Sample-size and bioinformatics independent, consistent patterns among diatom metabarcoding data from lake sediments

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Abstract

Diatoms (Bacillariophyceae) are widely used as bioindicators of present and past water quality because they inhabit the vast majority of aquatic ecosystems, are very diverse, highly sensitive to a variety of environmental conditions, and are characterized by silicified cell walls that favor their long-term preservation in sediments. Alongside with traditional morphological analyses, metabarcoding has become a valuable tool to study the community structures of various organisms, including diatoms. Here, we aimed to test whether the quantity of sediment sample used for DNA extraction is affecting the results obtained from high-throughput sequencing (metabarcoding) of the diatom rbcL region by isolating DNA from 10 g and 0.5 g (wet weight) of lake surface sediment samples. Because bioinformatics processing of metabarcoding data may affect the outcome, we also tested the consistency of the results from three different pipelines. Additionally, the agreement between metabarcoding data and morphological inventories of corresponding samples were compared. Our results demonstrate highly uniform patterns between the diatom rbcL amplicons from 10 g and 0.5 g of DNA extracts (HTS 10 and HTS 0.5, respectively). Furthermore, metabarcoding results were highly consistent among the data sets produced by different bioinformatics pipelines. Comparing results from metabarcoding and microscopy, we identified some taxonomic mismatches, which are related to the common issue of incompleteness of the sequence databases, but also to inconsistencies in diatom taxonomy in general and potential dissolution effects of diatom valves caused by high alkalinity of the investigated lake waters. Nevertheless, multivariate community analysis demonstrated highly similar results between data sets identified by microscopy and metabarcoding, further confirming that metabarcoding is a viable alternative for identifying diatom-environment relationships.

Introduction

Diatoms (Bacillariophyceae) rank among the most important components of aquatic food webs and play an important role in carbon fixation (Mann 1999). Because of their fast response and narrow optima for multiple environmental variables, diatoms are excellent indicators of ecosystem health (Dixit, Smol, Kingston & Charles 1992; Pan, Stevenson, Hill, Herlihy & Collins 1996), and may provide early warning signals for aquatic ecosystem changes in face of anthropogenic pressures such as eutrophication (Wang *et al.* 2012) or heavy metal contamination (Chen *et al.* 2015). The standard methods for assessing diatom communities rely on counting and identifying their silicified cell walls (valves) using mostly light microscopy (e.g. European-Committee-for-Standardization 2014). But with the rapid development and continuously decreasing costs of high-throughput sequencing (HTS) technologies, the metabarcoding approach, allowing simultaneous identification of multiple species from environmental samples, has become an alternative tool for fast

biodiversity assessment (Ruppert, Kline & Rahman 2019). Because morphological analyses of diatoms (and other microorganisms) are labor-intensive, require expertise and are prone to inter-investigator variation, metabarcoding, referred to as 'Biomonitoring 2.0' (Baird & Hajibabaei 2012), may have the potential to outperform the traditional, low throughput, monitoring methods.

Metabarcoding-based biodiversity studies, however, may face various difficulties, starting from DNA extraction to data processing in complex bioinformatics pipelines (Sinha *et al.* 2017; Anslan *et al.*2018; Hardge *et al.* 2018). Therefore, the suitability of metabarcoding approach for assessing diatom communities have been the research focus for several studies. Although the DNA barcoding library for accurate species level detection is still incomplete for diatoms, metabarcoding is a promising tool for biomonitoring of community assemblages of these organisms as it has been shown to produce similar results compared with morphological analyses (Zimmermann, Glöckner, Jahn, Enke & Gemeinholzer 2015; Apotheloz-Perret-Gentil *et al.*2017; Vasselon, Rimet, Tapolczai & Bouchez 2017; Keck, Vasselon, Rimet, Bouchez & Kahlert 2018; Rimet *et al.* 2018; Rimet, Vasselon, Barbara & Bouchez 2018; Rivera *et al.* 2018). The majority of diatom community studies are applied to biofilms of epilithic diatom species from rivers and lakes, with the goal of assessing current-state water quality. Because diatom silicified valves are usually well preserved in sediments, they also constitute important indicators for inferring paleo-environmental conditions such as water pH, nutrient dynamics, and temperature (Douglas & Smol 2010). However, only few studies have estimated the suitability of metabarcoding for identifying diatom communities directly from sediment samples and have assessed its consistency with microscopy (Dulias, Stoof-Leichsenring, Pestryakova & Herzschuh 2017; Piredda *et al.* 2017). Although morphological and metabarcoding data sets from these studies have demonstrated highly correlated results, it is not clear how this pattern is related to the quantity of sediment used for DNA extraction or affected by the use of different bioinformatics pipelines. The quantity of sediment used strongly depends on the approach taken for DNA extraction; it is common to use DNA isolation kits which allow input of 'large' quantities (usually up to 10 g) of environmental sample, to potentially capture the complete community represented in the sample. However, DNA extraction methods, for example the 'universal' Power Soil Kit (Hermans, Buckley & Lear 2018), which process much less material and thus use less chemicals, cost only a fractional amount and may represent attractive alternatives for DNA metabarcoding of large numbers of samples. Multiple publicly available tools exist for bioinformatics processing of large sets of sequencing data, amongst which QIIME (Caporaso *et al.*2010) and mothur (Schloss *et al.* 2009) are the most commonly used, but some studies have highlighted that an inappropriate choice of software and settings may heavily affect the final results (Majaneva, Hyytiäinen, Varvio, Nagai & Blomster 2015; Anslan *et al.* 2018). Also for diatom communities, recent studies have suggested that the choice of bioinformatics pipelines may affect the outcome of metabarcoding studies (Tapolczai, Keck, Bouchez, Rimet & Vasselon 2019; Rivera, Vasselon, Bouchez & Rimet 2020). Here, we investigate diatom communities from Nam Co, a saline lake on the Tibetan Plateau, and from nearby ponds and tributaries. Our aim is to explore whether the characterization of diatom community structure via metabarcoding is dependent on the quantity of sediment used for DNA extraction by comparing the two most commonly used DNA isolation kits, PowerMax Soil and Power Soil (Qiagen, Germany), and by applying those to 10 g and 0.5 g (wet weight) of surface sediment samples, respectively. We further tested the consistency of the metabarcoding results obtained via three different bioinformatics pipelines by applying exact sequence variants (ESV) and two OTU clustering approaches. In addition, we assess how the metabarcoding data sets (from 10 g *vs* . 0.5 g of sediments) compare with the morphological analyses of diatoms from the same samples, and how these datasets relate with environmental variables.

