nrw.de/elwas-web/index.jsf). In contrast, sample sites at the river Emscher were selected to reflect different stressor impact scenarios from being completely restored to open sewage channel. According the Water Framework Directive (WFD) the Emscher is a highly modified river systems which has to be accounted for ecological evaluation. Here the ecological potential, which describes the best possible condition while maintaining the main use of the water body, is assessed. Detailed information about sample locations and site-specific stressor impacts are given in table 1.

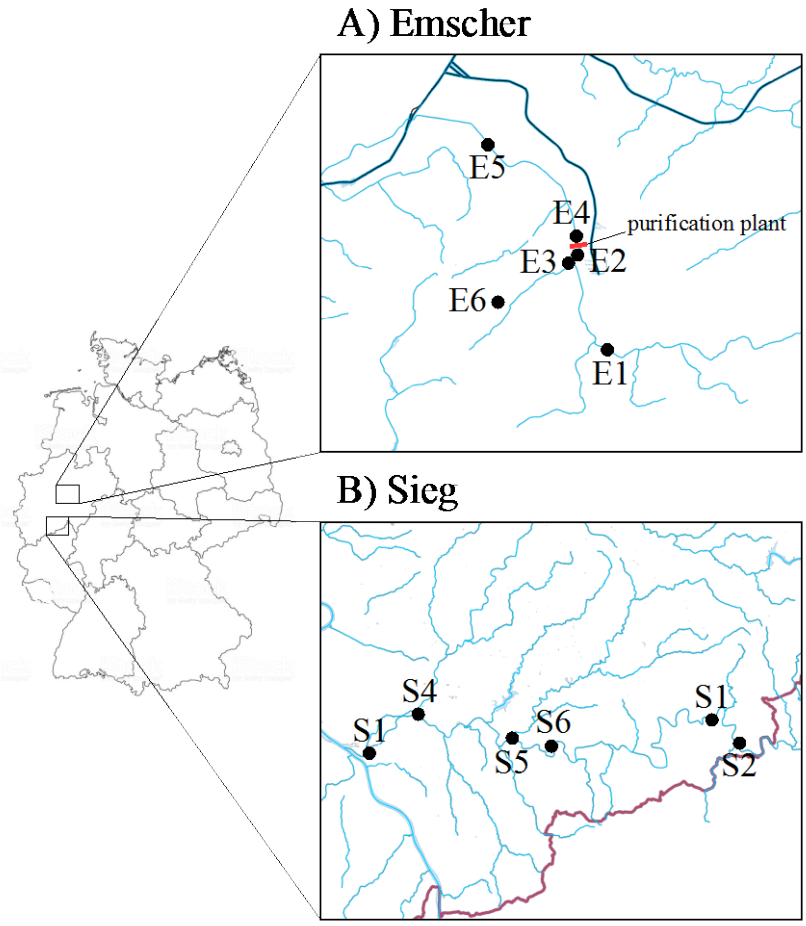

Figure 1: Sample sites of river A) Emscher and B) Sieg.

2.1 Sampling

Multi-habitat sampling was conducted according to the Water Framework Directive (WFD) conform protocol for German wadable streams (Meier et al., 2004), i.e., by taking 20 (10 at the Emscher due to a low habitat diversity) subsamples via kick-sampling at each sampling site covering all different microhabitats. Complete samples (containing organisms and substrate) were transferred to 96% denatured technical ethanol (permission from Amt für Natur- und Landschaftsschutz, Rhein-Sieg-Kreis: 67.1-1.03-19/2016KRO). For each sampling site, two bottles (1 L) were needed to capture the whole bulk sample, which combined the 20 (10) subsamples. The complete sample was equally divided between the two bottles (25% substrate per bottle). Immediately upon arrival at the laboratory (3–6 hrs after sampling), the ethanol of all samples was poured off through a sieve (mesh size: 0.5 mm) to retain animals and substrate. New 96 % denatured technical ethanol was added to the samples which were stored at – 20 °C until further processing.

Table 1: Coordinates of sample sites of river Emscher (E1-E6) and river Sieg (S1-S6) with characterization of stressor influence and ecological status based on morphologic identification (https://www.elwasweb.nrw.de/elwas-web/index.jsf).

River	Sample point	Latitude	Longitude	Stressor influence	Ecological status morphology (2013)
Emscher	E1	51.489473	7.447109	restored ~ 10 years, no specific stressor inflow	not evaluated
Roßbach	E2	51.531315	7.419964	clear water, concreted river bed	bad
Emscher	E3	7.419964	7.42317	occasional stressor inflow	bad
Emscher	E4	51.541686	7.447109	stressor inflow through purification plant	bad
Emscher	E5	51.584076	7.355304	stressor inflow through sewage	bad
Dellwiger Bach	E6	51.511027	7.365218	restored ~ 10 years, no specific stressor inflow	not evaluated
Sieg	S1	50.80103	7.58819	no specific stressor inflow	good
Sieg	S2	50.78136	7.62737	no specific stressor inflow	good
Sieg	S3	50.76520	7.10715	no specific stressor inflow	good
Sieg	S4	50.80075	7.17424	no specific stressor inflow	good
Sieg	S5	50.78194	7.30774	no specific stressor inflow	good
Sieg	S6	50.77494	7.36320	no specific stressor inflow	good

2.2 Laboratory protocols

All macroinvertebrates in each sample were separated from the substrate under a binocular (Leica S6E) and specimens from one sample were collected in a single tube filled with 96 % ethanol. Specimens were counted and categorised according to their size into two classes using standardized reference areas of 5 mm × 2 mm. Individuals fitting into this area were assigned to size class S, individuals exceeding this area were assigned to size class L. From extremely large animals (i.e. > 15 mm^2), which were rarely found in the samples (scattered Odonata, Lumbriculida), a leg or tissue part was removed and physically assigned to size class S. All specimens of the respective size class were homogenised to fine powder with an IKA Ultra Turrax Tube Disperser (BMT-20 S-M sterile tubes 20 ml, full speed for 30 min) and DNA was extracted following a modified salt extraction protocol (Sunnucks and Hales, 1996; ajdusted as in Weiss and Leese, 2016). After DNA extraction, the two size categories of the same sample were pooled in proportion to individual numbers (e.g., if 90 % of individuals were assigned to size class S and 10 % to size class L, for a 20 µL dilution, 18 µL of the extract of size class S were pooled with 2 µL of size class L). DNA concentration was quantified using a Qubit 2.0 Fluorometer (dsDNA BR Assay Kit, Thermo Fisher Scientific, Beverly, USA) and DNA diluted to 25 ng/µL. A two-step PCR was conducted for all samples

with one PCR replicate each. In the first step, the fragment was amplified using untailed BF2/BR2 primers (Elbrecht and Leese, 2017). PCR reactions consisted of 1× PCR buffer (including 2.5 mM Mg^{2+}), 0.2 mM dNTPs, 0.5 µM of each primer, 0.025 U/L of HotMaster Taq (5 Prime, Gaithersburg, MD, USA), and 1 µL DNA template filled up with HPLC H_2O to a total volume of 50 µL. The following PCR program was used: 94 °C for 180 s; 25 cycles of 94 °C for 30 s, 50 °C for 30 s, and 65 °C for 150 s; followed by a final elongation of 65 °C for 5 min in a Thermocycler (Biometra TAdvanced Thermocycler). For the second PCR step, 1 µL of each PCR product was used with uniquely tagged BF2/BR2 fusion primers (in-line barcode shift, (Elbrecht and Leese, 2017)). PCR conditions in the second step were identical to the first step, but only 15 cycles were used. A left-sided size selection of all samples was performed using magnetic beads with a ratio of 0.76x (Beckman Coulter, Krefeld, Germany) to remove short fragments (leftover primers and primer dimers). Concentration was measured on a Fragment Analyzer (Advanced Analytical, Ankery, USA) and all samples were equimolarly pooled. Paired-end sequencing was carried out by GATC Biotech AG (Constance, Germany) using one run on the Illumina MiSeq platform for samples of each season with a 250 bp paired-end v2 kit.

2.3 Data Analysis

Inline barcodes were used to assign reads to their original sample as implemented in JAMP v0.67 (https://github.com/VascoElbrecht/JAMP). Subsequent data processing was conducted for all samples in JAMP v0.67 using standard settings: paired-end reads were first merged (module U_merge_PE) and reverse complements were built where needed (U_revcomp) with usearch v10.0.240 (Edgar et al., 2011). Cutadapt 2.0 (Martin, 2011) was used to remove primer sequences and to discard sequences of unexpected length so that only reads with a length of 411– 431 bp were used for further analyses (module Minmax). The module U_max_ee was used to discard all reads with an expected error > 0.5. To achieve equal read numbers per sample, a subsampling was conducted using the module U_subsample as implemented in JAMP. Sequences were dereplicated, singletons were removed, and sequences with ≥ 97% similarity were clustered into OTUs using Uparse (U_cluster_otus). OTUs with a minimal read abundance of 0.01 % in each sample were retained for further analyses, while other OTUs were discarded. OTU sequences were compared with the database BOLD using the BOLD ID engine via the module BOLD_webhack in JAMP and taxonomies assigned after the following rules: for assignment to species level, a hit with 98 % similarity was required; 95 % similarity was required for assignment to genus level, 90 % for

family level, and 85 % for order level. Only OTUs present in both technical replicates per sample were kept for further analysis, OTUs present in only one replicate were discarded for the specific sample. Figures were created in R (R Core Team 2018) with the package ggplot2 (Wickham, 2016) and UpSetR (Conway et al., 2017). Diversity indices were calculated using the R package vegan (Oksanen et al., 2019) as well as NMDS analysis based on Bray-Curtis and Jaccard (dis)similarities. Seasonal effects on NMDS plots were assessed through a PERMANOVA (Permutational Analysis of Variance) through function adonis() in the package vegan. Effects on macroinvertebrate communities and diversity indices were demonstrated with a Kruskal-Wallis test and a Dunn-test was conducted to check for pairwise comparisons. Both tests were conducted with the package dunn.test() (Dinno, 2017) in R. We grouped OTUs into 'normal' (>0.1% frequency) and 'rare' (0.1-0.01%) ones based on the obtained ranked read abundance data and identified if OTUs significantly effected community dynamics reflected in NMDS plots using the function envfit() implemented in package vegan in R by Oksanen et al., 2019. The number of significantly effective OTUs was compared between the two abundance groups using a PERMANOVA as described above. The ecological status of sample sites based on metabarcoding was calculated through the assessment metric PERLODES anchored in the software Asterics (Hering et al., 2004a). Presence/absence data were used for status assessment following (Buchner et al., in review). Assessment values based on morphological identification of individuals were downloaded from ELWAS-WEB (https://www.elwasweb.nrw.de/elwas-web/index.jsf) where only data from the year 2013 were accessible for both river systems.

3. Results

After demultiplexing, one sample from river Sieg (primer combination BR20/BF24, S2 spring 2018) contained only 6 reads and was not included for further analysis. As we observed a very high correlation (R^2=0.98) of relative read distribution between technical replicates, the second sample of S2 spring 2018 was included for further analysis. For the Sieg data set, after quality filtering the lowest number per sample was 76,326 paired-end reads. Therefore, we subsampled the data set to 76,000 reads for all samples before clustering into OTUs. After OTU clustering and combining read numbers for OTUs present in both technical replicates, Sieg samples contained on average 143,305 (± 6,122 (S2 spring 2018 excluded)) reads. Sequences were clustered into 573 OTUs present in both replicates per sample with 397 OTUs assigned to aquatic macroinvertebrates with an identity ≥ 95 % by the comparison with

the BOLD database (Tab. 2, Tab. S1). On average 136,017 (± 17,753) reads per sample belonged to benthic macroinvertebrates, with the highest proportion belonging to Ephemeroptera (35.1 ± 19.6 %). For the Emscher data set, we subsampled libraries to 53,000 reads to achieve equal read numbers. After OTU clustering and combining of technical replicates, Emscher samples contained on average 102,854 (± 6,732) reads. In total, reads were clustered into 437 OTUs with 332 OTUs assigned to aquatic macroinvertebrates with an identity ≥ 95 % by the comparison with the BOLD database (for further information of OTU and read distribution within orders see Tab. 2 and Tab. S3).

Tab. 2: Number of OTUs and reads assigned to benthic macroinvertebrate taxa across all sample sites. Average values are given listed ± standard deviation. */** mark taxa or read numbers, which are significantly impacted by seasonality.

	Sieg				**Emscher**			
order	total		average per sample [%]		total		average per sample [%]	
	OTUs	reads	OTUs	reads	OTUs	reads	OTUs	reads
Amphipoda	10	127,487	3±1.7	3.8±5.8	5	311,984	5.6±4.5	12.8±15.1
Arhynchobdellida	1	3,208	0.4±0.7	0.1±0.4	4	6,419	1.2±2.1	0.3±0.7
Coleoptera	28	324,861	9.1±3	12.6±12.3	13	160,660	4.5±6.4	7.4±13.8
Diptera	175	681,483	39±11.3	22±16.3	152	555,096	36±10.8*	23.5±19.2
Enchytaeidae	12	3,616	1±2.6	0.1±0.3	20	64,523	3.1±6.6	7.7±18
Ephemeroptera	40	1,183,137	15.1±4.4	35.1±19.6**	11	330,558	4.8±4.6	13.5±21.3
Haplotaxida	57	180,480	10.3±8*	5.4±7.6*	59	374,813	25.6±14.1	16.5±24.2
Isopoda	5	46,053	2.3±1.7	1.5±2.5	10	184,526	3.9±5.1	7.7±21.1
Lumbriculida	6	200,772	2.2±0.9	6.1±6.3**	5	26,888	1.3±1.9	1.2±3.0
Mollusca	5	4,300	0.6±0.8	0.2±0.1	8	3,562	2.5±3.3	0.2±0.3
Odonata	4	96,039	0.8±1	2.9±9.4	1	3,596	0.2±0.6	0.1±0.7
Plecoptera	11	39,732	2.4±1	1.2±1.6**	1	30	0.2±0.6	0.001±0.01
Rhynchobdellida	2	714	0.2±0.5	0.02±0.07	9	60,895	1.7±3.8	3.2±12.3
Trichoptera	30	270,374	10.8±3.2	8.5±7.7	23	205,332	6.3±5.4	8.7±12.9
Trompidiformes	8	95,625	2.6±1.5	3.2±4.5	11	44,640	3.4±3.4	1.9±3.2*
Others	3	610	0.05±0.1	0.02±0.04	-	-	-	-

3.1 Temporal impact on community composition

Metabarcoding data aggregated at order level revealed a small, yet significant effect of seasonality on OTU number with more OTUs assigned to order Haplotaxida in autumn (average 14.4 OTUs) than in spring (6.2) at river Sieg ($p < 0.05$). At river Emscher, a higher OTU number was found for Diptera in spring (40.4 ± 9.9 %) compared to autumn (31.5 ± 9.7 %, $p < 0.05$, Fig. S1 B,D). No seasonal effect on OTU richness, Evenness and Simpson diversity was observed at river Sieg while significantly more OTUs were observed in spring than in autumn at river Emscher ($p < 0.05$) (Tab. S2). In contrast, non-metric multidimensional scaling (NMDS Fig. 2) based on Jaccard dissimilarity (presence/absence → p/a) revealed significant seasonal patterns ($p < 0.001$, $R^2 = 0.11$-0.16, Tab. 3) at the near-natural river Sieg when all OTUs assigned to a) benthic macroinvertebrates, b) EPT taxa, c) indicator taxa from the operational taxa list (taxa decisive for ecological status assessment), or

d) Chironomidae were compared. The seasonal effect was not driven by rare OTUs (0.1-0.01%) but rather by OTUs with average (or even high) abundance per sample > 0.1 % ($p < 0.05$, Fig. S3B, Tab. S4). Figure 4 A illustrates, that the overlap between seasons is 27 OTUs in autumn and 17 in spring looking at all sample sites together. NMDS analyses showed no seasonal impact on community composition at the urban river Emscher for EPT taxa ($p=0.14$, $R^2=0.05$) but an impact was determined when all macroinvertebrate OTUs ($p < 0.05$, $R^2=0.05$) as well as Indicator ($p < 0.01$, $R^2=0.05$) and chironomid taxa ($p < 0.05$, $R^2=0.06$) were included (Fig. 3, Tab. 3, S5). Effect size was low for all four analysis. As for river Sieg, also rare taxa did not drive the community partitioning pattern (see Fig. 3, $p = 0.076$, Fig. S3B, Tab. S4).

