Metabarcoding successfully tracks temporal changes in eukaryotic communities in coastal sediments

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Abstract

Metabarcoding is a method that combines high-throughput DNA sequencing and DNA based identification. Previously, this method has been successfully used to target spatial variation of eukaryote communities in marine sediments, however, the temporal changes in these communities remain understudied. Here, we follow the temporal changes of the eukaryote communities in Baltic Sea surface sediments collected from two coastal localities during three seasons of two consecutive years. Our study reveals that the structure of the sediment eukaryotic ecosystem was primarily driven by annual and seasonal changes in prevailing environmental conditions, whereas spatial variation was a less significant factor in explaining the variance in eukaryotic communities over time. Therefore, our data suggests that shifts in regional climate regime or large-scale changes in the environment are the overdriving factors in shaping the coastal eukaryotic sediment ecosystems rather than small-scale changes in local environmental conditions or heterogeneity in ecosystem structure. More studies targeting temporal changes are needed to further understand the long-term trends in ecosystem stability and response to climate change. Furthermore, this work contributes to the recent efforts in developing metabarcoding applications for environmental biomonitoring, proving a comprehensive option for traditional monitoring approaches.

Introduction

Metabarcoding has expanded our knowledge of the eukaryote community composition and diversity across marine habitats (e.g. Park et al. 2008, Massana et al. 2015, Forster et al. 2016). However, in general the benthic realm has received much less attention than the marine pelagic environments, even though the eukaryotes in sediments form complex and diverse assemblages (Bik et al. 2011, Forster et al. 2016, Kim et al. 2016) and respond to environmental change (e.g. Chariton et al. 2015, Zhang et al. 2018). In addition, the existing metabarcoding studies have focused more on the spatial diversity of benthic eukaryotes (e.g. Bik et al. 2011, Aylagas et al. 2016, Brannock et al. 2018) and so far metabarcoding application to track temporal changes in sediment eukaryote communities remains understudied, yet it could provide useful data for biomonitoring and environmental assessment applications.

Traditionally benthic biomonitoring has been based on morphological assessment of macrofauna (≥ 0.5 mm fraction), and many of the existing biodiversity indices used in biomonitoring are based on macrobenthos (e.g. Diaz et al. 2004). Including meiofauna (e.g. small metazoans) would increase the accuracy of monitoring, due to its high diversity and the fast response to anthropogenic impacts (Kennedy and Jacoby, 1999). However, such an approach is often neglected due to a number of practical reasons. For example, morphological environmental biomonitoring is already time-consuming, expensive and requires skilled taxonomic expertise. Inclusion of traditional meiobenthos approach to monitoring practices would make the work even more laborious and costly. Metabarcoding, however, has the advantage of being able to target macrofauna along with smaller eukaryotes (< 0.5 mm), as well as being cost-efficient, time-saving and readily applicable (Aylagas et al. 2018). Furthermore, recent studies suggest that metabarcoding can perform well as an environmental assessment tool (Lejzerowicz et al. 2015, Piredda et al. 2016, Aylagas et al. 2016, Lanzén et al. 2016, Aylagas et al. 2018) and it has been successfully applied to identify sediment eukaryote composition in a wide range of marine environments, such as the Norwegian continental shelf (Lanzén et al.2016), sandy beaches at the coast of China and USA (Zhang et al.2018) and marine sediments from shallow to deep waters in the Atlantic and the Pacific (Bik et al. 2011).

The use of metabarcoding in biomonitoring may be especially useful in environments, such as the Western Gulf of Finland, Baltic Sea, which are characterized by natural low biodiversity due to brackish waters (e.g. Bonsdorff, 2006, Ininbergs et al. 2015). The macrofaunal assemblages of the Baltic Sea have been intensively studied morphologically, whereas the general eukaryote diversity and community composition in sediments via metabarcoding has to our knowledge received no attention to date. In the Baltic Sea water column, however, Hu et al. (2016) showed that metabarcoding can be used to track spatial changes in eukaryote communities across salinity gradients, and even detect taxonomic groups previously un-observed in the Baltic Sea. In addition, sediment bacterial communities in the Baltic Sea have been shown to vary seasonally and annually (Vetterli et al. 2015). Yet, when it comes to eukaryote communities in sediments, the potential of metabarcoding remains until now unexplored.