Materials and Methods

Sample collection

Surface sediment samples were collected in Nam Co using an Ekman-Birge bottom sampler from water depths ranging between 0.2 m to 56 m (Table S1). A spatula was used to sample the oxygenized layer of sediments, i.e. about top 2 cm. Additional samples from shallow water sites such as rivers and lagoons were collected randomly from sandy substrate by using a hand-shovel and scraping the upper 2 cm. Approximate wet weight of a sample was 200 grams, which was mixed, split in half, and these two batches were then

transferred to Whirl-Pak bags for metabarcoding and morphological identification of diatoms. Limnological parameters were measured at each sampling site using a multi parameter probe WTW 3630 (Table S1). Water anions and cations were measured using ion chromatography (IC) and inductively coupled plasma optical emission spectrometry (ICP-OES), respectively, at the Institute of Geographical Sciences, Freie Universität Berlin (Table S1). The research permit was obtained via the Institute of Tibetan Plateau Research, Chinese Academy of Sciences from the Tibet Autonomous Region Government.

In the field, laboratory samples for the morphological identification of diatoms were stored at 4 °C. Samples for metabarcoding analyses were sieved through 2 mm sieves to remove coarser sediment components. Tap water was used for sieving, and therefore also as negative extraction (and PCR) control for metabarcoding analysis. Approximately, 50 g of sediment were divided between three 50 ml tubes and filled with 96% ethanol (4:1 ethanol:sediment ratio). All used equipment was bleached (10% sodium hypochlorite solution) after each step to avoid cross-contamination. All samples were stored and transported in a freezer (-20 ºC). The study design is illustrated in Figure 1.

Molecular analysis

Sediment samples were centrifuged at 4000 rpm for 10 minutes, supernatant was removed and subsamples were mixed. Wet samples were weighted to 10 g (in the following referred to as metabarcoding treatment HTS 10) and 0.5 g (HTS 0.5), and DNA was extracted using DNeasy PowerMax Soil Kit and DNeasy PowerSoil Kit (Qiagen, Germany), respectively (three samples that were processed with PowerMax Kit had $<$ 10 g input, see Table S1). Except for the amount of chemicals, these kits use identical chemistry and protocols. To enhance the cell lysis, we modified the initial step by adding Proteinase K (10 mg/ml) and 1M DTT (dithiothreitol) together with the C1 solution from the extraction kits. For the PowerMax Kit (10 g of sediments) 60 µl of Proteinase K and 100 µl of DTT, and for the PowerSoil Kit (0.5 g of sediments), 4 µl of Proteinase K and 25 µl of DTT was added, respectively; following overnight incubation at 56 ºC. For potentially higher DNA yield, the elution was performed twice by adding half of the recommended amount of the buffer onto a spin column membrane and incubated at room temperature for 3 minutes. The rest of the steps were performed following manufacturer's instructions.

PCRs were performed using uniquely tagged primers rbcL-646F (5'-ATG CGT TGG AGA GAR CGT TTC-3') and rbcL-998R (5'-GAT CAC CTT CTA ATT TAC CWA CAA CTG-3'), which amplify 331 base pairs (bp) of the large subunit of the ribulose-bisphosphate carboxylase/oxygenase (rbcL) gene (Kelly *et al.* 2018) (Table S2). We also tested the primers of Diat_rbcL_708F and R3 (Vasselon, Rimet, Tapolczai & Bouchez 2017), which amplify a shorter fragment (312 bp) of the same region. However, the PCR results were visually superior for rbcL-646F and rbcL-998R primer pair (data not shown), thus here, we decided to proceed only with the latter primers. The 25 µl PCR mix consisted of 5 µl of Hot Start FirePol Master Mix (Solis BioDyne, Estonia), 0.5 µl forward and reverse primer, and 1-3 µl of template DNA. The rest of the volume was filled with nuclease-free water. PCR conditions were as follows: 95 ºC for 15 minutes (hot start), 32-35 cycles of 95 ºC for 20 s, 55 ºC for 45 s, 72 ºC for 60 s, and final extension at 72 ºC for 5 minutes. Three replicate PCRs were performed per sample, following sample pooling and checking the yield of PCR products during gel electrophoresis by pipetting 5 µl PCR product on 1% agarose gel. Amplicons per sample were pooled as based on their relative quantity and purified using Favor-Prep Gel/PCR Purification Kit (Favorgen-Biotech Corp., Austria), following the manufacturer's instructions. Steps of DNA extraction, PCR and sequencing included both negative and positive controls. Sample preparations, as well as DNA isolations, were conducted under laminar flow clean bench, using 30 min UV sterilization prior analyses. Sequencing was performed on the Illumina MiSeq (2x250) using MiSeq Reagent Kit v2.