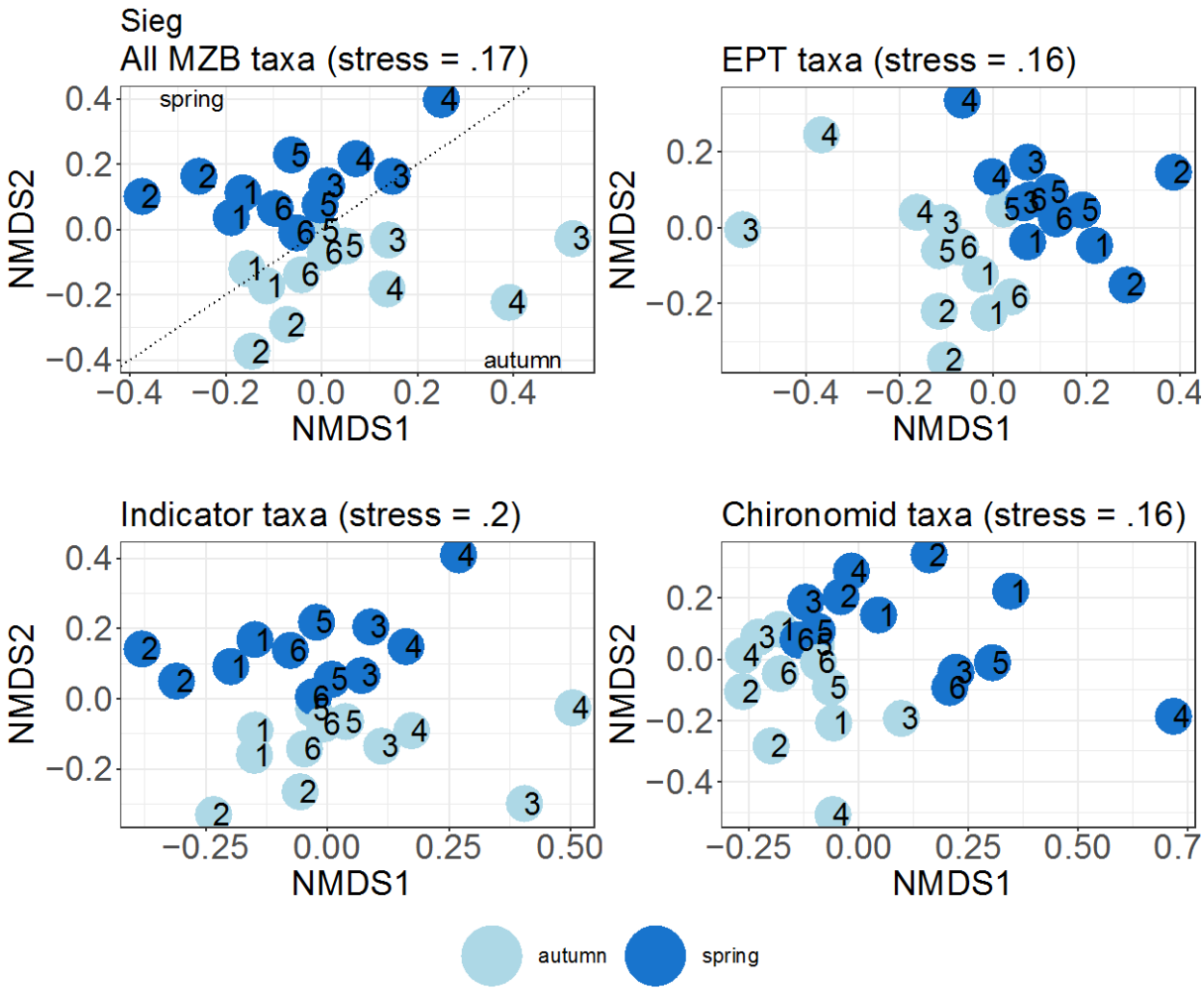

Figure 2: Non-metric multidimensional scaling of OTU composition of Sieg samples based on Jaccard-dissimilarity (presence/absence). Analysis include a) all OTUs assigned to macrozoobenthos taxa (MZB) with an assignment >95 % with BOLD, b) a subset of dataset (a) including all OTUs assigned to the orders Ephemeroptera, Plecoptera and Trichoptera (EPT), c) a subset of dataset (a) including all OTUs assigned to Indicator taxa regarded in ecological status assessment and d) a subset of dataset (a) including all OTUs assigned to chironomid taxa.

Aggregated at order level, a higher number of reads ($p < 0.05$) was assigned to Ephemeroptera in spring (44 ± 18.8 %) than in autumn (26.2 ± 16.8 %) while to Haplotaxida (5.4 ± 7.44 %, $p < 0.05$), Lumbriculida (6.1 ± 6.3 %, $p < 0.01$) and Plecoptera (1.2 ± 1.5 %, $p < 0.01$) higher read numbers were assigned in autumn (Haplotaxida: 8.9 ± 9 %, Lumbriculida: 10.1 ± 6.5, Plecoptera: 2.1 ± 1.8 %) than in spring (Haplotaxida: 1.9 ± 3.45 %, Lumbriculida: 2.1 ± 1.8 %, Plecoptera: 0.3 ± 0.5 %) at river Sieg. Only a minor effect of seasonality ($p < 0.05$) was detected on the order Trombidiformes at river Emscher. NMDS analyses based on Bray-Curtis dissimilarity (abundances) indicate similar pattern to p/a data for river Sieg, when all macroinvertebrates are included ($p < 0.01$, R^2=0.14, Fig. S2 A, Tab. 3). Impact of seasonality is still significant ($p < 0.01$, R^2=0.13-0.2, Tab. 3), but a less clear separation of samples is observed, when only EPT, indicator or chironomid taxa are included (Fig. S2 B, C, D). NMDS analyses based on Bray-Curtis dissimilarity indicate no impact of seasonality on community composition at river Emscher for EPT and chironomid taxa (Fig. S2, $p = 0.15$-0.34, $R^2 = 0.03$-0.04), while a weak effect is detected for complete macroinvertebrates ($p < 0.05$, $R^2 = 0.05$) and Indicator taxa ($p < 0.05$, $R^2 = 0.05$, Tab. S5).

3.2 Spatial impact on community composition

Sampling location had a significant impact on beta diversity for the river Sieg. NMDS analysis based on Jaccard dissimilarity indicated an influence of sample location ($p < 0.01$, R^2=0.3) including all macroinvertebrate OTUs. This, however, was strongest within seasons which is clearly visible in figure 2. This held true also for the significant effects reported for EPT ($p < 0.01$, R^2=0.29), Indicator taxa ($p < 0.01$, R^2=0.3) and Chironomidae ($p < 0.05$, Fig. 2 A-D). At order level, only OTU number assigned to the order Lumbriculida ($p < 0.01$, Fig. S1B) and Haplotaxida ($p < 0.01$) differed between sampling sites. Figure 4 C supports those results and demonstrates the high overlap of 60 OTUs between all sample sites.

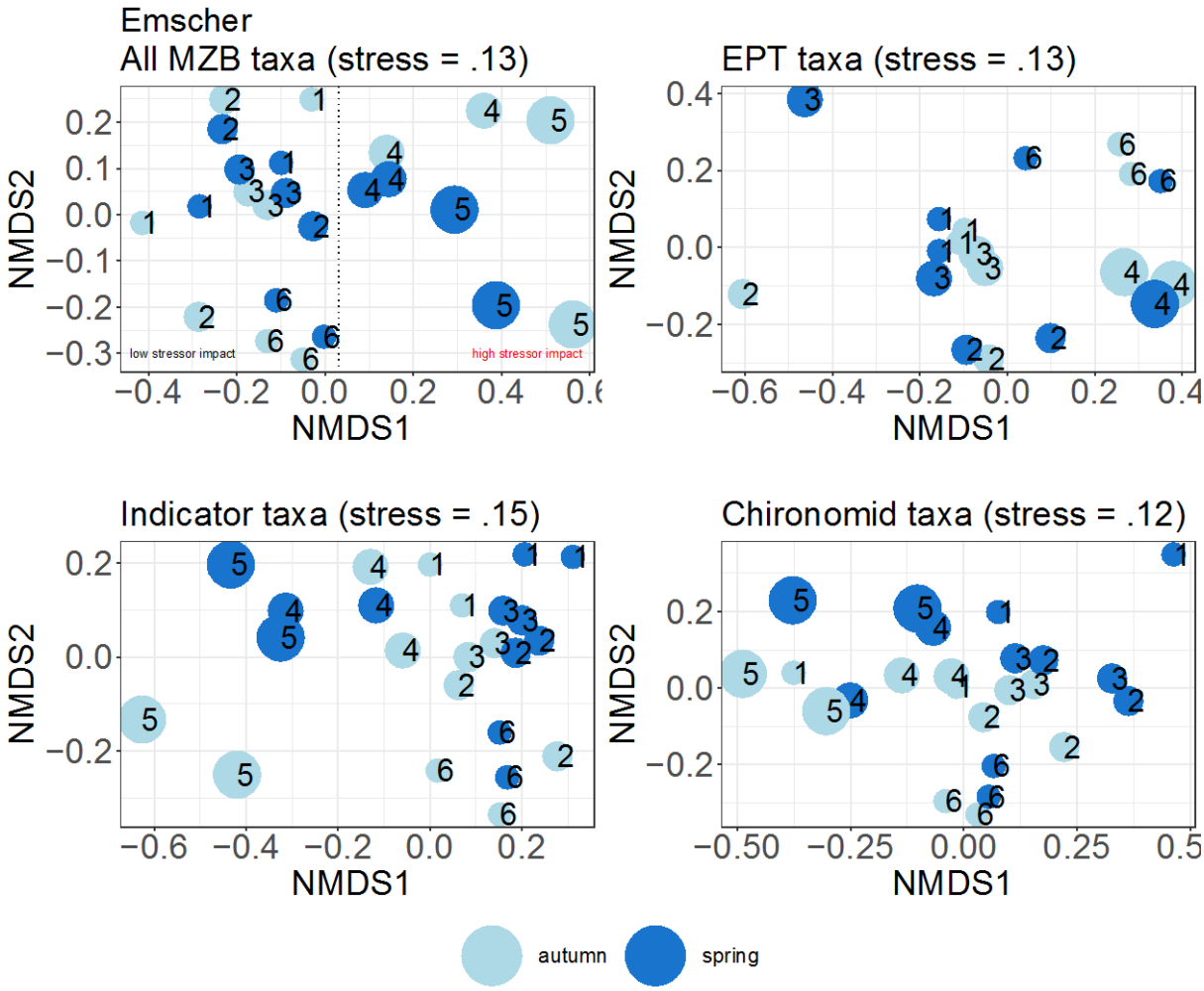

Figure 3: Non-metric multidimensional scaling of OTU composition of Emscher samples based on Jaccard-dissimilarity (presence/absence). Increasing dot size is related to increasing stressor impact on site (see table 1). Analysis include a) all OTUs assigned to macrozoobenthos taxa (MZB) with an assignment >95 % with BOLD, b) a subset of dataset (a) including all OTUs assigned to the orders Ephemeroptera, Plecoptera and Trichoptera (EPT), c) a subset of dataset (a) including all OTUs assigned to Indicator taxa regarded in ecological status assessment and d) a subset of dataset (a) including all OTUs assigned to chironomid taxa.

Tab. 3: p and R^2 values for datasets analysed on Figure 2 and 3 (Jaccard) and figure S2 (Bray-Curtis)

Sieg	All MZB taxa		EPT taxa		Indicator taxa		Chironomid taxa	
Jaccard	p value	R^2	p value	R^2	p value	R^2	p value	R^2
Season	0.001	0.11273	0.001	0.16042	0.001	0.11331	0.001	0.1157
Sample	0.001	0.2999	0.001	0.28754	0.001	0.29703	0.012	0.23254
year	0.001	0.08	0.11	0.06766	0.001	0.09195	0.001	0.1206
Bray-Curtis								
Season	0.001	0.13532	0.001	0.21201	0.001	0.13596	0.001	0.16722
Sample	0.001	0.29274	0.001	0.32171	0.002	0.29128	0.105	0.22048
year	0.023	0.05534	0.007	0.07108	0.041	0.05416	0.001	0.10142
Emscher								
Jaccard								
Season	0.011	0.05477	0.137	0.05032	0.005	0.04922	0.011	0.05781
Sample	0.001	0.45593	0.001	0.49062	0.001	0.43342	0.001	0.45672
year	0.033	0.04522	0.455	0.03373	0.002	0.05475	0.002	0.08128
Bray-Curtis								
Season	0.049	0.04525	0.152	0.04553	0.016	0.05005	0.34	0.03444
Sample	0.001	0.50279	0.001	0.57393	0.001	0.47481	0.001	0.40085
year	0.019	0.04895	0.556	0.0251	0.031	0.04837	0.014	0.06521

While season had no obvious impact on beta diversity for the heavily impacted river Emscher, spatial information strongly did. NMDS analysis based on Jaccard dissimilarity supported a strong impact of sample location on community composition regarding all four datasets ($p < 0.01$, R^2=046-0.49). Fig. 3 indicates a separation of locations with no/low (E1, E2, E3) and strong stressor inflow (E4, E5). Samples taken at E6, a restored tributary of the Emscher ('Dellwiger Bach') represent a distinct cluster. Regarding only EPT taxa (Fig. 2B), the sample set was reduced to 19 samples because E5 (sewage channel, all sampling seasons) and E4 (after purification plant, spring 2018) did not contain any OTU assigned to those taxa. Aggregated at order level, OTU number differed significantly between sample sites with effects on Amphipoda, Coleoptera, Enchytraeida, Ephemeroptera, Haplotaxida, Isopoda, Mollusca and Trichoptera ($p < 0.05$). The dominant pattern here is a higher number of Ephemeroptera and Trichoptera at E1, E2 and E3 compared to E4 and E5. In opposite more OTUs are assigned to the Haplotaxida and Enchytraeida at E4 and E5 (Tab. 2, Fig. S1D). Figure 4 D illustrates the high differences in taxa composition at river Emscher, where only 6 OTUs were found at all sampling sites.

3.3 Community dynamics and read abundance

Sampling location had a small effect ($p < 0.05$) on read numbers assigned to order Coleoptera at river Sieg. NMDS analysis based on Bray-Curtis dissimilarity on OTU level revealed a significant impact of sample location including all benthic macroinvertebrates, EPT and Indicator taxa ($p < 0.01$, R^2=0.22-0.32) which is however strongest within seasons (Fig. S2 A-D). At river Emscher, an effect of sampling location was visible on read number distribution in Amphipoda, Coleoptera, Enchytraeida, Ephemeroptera, Haplotaxida, Isopoda, Rhynchobdellida and Trichoptera ($p < 0.05$), where differences were visible between the sample sited with high stressor inflow (E4/E5) and the other 4 sample sites (E1-E3, E6). This pattern is supported by NMDS analysis based on Bray-Curtis analysis (Fig. S2 E-H), where a clear separation is visible between sample sites of high and low stressor inflow.

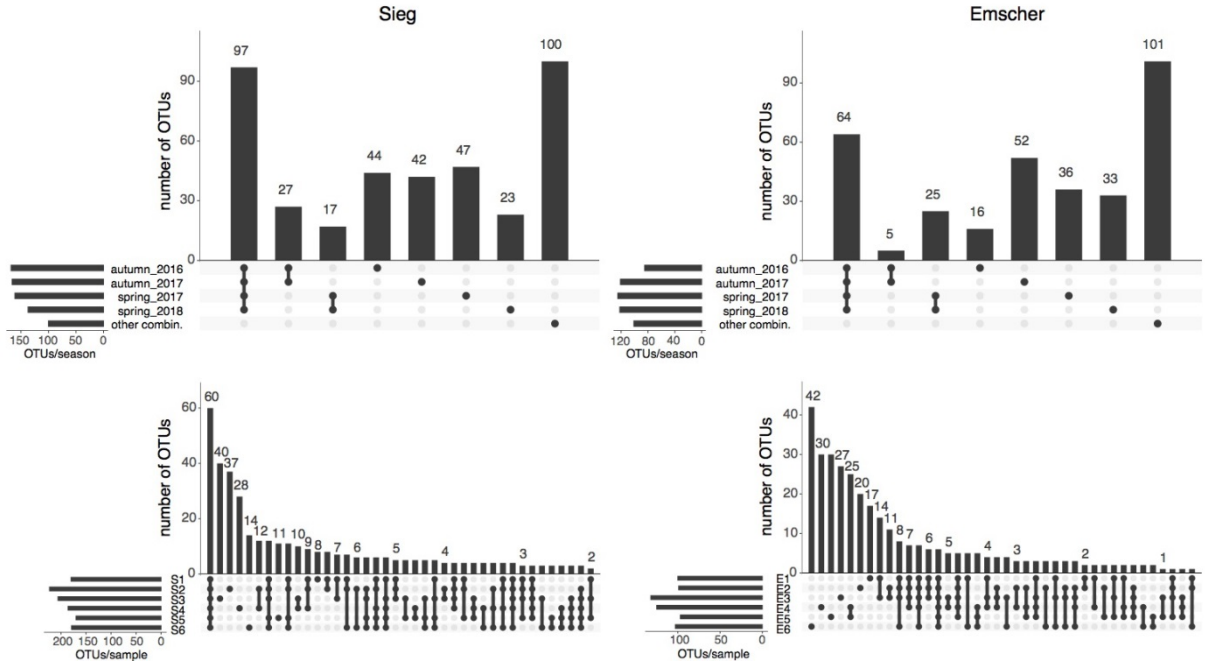

Figure 4: Number of OTUs shared between A) seasons at river Sieg, B) seasons at river Emscher, C) samples at river Sieg and D) samples at river Emscher.