One of the crucial steps in metabarcoding studies is the choice of the targeted region, since this may affect the community composition obtained (Dunthorn et al. 2012, Aylagas et al.2016, Giner et al.2016, Piredda et

al. 2016, Tragin et al. 2018). In the 18S rRNA gene, the short (around 150 bp) hypervariable region V9 is one of the most commonly targeted and thus well-represented in reference databases (Amaral-Zetterel et al. 2009). Improvements in high-throughput sequencing technologies allow for bigger amplicon sizes, thus the use of the longer V4 region of 18 S rRNA gene is continuously increasing (van Dijk et al. 2014). Metabarcoding studies comparing both of these regions have been conducted (e.g. Dunthorn et al. 2012, Piredda et al. 2016, Giner et al. 2016, Tragin et al. 2018) but, so far, a consensus of the most suitable 18S hypervariable region remains a matter of debate.

In this study, a metabarcoding approach, targeting both the V4 and V9 hypervariable regions of the 18S rRNA gene, is used to investigate the eukaryote communities in two localities from the Western Gulf of Finland, Baltic Sea, coastal sediments over a period of two years. The aim of the study is to identify temporal changes at these two localities and evaluate the overdriving factors in shaping the sediment eukaryotic communities through time. In addition, we demonstrate the potential use of the metabarcoding approach for environmental assessment in a coastal settings.

Materials and methods

Study site and sampling

The samples were collected from two sites, located less than one kilometer apart from each other near Tvärminne Zoological Station, on the Finnish coast of the Gulf of Finland, the Baltic Sea (Vetterli et al. 2015). Storfjärden sampling site is a muddy accumulation basin (59˚51.310’ N, 23˚18.810’ E) with a depth of 33m. In contrast, Muncken is a sandier, shallow (11 m) transportation channel (59˚51.140’ N, 23˚14.700’ E). The samples were collected three times a year during two consecutive years, in 2008 (April, August and November) and 2009 (April, August and December). From here on, we refer to the sampling seasons as spring, summer and winter. Intact, undisturbed sediment cores were retrieved with a Gemax twin corer. Surface sediment (0.0-0.5 cm) was collected for molecular analysis and immediately after sampling frozen at -70˚C. Several replicate cores were taken at each sampling moment. Salinity and temperature were measured using a conductivity, temperature and depth device (CTD). In addition to molecular samples, surface

sediments were sampled for organic matter content and bottom water samples were collected from 5 cm above the sediment surface for measurements of dissolved oxygen, ammonium and nitrate. Sedimentary organic matter content was measured as loss on ignition (LOI) and diffusive oxygen utilization (DOU) in bottom waters was inferred from triplicate oxygen micro sensor profiles (Jäntti et al. 2011, Vetterli et al.2015). For additional details on the sampling protocols and site descriptions, see Jäntti et al. 2011 and Vetterli et al. 2015.

DNA extraction and amplification

DNA was extracted from 0.25 g of sediment with the MoBio Powerkit for soil (MoBio, Carlsbad, Germany) according to the manufacturer's instructions. Three replicate DNA extractions were done for each sample moment, all of which were taken from different replicate cores, with the exception of summer 2008 sample from Storfjärden in both datasets and winter 2009 sample from Storfjärden in the V4 dataset, which had only 2 replicates available.

Two sets of primers were used for DNA amplification, targeting either the V4 (Comeau et al. 2011 and Hugerth et al. 2014) or the V9 (Amaral-Zettler et al.2009) region of the 18S rRNA gene (Supplementary information Table S1). Both forward and reverse primers were modified at the 5' end to include overhang sequences for the downstream sequencing. DNA was amplified with Polymerase Chain Reaction (PCR) using Phusion Mastermix (ThermoFisher) according to manufacturer's instructions. Duplicate PCR products in equal volumes were pooled after amplification and quality-checked with agarose gel electrophoresis.