Bioinformatics

Three different bioinformatics workflows (pipelines) were used to process raw paired-end Illumina data: 1) ESVs (exact sequence variants) pipeline as implemented in DADA2 (Callahan *et al.* 2016); 2) 95% OTUs (operational taxonomic units) pipeline, where OTUs are clustered at 95% sequence identity; and 3) a pipeline based on OTUs clustered at 97% identity. The processing of sequencing data to generate ESVs and 95% OTUs

followed the workflows as described in Tapolczai, Keck, Bouchez, Rimet and Vasselon (2019) and Rivera, Vasselon, Bouchez and Rimet (2020), respectively, except that taxonomy assignment of the representative sequences was performed using blastn algorithm (instead of Naïve Bayesian classifier) (Camacho *et al.* 2009) with e-value = 0.001, word size = 7, reward = 1, penalty = -1, gap open = 1 and gap extend = 2 (against R-Syst v.7.2 diatom database (Rimet *et al.*2016)). Based on our positive and negative controls, the 95% OTUs data set was further filtered to exclude low occurrence ([?] 3) reads per OTU per sample to alleviate to 'tag-switching' error. The latter was not performed for the ESVs data set as no sequences were observed in the negative controls and no 'read-leakage' from the positive control sample. Singleton ESVs/OTUs were discarded from the data sets (i.e. ESVs/OTUs that had only one read across samples).

To generate 97% OTUs, raw paired-end Illumina sequencing data was processed in PipeCraft (Anslan, Bahram, Hiiesalu & Tedersoo 2017), which incorporates all the following tools (except LULU). Reads were assembled and quality filtered using vsearch (fastq_minoverlen 15, maxdiffs 45, fastq_minmergelen 200, fastq_-maxee 1, fastq_maxns 0, fastq_truncqual 5, fastq_allowmergestagger) (Rognes, Flouri, Nichols, Quince & Mahe 2016). Chimera filtering step included vsearch uchime_denovo algorithm with options id 0.97 and abskew 2. The filtered sequences were clustered using UPARSE (Edgar 2013) with 97% similarity threshold and minimum cluster size of 2 (i.e. singletons excluded). The obtained OTU table was further curated with post-clustering algorithms as implemented in LULU (Froslev *et al.* 2017) to merge consistently co-occurring 'daughter' OTUs (minimum_ratio = 1, minimum_ratio_type = "avg", minimum_relative_cooccurence = 0.8, minimum_match = 96.97). Potential tag-switching errors were also corrected based on negative and positive controls based on relative abundances of sequences in the control samples (Taberlet, Bonin, Coissac & Zinger 2018). To account for unequal sequencing depth, we rarefied samples to common depth of 7000 sequences using the mothur software (Schloss *et al.* 2009). The latter removed five samples from the data set. Representative sequences per OTU were compared against R-Syst v.7.2 diatom database using blasn as stated above.

The used primers (rbcl-646F and rbcL-998R) amplify DNA also from other algae and bacteria (especially Proteobacteria and Cyanobacteria). To exclude the non-target taxa, only OTUs that demonstrated the match coverage and identity of [?] 90% against a reference database, were considered as diatom OTUs and included in the final tables produced by each pipeline. According to additional blastn search against NCBI database (Geer *et al.* 2009), the above threshold was confirmed to include only diatom taxa into the final OTU table. OTUs with lower thresholds to reference diatom sequences (in R-Syst) were often more closely related (based on e-value, sequence similarity and coverage) to other micro-algae (e.g. taxa from Mischococcales, Tribonematales, Eustigmatophyceae), thus excluded from the downstream analyses.

Because of the uncertainty of the most adequate species-level sequence similarity threshold for diatoms, the taxonomic composition comparisons between metabarcoding treatments (HTS 10 and HTS 0.5) and microscopy was performed on genus level. Reliable genus level classification of the OTUs in the HTS data set was here defined when sequence similarity and coverage was [?] 95% against a reference sequence in the R-Syst database. OTUs that displayed lower values against the R-Syst database sequences were blastn-searched against the NCBI database to check for additional genus-level annotations. Synonym names for genera were also explored in the case of mismatches between microscopy and metabarcoding data sets.

Morphological analysis

Sediment samples for morphological diatom analyses were treated and examined using standard methods (Battarbee *et al.* 2001). Specifically, approximately 0.1 g of dry sediment (freeze dried) for each sample was treated with 30 % hydrogen peroxide (H_2O_2) and 10 % hydrochloric acid (HCl) for 2-3 hours to remove organic matter and carbonates, respectively. The resulting slurries were then washed repeatedly with distilled water until a neutral pH was reached and strewed onto glass coverslips to dry at room temperature. The dry samples were then fixed onto microscope slides with Naphrax(r), a highly refractive mountant. At least 400 valves were identified (range 400-407) and enumerated along transects using a light microscope (Leica DM6B, magnification x1000) with oil immersion objective. Diatom identification and taxonomy were based primarily on general floras such as Krammer and Lange-Bertalot (1986-1991) and Lange-Bertalot,

Hofmann, Werum, Cantonati and Kelly (2017). We also used specific taxonomic publications to aid with the identification of difficult groups of diatoms, such as Mohan *et al.* (2018) for*Lindavia biswashanti* , and Pavlov, Levkov, Williams and Edlund (2013) for species belonging to the *Hippodonta* genus. To help further with morphological identification, scanning electron microscope (SEM) photographs were taken using a Zeiss MERLIN instrument at the Institute of Geology and Geophysics, CAS, Beijing.