3.4 Ecological status assessment

The ecological status based on metabarcoding of sample sites at river Sieg was determined as 'good' through all seasons with two exceptions (Tab. 1, Fig. 5). S2 sampled in spring 2017 was assessed with a 'high' ecological status while S3 of the same season was determined as 'moderate'. Comparisons with assessments based on morphological data (assessed 2013) are largely congruent. Here, the whole river section covering all locations included in the present study was determined as 'good', rendering only S2 and S3 (2017) with a divergent assessment through metabarcoding (Tab. 1, Fig. 5). River Emscher is classified as a highly modified water body, which needs to be considered when ecological status or potential is determined. Sample site E1, which prevails in restored conditions was determined as 'moderate' in autumn 2016 and spring 2017 whereas potential was assessed as 'good' in autumn 2017 and spring 2018. A 'good' ecological potential was assessed at all seasons but spring 2017 (moderate) for sample site E6 ('Dellwiger Bach'). Sample point E2, which is influenced by a punctual stressor impact of sewage mixed with rain water, released at heavy rainfalls is evaluated with 'moderate' conditions through all seasons (Tab. 1, Fig. 5). Sample point E3 is located at the Emscher tributary 'Roßbach' and contains clear water while the river bed is still artificial and made of concrete. Samples at this point are assessed as 'good' in autumn 2016 and 'moderate' in autumn 2017 and spring 2018 while a 'poor' condition is determined in spring 2017. Sample site E4, which is located after the inflow of a purification plant showed

‘poor’ ecological potential in autumn 2017 while the assessment reveals ‘bad’ conditions in autumn 2016 and spring 2017/2018. Sample site E5, which was located at a stretch of the Emscher, which still functions as a sewage channel, is assessed with a ‘bad’ potential in autumn 2017 and spring 2018, while no evaluation was possible in autumn 2016 and spring 2017. Comparisons assessments based on morphological examination were unsuccessful, because no values were accessible for the restored sample sites. Based on published data from 2013, ecological potential of sites E2-E5 was determined as ‘bad’, yet no recent updates were available for a more direct comparison.

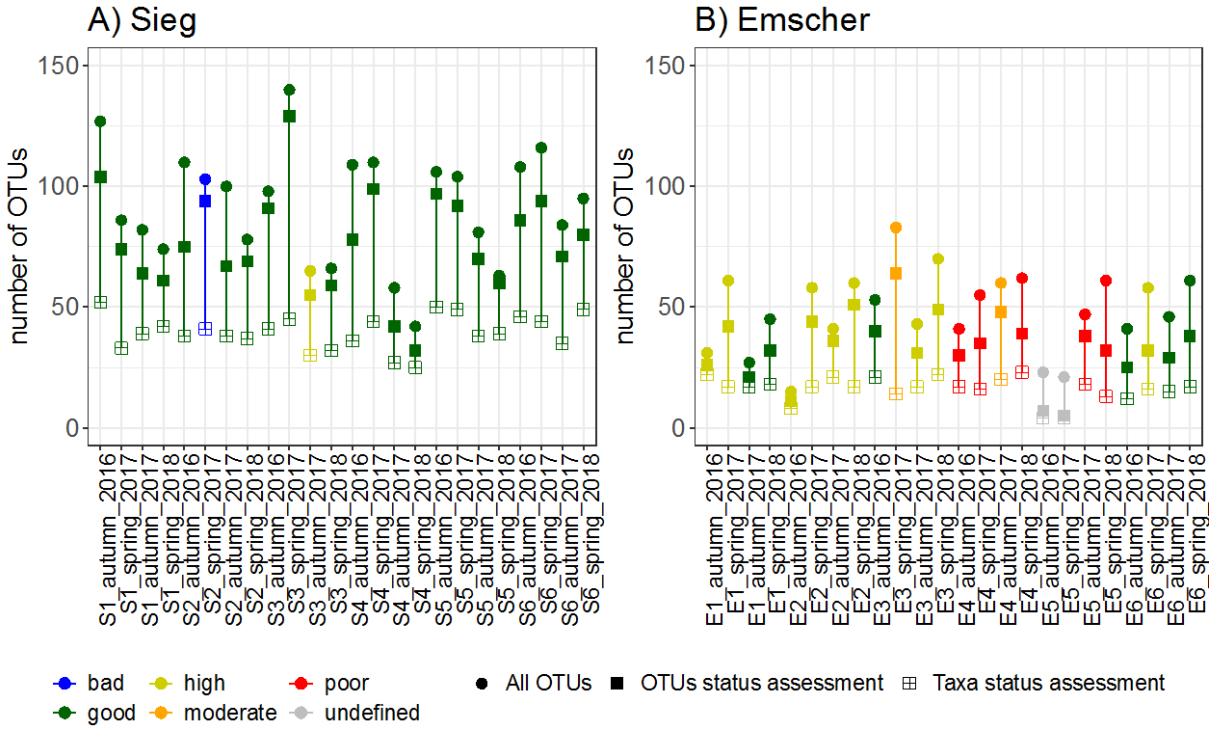

Figure 5: Circles indicate macroinvertebrate OTUs with an assignment > 95 % by the comparison with BOLD for both rivers. Filled squares symbolize number of OTUs assigned to taxa for ecological status/potential assessment (e.g. 42 OTUs were assigned to chironomids → 42 included in calculation), while empty squares symbolize the number of actual taxa included in ecological status/potential assessment (e.g. 42 OTUs were assigned to chironomids → 1 included in calculation).

4. Discussion

Seasonal and spatial variation of community composition

DNA metabarcoding revealed an enormous and taxonomically broad diversity of taxa in both studied river systems. In line with hypothesis I, which assumed a largely constant community composition of macroinvertebrates between seasons, we found a minor impact of seasonality on OTU number for both river systems when addressing the order or family level. In contrast analyses based on OTU level (NMDS analysis) assigned to all benthic macroinvertebrates at

river Sieg revealed clear seasonal differences including presence/absence (Jaccard) data. This is also largely supported by analyses based on subsamples including EPT, indicator and chironomid taxa. Sampling site also explained a large proportion of variation in the data, while year showed a minor influence (Fig. 2 A-D, Tab. 3). Results indicate extreme variation in individual numbers or biomass on OTU level between seasons: taxa highly abundant in spring seem to be almost absent or present only as eggs or overwintering stage in autumn (or the other way around). Initial expectations, that different life stages or fluctuations in individual abundance will not affect community composition based on presence/absence data are therefore not confirmed with the present results. We expect that shifts in biomass were substantial for many taxa, which were therefore not picked (reported as absent) by metabarcoding in both seasons, despite reasonable sequencing depths. Our strict filtering approach that aimed to omit false positives may have inflated this signal: The filtering threshold of 0.01 % abundance of an OTU/per sample and the necessary occurrence in both technical replicates was applied to exclude sequencing mistakes or tag-switching from the dataset. It has most likely also led to the exclusion of 'real' positives, mainly of low biomass. Supposing seasonal changes in biomass of specific taxa, strict filtering will lead to the exclusion of OTUs with low read proportion in one season but the presence in the other due to a higher biomass of assigned taxa. With less stringent filtering, a somewhat greater overlap in community composition between seasons is expected. Results also support the assumption that a high taxonomic resolution is required to reveal detailed diversity patterns in aquatic macroinvertebrate communities and to detect natural or anthropogenic stressor effects (Beermann et al., 2018; Leese et al., 2018; Macher et al., 2016; Taberlet et al., 2012).

Taxa with high biomass are typically dominating environmental bulk samples (Elbrecht et al., 2017a). We expected EPT and other merolimnic taxa with a hatching period in spring/early summer to dominate the spring sample read abundances. This, however, was not entirely found. Especially the mayflies dominated spring samples in terms of read abundances (Heptageniidae, Caenidae, Ephemerellidae, Baetidae), with the most obvious observed seasonal variation in reads found for the most abundant mayfly, i.e. *Ecdyonurus insignis* (OTU1). Representatives of the Ephemeroptera in temperate regions often have synchronized emergence patterns in early summer (Bêche et al., 2006; Füreder et al., 2005; Jackson and Füreder, 2006a; Obach et al., 2001; Wise, 1980). As predicted by hypothesis II, the rapid development and large body size in spring leads presumably to a higher proportion of tissue and consequently DNA in bulk samples, leading to higher read numbers assigned to

ephemeropterans. In contrast, Plecoptera show higher read numbers in autumn - mainly represented by *Leuctra fusca* - with the highest read number assigned to this taxon. Results are congruent with the literature, indicating an average nymphal developmental period of 3 month, with the highest nymph size and emergence in autumn (Elliott, 1987; López-Rodríguez et al., 2012). We find an opposite pattern for the stonefly *Leuctra geniculata,* which was represented with much lower reads than *L. fusca* but shows a higher read distribution in spring than in autumn. This species is known to have an earlier emergence time than *L. fusca*, which is likely also a mechanism to avoid possible competition between co-occurring species (Elliott, 1987; López-Rodríguez et al., 2009). Other stonefly species in the present study showed extremely low read numbers and were uniquely found at specific sample sites (Tab. S1). Most diverse families within the Haplotaxida are the Naididae and Lumbricidae in which almost all species (exception *Nais elingius*) show higher read numbers in autumn than in spring. For family Naididae, this is congruent with previous studies, indicating the highest abundance of representatives in summer to autumn due to higher temperatures and food supply leading to a higher growth rate and asexual reproduction (Learner et al., 1978), with *N. elinguis* as an exception and higher individual numbers in spring. For the Lumbricidae, the species *Eiseniella tetraedra* and *Allolobophora chlorotica* showed the highest differences in assigned reads between autumn and spring (Tab. S1). Those distinct differences cannot be explained with evidence from earlier studies, where mainly a lower abundance of Lumbricidae is observed in summer with no differences between the other seasons (Bundy et al., 2004; Spurgeon and Hopkin, 1999). As a general limitation, we cannot infer whether high levels of reads resemble many small or few large individuals (Elbrecht and Leese, 2015; Krehenwinkel et al., 2017).

In line with hypothesis II, seasonal effects on read numbers were higher for the near natural river system Sieg than for the urban river Emscher, where the site-specific stressor conditions lead to much stronger spatial than temporal signatures. Here it was due to the high variation in general condition and stressors present along the river difficult to assess the magnitude of seasonal effects. Additionally, figure S1C/D shows high variation in read proportion at the same sample site between different sampling dates, which is however not congruent with seasonality, but illustrates a rather ‘chaotic’ structure. The observed pattern could be induced through instable population dynamics, which are likely to occur in stressed ecosystems (Lorenz et al., 2004; Miller et al., 2007; Rapport et al., 1998). Also at restored sample sites (E1/E3/E6) high variation between sample dates occur and can be caused by short-term

disturbances influencing the not yet completed natural diversity and stability of populations at those locations (Chapman, 1998; Lake et al., 2007; Power, 1998) or an ongoing community change due to sequential recolonization. Especially sample site E5 and E6 were difficult to assess due to the canalization and sewage inflow and sampling had to take place under special health safety measurements. Additional changes e.g. in water level made sampling more difficult and could have biased the process, inflating the inferred temporal variation in community composition and read numbers at identical sampling sites.

Still, analyses at the Emscher revealed differential stressor impacts at the sites. Sample sites E1, E2 and E3 (low or no stressor impact) clustered together whereas the group of highly impacted sample sites E4 and E5 (Fig. 3, Fig. S2 C, D) were distinctly different. Marked differences were observed especially for mayflies, caddisflies and beetles, which showed a moderate diversity and read abundance at the restored sites, but were completely absent at stressed sites E4 and E5. For E4 macroinvertebrate communities were mainly composed of Haplotaxida, Enchytraeida, Rhynchobdellida, Isopoda and a few dipterans while E5 was dominated by OTUs assigned to the two worm orders Haplotaxida and Enchytraeida with considerably higher read numbers than at the other sampling sites. These most abundant taxa are known to be generalists with moderate to high tolerance against pollution and other stressors (Chapman and Brinkhurst, 1981; Khangarot, 1991). Therefore, their dominance at these sampling sites is expected.

With respect to ecological status assessment we expected minor variation between seasons using p/a-based metabarcoding. With the exception of two samples, metabacoding-based ecological status assessment was identical for all sample sites of the river Sieg and largely congruent with traditional morphological assessments. One sample was classified one status class higher (‘high’, S2 spring 2017) and one sample as one lower (‘moderate’, S3 autumn 2017). For sample site S3 in autumn 2017, the lower classification could be due to an extraordinarily high water level associated with sampling difficulties in this season, while a random capture of individuals belonging to rare, yet ‘high status class indicator taxa’ at S2 in spring 2017 could have led to a higher status classification. For the other samples, a ‘good’ ecological status is determined throughout all seasons, and variation of status class of max. 1 is also not uncommon for traditional sites (see Buchner et al. in review and discussion therein). The consistent assessment results seem first surprising at first in view of the distinct differences between seasons reported at OTU level, which still remained even if only taxa

were included in analysis that are considered for ecological status assessment (operational taxa list). To understand this, it is important to consider how ecological status is inferred in Germany. The final determination of the ecological status is based of two different modules ('general degradation', 'saprobic index'), computed through different core metrics, where the worst value of both modules is decisive for the ecological status (see Buchner et al., in revision). It is therefore possible that differences displayed in figure 1 are due to changes in only one of the two modules, which do not translate to the final assessment value through the robustness of the underlying metrics (Hering et al., 2018b, 2018a). This assumption is supported by the fact that the 'general degradation' and 'saproby' module values sometimes differ for sample sites (not shown). However, assessment values are, except for two sample sites and points, in line with published information based on morphological examination. Thus, seasonal patterns detect by metabarcoding are not translated into different status assessment. Results highlight the reliability of the method metabarcoding and its functionality for ecological status assessments. Additionally, figure 5 clearly indicates a distance between the three shown data points, including all detected OTUs with a taxonomic assignment > 95 % with BOLD, all OTUs assigned to taxa that are regarded for ecological status assessment (taxonomically described with an assigned ecological trait/value) and the actual number of taxa included for assessments for each sample site. This again shows the gain of information through metabarcoding and molecular approaches in general.

Our data from the urban river Emscher show that ecological conditions at the different sample sites exposed to stressor impacts differ and this is well-reflected in the metabarcoding data. Assessments spanned from 'good' to 'bad' potential and were also variable within some sample sites. Potential variation at restored sites without known stressor influence could be explained by a reduced stability of macroinvertebrate communities in fragmented habitats and the concerned changes due to short-time disturbances and the lack of recolonization from surrounding intact regions (Winking et al., 2014). At stressed sites, ecological potential assessments indicate an influence on macroinvertebrate communities immediately after the inflow of mixed systems (E2, 'moderate' – 'bad') and purification plants (E4, 'poor' – 'bad'). Unfortunately, no updated assessment results based on morphological identification for the investigated sample sites are published so that no direct comparison between the two methods is possible.

It should be noted that the availability of many reference barcodes for macroinvertebrates from Sieg was of clear advantage with respect to robustness of the status class assessment compared to Emscher. Thus, while reference barcode libraries are already good for Germany stream assessment (Weigand et al. 2018), further improvements are still possible.

5. Conclusion

The present study demonstrates that presence-absence metabarcoding can robustly assess site-specific taxa composition of aquatic macroinvertebrate communities and also reveal temporal changes in communities. These changes were most obvious when using the full resolution down to species and OTU-level. Ecological status assessment in the heavily impacted urban stream revealed differing quality values between seasons, likely reflecting varying punctual stressor inflow and a higher susceptibility to short-time disturbances. For the near-natural stream, assessments were stable through sample sites and seasons and metabarcoding data were largely congruent with assessment data based on morphological determination. Our data encourages the wider adoption of the powerful technique in applied bioassessment and -monitoring.

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Data Availability Statement

Raw data will be accessible on GenBank (Short Read Archive; submission number XX); processed data are available in the Supporting Information.

Conflict of Interest Statement

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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Can metabarcoding resolve intraspecific diversity changes to environmental stressors? A test case using river macrozoobenthos

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Contributions to this manuscript

Experimental design and planning: 75 %

Sampling: 70 %

Laboratory work: 100 %

Data analysis: 100 %

Figures: 100 %

Manuscript writing: 75 %

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doctoral candidate supervisor

Can metabarcoding resolve intraspecific diversity changes to environmental stressors? A test case using river macrozoobenthos

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Key words

Genetic diversity, haplotypes, denoising, genetic erosion, environmental impact

Abstract

Genetic diversity is the most basal level of biodiversity and determines the evolutionary capacity of species to adapt to changing environments, yet it is typically neglected in routine biomonitoring and stressor impact assessment. For a comprehensive analysis of stressor impacts on genetic diversity, it is necessary to assess genetic variants simultaneously in many individuals and species. Such an assessment is not as straight-forward and usually limited to one or few individual species. However, nowadays species diversity can be assessed by analysing thousands of individuals of a community simultaneously with DNA metabarcoding. Recent bioinformatic advances also allow for the extraction of exact sequence variants (ESVs or haplotypes) rather than of Operational Taxonomic Units (OTUs) only. By using this new capability, we here evaluated if the analysis of haplotype diversity as a proxy of genetic diversity in addition to species diversity can provide new insights into responses of stream macrozoobenthos communities to environmental stressors. For this purpose, we analysed macroinvertebrate bulk samples of three German river systems with different stressor levels using DNA metabarcoding. While OTU number and composition were clearly affected by the stressor level, this association was not as clear when looking at haplotype diversity. Here, stressor responses were only found for sensitive EPT (Ephemeroptera, Plecoptera, Trichoptera) taxa, and those exceedingly resistant to organic stress. An increase in haplotype number per OTU and haplotype diversity of sensitive taxa was observed with an increase in ecosystem quality and stability, while opposite pattern was detected for pollution resistant taxa. However, this pattern was less prominent than expected based on the strong differences in stressor intensity between sites. To compare genetic diversity among river systems, only OTUs could be used which were present in all systems. As OTU composition differed strongly between the rivers, this led to the exclusion of a high number of OTUs especially in diverse river systems of good quality, which then could have diminished the genetic diversity patterns. Altogether, this study shows that the extraction of haplotypes from DNA metabarcoding datasets is a promising tool to assess genetic diversity and investigate stressor impact at a large scale. However, to better understand responses of genetic diversity in i.e. river ecosystems to environmental stressors, it would be important to increase OTU overlap between sites of comparisons, i.e. by sampling a narrower stressor gradient.