Negative controls were made for the extraction kit used (to test for contamination in the kit reagents) and for all the PCR reactions (to test the contamination in the PCR master mix). All negative controls except one in the V9 dataset did not show a product on agarose gel electrophoresis. The negative control that was visible in gel electrophoresis was subsequently sent for sequencing and analyzed. It contained 110 reads assembled in 33 OTUs (< 0.1 % of reads in an average sample in the V9 dataset). The OTUs of the negative control were removed from the final V9 dataset. Furthermore, negative controls were made during the PCR purifications and attachment of barcodes during the MiSeq library preparations. The V9 sequencing control consisted of 3

OTUs with > 100 reads which were present in low numbers in the actual samples, and they were removed from the dataset.

Sequencing and sequence analysis

Samples were sequenced in the Laboratory of DNA sequencing and Genomics in the Institute of Biotechnology at the University of Helsinki (http://www.biocenter.helsinki.fi/bi/dnagen/index.htm). PCR products were purified prior to sequencing and custom barcodes for later sample de-multiplexing were attached in a second PCR reaction. Samples were sequenced on the Illumina MiSeq platform. Raw reads were grouped into samples and primers, MiSeq overhangs and barcode sequences removed. Sequences were assembled to paired-end reads and quality-filtered in Mothur version 1.36.1 (Schloss et al. 2009). Maximum length was set to 349 and 150 base pairs (bp) in the V4 and V9 datasets, respectively. No ambiguous sequences were allowed and maximum number of homopolymers was set to 8. Quality filtered reads were aligned against the SILVA database (release 132) and chimeric sequences were removed in Mothur with the UCHIME tool (version 4.2.40, Edgar et al. 2011). Operational taxonomic units (OTUs) were created using 95% similarity as threshold (as suggested by Caron et al. 2009). Taxonomic classification of OTUs was performed in Mothur against the SILVA database. The distance matrix created at the OTU generation stage was used to define representative sequences for each OTU, by selecting the reads with the smallest maximum distance to other sequences. In case of a tie, the read with the smallest average distance was selected. Sequence data is available in the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra/docs/) under BioProject accession number PRJNA459491.

Before further analysis, we removed taxa (OTUs) likely to create noise to our dataset, e.g. OTUs that are unlikely to provide any useful information because they are very rare in our samples or they contain only a small amount of reads (singletons, doubletons etc.). Additionally, we reasoned that the presence of OTUs with low amounts of reads may be the result of the clustering process rather than the presence of real unique/rare taxa, thus leading to overestimates of the community diversity. In order to determine a filtering threshold, the total counts (total number of observations of an OTU across all samples) in both datasets were calculated. This indicated that many of the OTUs obtained contain only a small amount of the total reads, whereas the majority of reads are spread across a few OTUs. Subsequently, the cumulative sum of OTUs that

would be filtered was plotted against the total counts (Supplementary information Fig. S1), which plateaus at about total counts 50 in both datasets. In order to be as inclusive as possible, a lower filtering threshold of >24 reads per OTUs was applied for both datasets. This means that by excluding OTUs observed less than 25 times across our samples, we excluded 2 872 OTUs in the V4 dataset and 5 148 in the V9, consisting only of < 1% of the total reads.

Statistical analysis

Statistical analysis was conducted in R (version 3.4.2, 2017-09-28). Alpha diversity and rarefaction analysis were performed using the package Vegan (version 2.4-5, Oksanen et al. 2017). Principal coordinate analysis (PCoA) and non-metric multidimensional scaling analysis (NMDS) were computed with the package Phyloseq (version 1.22.3, McMurdie and Holmes, 2013). The analysis was based on the weighted Unifrac metric with Bray-Curtis distance, including a phylogenetic tree built in Mothur. Canonical correspondence analysis (CCA) was conducted using the Phyloseq package and including the variables of year, season and site and the environmental parameters, including bottom water NH_4^+, salinity and temperature, diffusive oxygen utilization (DOU) and sediment organic matter content based on loss on ignition (LOI). Significance of these variables was determined using the ANOVA function in Vegan (Oksanen et al. 2017).