Statistics

Differences in the ESVs/OTUs/morphospecies richness and correlation analysis between the three treatments (HTS 10, HTS 0.5 and microscopy) were tested using Kruskal-Wallis Analysis of Variance (ANOVA) by Ranks and Spearman Rank Order Correlations in STATISTICA 7 (StatSoft 2004). Permutational Analysis of Variance (PERMANOVA, with 9999 permutations) and Principal Co-ordinate Analysis (PCoA) of Bray-Curtis similarity matrices of log transformed data were performed in PRIMER v6 (Clarke & Gorley 2006). The values of the PCoA first axis were selected as a response variable for Random Forest analysis to identify most important environmental variables for diatom community assembly. The latter was performed in R (R-Core-Team 2015), using the package 'randomForest' (Liaw & Wiener 2002). The variables included for Random Forest analyses were maximum depth, conductivity, and concentration of zinc (Zn), calcium (Ca), silica (Si), chromium (Cr), nitrate (NO_3), copper (Cu), manganese (Mn), iron (Fe), strontium (Sr), vanadium (V) (Table S1). Variables, selected by Random Forest model per treatment, were used in marginal test analysis (with 9999 permutations), in PRIMER. Only variables with P-values < 0.05 were displayed as vectors in the PCoA ordination plots.

Bray-Curtis similarity matrices of Hellinger-transformed data per treatment were compared with Mantel tests (with 9999 permutations, method="spear") to assess the correlations between sample similarities as implemented in the 'vegan' package (Oksanen *et al.* 2015). In addition, Procrustes analyses were used to compare the similarity of PCoA ordinations between different treatments (in 'vegan'). To examine the presence of diatom OTUs that were consistently detected either by the HTS 10 or the HTS 0.5 treatment, an indicator species analysis (with 9999 permutations) was performed using the 'indicspecies' package (De Caceres, Jansen & De Caceres 2016).

The analyses for comparing metabarcoding treatments (HTS 10 and HTS 0.5) were performed for 20 corresponding samples. Because of smaller sample size for the microscopy data, analyses for HTS 10 *vs* . microscopy and HTS 0.5 *vs* . microscopy were performed with 14 and 11 samples, respectively (see Table S1).

Results

Richness and composition

The numbers of ESVs/OTUs/morphospecies per sample for metabarcoding and microscopy data are summarized in Table 1. The numbers of ESVs/OTUs per sample between HTS 10 and HTS 0.5 treatments demonstrated a strong positive correlation (Spearman R > 0.863; Fig. 2a-c; Fig. S1), and no significant differences in the (ESV/OTU) richness (Fig. 2d). Accordingly, the intra-pipeline comparisons of HTS 10 and HTS 0.5 data (20 comparable samples; Table 1) demonstrated high proportions of shared ESVs/OTUs (63.9-87%; Fig. 3). For the genus level comparisons across metabarcoding data sets, ESVs and OTUs were annotated to genus level only when the similarity and coverage of the representative read of ESV/OTU was [?] 95% against a reference sequence. Similarly, a large proportion of genera were shared between HTS treatments (87.7-92.2%; Fig. 3). Interestingly, inter-pipeline comparisons revealed that higher proportion of genera was shared between HTS 0.5 treatments compared with HTS 10 (87.7% *vs* . 76.4%; Fig. S2). Compared to data generated with the OTUs pipelines, the data from the ESVs pipeline harbored a higher number of different genera (67 *vs* . 62 for HTS 10 and 69*vs* . 65 for HTS 0.5; Fig. S2). For the HTS 10 data, the unique genera (8 genera; i.e. genera that were identified only in the corresponding data set) of the ESVs data set represented a total of only 2.67% of sequences (range of <0.001% to 1.44%; Table S3). For the HTS 0.5 data, the unique genera (4 genera) of the ESVs data set represented total of less than 0.1% of sequences (range of $< 0.001\%$ to 0.016%; Table S3). The data set of 97% OTUs did not contain any unique

genera, and there was only one unique genus for the 95% OTUs data (*Sternimirus* , sequence abundance < 1%; Fig. S2; Table S3).

Morphological examination of the sediment samples recovered a total of 189 diatom taxa from 11 surface sediment samples (Table 1), which included 59 genera (Fig. 4; Table S3). Unlike the per sample richness correlations observed between the metabarcoding treatments (Fig. 2a-c), correlations were not obvious between richness values from the microscopy and metabarcoding data ($P > 0.398$ for all cases; Fig. S3). Across treatments, detected species richness by microscopy differed significantly only from the ESVs data (Fig. 2d). The detected composition of genera by microscopy were compared with metabarcoding data, which harbored 54 different genera for the ESVs data, 49 and 50 genera for the 97% and 95% OTU data, respectively. The genus level comparisons (among 11 corresponding samples) revealed that 50.7-54.3% of genera were shared between microscopy and metabarcoding treatments (Fig. 4). Compared with the metabarcoding inventories, the microscopy data set harbored larger proportion of unique genera (Fig. 4). From these, the majority were represented in low abundances in the microscopy data set (< 9 counted valves per sample). However, counts of the valves assigned to *Pseudostaurosira* , one of the most abundant genera that were completely missing from metabarcoding data, was 519 (11.77%) across the microscopy data set.