Introduction

Degradation, pollution, and exploitation of freshwater ecosystems has resulted in a drastic decline of biodiversity (Vörösmarty et al., 2010; WWF, 2018). The magnitude of biodiversity loss depends on stressor intensities as well as on resistance and resilience of the biotic communities (Dobson et al., 2006; Elmqvist et al., 2003). So far, degradation and recovery processes have mostly been studied at the level of species diversity (alpha diversity). However, the underlying genetic diversity within species is an essential variable to consider in this context, as it showcases the effective population size and determines the evolutionary capacity of a species to adapt to changing environments. Genetic diversity in ecosystems is influenced by four major processes: mutation, drift, migration, and selection (Vellend and Geber, 2005). A high level of genetic variation is assumed to occur in intact and stable ecosystems, where effective population sizes are large and relatively constant over time. Under stressor impacts, genetic diversity declines ('genetic erosion hypothesis') primarily due to reduced population sizes resulting in enhanced genetic drift (Amos and Balmford, 2001; Reusch et al., 2005; Reynolds et al., 2012; Ribeiro and Lopes, 2013; van Straalen and Timmermans, 2002). Genetic diversity is the most basal level of biodiversity and typically the first to decrease under and the last to regenerate after stressor impact. It consequently provides a proxy for ecological processes long before or even if never visible on the species diversity level (Bazin et al., 2006; Guttman, 1994; Hughes et al., 2008; Reynolds et al., 2012; Vellend and Geber, 2005). In conservation biology, genetic diversity is therefore used as a measure for population and habitat stability. This information is typically neglected in the legally binding species assessment, or species diversity of a habitat is regarded as a proxy for genetic diversity (Vellend, 2005; Vellend and Geber, 2005). However, this concept has been rarely tested and has been questioned for example by Taberlet et al. (2012).

Nowadays, alpha diversity can be assessed with great resolution using DNA metabarcoding (Deiner et al., 2016; Hänfling et al., 2016; Macher et al., 2018). With these data, stressor impacts can be analysed simultaneously for many taxa not distinguishable by morphological determination methods (Bagley et al., 2019; Beermann et al., 2018; Pfrender et al., 2010; Theissinger et al., 2019). Typically, DNA metabarcoding infers responses at Operational Taxonomic Unit (OTU) level. In most cases, distance-based thresholds are used to define OTUs with the aim that these reflect as closely as possible biological species. While OTU classification can drastically improve the taxonomic and ecological resolution in comparison to classical morphological taxa assignment (Beermann et al., 2018; Macher et al., 2016; Sturmbauer et al., 1999), the level of intraspecific diversity still goes unnoticed, when distance-based clustering is used to analyse the sequencing data. As an alternative, bioinformatic denoising approaches can be used to obtain 'exact sequence variants' (ESVs) (Callahan et al., 2016; Frøslev et al., 2017). Here, additional bioinformatic filtering steps are used that allow to separate biological template sequences from noisy reads caused by PCR and sequencing mistakes, or tag-switching. With ESVs it is possible to explore intraspecific diversity patterns in eukaryotes (Elbrecht et al., 2018; Tsuji et al., 2019;

Turon et al., 2019). For animals, commonly the mitochondrial cytochrome c oxidase I gene (COI) is used for DNA barcoding and a shorter fragment also for metabarcoding (Hebert et al., 2003). While the use of mitochondrial markers has certain limitations for detailed population genetic analyses (Ballard and Whitlock, 2004; Leese and Held, 2011), its utility to infer insights into ecological processes acting at local or regional level has often been demonstrated (Pauls et al., 2006; Weiss and Leese, 2016; Witt and Hebert, 2000). Furthermore, extracting intraspecific variation data from metabarcoding datasets that typically operate only on species diversity is regarded as a promising tool to gain better understanding of ecosystem structure and stressor impacts to eventually construct better conservation policies than possible with species diversity data alone (Adams et al., 2019; Geist, 2011; Hughes et al., 2008; Reusch and Hughes, 2006; Reynolds et al., 2012).

In our study, we wanted to further evaluate the potential of analysing ESV data additionally to OTU data obtained from bulk sample metabarcoding datasets for analysing the impact of different stressor levels on macrozoobenthos (MZB) communities in river ecosystems. MZB organisms play a key role in freshwater ecosystem functionality and include a wide range of taxonomic groups with often narrow and specific demands with respect to habitat conditions (Jackson and Füreder, 2006; Usseglio-Polatera et al., 2000; Wallace and Webster, 1996). As river ecosystems, three German rivers systems with differing stressor levels were chosen: Emscher – high stress, Ennepe – moderate stress, and Sieg – low stress. The main branch of the Emscher has been used as an open sewage channel for the past hundred years and is now part of one of the biggest restoration projects in Europe. Sample sites in this stream were chosen to be in conditions with variable stressor inflow, ranging from completely restored sites, to canalized sites with purification plant inflow, and to sites in unrestored sewage channels. Heterogeneous conditions were also present at the Ennepe, and included near-natural sites, sites stressed through rainwater retention basins, which overflow at heavy rainfall, and sites with sewage treatment plant inflow. In comparison, the river Sieg is considered as a stable, near-natural system with a good ecological and chemical status. Punctual stressor inflow is present through rainwater retention basins, but was not present immediately at sampling sites.

By comparing communities between the different streams, we want to test, if species and genetic diversity is correlated with present stressor gradients. Following predictions from the ecological habitat concept, which links presence and abundance of species over time to the available resources (see e.g. Van Dyck, 2012), we expected that OTU diversity is decreasing from river Sieg to Ennepe and Emscher. After the genetic erosion hypothesis, we assumed that genetic diversity co-varies with OTU richness, expecting the highest haplotype number and diversity at the Sieg due to its long-time stable good ecological conditions supporting large and stable population sizes. Lower values were expected at the river Ennepe, where communities are regularly affected by organic stressor influx and thus recurrent population decline, resulting in higher genetic drift. The lowest genetic variability was expected at river

Emscher due to the complete erasure of MZB diversity in the history of this river system caused by the usage as sewage transport system, and the still prevalent stress level in many parts. Further, separating taxa into groups sensitive (EPT – Ephemeroptera, Plecoptera, Trichoptera) or resistant to organic pollution (PR – 'Pollution Resistant' – Arhynchobdellida, Enchytraeida, Haplotaxida, Isopoda, Rhynchobdellida), we assumed that OTU and haplotype diversity for EPT taxa will be distributed as described above for whole communities. In contrast, we expected that PR taxa will show opposing patterns, because they are resistant to organic stress and can even use particles as a source, which can lead to large population sizes and an associated positive effect on genetic diversity.

Material and Methods

Sampling

Macroinvertebrates were sampled at six sites in river Emscher and Sieg, respectively, in autumn 2016 & 2017, and spring 2017 & 2018 according to Water Framework Directive compliant sampling of the respective stream types (Meier et al. 2006). In short, kick-net sampling of different habitats with 20 subsamples in the Sieg, and 10 subsamples in the Emscher due to fewer available microhabitats, was executed. The seven sites at the Ennepe were sampled in autumn 2017 and spring 2017 & 2018 in a similar fashion with 10 subsamples. Subsamples were pooled, large parts of substrate discarded (e.g. stones, leafs, small branches), and samples, including Macrozoobenthos specimens and remaining substrate, were transferred to 1L bottles filled up with ethanol. Approximately 1/3 of bottle volume was filled with the sample and 2/3 with 96 % technical ethanol. If volume of sampled material was too large, it was divided into multiple bottles. Samples were transported to the laboratory, and old ethanol was changed with new 96 % technical ethanol on the same day.

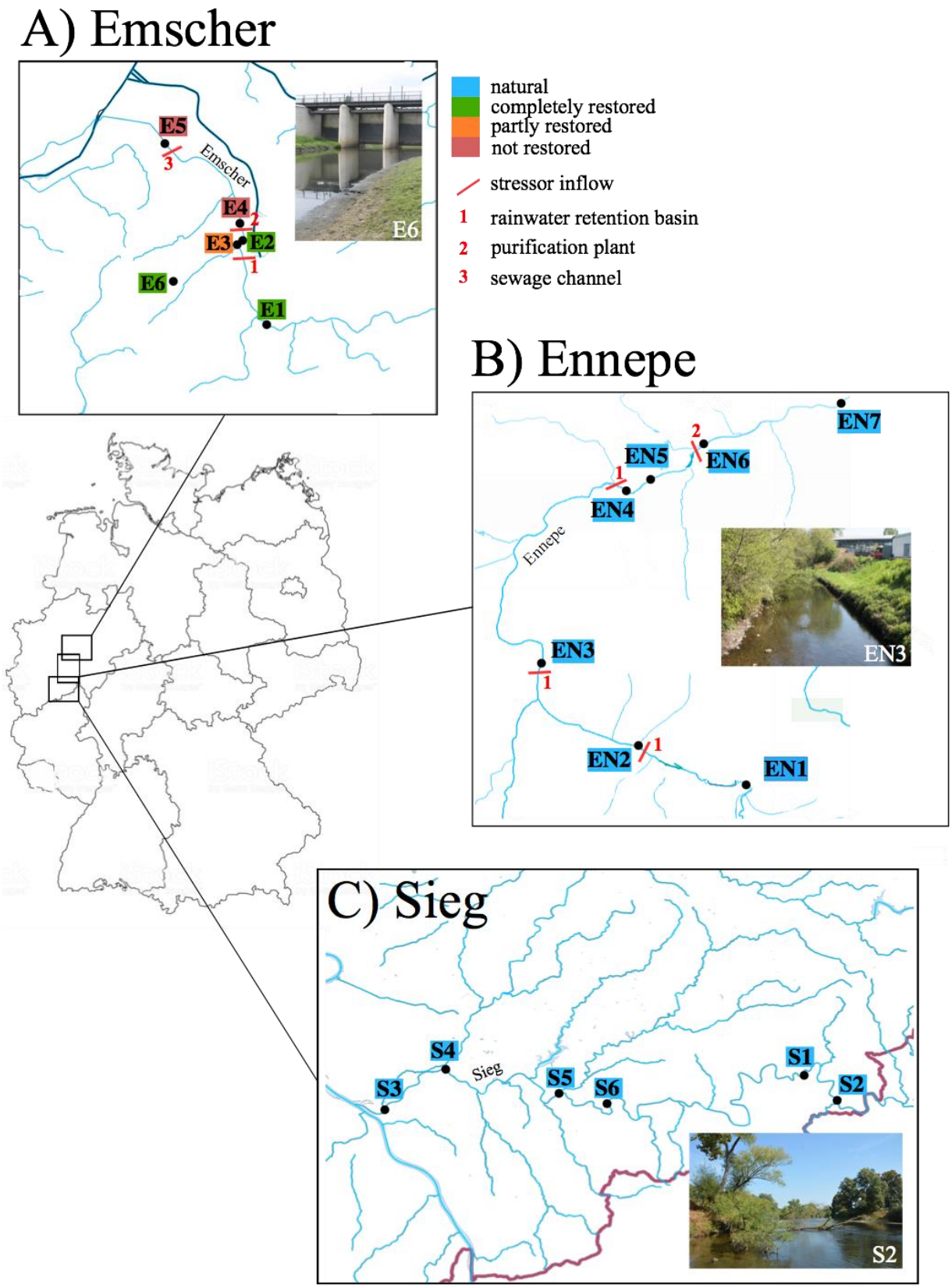

***Figure 1**: Sample sites at river A) Emscher, B) Ennepe and C) Sieg.*

Laboratory Protocols

Samples were examined under a binocular (Leica S6E) to separate individuals from substrate. Substrate was discarded and individuals were counted and separated in two size categories (size class A: ≤ 25 mm, size class B: ≥ 25 mm) (see Elbrecht et al. 2017b for the principle of the procedure). Separated size classes were dried in petri dishes overnight and homogenized to fine powder with an IKA Ultra Turrax Tube Disperser (BMT-20-S-M sterile tubes, full speed for 30 min). Two times half of a spatula (0.02 g) was transferred into two Eppendorf tubes and 600 µl TNES buffer and 15 µl Proteinase K (10 mg/ml) were added per tube and incubated over night at 36 °C shaking at 250 rpm in a Thermoshaker (ThermoMixer C, Eppendorf). A salt extraction protocol (after Sunnuks and Hales 1996, adjusted as in Weiss and Leese, 2016) was used to isolate DNA from powder. After DNA extraction and subsequent RNA digestion (1 µl RNAse per sample, heated at 37 °C for 30 min), samples were cleaned up (NucleoSpin gel and PCR clean up kit, Macherey-Nagel), and size groups per sample were pooled according to individual numbers per group. DNA was quantified with a Qubit Fluorometer (ds DNA BR Assay kit, Thermo Fisher Scientific, Beverly, USA) and adjusted to 25 ng/µl. A two-step PCR (for futher information see Zizka et al., 2019) was conducted with one technical (PCR) replicate per sample. The universal BF2/BR2 primers were used (Elbrecht and Leese, 2017). PCR reactions included 1× PCR buffer (including 2.5 mM Mg^{2+}), 0.2 mM dNTPs, 0.5 µM of each primer, 0.025 U/L of HotMaster Taq (5 Prime, Gaithersburg, MD, USA), and 1 µL DNA template filled up with HPLC H_2O to a total volume of 50 µL, and was processed with the following PCR protocol: 94 °C for 180 s; 25 cycles of 94 °C for 30 s, 50 °C for 30 s, and 65 °C for 150 s; followed by a final elongation of 65 °C for 5 min in a Thermocycler (Biometra TAdvanced Thermocycler). A left-sided size selection was conducted per sample with magnetic SpriSelect beads (Beckman Coulter, Krefeld, Germany), using a ratio of 0.76x to remove small fragments (primers, primer dimers). DNA concentration after PCR was measured on a Fragment Analyzer (Advanced Analytical, Ankery, USA) and samples were pooled equimolarly. Library pools were sent for paired-end sequencing to Eurofins GATC Biotech GmbH (Constance, Germany) using four Illumina MiSeq runs with a 250 bp paired-end v2 kit, one for each sampling season.

Data Analysis

Delivered sequences were analysed with JAMP (https://github.com/VascoElbrecht/JAMP) including demultiplexing of data, paired-end-merging and primer trimming following standard settings. For haplotype extraction only reads of excepted fragments length (421 bp) were. A strict quality filtering was applied (maximal expected error *max_ee = 0.3*) and reads with an abundance < 0.003 % in at least one sample were discarded. The algorithm Unoise3 (Edgar, 2016) implemented in JAMP, was used to denoise the dataset (*alpha = 5*) and to separate common haplotypes from chimeras and sequencing noise. The denoising approach is based on the assumption, that high abundant unique reads (centroids) are real sequences amplified from biological template. Defined by distance (d), other unique sequences (neighbours) are grouped around these high abundant sequences. Based on the Levensthein distance and abundance (defined by *alpha*), neighbours showing a small difference and abundance compared to the

centroid are predicted to be erroneous. Denoised reads were clustered into OTUs by 3 % distance and all sequences per sample were mapped against the completed OTU list. As a further filtering step, OTUs with an abundance below 0.01 % (*OTUmin = 0.01*) in at least one sample and haplotypes with an abundance below 0.003 % (*minhaplosize = 0.003*) in at least one sample were discarded. This step is included, to filter also low abundant unique sequences, which are not integrated in the filtering through *alpha*. Taxonomic assignment of haplotypes was conducted through a comparison with the database BOLD (Ratnasingham and Hebert, 2007) using an in-house python script. Haplotypes with similarity < 95 % to a deposited sequence in the database were excluded from further analysis to prevent incorrect assignments potentially leading to the assessment of erroneous diversity patterns. Read numbers per haplotype of technical PCR replicates were fused and the average was calculated. Further analyses were carried out with the average read number per haplotype.

To assess haplotype richness per OTU, we used count data. However, in order to approximate also traditional population genetic measures, we calculated haplotype and nucleotide diversity per sample side and season with the software Arlequin 3.5 (Excoffier and Lischer, 2010) using read depths as proxy for haplotype abundance. Data were not normally distributed, and therefore the non-parametric Kruskal-Wallis test was used to check for effects of river system on diversity variables. A post-hoc Dunn test (package dunn.test()), Dinno, 2017) was used to conduct pairwise comparisons for significant differences. All statistical analysis were conducted in R (R Development Core Team, 2008). Table modification and figure preparation was done using the packages vegan (Oksanen et al., 2019), tidyverse (Wickham et al., 2019) and ggplot2 (Wickham, 2016) implemented in R.

Results

Tab. 1: Average number of individuals per sample site (Emscher, Sieg: autumn 2016/2017, spring 2017/2018, Ennepe: spring 2017/2018, autumn 2017). For detailed information see table S1.