Results

The number of total sequence reads before / after quality filtering was 11 237 993 / 7 708 041 in the V4 dataset, and 6 409 150 / 4 169 688 in the V9 dataset. UCHIME (Edgar et al. 2011) removed 3.4 % of the V4 and 0.03% of the V9 sequences. Clustering at 95% similarity produced 3717 operational taxonomic units (OTUs) using the V4 region and 5 194 OTUs using the V9 region. After OTUs with <25 reads were removed (Supplementary information Fig. S1), the V4 dataset contained 885 OTUs (retaining 99.7% of total reads in the dataset) and the V9 dataset contained 613 OTUs (retaining 99.7% of total reads in the dataset).

Community structure and diversity

The majority of samples the in the V9 dataset reached a satisfactory sequencing depth, as indicated by the leveling rarefaction curves (Fig. 1). In the V4 dataset, 7 replicates (all from year 2009) were relatively low in the number of reads (Fig. 1). Some variation was also seen in the distribution of these replicates in non-metric multidimensional scaling (NMDS) analysis of the V4 dataset, which shows that the replicates from 2008 generally plot closer to one another than the replicates from 2009 (Supplementary information, Fig. S3a). However, replicate was found to be significant parameter for all samples (PERMANOVA, p=0.001), even when V4 samples from the year 2009 were analyzed separately (PERMANOVA, p=0.049). Therefore, all replicates were included in the subsequent analysis.

The V4 region targeted 60 eukaryote classes, and the V9 region 68 (Supplementary information Table S3). The most dominant class in all samples was on average Dinophyceae (70% V4, 42.6% V9), of which the genus *Biecheleria* accounted for 87.5% in the V4 dataset and 74.4% in the V9 dataset (Supplementary information Fig. S2). Other relatively abundant classes were Maxillopoda (average 12.9% V4, 31.6% V9), and Diatomea (average 6.4% V4, 17.9% V9) (Fig. 2). In the year 2008, Dinophyceae had 89.5% relative abundance on average across all seasons and both sites in the V4 dataset (Fig. 2a), and 67.9% in the V9 dataset (Fig. 2b). Diatomea was the second most abundant class (average 3% in V4 dataset (Fig. 2a), and 16.7% in V9 dataset (Fig. 2b). In the year 2009, based on V4 region, the class Dinophyceae was still the relatively most abundant class in Muncken and Storfjärden (48.1% average of all samples, Fig. 2a). However, based on the V9 region (Fig. 2b), the most relatively abundant class was Maxillopoda, with the average of 57.7% in all 2009 samples. Other differences between the primers observed at the class level were noticed in classes, such as Ostracoda, Perkinsidae and Ulvophyceae (Fig. 2a), which were more clearly targeted by V4 region and only observed in low (<1%) relative abundance in the V9 dataset. In V9 dataset, fungal class Agaricomycetes and metazoan class Chromadorea were more abundant than in the V4 dataset (Fig. 2b).

Altogether 101 eukaryote orders were found using the V4 region, and 112 using the V9 region (Supplementary information Table S3). The most relatively abundant order was Gymnodiniphycidae, which accounted for 61.8% of the V4 and 32.6% of the V9 dataset. Gymodiniphycidae was particularly common in the year 2008 (81.4% V4, 53.1% V9, Fig. 2c-d), with the greatest relative abundance in winter (84.6% in V4

dataset (Fig. 2c) and 66.4 % in V9 dataset (Fig. 2d), average of both sites). At order-level, V9 region was able to identify three dominant orders in the class Maxillopoda, namely Harpacticoida (25.5% average in all samples, Fig. 2d), Calanoida (5.7%) and Cyclopoida (0.5%), whereas the V4 region could not resolve the lower taxonomic levels of Maxillopoda (mentioned as "unclassified Maxillopoda", Fig. 2c). In total 4.9% of all reads were classified as "unclassified eukaryotes" when using V4 region. Using V9 region none of the reads were classified as "unclassified".