Comparing the relative abundance of valves and sequences of the matching genera between microscopy and metabarcoding data, revealed overall significant positive correlations (Spearman $R > 0.317$ and $P < 0.023$; except for 97% OTUs HTS 10 *vs* . microscopy data, where $P = 0.067$; Fig. S4). The outstanding exceptions were*Pantocsekiella* and *Achnanthidium* , which had high relative abundance in microscopy, but low abundance in metabarcoding data (Fig. S4). *Vice versa* , *Staurosira* and *Aulacoseira* were found to have high relative abundance in metabarcoding data, but low in microscopy data (Fig. S4).

Community analyses

Mantel test and Procrustes analyses revealed that the inter-sample community similarities between metabarcoding treatments were greatly correlated (Fig. 5; Table 2). For all cases, the Mantel correlation (i.e. Mantel R) between HTS 10 and HTS 0.5 was higher than 0.851 and $P < 0.001$ (Fig. 5). Among intra-pipeline treatments (HTS 10*vs* . HTS 0.5), 95% OTUs exhibited highest correlation (Mantel $R = 0.969$; Table 2; Fig. 5). Procrustes correlations, however, revealed highest values between ESVs treatments (HTS 10 *vs* . HTS 0.5; Procrustes correlation = 0.969, $P < 0.001$; Table 1), but a much lower value between 95% OTUs treatments (Procrustes correlation = 0.688; Table 1). The high community similarity among the metabarcoding data sets was also demonstrated by PERMANOVA analyses using treatment as fixed variable ($P > 0.999$ for all cases). Moreover, no group-specific indicator OTUs were assigned to neither HTS 10 nor HTS 0.5 treatments by indicator species analysis for any of the metabarcoding data sets.

The inter-sample community similarities (Mantel correlations) and Procrustes analyses between microscopy and HTS data also demonstrated highly correlated patterns (microscopy *vs* . all HTS treatments: Mantel R [?] 0.800, $P < 0.001$; Procrustes correlations [?] 0.681, P [?] 0.001; Fig. 6, Table 1). Compared with other treatments, the 95% OTUs data set demonstrated slightly higher Mantel correlations with microscopy data (Table 1), whereas the highest Procrustes correlations were found for the ESVs HTS 0.5 data set (Table 1). Based on Random Forest analysis, the diatom assemblages in all treatments were most strongly affected by conductivity, water depth, Si, Ca, Sr, Mn and Fe, however, with different orders in variable importance (Fig. S5). Marginal tests (the significance of an individual variable when considered alone and ignoring all other variables) showed consistent patterns for the most important variables for all treatments (Table S4). The highly correlated community structures were also demonstrated in the ordination plots (Fig. 7).

Discussion

In the current study, regardless of sample size (10 g, 0.5 g) and bioinformatics pipeline (ESVs, 97% OTUs, 95% OTUs) applied, we found highly comparable diatom community patterns between metabarcoding data and microscopic inventories from lake sediment samples. This was especially pronounced between the metabarcoding treatments, i.e., amplicons from 10 g and 0.5 g of DNA extracts (HTS 10 and HTS 0.5, respectively). Although the per-sample diatom richness was not significantly correlated between microscopy

and metabarcoding data, community analyses indicated consistent patterns of environmental variables shaping the diatom community structures, irrespective of sample-size and bioinformatics pipeline used. This further demonstrates that metabarcoding is a viable alternative to detect diatom-environment relationships.

Sample size

Although the DNA extraction method may have a strong impact on taxa recovery in metabarcoding studies (Schiebelhut, Abboud, Gomez Daglio, Swift & Dawson 2017; Sinha *et al.* 2017), previous comparisons of different DNA isolation kits for diatoms have demonstrated compatible patterns for diversity and community assembly (Vasselon, Domaizon, Rimet, Kahlert & Bouchez 2017). The present study also shows that richness and community structure of diatoms are highly correlated between metabarcoding data of 10 g *vs* . 0.5 g of sediment samples. Interestingly, the correlations of relative abundances of matching diatom genera between metabarcoding and microscopy data sets, resulted in higher correlation values for the HTS 0.5 data (Fig. S4). However, other studies comparing metabarcoding results from DNA extracts of various amounts of substrate have reported contrasting results. For example, Penton, Gupta, Yu and Tiedje (2016) reported significant effects of sample size (in terms of input quantity) on the community structure of fungi and bacteria from soil. Higher diversity estimates were associated with 10 g of soil DNA extracts compared with 5 g, 1 g and 0.25 g. Studying meiofaunal communities from marine sediment samples, Brannock and Halanych (2015) found that different extraction quantities did not result in significantly different diversity estimates, however, the OTU community compositions were different. Exploring various eukaryotes from sediment samples, Nascimento, Lallias, Bik and Creer (2018) also found significantly different diversity metrics and community compositions for various sample sizes. They suggested that larger volumes of sediment are necessary to capture the representative metazoan communities compared to the non-metazoan eukaryotes. Therefore, the choice of a quantity of sediment for DNA extraction may depend on the expected distribution of the target groups in the substrate, where the detection of more patchily distributed metazoan communities requires larger quantities of sediment for analyses. Although it could be hypothesized that sample size may affect also the recovery of some microbial groups, here we demonstrate that this was not the case for diatoms from lake sediment samples (when comparing sample size on 10 g *vs* . 0.5 g). The benefits of using only up to 0.5 g of the sample include a more time- and cost-effective DNA extraction procedure, and the possibility to conduct meaningful analyses when only a limited amount of sediment is available.