Emscher	E1	507 ± 205	Sieg	S1	2434 ± 530	Ennepe	En1	606 ± 269
	E2	1078 ± 1010		S2	746 ± 386		En2	1011 ± 770
	E3	670 ± 320		S3	1877 ± 1369		En3	571 ± 209
	E4	5454 ± 8930		S4	2186 ± 2842		En4	1099 ± 371
	E5	533 ± 274		S5	2666 ± 1202		En5	1802 ±1242
	E6	680 ± 234		S6	2090 ± 934		En6	476 ± 277
							En7	1178 ± 487

The number of individuals differed between all river systems ($p < 0.001$) and seasons ($p < 0.05$) (Table 1). Within streams, individual numbers did not significantly differ between sampling sites, while an effect of sample site was observed comparing all three rivers $p < 0.05$. Emscher samples included on average 735 ± 539 individuals, while 988 ± 686 and 1992 ± 1332 individuals were counted for river Ennepe and Sieg. However, no correlation was detected between total individual number per site and season, and average haplotype number per OTU (Fig. S1). Samples contained 228 – 694 haplotypes

after denoising (Emscher: 348 ± 84, Ennepe: 500 ± 87, Sieg: 569 ± 73) which clustered into 70 – 155 OTUs (Operational Taxonomic Units, Em: 87 ± 9, En: 122 ± 6, S: 134 ± 13). OTU and haplotype numbers were higher at river Ennepe and Sieg than at river Emscher ($p < 0.01$, Fig 2). Splitting the dataset in EPT (Ephemeroptera, Plecoptera, Trichoptera) and PR ('Pollution Resistant') taxa revealed 13 - 273 haplotypes (Em: 22 ± 17, En: 62 ± 53, S: 60 ± 49) clustered into 2 – 46 OTUs (zeros excluded e.g. E5, Em: 5 ± 3, En: 12 ± 7, S: 11 ± 7) for EPT taxa, and 8 – 257 haplotypes (Em: 26 ± 42, En: 7 ± 12, S: 12 ± 20) clustered into 4 - 55 OTUs (Em: 7 ± 9, En: 3 ± 4, S: 4 ± 5) for PR taxa. No plecopterans were found at river Emscher throughout the whole analysis. Total OTU and haplotype numbers of EPT taxa were affected by the river system with more OTUs and haplotypes at Ennepe and Sieg than at Emscher ($p < 0.001$). Sample site E4 and E5 showed remarkably high OTU and haplotype numbers assigned to PR taxa compared to all other sample sites. However, no effect of the river system was detected on PR taxa (haplotype number $p = 0.09$) (Fig. 2).

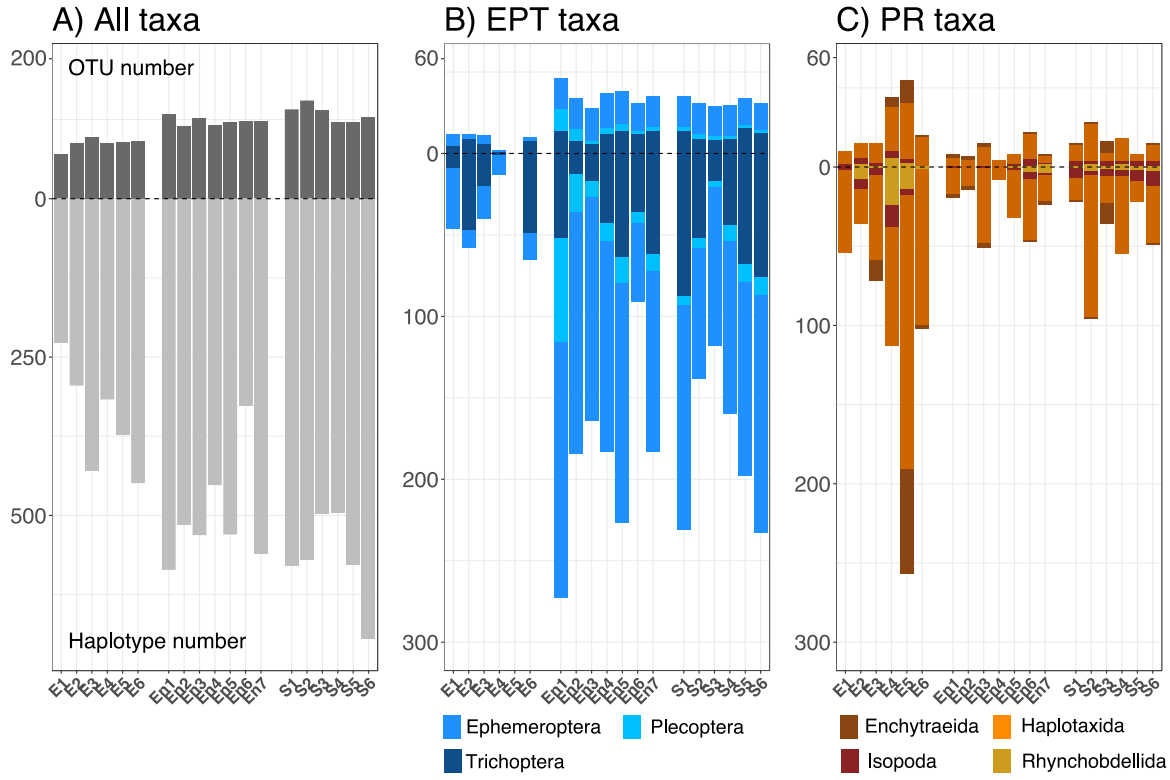

Fig. 2: *Total OTU (upper part) and haplotype number (lower part) summed up over all seasons for all river systems (Em 1-6 – Emscher, En 1-7 – Ennepe 1-6, S - Sieg). A) All benthic macroinvertebrate taxa assigned to a reference in BOLD with > 95 %; B) EPT taxa (Ephemeroptera, Plecoptera, Trichoptera); C) PR taxa (Pollution Resistant → Enchytraeida, Haplotaxida, Isopoda, Rhynchobdellida).*

To compare average haplotype number per OTU and haplotype diversity between river systems we searched for OTUs present in most samples. The five most common OTUs, all occurring at more than 50% of the analysed samples, were: OTU2 (*Baetis rhodani*, 63 %), OTU9 (*Asellus aquaticus*, 52 %), OTU12 (*Stylodrilus heringianus*, 63 %), OTU65 (*Esolus parallelepipedus*, 55 %), OTU107 (*Microtendipes pedellus*, 52 %). To increase overlap of OTUs between sites further, samples collected

at different seasons were merged (E1-E6, En1-En7, S1-S6). By this, we found six OTUs occurring in more than 80 % of all sites (OTU2: *Baetis rhodani*, 84 %; OTU5: *Orthocladius*, 89 %, OTU9: *Asellus aquaticus*, 84 %; OTU45: *Orthocladius*, 84 %; OTU67: *Tanytarsus eminulus*, 84 %, OTU107: *Microtendipes pedellus*, 95 %). To increase number of OTUs for analysis, all OTUs present in at least one of the samples per river system were included for further analysis, resulting in four different data sets (Em-En-S: 78 shared OTUs, Em-En: 110 shared OTUs, Em-S: 125 shared OTUs, En-S: 155 shared OTUs). Per dataset > 47 % of shared OTUs were assigned to dipterans, of which the majority (> 90 % were chironomids (Figure S2). Comparisons of average haplotype number per OTU and haplotype diversity revealed no differences between river systems for all four datasets when all taxa were included (Fig. 3). Dividing the datasets into OTUs assigned to EPT (Ephemeroptera, Plecoptera, Trichoptera, pollution sensitive) and PR (pollution resistant) taxa, revealed a significant effect of the river system on the average haplotype number per shared OTU for both groups (EPT: $p < 0.05$, PR: $p < 0.05$) (Fig. 3). Ennepe and Sieg showed a higher average haplotype number per OTU for EPT taxa than the Emscher (En: 4; S: 3.3; Em: 2.7, $p < 0.05$), while the ratio for PR taxa was higher at Emscher (3.1) than at the other two rivers (En: 2.7, S: 2.6). When comparing only shared PR OTUs between Emscher and Sieg, more haplotypes per OTU were found at the Emscher (Em: 3.2; S: 2.4, $p < 0.05$). Similar patterns were observed for haplotype diversity (Fig. 3, Fig. S5). Comparing all river systems, a higher haplotype diversity was found at the Ennepe (0.4513) in comparison with the Emscher (0.2857) for EPT taxa ($p < 0.05$) but not in comparison with the Sieg (0.3784), while PR taxa showed a higher haplotype diversity at the Emscher in comparison to both other streams (Em: 0.374, En: 0.303, S: 0.249). When comparing shared OTUs only between Emscher and Sieg, a significantly higher haplotype diversity of PR taxa was observed at the Emscher (Em: 0.3338; S: 0.1934, $p < 0.05$) (Fig. 3). Detailed information of average haplotype number per OTU and haplotype diversity per sample site are illustrated in figure S3 (average haplotype number per OTU) and S4 (nucleotide diversity).

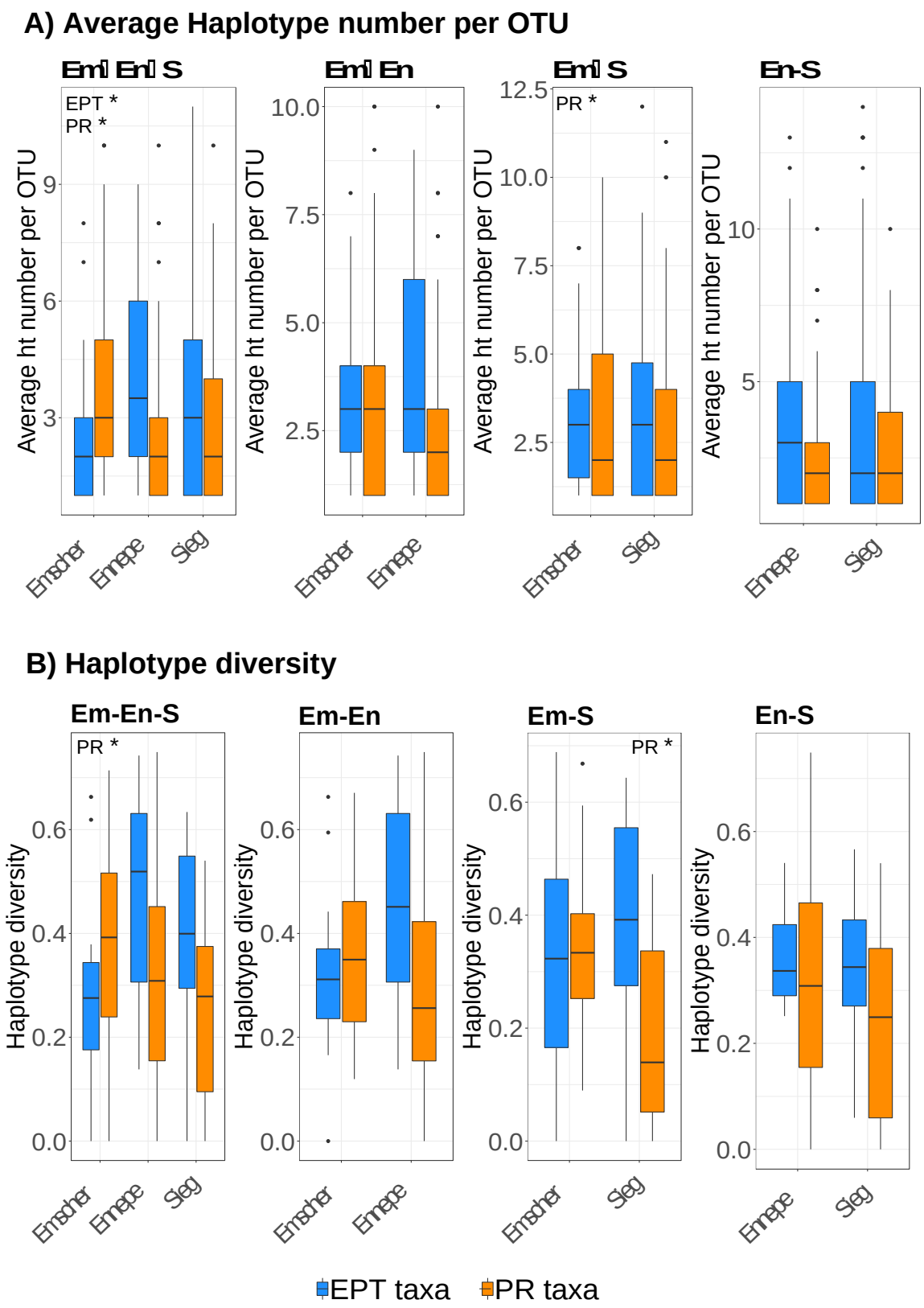

Fig. 3: *A) Average haplotype number per OTU for the four datasets of shared OTUs. B) Haplotype diversity for the four datasets of shared OTUs. EPT and PR taxa are shown.*

Further, the comparison of shared OTUs between the three river systems revealed an effect of river system on nucleotide diversity for EPT taxa ($p < 0.05$). Average nucleotide diversity of taxa was higher at river Ennepe (0.00215) and Sieg (0.00266) than at the Emscher (0.00104). In contrast, PR taxa showed

a higher nucleotide diversity at the Emscher (0.00143) than at the Ennepe (0.00215), but only when comparing OTUs shared between those two rivers ($p < 0.05$, Fig. S6).

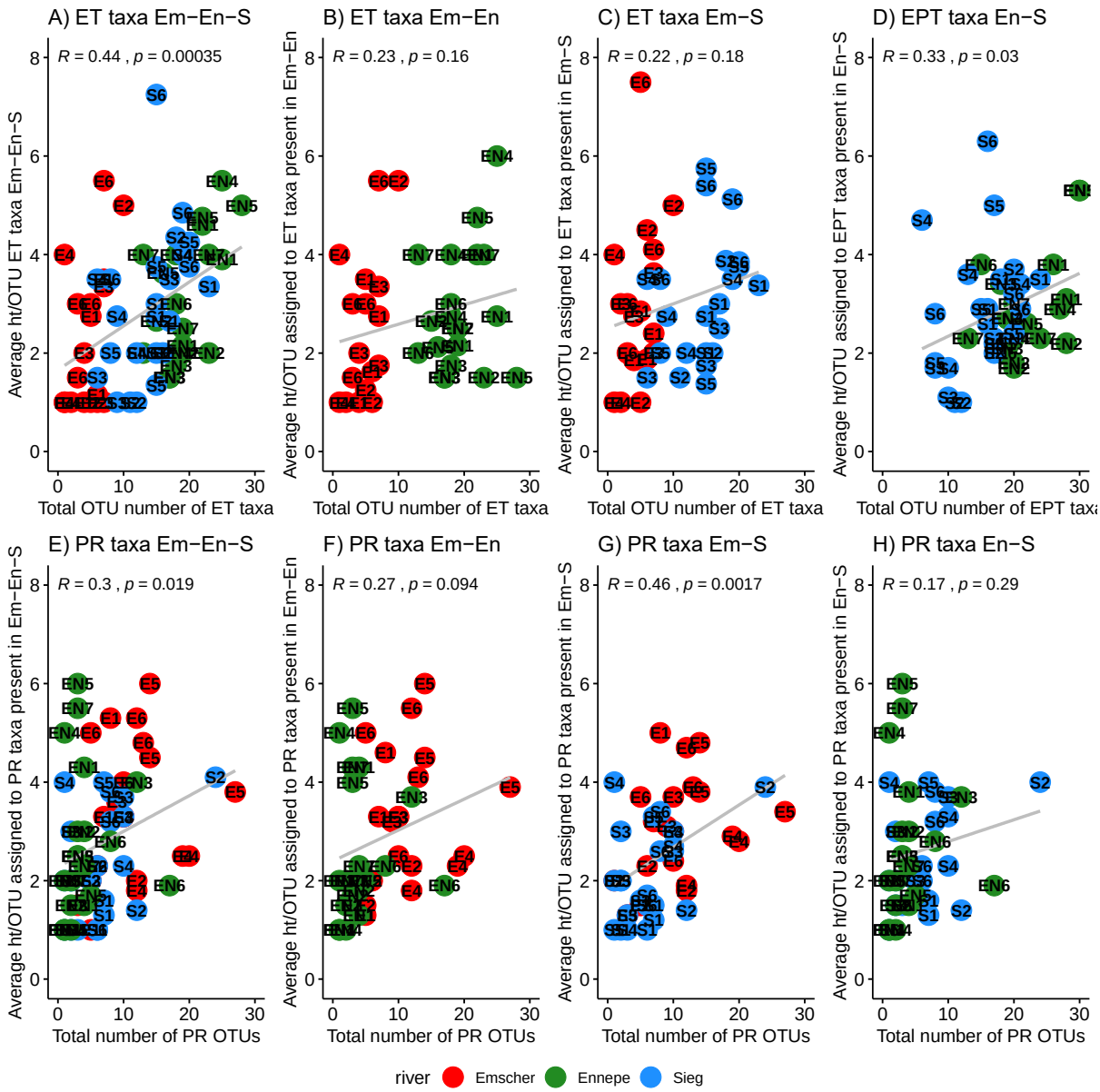

Fig. 4: *Correlation analysis of total number of OTUs assigned to EPT/PR taxa and average haplotype number per OTU for those groups. A-D four datasets of shared OTUs for EPT taxa. E-H four datasets of shared OTUs for PR taxa.*

We plotted total OTU number assigned to EPT and PR taxa against the average haplotype number per OTU and sample site for the four datasets of shared OTUs (Fig. 4 A-H) to test if OTU and genetic diversity are linked and indirectly, if ecosystem quality effects genetic variability. A distinct signal was only detected for OTUs assigned to EPT taxa shared between all three river systems. Figure 4A shows a clear increase in total OTU number from Emscher to Sieg and Ennepe and a significant correlation between OTU number and haplotype number/OTU. A significant correlation was also observed for EPT OTUs shared between Sieg and Ennepe, but here, no clear distinction in total number of EPT taxa was

visible between rivers. A significant correlations for the dataset including all river systems was observed for PR taxa (Fig. 4E) as well as in the dataset comparing Emscher and Sieg (Fig. 4G).