Bioinformatics

Here, three different bioinformatics pipelines were used to analyze metabarcoding data. Combination of analyses among metabarcoding data sets and between microscopy *vs* . metabarcoding data demonstrated highly correlated patterns (Table 2, Fig. 3-7). Although the comparisons between microscopy and metabarcoding data demonstrated highest Mantel correlation of the former with the 95% OTUs data set, Procrustes correlation was highest with the ESVs data set (Table 2). Thus, the identification of the best performing pipeline, in terms of consistency with microscopic inventories, may depend on the applied statistical method. Nevertheless, considering the genus level comparisons from metabarcoding, the ESVs data set had the slightly higher number of genus level identifications, and therefore a (marginally) higher match proportion with microscopy data. But interestingly, the ESVs data set harbored also one diatom taxon that is considered to be purely marine, *Trachyneis* sp. (Fig. 4; Table S3). ESV assigned to the latter taxon, however, consisted of only three reads across the whole data set. It is uncertain whether this low abundance ESV assigned to *Trachyneis* sp. represents remaining sequencing errors or real occurrences, but raises caution in evaluating data quality based on highest taxonomic richness alone. In any case, the differences between bioinformatics workflows in this study were minor and indicated highly similar signals among all of them.

Although this study displayed an overall consistency of results obtained by different pipelines, several studies have demonstrated the influence of bioinformatics in metabarcoding studies targeting other organisms (Majaneva, Hyytiainen, Varvio, Nagai & Blomster 2015; Sinha *et al.* 2017; Anslan *et al.* 2018; Pauvert *et al.* 2019). The impacts may originate from inadequate error filtering processes (Edgar 2017), inaccurate taxonomic annotation (Anslan *et al.* 2018) or inappropriate clustering methods in the specific case of diatoms (Tapolczai *et al.* 2019). Towards standardization of analyzing short (312 bp) rbcL metabarcoding

data of diatoms for biomonitoring purposes, the studies of Tapolczai, Keck, Bouchez, Rimet and Vasselon (2019) and Rivera, Vasselon, Bouchez and Rimet (2020) found that individual sequence units (ISU) approach tend to outperform operational taxonomic units (OTU) based approaches. Nevertheless, furthest neighbor OTU clustering and ESVs approach showed to perform equally well (Rivera, Vasselon, Bouchez & Rimet 2020). Our study also resulted in highly similar results across three applied pipelines (ESVs *vs* . two OTU approaches), which demonstrates that the appropriate filtering of erroneous sequences and critical taxonomic assignment of the target taxa may be a key step, with the potential of mitigating the otherwise considerable effect of bioinformatics.

Taxonomic composition

Because different data sets in this study included ESVs, OTUs or morphospecies, the direct comparisons of their taxonomic unity were performed at the genus level. In this study, metabarcoding results from 10 g and 0.5 g of DNA extracts exhibited highly concurring taxonomic composition, with only few mismatched taxa, which were represented by a low relative abundance of reads. In accordance with the community analyses, this indicates sample-size independent patterns when detecting diatoms via metabarcoding from lake sediments. However, comparisons between microscopy and metabarcoding data resulted in a higher number of mismatched taxa (Fig. 4). Not completely matching identifications from microscopy *vs* . metabarcoding have been reported in several previous diatom-related studies (e.g. Visco *et al.* 2015; Rivera*et al.* 2018; Tapolczai *et al.* 2019), with the possible reasons discussed within. One of the main reasons of such mismatches is the incompleteness of the reference sequence databases, which consists of a limited number of annotated taxa. For example, *Sichuaniellalacustris* , discovered only by morphological analyses, is the unique representative of the genus *Sichuaniella,* which was originally described from Sichuan Province on the southeastern Tibetan Plateau (Li, Lange-Bertalot & Metzeltin 2013) and has no genetic information in the public databases. Therefore, the identity of this species in the metabarcoding data set cannot be confirmed. Additionally, there are no reference sequences for genera such as *Platessa* ,*Odontidium* and *Gomphosinica* in the public databases.*Gomphosinica* has been separated from *Gomphonema* and described as a new genus based only on their morphological differences (Kociolek, You, Wang & Liu 2015). Thus, *Gomphosinica* in the microscopy data set could potentially be represented as*Gomphonema* in the metabarcoding data set.

The inter-investigator variation depending on changes in diatom taxonomy and the use of synonym names could add additional layers for the mismatches between microscopy and metabarcoding data. In this study, it is difficult to consistently separate *Staurosirella* and*Pseudostaurosira* (missing from metabarcoding data) from*Staurosira* (present in metabarcoding data) under the light microscope and even with support from SEM images. Although*Pseudostaurosira* was one of the most abundant genera in the microscopy data, it was missing from the metabarcoding inventories, whereas the relative abundance of *Staurosira* was high in latter data sets (Table S3). Medlin, Yang and Sato (2012) have pointed out that the molecular separation of *Pseudostaurosira* and*Staurosirella* from *Staurosira* is arguable. On the other hand, in the few studies that have attempted to merge morphological- and molecular-based phylogenies of the Fragilariaceae, the morphological characterization is often poorly done (Morales *et al.* 2019). We speculate that morphologically identified *Pseudostaurosira*(especially *Pseudostaurosira brevistriata*) corresponds to*Staurosira* in the metabarcoding data, as their presence-absence patterns in our sediment samples correlates well (Table S3). Furthermore, identification of *Pseudostaurosira* in the metabarcoding data sets was also limited due to a fact that almost all originally named *Pseudostaurosira* were re-assigned to*Staurosira* in the curated R-Syst diatom database (Rimet *et al.* 2016).