Discussion

OTU and haplotype number

Assuming a correlation between species (OTU) and genetic (haplotype) diversity, we expected an effect of stressor intensity on both diversity levels at the three river systems. The highest diversity was expected at river Sieg due to long time stable and ecological conditions. Due to intermediate stressor inflow at river Ennepe we expected moderate diversity pattern at this system and the lowest species and genetic diversity at the highly stressed river Emscher. In line with those expectations river Emscher showed the lowest OTU number compared with the other two systems. However, no distinct differences in OTU number between Ennepe and Sieg were detected and an even higher number of highly sensitive stoneflies (Plecoptera) was assessed at the Ennepe. We therefore conclude, that intermediate stressor inflow at Ennepe sample sites had no or only a small effect on MZB communities. As expected, haplotype number per sample site revealed a similar pattern with the lowest haplotype number at river Emscher, but no distinct differences between the other two river systems. The separation of OTUs into EPT and PR taxa supported those findings. Results based on EPT taxa were similar to results based on the whole taxa number (lowest at Emscher, no differences between the other to rivers), while PR taxa showed an opposed pattern which was however, less clear than expected and not significant.

Genetic diversity

Genetic variability, defined here by the average haplotype number per OTU and haplotype diversity, only partly met previous assumptions, because a less clear distinction between river systems according to stress level was detected than on OTU level. Besides biological reasons, the lack of a clear correlation could have been influenced by methodical issues, as the results could have been weakened by the underlying datasets that included only OTUs present in at least one sample site of all compared river systems. Analyses were therefore based on subsamples of actual diversity per river system, which excludes genetic variability primarily to the disadvantage of highly diverse systems (Sieg, Ennepe). Variable stressor levels and time since restoration at Emscher sample sites lead to high variations in OTU composition already within the river, which made comparisons of genetic variability based on high frequent OTUs difficult. We decided to base comparisons on those subsamples to circumvent differences in genetic variability due to species specific variations.

However, results of shared OTUs between all three rivers showed a higher genetic variability for sensitive EPT taxa at the Ennepe and the Sieg compared to the Emscher, while an opposing pattern was observed for PR taxa. This is congruent for the average number of haplotypes per OTU, haplotype diversity and nucleotide diversity. Results support the hypothesis, that sustaining good ecological

conditions and stability at river Sieg induce stable and large population size, favouring an increase in genetic diversity in sensitive MZB communities (Reynolds et al., 2012; Ribeiro and Lopes, 2013). The opposite is shown for pollution resistant (PR) taxa, which are able to handle a high level of organic? stress or even use it as a source. Prevailing conditions at stress intensive sample sites at the Emscher (mainly E4 and E5) favour population stabilisation and growth within these taxa with a low competition pressure, due to the absence of less resistant taxa (Gaufin and Tarzwell, 1952; Gray et al., 1979). Here, the significant difference of genetic diversity between Sieg and Emscher, supports the expectation, that the Sieg prevails with the lowest amount of organic stress suppressing high population sizes of PR taxa. Results further support the assumption, that stressor impact at the Ennepe does not affect the sensitive community composition of MZB taxa and according genetic variability since the highest average haplotype diversity was found at this river system for the three data sets. Additionally, results underline the relation between total number of EPT taxa and average haplotype diversity per OTU, which is strongly correlated for the Em-En-S dataset emphasising, that species (OTU) diversity and genetic variability are linked (Vellend and Geber, 2005).

Methodology

As outlined in previous studies (Elbrecht et al., 2018; Tsuji et al., 2019; Turon et al., 2019), the main challenge in extracting haplotypes from metabarcoding datasets is the separation of 'real' environmental sequences from those produced by PCR or sequencing errors. New programs enable the denoising of datasets with the optimisation of filtering steps, to efficiently separate real sequences from erroneous ones. However, a decision has to be made concerning the strictness of filtering. By using high filtering thresholds erroneous sequences are excluded with a higher probability, but at the same time it is more likely to exclude real sequences of low abundance. In comparison, a lower filtering increases the number of rare real sequences, but also includes a higher number of erroneous sequences into diversity analysis. For the present study we followed denoising as recommended in Elbrecht et al., 2018. The study investigated the genetic variability of benthic macroinvertebrates in Finland streams, implementing an α-value of 5, which was also applied in Turon et al., 2019. The additional percentual abundance threshold filtering for OTUs and haplotypes was set after suggestions in Elbrecht et al., 2018 and applied in Laini et al., in review. We found a high number of unique haplotypes per sample site (exemplary networks of the two most frequently found EPT and PR taxa in S7), which is similar to other metabarcoding studies (Elbrecht et al., 2018; Laini et al., in review), but exceeds those found in studies based on single specimen barcoding (Lucentini et al., 2011; Weiss and Leese, 2016; Williams et al., 2006). Higher numbers of unique haplotypes could be induced into the dataset through sequencing errors, which would emphasize the application of an even higher filtering threshold on metabarcoding datasets. However, the increased number of haplotypes found through metabarcoding can also be real haplotype variants in one specimen which cannot be determined through single specimen barcoding due to the underlying sequencing method (Phillips et al., 2019).

Conclusion

The potential of haplotype extraction from metabarcoding datasets to assess effects on genetic variability was shown in the present study using the example of three German river systems. Even if the choice of data filtering thresholds is still a trade-off between rare 'real' sequences and erroneous, artificially generated ones, we were able to link stressor effects to genetic variability of aquatic macroinvertebrate communities. Sites with good and stable ecological conditions showed higher genetic diversity than stressed sites, which is coupled with OTU diversity. However, due to a low OTU overlap between river systems, genetic diversity analyses were based only on subsets, including all shared OTUs. This subsampling induces the exclusion of variability and ecological specialists, especially at highly diverse sample sites and might skew actual differences. Further analysis of stressor level or restoration events on genetic diversity should include several replicates of similar conditions and presuppose a threshold of overlapping OTUs in compared river systems.

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Chapter 3

Beyond DNA metabarcoding

A simple centrifugation protocol for metagenomic studies increases mitochondrial DNA yield by two orders of magnitude

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APPLICATION

Methods in Ecology and Evolution BRITISH ECOLOGICAL SOCIETY

A simple centrifugation protocol for metagenomic studies increases mitochondrial DNA yield by two orders of magnitude

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Abstract

1. DNA (meta)barcoding is applied to study biodiversity and is available for standardized biodiversity assessments. However, it suffers from PCR bias, which can lead to the loss of specific taxa. PCR-free techniques such as shotgun metagenomics are therefore thought to be more suited for biodiversity assessments, but are currently limited by incomplete reference libraries.
2. The technique of "mitogenome-skimming" or "mitogenomics," in which complete mitochondrial genomes are sequenced, is ideal to bridge the techniques of (meta) barcoding and metagenomics. However, without the enrichment of mitochondria, roughly 99% of all sequencing reads are of non-mitochondrial origin and mostly useless for common applications, e.g. species identification.
3. Here, we present a simple centrifugation protocol that leads to an average 140-fold enrichment of mitochondrial DNA. By sequencing six 'mock'-communities—comprising the freshwater taxa *Corbicula fluminea*, *Gammarus roeselii* and *Hydropsyche exocellata* each—we recovered whole mitochondrial genomes of these species and the acanthocephalan endoparasite *Pomphorhynchus laevis*.
4. The enrichment protocol will speed up building reference libraries for whole mitochondrial genomes, as dozens of species can be sequenced on a single MiSeq run. Subsequently, it will also allow biodiversity assessments, using mitogenomics at greatly reduced costs in comparison to mitogenomic approaches without prior enrichment for mitochondria.

KEYWORDS
Amphipoda, cost-effective, freshwater mock community, genome skimming, mitogenomics, PCR-free, Trichoptera, Veneroida

1 | INTRODUCTION

Biodiversity is highly important for intact ecosystems and inevitable for human well-being (Rockström et al., 2009). Molecular techniques such as DNA barcoding (Hebert, Ratnasingham, & deWaard, 2003) and metabarcoding (Hajibabaei, Shokralla, Zhou, Singer, & Baird, 2011) are increasingly used to study biodiversity, but suffer from PCR stochasticity and primer bias (Elbrecht & Leese, 2015). The same bias can be introduced by the use of baits or probes (e.g. Liu et al., 2015; Mayer et al., 2016). Therefore, PCR and primer/probe-free techniques harbour the potential for future biodiversity assessments (Coissac, Hollingsworth, Lavergne, & Taberlet, 2016; Crampton-Platt, Yu, Zhou, & Vogler, 2016; Elbrecht & Leese, 2015; Tang et al., 2014; Zhou et al., 2013) by circumventing taxon-dependent PCR amplification biases and offering the possibility to correlate read numbers with biomasses. Since reference libraries are still largely incomplete

for nuclear genomic information, but relatively comprehensive for mitochondrial genes—such as the cytochrome c oxidase subunit I (COI) gene for animals– the consequent step towards a PCR-free analysis of biodiversity samples could be seen in "mitochondrial metagenomics," "mitogenomics" or "mitogenome-skimming" (e.g. Crampton-Platt et al., 2015; Tang et al., 2014). This technique enables the comparison of newly generated mitogenomes or mitogenome fragments with reference databases and thereby links genomic information to taxonomic knowledge. However, the currently applied approaches are relatively ineffective in terms of sequencing capacity, with most "PCR-free" mitogenomic libraries comprising less than 1% sequences of mitochondrial origin (Crampton-Platt et al., 2016). The major methodological disadvantages thus are the great sequencing depth needed, and the associated high costs.

A potential solution is the enrichment of mitochondria prior to DNA extraction and library sequencing, shifting the initial ratio of mitochondrial versus nuclear DNA towards a higher mitochondrial DNA proportion. It is known that ultracentrifugation in CsCl-gradients can enrich for the typically AT-rich mitochondrial genomes (e.g. Garber & Yoder, 1983). However, this approach grounds on cost- and labour-intensive ultracentrifugation and mitochondria can have highly variable AT-contents, rendering extractions from bulk biodiversity samples in CsCl-gradients less straightforward. The enrichment of mitochondria is most promising when organelles are intact, i.e. when living tissue is used (Tamura & Aotsuka, 1988). Until now, this approach has not been tested for environmental bulk samples, mainly because most specimens used for biodiversity assessments and genome sequencing are commonly stored in preservation fluids, which damage or destroy mitochondria. Here, we use a "mock"- community of three freshwater species to test a simple centrifugation protocol for mitochondrial enrichment in a metagenomic context. We demonstrate that our protocol strongly enriches mitochondrial DNA and therefore can greatly reduce costs of future mitogenomic approaches, e.g. when (1) constructing mitochondrial reference libraries and (2) assessing biodiversity by an approach which omits biases introduced by primers, probes and PCR reactions. The approach also confirms previous work (Choo, Crampton-Platt, & Vogler, 2017; Tang et al., 2014), showing that mitochondria can reliably extracted from bulk samples, making the process feasible for standard biodiversity assessments.

2 | MATERIALS AND METHODS

2.1 | Sampling and laboratory protocols

Sampling was conducted at two locations (51°00′52.6″N 6°41′04.5″E; 51°05′23.4″N 6°41′17.0″E) of the Gillbach (Germany) in December 2016. Twenty individuals of each of the three macrozoobenthic freshwater species *Corbicula fluminea*, *Gammarus roeselii* and *Hydropsyche exocellata* were sampled with a dip net or collected from stones. Specimens were transferred into water (500 ml) and transported to the laboratory for immediate processing. Specimens were weighed (Mettler Toledo XS105, Table S1) and assembled to six 'mock'-communities, each containing three individuals of *G. roeselii* and *H. exocellata* and a single *C. fluminea* specimen. 'Mock'-communities were separately transferred into 3 ml 5°C cold homogenization buffer (0.25 M sucrose, 10 mM EDTA, 30 mM Tris-HCl, pH 7.5; Tamura & Aotsuka, 1988) in a mortar and crushed with a pestle until tissue was homogenized (70 strokes each). Two millilitres of homogenate was pipetted into a 2 ml Eppendorf tube and samples were treated after the following centrifugation protocols (4°C, centrifuge Eppendorf 5427 R) (see Supporting Information 1 for short protocol).

1. Samples 1–3 ("Complete"-no enrichment of mitochondria): samples were centrifuged for 1 min at 1,000 g. This step was repeated four times. Final centrifugation was conducted for 10 minutes at 14,000 g. Supernatant was discarded and 600 µl TNES (50 mM Tris Base, 400 mM NaCl, 20 mM EDTA, 0.5% SDS) buffer was added to the pelleted material. Samples were then homogenized by vortexing.
2. Samples 4–6 ("Mito"-enrichment of mitochondria): samples were centrifuged for 1 minute at 1,000 g. Pelletized material was discarded, the supernatant transferred to a new tube and again centrifuged for 1 minute at 1,000 g. This step was repeated three times. Final centrifugation was conducted for 10 minutes at 14,000 g. The supernatant was discarded, 600 µl TNES buffer was added to the pelleted material and samples were homogenized by vortexing.

A total volume of 40 µl Proteinase K (300 U/ml, 7Bioscience, Hartheim, Germany) was added to each sample, which were than vortexed and incubated at 37°C for 12 hours (Eppendorf Thermomixer C). DNA was extracted using a salt precipitation protocol as described in Weiss and Leese (2016). For RNA digestion, 1.5 µl RNAse (1.5 µg, Thermo Fisher Scientific, Oberhausen, Germany) was added to each reaction and incubated at 34°C for 30 minutes on a Thermomixer, followed by a clean up using the MinElute Reaction CleanUp Kit (Qiagen, Hilden, Germany). For DNA fragmentation, samples were placed in an ultrasonic bath (Bandelin SONOREX, RK 510 Hz) for 8 hours. Library preparation was performed with a TruSeq Nano DNA LT Library Prep Kit [Set A, step (2) "Repair Ends and Select Library Size" – (5) "Enrich DNA Fragments"]. After each step, fragment lengths and concentrations were quantified on a Fragment Analyzer (Advanced Analytical, Automated CE Systems). Samples were equimolar pooled and sent for sequencing on a MiSeq sequencer (v2 chemistry, 250 bp paired-end) at GATC-Biotech (Konstanz, Germany).

2.2 | Sequence analysis

Raw sequences were checked for remaining adapters and trimmed with BBDuk as implemented in Geneious v.10.0.9 (Kearse et al., 2012). The complete mitochondrial genomes of *C. fluminea*, *G. roeselii*, *H. exocellata* and *Pomphorhynchus laevis* (an acanthocephalan endoparasite) were assembled using MIRA 4.0.2 (Chevreux, 2014) as implemented in Geneious (Settings: Genome, accurate). Contigs were elongated by mapping reads against contigs with the Geneious mapper (Settings: no gaps allowed, 3% maximum mismatch per read, word length 40).

Annotations were performed with the MITOS (Bernt et al., 2013) web server and corrected manually.

Analyses of mitochondrial enrichment were conducted after strict quality filtering of raw reads, i.e. discarding reads with bases with a Phred Score <30 (-fastq_truncqual 30) and a length <200 with usearch (Edgar 2010, v9.0.2132_i86linux32). This very strict quality filtering approach was used to get the most reliable data possible. From each sample (Mito 1–3, Complete 1–3), 100,000 reads were randomly selected with usearch and mapped against the assembled reference genomes using Bowtie (settings: Seed length 100, min insert size 200, max insert size 251, max mismatches 3, best match only). Random selection of 100,000 and mapping were repeated five times. This was done to allow for a standardized comparison to test for variation within samples (See Supplementary Tables S4, S5, S8 and S9). After mapping, control regions were manually excluded and the number of remaining mapped sequences was determined. Enrichment factors were calculated for each species by comparing weight-corrected read numbers in the three enriched samples (Mito 1–3) to read numbers in the non-enriched samples (Complete 1–3) for all combinations (3 × 3). Total mitochondrial sequence enrichment was estimated by averaging enrichment factors across all combinations and species (3 × 3 × 3).

3 | RESULTS

A total of 7,707,640 reads were obtained, with 1,191,468 reads for samples "Complete 1–3" and 6,516,172 reads for samples "Mito 1–3." Samples enriched for mitochondria (Mito 1–3) were sequenced with 2.17 million reads on average, while the samples not enriched for mitochondria (Complete 1–3) were sequenced with 0.4 million reads on average. After strict quality filtering with usearch 483,778 (Complete 1–3) respectively 2,305,181 (Mito 1–3) reads were retained. On average, 40.17% of reads were retained for the unenriched samples, and an average of 35.14% of reads were retained for enriched samples (raw reads, quality filtered reads and percentage of retained reads after quality filtering per sample: Supporting Information 2, Table S2, Figure S1). These were assembled to the complete mitochondrial genomes of *C. fluminea* (17,575 bp with and 15,660 bp without control region), *G. roeselii* (14,906; 13,927), *H. exocellata* (15,789; 14,958) and *P. laevis* (13,886; 13,422) (Supporting information 2: Table S3, Mitogenomes: Supporting information 3). Mapping of five subsets of 100,000 high quality reads each against reference genomes for which control regions were removed after mapping showed that samples not enriched for mitochondria (Complete 1–3) contained on average 0.16% mitochondrial reads. In comparison, samples enriched for mitochondria comprised 9.47% mitochondrial reads on average (Supporting Information 2: Tables S4, S5). Variation within samples was found to be minimal. Species-specific enrichment factors (corrected for the relative weight per species in each sample, Supporting Information 2: Table S6) were 88.1 (*SD*: 56.6) for *G. roeselii*, 163.3 (*SD*: 141.6) for *H. exocellata* and 168.8 (*SD*: 121.4) for *C. fluminea*. Overall enrichment for the whole mock communities was 140.1 (*SD*: 114.4) (Figure 1, Supporting Information 2: Table S7). Further relaxing the quality filtering to retain more reads did not significantly impact the differences in enrichment between treatments.