The majority of other missing genera from metabarcoding data sets were represented in very low abundances in the microscopy data. Similarly, Kermarrec *et al.* (2013) reported that morphologically identified low abundance taxa ($< 1\%$ from 450 valve counts) were often not detected in the DNA metabarcoding data set. These low abundance taxa may indicate the transport of diatom valves with highly degraded DNA from other locations (thus non-detectable with herein used primers). On the other hand, environmental DNA could be carried along large distances (Deiner & Altermatt 2014), which also could contribute to the observed 'extra' diatom taxa in metabarcoding data sets, which were not detected via microscopy. More-

over, some of the diatom taxa with fragile and weakly silicified valves, such as *Cylindrotheca* ,*Entomoneis* , *Fistulifera* , *Reimeria* ,*Seminavis* , that were detected only in the metabarcoding data sets, might be sensitive to the chemical treatment (e.g. HCl and H_2O_2) during sample preparation for microscopy. Based on personal observations of water samples from Nam Co, we have confirmed the presence of several *Entomoneis* and*Fistulifera* species (data not shown), which further supports the assumption that valves of fragile diatom species may be more prone to dissolution and therefore undetectable in sediment samples. Thus, incompleteness of the reference databases, together with the continuously changing diatoms classification system and DNA transportation characterizations contribute to at least some extent to the issue of non-matching taxa between microscopy and metabarcoding results.

Perspectives

Assemblages of diatom communities reflect environmental parameters (Dixit, Smol, Kingston & Charles 1992), and therefore they are widely used as paleo-ecological indicators of lake ecosystems (Douglas & Smol 2010). We found that the diatom communities within sediment samples from Nam Co can be related to the same environmental variables for both, the morphological and metabarcoding data sets, which is in accordance with the study by Dulias, Stoof-Leichsenring, Pestryakova and Herzschuh (2017). This suggests that inferring (paleo-) environmental characteristics via, for example, diatom-based transfer functions would produce similar results using either method, where the high-throughput nature of metabarcoding analyses, however, enables simultaneous processing of much larger numbers of samples in a time-effective manner. Although not tested here, the additional 'fine-tuning' of the metabarcoding data with e.g. quantification correction factors or including phylogeny of the OTUs has been suggested to further improve the (biomass) correlations between microscopy and metabarcoding results (Vasselon *et al.* 2018; Mortagua *et al.* 2019) as well as boost the applicability of the latter for biomonitoring purposes (Keck, Vasselon, Rimet, Bouchez & Kahlert 2018). Because of the incompleteness of available DNA barcode databases, taxonomy-independent methods for molecular taxa are another promising advancement towards the applicability of metabarcoding in environmental surveys (Apotheloz-Perret-Gentil *et al.* 2017; Tapolczai *et al.*2019). Moreover, when the preservation of diatom valves is poor, as for example in saline, high pH lakes with low sediment accumulation rates (Flower 1993), DNA may still preserve in sediments as for example has been demonstrated by studying 'non-fossilizing' phytoplankton by means of sedimentary ancient DNA (Li *et al.* 2016). The strong similarity of our metabarcoding results from 10 g and 0.5 g of DNA extracts implies that 'small' DNA isolation kits (for ˜0.5 g) may serve as an alternative approach when the amount of sediments is limited in sedimentary ancient DNA (sedaDNA) studies.

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Data accessibility

Illumina data sets have been deposited in the Sequence Read Archive (SRA) under BioProject PRJNA558871.

Authors' contributions

W.K., N.B. and A. Schwarz performed sampling. R.S. and S.A. performed molecular analyses, S.K. ran high-throughput sequencing, S.A. processed the sequence data. W.K. and P.R. worked on morphological analyses of the diatoms. S.A. and W.K. analyzed the data and performed statistical analyzes. A. Schwalb developed the program (DFG-GRK 2309) that funded this research. S.A. prepared the manuscript with the contributions from all authors. All authors approved the final version.

Competing interests

The authors declare no competing interests.

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Tables

Table 1 . Number of ESVs/OTUs/morphospecies per sample for metabarcoding and microscopy data. TOTAL11 denotes the sum of ESVs/OTUs/species for 11 comparable samples of all data. TOTAL 20 denotes the sum of ESVs/OTUs for 20 comparable samples of metabarcoding data. N.a denotes 'not available' data; thus the last three samples were not included to HTS 10 *vs* . HTS 0.5 comparisons, and the analyses of microscopy *vs* . HTS treatments were conducted using corresponding samples with available data.

Sample	ESVs (HTS10/HTS0.5)	95% OTUs (HTS10/HTS0.5)	97% OTUs (HTS10/HTS0.5)	Micro
1	134/125	73/76	53/49	32
2	107/39	63/32	53/23	41
3	127/112	71/69	55/50	42
4	273/261	145/137	120/128	n.a
5	60/88	45/59	36/48	n.a
6	101/133	70/77	52/60	n.a
7	38/28	27/26	18/14	28
8	219/273	117/136	103/118	n.a
9	221/251	116/122	103/102	n.a
10	273/272	131/133	115/120	n.a
11	182/169	111/100	101/91	n.a
12	137/139	79/81	67/64	n.a
13	82/81	53/57	47/48	n.a
14	187/193	101/100	82/79	41
15	41/69	32/46	20/28	38
16	102/29	64/21	54/13	34
17	185/175	105/98	81/82	43
18	73/87	53/66	47/52	46
19	139/110	86/78	64/53	41
20	53/68	37/44	29/35	57
21	92/n.a	59/n.a	44/n.a	42
22	50/n.a	41/n.a	34/n.a	44
23	11/n.a	64/n.a	46/n.a	28
TOTAL 11	**474/433**	**175/178**	**165/161**	**189**
TOTAL 20	**1011/1018**	**297/307**	**306/318**	n.a

Table 2 . Mantel test and Procrustes analyses results between all treatments. HTS 10 and HTS 0.5 represent metabarcoding data from 10 grams and 0.5 grams of sediments, respectively.

Treatment 1	Treatment 2	Mantel R	Mantel P value	Procrustes correlation	Procrustes P
95% OTUs HTS 10	95% OTUs HTS 0.5	0.9688	<0.001	0.688	<0.001
95% OTUs HTS 0.5	ESVs HTS 0.5	0.936	<0.001	0.933	<0.001
97% OTUs HTS 0.5	95% OTUs HTS 0.5	0.96	<0.001	0.831	<0.001
97% OTUs HTS 0.5	ESVs HTS 0.5	0.902	<0.001	0.888	<0.001
ESVs HTS 10	ESVs HTS 0.5	0.951	<0.001	0.969	<0.001
ESVs HTS 10	95% OTUs HTS 0.5	0.918	<0.001	0.929	<0.001
ESVs HTS 10	97% OTUs HTS 0.5	0.875	<0.001	0.821	<0.001
95% OTUs HTS 10	ESVs HTS 10	0.949	<0.001	0.777	<0.001
95% OTUs HTS 10	97% OTUs HTS 0.5	0.935	<0.001	0.689	<0.001
95% OTUs HTS 10	ESVs HTS 0.5	0.914	<0.001	0.705	<0.001
97% OTUs HTS 10	95% OTUs HTS 10	0.9686	<0.001	0.827	<0.001
97% OTUs HTS 10	97% OTUs HTS 0.5	0.937	<0.001	0.586	0.0015
97% OTUs HTS 10	95% OTUs HTS 0.5	0.934	<0.001	0.766	<0.001
97% OTUs HTS 10	ESVs HTS 10	0.901	<0.001	0.756	<0.001
97% OTUs HTS 10	ESVs HTS 0.5	0.852	<0.001	0.71	<0.001
Microscopy	ESVs HTS 0.5	0.865	<0.001	0.818	0.003
Microscopy	ESVs HTS 10	0.800	<0.001	0.777	0.001
Microscopy	95% OTUs HTS 0.5	0.889	<0.001	0.813	0.002
Microscopy	95% OTUs HTS 10	0.893	<0.001	0.681	0.007

Treatment 1	Treatment 2	Mantel R	Mantel P value	Procrustes correlation	Procrustes P
Microscopy	97% OTUs HTS 0.5	0.879	<0.001	0.788	0.007
Microscopy	97% OTUs HTS 10	0.835	<0.001	0.684	0.008

Figure legends

Figure 1 . Illustration of the study design. HTS 10 and HTS 0.5 represent 10 g and 0.5 g (wet weight) treatments for metabarcoding. Sequencing data from metabarcoding were subjected to three different bioinformatics pipelines. Morphological analyses of the diatoms included light microscopy (analyses of 400 valves) and scanning electron microscopy (SEM).

Figure 2. a-c) scatterplots between per-sample richness of taxonomic units from HTS 10 and HTS 0.5 treatments. d) box plot for the number of ESVs/OTUs/morphospecies (log transformed) per treatment. Different letters above the whiskers indicate significant differences according to Kruskal-Wallis pairwise test.

Figure 3. Venn diagrams of the diatom genera relations between HTS 10 and HTS 0.5 treatments (20 corresponding samples).

Figure 4. Venn diagrams of the diatom genera relations between treatments of HTS 10, HTS 0.5 and microscopy (11 corresponding samples).

Figure 5. Mantel correlation plots between HTS 10 and HTS 0.5 treatments.

Figure 6. Mantel correlation plots between microscopy data and metabarcoding treatments (HTS 10 and HTS0.5).

Figure 7. Ordination plots of diatom communities for all treatments. a-d) plots for corresponding 11 samples between microscopy and HTS 0.5 data; e-h) plots for corresponding 14 samples between microscopy and HTS 10 data. Vectors on the plots denote most important variables as based on Random Forest modelling and DistLM marginal tests. Length of the vector represents the importance of variable.

Supplementary material

Figure S1. Scatterplots between per-sample richness of taxonomic units from all metabarcoding treatments.

Figure S2. Venn diagrams of the diatom genera relations between pipelines (ESVs, 97% OTUs, 95% OTUs).

Figure S3 . Scatterplots between per-sample richness of taxonomic units from all metabarcoding treatments and microscopy.

Figure S4 . Scatterplots between relative abundance of genera from metabarcoding treatments and microscopy. Relative abundance is calculated as based on the number of sequences and valves of the matching genera between metabarcoding and microscopy data sets, respectively.

Figure S5 . Most important community assembly variables as selected by Random Forest analyses. Graphs a-d) represent analyses results for 11 corresponding samples from microscopy and HTS 0.5 data sets. Graphs from e) to h) represent 14 corresponding samples from microscopy and HTS 10 data sets.

Table S1. Sediment samples used in this study, and their measured environmental parameters.

Table S2. Primer used in this study. PCR primers = adapter+tag+pad+link+primer.

Table S3. ESV/OTU (and genus level) occurrence tables per samples for sequence data, and species occurrence table for microscopy data.

Table S4. Marginal tests for all data sets. Included variables are the ones selected as most important by Random Forest analyses.