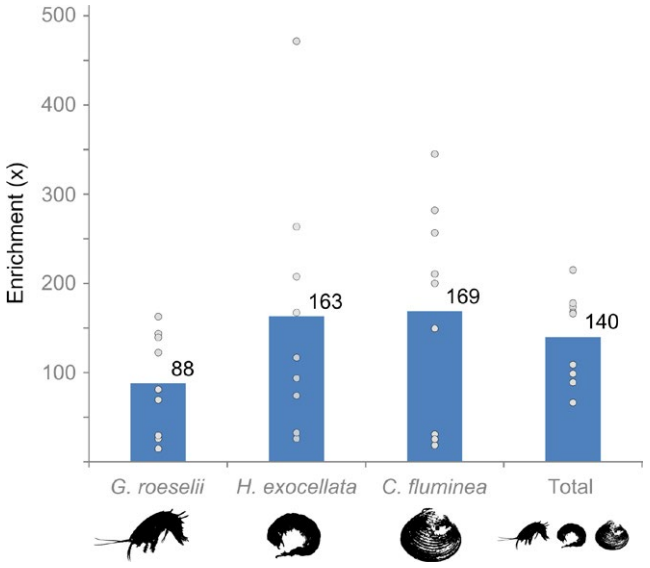

FIGURE 1 Mitochondrial DNA enrichment factors for the studied species and the complete mock community. Grey circles represent the enrichment factors calculated for every combination of non-enriched (Comp 1–3) and enriched (Mito 1–3) samples

Mapping against whole mitochondrial genomes including control regions showed a 129.1-fold (*SD*: 92.6) enrichment of mitochondrial reads (Supporting Information 2: Tables S8-S10). Mapping of all 2,305,181 high quality reads from samples Mito 1–3 against mitogenomes without control regions showed that *G. roeselii* was sequenced with a mean coverage of 1028.8 ± 182.1, *H. exocellata* with 1109.9 ± 371.7, *C. fluminea* with 1114.4 ± 283.5 and *P. laevis* with a coverage of 132.7 ± 46.1. The full *P. laevis* mitogenome could only be recovered from samples enriched for mitochondria (Coverage calculated with Geneious, Supporting Information 2: Table S11).

4 | DISCUSSION

We developed and tested a simple centrifugation protocol for the enrichment of mitochondrial DNA from "mock"-communities. By using this technique, the full mitochondrial genomes of the amphipod *G. roeselii*, the caddisfly *H. exocellata* and the clam *C. fluminea* were sequenced. In addition, we unintentionally recovered the full mitogenome of the acanthocephalan endoparasite *P. laevis*, being present in *G. roeselii*.

We assume that for unknown reasons, sequencing worked better for the enriched samples or that FragmentAnalyzer measurements were not exact and unequal amounts of DNA were therefore pooled in the final library. Thus, an average of 2.17 million reads was obtained for the enriched samples and an average of 0.4 million reads for the unenriched samples after quality filtering, corresponding to 40.17% (unenriched samples) and 35.14% (enriched samples) of raw reads.

We demonstrated that samples enriched for mitochondria contained 9.47% high quality mitochondrial reads on average, while samples not enriched for mitochondria contained on average 0.16%. Overall, the

enrichment was 140-fold when mitogenomes without control regions were analysed, and 129-fold when control regions were included in the analyses. However, standard deviations were high (114 with control region excluded and 93 with control region included), and several potential explanations exist for this observation: First, different specimens were used for control and enrichment samples and enrichment factors had to be estimated by comparing all control to all enrichment samples (n = 9). As mitochondrial copy number varies naturally due to various factors such as different biomasses, life stages or environmental conditions (Okie, Smith, & Martin-Cereceda, 2016; Salminen et al., 2017; Tang et al., 2015), our estimation approach artificially inflates standard deviation and we expect less variation when using the same tissue for control and enrichment. Second, due to the manual crushing of specimens, homogenization of the different samples might have been unequal. We thus recommend an automated method for homogenization in the future.

The weight-adjusted calculations of enrichment factors via permutations reflect a proxy but likely overestimate variation because sampling of control and treatment used different communities, albeit from the same population/streams. The minimum mean coverage for one of the target species was 1028.8 ± 182.4 (*G. roeselii*) and still 132.7 ± 46.1 for the acanthocephalan parasite *P. laevis*. This highlights the great potential of the applied technique for fast sequencing of whole mitochondrial genomes, as even the relatively small parasite species was sequenced with an appropriate coverage. By using our protocol, bulk samples can be expected to be sequenced with a high enough coverage that allows the detection of mitochondrial sequences for many hundreds to thousands of specimens in parallel. However, as mitochondrial enrichment and resulting coverage will greatly depend on number and biomass of specimens in a sample, more research on this topic is needed. Taxonomic assignments of these reads require the completion of reliable, well-curated full mitochondrial reference libraries. Our technique for mitochondrial enrichment can greatly speed up this process, and it can be expected to obtain full mitochondrial genomes of at least several dozens of species with a single MiSeq lane if specimens are carefully selected and varying biomasses are accounted for (Elbrecht, Peinert, & Leese, 2017). The latter step is important as specimens with a high biomass can potentially prevent smaller specimens from being sequenced with a high enough coverage. However, different mitochondrial copy numbers per cell can also influence sequencing results (Tang et al., 2015). Recovery of diverse taxonomic groups from mitogenomic datasets has been shown to work well with samples not enriched for mitochondrial DNA (Arribas, Andújar, Hopkins, Shepherd, & Vogler, 2016; Gillett et al., 2014), and efficiency is expected to greatly increase by the application of mitochondrial enrichment. Although our protocol already leads to a high enrichment of mitochondrial DNA, the technique can be further improved in order to make the procedure of extracting mitochondria from tissue more reliable and standardized. We propose an automated homogenization technique using machines instead of manual homogenization with mortar and pestle, which is thought to often not break cells and release intact mitochondria. A refined technique is expected to lead to an even higher and more even enrichment of mitochondrial DNA—a desirable goal since still around 90% of all produced reads are of putative nuclear origin and enrichment is uneven for different species. Also, further studies addressing the enrichment efficiency for different species, the biomass to reads ratio and effect of different mitochondrial copy numbers per cell and species are needed to further explore the potential of mitogenomic approaches. As shown by Gómez-Rodríguez, Crampton-Platt, Martijn, Baselga, and Vogler (2015), extracting mitochondrial genomes from bulk biodiversity samples can lead to the assembly of hybrid genomes of closely related species, although to a minor extent (0.3% of assemblies). "Merged mitogenomes," i.e. mitochondrial genomes that merge haplotype diversity of a species into a single mitogenome, will inevitably be assembled if several haplotypes of a species are present in a bulk sample as in our study. However, mapping reads against assembled reference genomes and extracting haplotype diversity data allows estimating intraspecific genetic variability (Gómez-Rodríguez, Martijn, Crampton-Platt, & Vogler, 2017). Minor intraspecific variability was also observed in this study, but any in-depth analysis is out of the scope of this work and needs to be addressed in future studies.

In conclusion, our study demonstrates that the application of a simple centrifugation protocol enriches mitochondrial DNA 140-fold on average from mock-communities containing several species. The achieved coverage of complete mitochondrial genomes of minimum 1028.8 for our target species from 2.3 million sequences makes it obvious that even with 10% of resulting high quality mitochondrial reads, many specimens could be sequenced and their mitogenomes assembled in a single MiSeq run. The unintentional discovery and effective mitogenome assembly of the acanthocephalan parasite *P. laevis* further strengthens the conducted approach, as PCR primers/probes often do not capture unexpected taxa for which primers/probes have not been designed. Finally, our protocol can easily be applied in the field if a cooling centrifuge can be made available, allowing to process the fresh tissue material needed for high(er) rates of mitochondrial enrichments. The ease of application in combination with (1) a minimized laboratory workload, (2) greatly reduced costs compared to mitogenomic approaches without mitochondrial enrichment and (3) the high sequencing coverage per recovered mitogenome renders our mitochondrial enrichment protocol ideal for the fast generation of reference libraries ("mitogenome skimming") and subsequently also for biodiversity assessments.

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AUTHORS' CONTRIBUTIONS

J.-N.M., V.M.A.Z., A.M.W. and F.L. designed the study. J.-N.M. and V.M.A.Z. sampled the specimens and performed laboratory work. J.-N.M., V.M.A.Z., A.M.W. and F.L. analysed the data. J.-N.M., V.M.A.Z., A.M.W. and F.L. wrote the manuscript. All authors read and approved the final version of the manuscript.

DATA ACCESSIBILITY

All data was deposited in the NCBI Short Read Archive (https://www.ncbi.nlm.nih.gov/sra; Accession numbers: SAMN07828199 - SAMN07828210).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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Discussion

Methodological advancements in DNA metabarcoding

To fully exploit advantages of DNA metabarcoding for biodiversity assessment, current limitations have to be overcome to finally prepare a best-practice protocol that can be used by the applied sector. A faster alternative to the up to now applied but time-consuming sorting of samples is potentially present through the extraction of DNA from bulk sample fixative, which would also circumvent homogenisation of samples (Zizka et al., 2018). However, various extraction procedures are at hand which still need strict validation tests (Blackman et al., 2019) in comparison to tissue-based metabarcoding, morphological assessments and the recently tested application of unpicked sample homogenisation (Pereira-da-Conceicoa et al., 2019). Taxa detection inconsistencies through primer bias have been intensively investigated and reduced by the development and evaluation of highly degenerated primers (Elbrecht et al., 2019; Elbrecht and Leese, 2017a, 2017b; Leray et al., 2013; Miya et al., 2015) or the use of various primer sets (Hajibabaei et al., 2019b). Comparatively little attention has been paid to inconsistencies caused by PCR-based sample tagging. Extremely high inconsistencies in biodiversity assessment were found when template amplification and tagging occurred in a single PCR step in a study by O´Donnell et al. (2016). These results are not be confirmed by Zizka et al. (2019) (see chapter below).

DNA Metabarcoding from sample fixative

In line with results in the literature it is shown here that DNA extraction and subsequent DNA metabarcoding from bulk sample fixative is feasible to investigate community composition of samples (Carew et al., 2018; Hajibabaei et al., 2012; Shokralla et al., 2010). Different to previous and following studies on aquatic ecosystems (Erdozain et al., 2019; Gauthier et al., 2020; Martins et al., 2019) DNA extraction in Zizka et al. (2018) is based on the preceding step of filtering of the total fixative and the isolation of DNA from the filter residue. In all studies except Zizka et al. (2018) and Martins et al. (2019) the method is tested on clean samples separated from substrate or mock communities. Studies based on cleaned samples give important information about general functionality and, partly, about the reliability of approaches based on PCR-free target enrichment or genome skimming (Gauthier et al., 2020; Linard et al., 2016). They have also produced interesting results in applied projects as the

assessment of forest management impact on macroinvertebrate communities (Erdozain et al., 2019). However, they are hardly transferable to standardised biodiversity assessment. The separation of individuals from bulk sample substrate is an extremely time-consuming step and therefore not applicable in applied biomonitoring. As clearly indicated by Zizka et al. (2019) metabarcoding based on the extraction from complete bulk sample fixative including plant material, detritus, stones and untargeted taxa successfully detects especially weakly sclerotized and EPT taxa (Ephemeroptera, Plecoptera, Trichoptera), while it fails to reliably assess sclerotised and small ones. This is supported by Martins et al. (2019), Carew et al. (2018), and Erdozain et al. (2019). Martins et al. (2010) focus on EPTO (EPTOdonata) taxa but use an alternative way for sample processing by implementing the evaporation of much smaller volumes of ethanol (2 ml), different extraction methods and compare the results to morphological identifications. These authors conclude that larger volumes of investigated fixative would increase the method efficiency, but this has not been tested yet. Investigations on the ethanol phase of Malaise traps, also based on the filtering of the complete fixative, detect a highly different community composition than obtained through tissue-based (Malaise trap) and eDNA (soil) metabarcoding (Marquina et al., 2019).

All studies investigating fixative-based DNA metabarcoding and its application for biodiversity assessment of aquatic ecosystems see a high potential in this method due to the avoidance of picking of individuals and the possible preservation of voucher specimens. However, protocols applied in different studies are largely inconsistent and rely on different approaches to increase DNA release of targeted organisms into the fixative. This diversity slows down the potential transfer into applied biomonitoring. As implied in Blackman et al. (2019) the current state clearly indicates the need for strict validation tests to agree on the most efficient protocol (concerning fixative volume, pre-processing of samples, primer choice) which should then be compared to biodiversity assessments based on morphological identification but also tissue-based metabarcoding. Another promising alternative has been investigated recently by Pereira-da-Conceicoa et al. (2019) who tested the homogenisation of complete freshwater bulk samples without any pre-sorting and then subsequent DNA metabarcoding. Results show a much higher diversity score of aquatic macroinvertebrates than achieved through homogenisation of picked samples and eDNA and also determined the same water quality score as morphology-based assessments. This approach successfully circumvents the time-consuming step of sample sorting but still includes homogenisation of

samples, preventing the preservation and reidentification of voucher specimens after DNA metabarcoding (Pereira-da-Conceicoa et al., 2019).

Influence of sample tagging and library preparation on DNA metabarcoding

The labelling of sequences according to the original sample is crucial for the efficient use of available sequencing depth and the parallel processing of multiple samples in one sequencing run (Son and Taylor, 2011). Labelling with manufactured kits has been shown highly consistent and effective in taxa detection by studies on the ligation based TruSeq Library Preparation Illumina kit (Zizka et al., 2019). According to our results, it is the most reliable tool for library preparation if low or moderate numbers of samples need to be processed and the necessary financial resources are available. With limited financial possibilities, PCR-based approaches can, in contradiction to assumptions of previous studies (Berry et al., 2011; O'Donnell et al., 2016), be used as an alternative tagging approach, being less time and money consuming and showing less tag-switching (Zizka et al., 2019). While the one-step PCR approach performs worse than the two-step approach and shows a higher susceptibility to inhibitors, both methods are applicable regarding consistency and taxa detection efficiency, with the one-step approach slightly cheaper, faster and less prone to contamination. The extreme inconsistency of the one-step approach found by O´Donnell et al. (2016), who observed dissimilarities between PCR replicates of almost 1, are not confirmed in Zizka et al. (2019). Differences between the two studies need to be considered, which are I) a different length of index sequences (0 - 4 in Zizka et al., 2019, 6 plus 3 ambiguous bases (NNN) in O'Donnell et al., 2016) and II) deviant attachment of the flow cell binding Illumina adapters (through PCR in Zizka et al., 2019, through ligation with KAPA High-Throughput Library Preparation kit in O´Donnell et al., 2016). Especially the longer index sequences can induce higher inconsistencies in PCR, which can only be proven by a direct comparison of the methods.

In recent DNA metabarcoding studies the two-step PCR is dominating PCR-based labelling of sequences but with slightly different protocols than introduced before (Beermann et al., 2018; Elbrecht et al., 2017b; Zizka et al., 2019). The first PCR step is conducted with target-specific primers which carry only an overhang adapter (part of the sequencing primer binding site sequence) at the 5´-end. This overhang is complementary to the primer applied in the second PCR step, which carries the other part of the sequencing primer binding site sequence, a

unique Illumina index sequence (8 bases) and the Illumina adapter sequence at the 5'-end (Bista et al., 2017; Miya et al., 2015). An N-shift (addition of ambiguous bases) is inserted between first step primer and template to increase sequence diversity and ensure balanced fluorescence signals and effective cluster location during sequencing. The method is advantageous to the two-step applications in Zizka et al. (2019) because primers used in the second step PCR are complementary to the primer overhang of the primer used in the first step and can therefore be applied on different primer sets. Especially if DNA metabarcoding is used to investigate a broad range of taxonomic groups consequently using various primers, this approach is more cost efficient. The use of the same index sequences implemented in forward and reverse primers will exclude the risk of tag-switching but also increases the costs compared to the use of different indices.

Application of DNA metabarcoding

DNA metabarcoding is successfully applied to reveal detailed biodiversity patterns of aquatic macrozoobenthos in the rivers Emscher, Ennepe, and Sieg reflecting also diffuse stressor impacts. Furthermore, the effect of defined multiple stressors is investigated on the highly diverse family Chironomidae within an experimental setup at the Felderbach. Temporal and spatial effects of stressors on the community composition are shown to differ in strength between the near-natural river Sieg and the urban river Emscher while ecological status assessment based on metabarcoding presence/absence data is largely congruent with assessments based on morphology abundance data and clearly reflects stressor level. Genetic diversity is extracted from metabarcoding datasets revealing the potential of this approach to also investigate stressor impact on the diversity on population level. However, studies clearly underline problems of incomplete reference databases and inaccurate biomass assessments which need to be dealt with and upcoming methods are already introduced as solutions.

DNA metabarcoding and the responses of chironomids to multiple stressors

The clustering of High Throughput Sequencing (HTS) datasets into Operational Taxonomic Units (OTUs) based on a fixed similarity threshold (97 % similarity in COI based analyses) is widely accepted in DNA metabarcoding studies to handle high data volumes in reasonable amount of time. Because intraspecific divergence is highly variable between taxonomic groups, any particular threshold value for species delimitation will affect detected taxon diversity and will probably be differently appropriate for different groups. Differences in

genetic divergence within and between species are known across benthic macroinvertebrate orders (Hajibabaei et al., 2006; Meier et al., 2006; Monaghan et al., 2005; Zhou et al., 2010) and also within the family Chironomidae (Lin et al., 2015). To avoid splitting of genetically diverse species two different clustering thresholds were applied in Beermann et al. (2018) revealing 183 (3 % clustering threshold) and 142 (5 %) different chironomid OTUs. As demonstrated for the two species *Brillia bifida* and *Polypedilum convictum*, the application of a broader filtering threshold can prevent the splitting of species into various OTUs with identical stressor responses but can also merge OTUs which different stressor reactions leading to divergent biological interpretation (Beermann et al., 2018). Comprehensive investigations on intraspecific divergence across various taxonomic groups as for example occurring in aquatic macroinvertebrate bulk samples are not applicable for DNA metabarcoding studies. Therefore, alternative clustering algorithms (Ghodsi et al., 2011; Mahé et al., 2014) have been proposed which avoid a fixed global clustering threshold and are therefore more appropriate for taxonomic groups with a high intraspecific divergence (e.g. Chironomidae). Moreover, the investigation of Exact Sequence Variants (ESVs) can give even finer information on intraspecific stressor responses independent from clustering thresholds (Turon et al., 2019).

More than 85 % of the detected chironomid OTUs in Beermann et al. (2019) could not be assigned to a formally described species due to the lack of accurate reference database entries. This is in line with a comprehensive gap-analysis, revealing a rather low coverage in reference databases (BOLD and NCBI GenBank) for the order Diptera (Weigand et al., 2019). Complete reference databases of high quality are essential for biodiversity assessment through DNA metabarcoding, ideally providing several verified sequences per taxon. Even if the complementation of reference databases is a high priority goal receiving much attention, the taxonomic assignment of chironomid OTUs will most probably be an ongoing challenge due to the extreme diversity of this group accompanied by difficult identification and available taxonomic expertise (Milošević et al., 2020; Weigand et al., 2019). This is not only the case for Chironomidae, but refers also to other diverse taxonomic groups with relevance for standardised biomonitoring. Automated image-based identification of specimens has been introduced in recent years (Wäldchen and Mäder, 2018) and successfully tested on chironomids. This approach has high potential to increase identification speed and accuracy for taxonomically challenging groups and can thus also contribute to complement reference databases (Milošević et al., 2020). Further investigation on supervised machine learning

(SML) even envisages the application of molecular methods in biomonitoring largely independent from taxonomic assignments or biomass values (Cordier et al., 2018, 2017). Here the ecological status of a studied sample is derived from comparisons with known morphotaxonomic-derived status values and coupled molecular data. After the establishment of training datasets, the applied procedure for conservation status assessment is "taxonomy free" and does not require additional abundance measurements. This method is a potential solution to overcome the problems in taxonomic identification and biomass detection. Further investigations are needed to evaluate the method and its applicability to target a broader spectrum of biodiversity (Cordier et al., 2018, 2017).

DNA metabarcoding of stream invertebrates in a natural and urban river

DNA metabarcoding reveals clear seasonal patterns in OTU-based community composition (presence/absence) for the near-natural river Sieg, while site-specific conditions in an urban stream (Emscher) indicate a much stronger spatial than temporal signal. Seasonal variability in community composition at the Sieg, is constantly detected in analyses based on presence/absence data including all OTUs assigned to aquatic macroinvertebrate taxa but also when only EPT taxa, indicator taxa or chironomids are considered. Results strongly contradict preliminary assumptions, that DNA metabarcoding efficiently assesses community composition without being affected by seasonal fluctuations in biomass or occurring life stages. In our study, the high sequencing depth is regarded sufficient to reliably depict prevailing diversity (except extremely rare taxa, Singer et al., 2019) and the initial size sorting of individuals should prevent overrepresentation of extremely big ones. Nevertheless, results indicate that fluctuations in biomass do effect taxa detection success through DNA metabarcoding more than expected. Here it needs to be noted, that datasets were strictly filtered before analysis to remove error sequences. Filtering steps excluded all OTUs with an assigned read number < 0.01 % per sample and present in only one PCR replicate. Both filtering steps bias the dataset towards OTUs with high read numbers. Even if read number is no exact proxy for taxon biomass, correlations between these two parameters have been demonstrated (Elbrecht and Leese, 2015). The application of a strict filtering threshold therefore increases the proportion of high-biomass OTUs in metabarcoding datasets and reduces the chance to detect rare or low-biomass taxa. Changes in seasonal community composition can therefore be enhanced through strict filtering thresholds.

Various studies on DNA metabarcoding show, that the method is highly applicable for presence/absence biodiversity assessment but does not provide robust abundance data (Elbrecht and Leese, 2015; Krehenwinkel et al., 2017; Piñol et al., 2019). Taxon abundance is anchored in ecological status assessment in Germany which hinders the application of DNA metabarcoding in this field, despite the possibility of higher taxonomic resolution and speed. A high congruence between ecological status assessments of freshwater systems based on presence/absence and abundance data has been shown by Beentjes et al. (2018) and Wright-Stow and Winterbourn (2003) and is also supported by Zizka et al. (in review) on a small scale. Buchner et al. (2019) investigated more than 13.000 assessments of German streams on abundance and presence/absence data resulting in 77 % congruent assessments. Results indicate a realistic standardised translation of presence/absence data to the prevailing assessment metrics. This would still by far not exploit the complete potential of DNA metabarcoding for biodiversity assessment as indicated through the high number of detected OTUs neglected for ecological status determination (Zizka et al., in review). However, a conservative approach (adapting to currently used assessment schemes) is far more realistic than the establishment of completely new metrics tailored to molecular datasets including high costs and legislation changes even if those would ensure a more precise assessment of ecosystem integrity. Comprehensive and systematic comparison of ecological status assessments based on morphological investigations (abundance) and DNA metabarcoding (presence/absence) of identical samples of different river types are the next necessary step to finally evaluate the applicability of DNA metabarcoding in legally binding monitoring despite the weaknesses in robust abundance measures.

Resolving intraspecific diversity by DNA metabarcoding

Investigations on genetic diversity through the analysing of haplotypes from metabarcoding datasets has largely focused on single species (Pedro et al., 2017; Stat et al., 2017). Especially for taxa, for which it is complicated to obtain tissue material (e.g. large mammals, endangered or rare taxa), the sampling of non-invasive eDNA from water or faeces and subsequent metabarcoding to obtain genetic information is very practicable to investigate population structures (Baker et al., 2018; Parsons et al., 2018; Russello et al., 2004; Smith and Wang, 2014). Two approaches investigated the extraction of whole community genetic diversity from mixed samples (Elbrecht et al., 2018; Turon et al., 2019), both revealing the separation

of erroneous sequences from true intra-OTU variation as the main challenge for reliable analyses. Both studies suggest strict filtering processes to tackle this challenge which however will also lead to the exclusion of "real" haplotypes present in the sample. The authors also emphasize, that settings regarding replication level, number of habitats, and number of localities need to be customized to particular studies. Analysis of Zizka et al. (in preparation) comply with settings in Elbrecht et al. (2018) but do not implement a final filtering step, thus excluding all haplotypes with not at least 200 sequences per sample. A re-analysis of data including this filtering step and resulting in not including rare haplotypes might reveal more clearly the difference between streams with different stressor impact. The huge advantage of genetic diversity information obtained from metabarcoding datasets is the fact, that information on complete communities is extracted that can be mined from already existing data. This is contrasting to usual population genetic studies, mainly focussing on one species and being very elaborated. However, metabarcoding studies for animals mainly focus on a single gene fragment that is of mitochondrial origin (Cytochrom c Oxidase I gene). Even if the COI gene meets the requirements for molecular species identification and various studies have also used it for population genetics, the applicability for the latter purpose is debatable mainly due to its maternal inheritance and unusual evolutionary history (but see Ballard and Whitlock, 2004; Leese and Held, 2011 for detailed information). For deeper understanding of population structure, the consideration of nuclear DNA markers is highly recommended, potentially showing different divergence patterns than mitochondrial DNA data (Ballard and Whitlock, 2004; Carranza et al., 2016; Pratlong et al., 2017). As nuclear markers are rarely used for DNA metabarcoding approaches on animal communities, these markers would be especially applicable for investigations on single species but less for community samples because taxonomic assignments are rarely possible. In addition, it is not possible to assign sequences to individuals if extracted from community metabarcoding datasets so that haplotypes could indicate one individual or multiple individuals with the same haplotype further complicating detailed analysis of population genetics. The current status indicates, that the extraction of haplotypes from community metabarcoding datasets can provide proxies of genetic diversity by the total haplotype number or the average haplotype number per OTU, also indicating stressor intensity effects (Zizka et al., in prep.). However, these data should cautiously be used for analysis of genetic mechanisms as gene flow, inbreeding or mating systems.

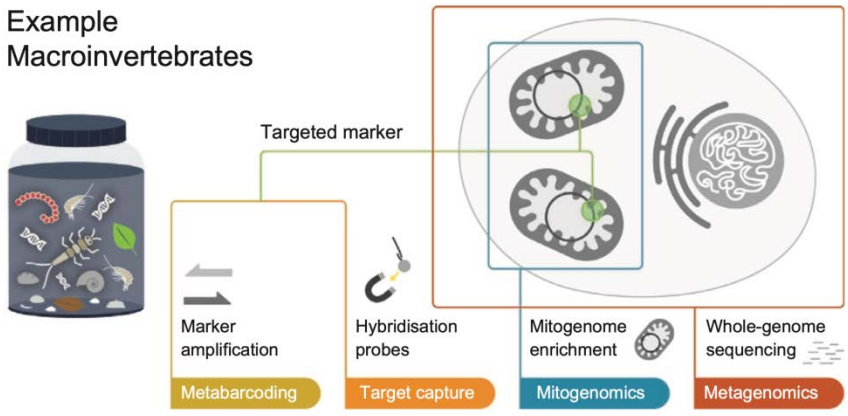
Beyond DNA metabarcoding

To circumvent the error prone PCR step implemented in DNA metabarcoding, PCR-free methods (metagenomcs or mitogenomics) have a high potential to assess abundance patterns from mixed environmental samples. To overcome a “bottleneck” and to enrich informative mitochondrial DNA in samples, a centrifugation protocol was applied before shotgunsequencing of a mixed mock sample. This additional step increased mitochondrial yield up to 10 % of the total read number. The protocol can be applied in mitogenomic approaches increasing the usability for biodiversity assessment, eventually implying biomass calculations. Further investigation is needed on the variation in mitochondrial copy numbers per cell and species and due to biomass, life stage and environmental condition before the final usability can be stated.

A centrifugation protocol for increasing mitochondrial DNA yield

To circumvent taxon-dependent PCR amplification biases and resulting stochasticity in read distribution in DNA metabarcoding, shotgun sequencing or shotgun metaganomics is discussed as an alternative method for biodiversity assessment (Bista et al., 2018; Crampton-Platt et al., 2016). Due to incomplete reference libraries for nuclear genomic information, studies at hand focus on the sequencing of complete mitochondrial genomes also called mitochondrial metagenomics, mitogenomics or mitogenome skimming (Figure 3). Since most PCR-free obtained mitogenomic libraries comprise less than 1 % sequences of mitochondrial origin, enrichment steps need to be conducted (Crampton-Platt et al., 2015; Gómez-Rodríguez et al., 2015; Tang et al., 2014). In Macher et al. (2018), a method for enrichment of mitochondrial genomes is developed and successfully applied on a mock community, increasing mitochondrial read numbers up to 10 % and encouraging its application for the setup of complete mitochondrial genome databases. Furthermore, it increases the applicability for biodiversity assessments that has already been tested in several studies (Arribas et al., 2016; Gillett et al., 2014). Due to its independence from PCR, mitogenomic approaches are a promising approach for biomass or even individual number assessment from mixed samples and strong correlations between biomass and final read number has been identified (Bista et al., 2018; Coissac et al., 2016; Tang et al., 2014; Zhou et al., 2013). However, studies up to now focus on mock communities or selected taxonomic groups. This demands for more detailed investigations on the variation in mitochondrial copy numbers per cell and species

and due to biomass, life stage and environmental condition before the usability can be finally judged. Also metatranscriptomics have been introduced for biomonitoring purposes. Different to metagenomics, this method targets RNA molecules and thereby assesses expressed genes in an ecosystem at the moment of sampling revealing some contigs and functional transcripts as efficient indicators for anthropogenic disturbances (Cheaib et al., 2018; Thompson et al., 2016). The investigation of RNA from bulk samples and especially eRNA (environmental RNA) instead or in addition to DNA has also been suggested for diversity identification as a better indicator of the living biotic assemblage at a sampling site, providing a more local signal. However, RNA molecules show a lower stability compared to DNA molecules, rendering the handling more challenging and more difficult to transfer to applications in biomonitoring (Cristescu, 2019; Cristescu and Hebert, 2018; Pochon et al., 2017)



Taxonomic resolution	Good	Good	Very good	Very good
Needed sequ. depth	Medium	Low	Low	Very high[a]
Primer bias	Yes	Maybe	No	No
↳ Taxa missed	~20%	20% ?	0%	0%
↳ Biomass/abundance	p/a only	Maybe	Maybe	Maybe
Reference data	Good	Good	Medium[b,c]	Poor[b]
Method validation	Good	Poor	Poor	Good
Constraints			**Fresh tissue needed**	

[a] Without genomic references. [b] Can use e.g. COI reference databases. [c] Reference genomes can be assembled de novo from HTS dataset.

Figure 3: *Evaluation of molecular methods applied in biodiversity assessment (after Leese et al., 2018)*

Conclusions

DNA metabarcoding is nowadays widely used for biodiversity assessment of aquatic ecosystems and its advantages over traditional identification methods in taxonomic resolution and speed are out of question. Tremendous effort has been made to further develop methodology and to establish DNA metabarcoding in standardized legally binding biomonitoring. Despite the progress achieved, technical limitations and conceptual issues still exist. While some of them seem to be solved in the near future, others – like a complete reference database or the assessment of specimen biomass or abundance - are still far from being settled or seem unsolvable. However, the establishment of DNA metabarcoding in standardised biomonitoring most probably will be accomplished in the near future. Generally, the capability of the molecular approaches in biomonitoring and conservation assessments will increase considerably with new complementary methods as shotgun metagenomics or transcriptomics and significant developments in machine learning, potentially decoupling biomonitoring assessment from permanent taxonomic assignment and biomass measurements.

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Abbreviations

BLAST	Basic Local Alignment Search Tool
BOLD	Barcode of Life Data Systems
BQE	Biological Quality Element
DNA	Deoxyribonucleic Acid
eDNA	environmental Deoxyribonucleic Acid
EPT	Ephemeroptera, Plecoptera, Trichoptera
EPTO	Ephemeroptera, Plecoptera, Trichoptera, Odonata
EU	European Union
ESV	Exact Sequence Variants
HTS	High-Throughput Sequencing
MZB	Macrozoobenthos
NCBI	National Center of Biotechnology Information
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
eRNA	environmental Ribonucleic Acid
SML	Supervised Machine Learning
WFD	Water Framework Directive
WRRL	Wasserrahmenrichtlinie
WWF	World Wide Fund for Nature

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Appendix I – digital supplements

Chapter 1 –Methodological advancements in DNA metabarcoding

DNA metabarcoding from sample fixative

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Influence of sample tagging and library preparation

File: men13018-sup-0001-figs1

File: men13018-sup-0002-figs2

File: men13018-sup-0003-figs3

File: men13018-sup-0004-figs4

File: men13018-sup-0005-tables1-2

File: men13018-sup-0006-tables2

File: men13018-sup-0007-tables3

File: men13018-sup-0008-tables4

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Chapter 2- Application of DNA metabarcoding

DNA metabarcoding and the responses of chironomids to multiple stressors

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DNA metabarcoding of stream invertebrates in a natural and an urban river

File: FigureS1

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Resolving intraspecific diversity by DNA metabarcoding

File: FigureS1

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File: FigureS5

File: FigureS6

File: FigureS7

Chapter 3 – Beyond DNA metabarcoding

A centrifugation protocol for increasing mitochondrial yield

File: mee312937-sup-0001-figs1

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Appendix II – additional work

abarcoding & Metagenomics

Forum Paper

Curriculum vitae

Aus datenschutzrechtlichen Gründen ist der Curriculum Vitae nicht in der Online-Version enthalten

Erklärungen

Erklärung:
Hiermit erkläre ich, gem. § 7 Abs. (2) d) + f) der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient, bei der Abfassung der Dissertation nur die angegeben Hilfsmittel benutzt und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

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