Overall the Shannon diversity index (H') and Species richness estimate (S') were higher in average of all samples in the V9 dataset than in the V4 (Fig. 3). The general tendency in both of these diversity indices suggests that the average diversity was overall higher in summer (median H': 1.3 V4, 2.2 V9; S': 200 V4, 315 V9) than in winter (median H': 1.1 V4, 1.4 V9; S': 194 V4, 280 V9) or in spring (median H': 1.2 V4, 1.6 V9; S': 199 V4, 271 V9). In V4 dataset exceptions to the medians were seen in 2008, where in Storfjärden both S' and H' indices were higher in spring and winter in Storfjärden, and in Muncken S' index was highest in winter. In the V9 dataset the S' index of the year 2008 was also higher in winter and spring than in summer at site Storfjörden (Fig. 3). Pielou's evenness values in the V9 dataset were also greater in summer compared with other seasons (median 0.4) with highest values recorded in Storfjärden in summer 2009 (0.50), but in the V4 dataset the median for all season was the same (0.2) (Fig. 3).

Community response to temporal environmental changes

Principal coordinate analysis (PCoA) based on Unifrac metric revealed that most of the observed community variance, in both the V4 and V9 datasets, can be explained by the year (Fig. 4). Differences between the two years are mainly highlighted by the separation of 2008 and 2009 samples on the first axis, which explained 69.3% of the variance in the V4 dataset and 70.8% of the variance and in the V9 dataset (Fig. 4). The second axis of the PCoA plot appears to depict seasonal changes with summer samples clustering separately from winter samples, explaining 9% of the variance in the V4 dataset (Fig. 4a) and 10.7% of the variance in the V9 dataset (Fig. 4b).

The influence of temporal variation on eukaryotic sediment community was confirmed with canonical correspondence analysis (CCA), where 51.6% of the total observed community variance was explained by

constrained variables in the V4 dataset and 72.3% of the variance in the V9 dataset, respectively (Fig. 5). The most significant factor for the V4 dataset was year ($p < 0.001$) followed by season ($p < 0.004$). For the V9 dataset, the most significant factors were year, season, diffusive oxygen utilization (DOU) and loss on ignition (LOI, reflecting sediment organic matter content) ($p < 0.001$ for all), followed by site ($p < 0.002$).

Discussion

Temporal and environmental impact on eukaryote communities in sediments

Our data shows that in this study the driving factor for the observed eukaryote community variance was time, firstly the sampling year, followed by the season (Figs. 3, 4). The difference between the sampling years was observed as a change from a strongly phytoplankton (mainly dinoflagellate, class Dinophyceae) dominated community in 2008 to a more metazoan (class Maxillopoda) dominated community in 2009 (Fig. 2). However, this trend appears to be more evident in the V9 dataset then in the V4 dataset, which may be related to differences in the ability of the two regions to target various eukaryote groups (e.g. Giner et al. 2016, Piredda et al. 2016).

The temporal changes in the sediment eukaryote communities may be related to changes in prevailing weather/climatic conditions in the study region. Based on available Baltic Marine Environment Protection Commission – Helsinki Commission (HELCOM) monitoring data, the winter of 2007-2008 was warm and the ice cover season at the Baltic Sea started late and was exceptionally short (Vainio, 2008). By contrast, the ice season of 2008-2009 was colder (Finnish Meteorological Institute, http://en.ilmatieteenlaitos.fi/ice-winter-2008-2009). The ice-free conditions at the Gulf of Finland affected the community composition and the phytoplankton bloom biomass, which was higher-than-average especially in July in year 2008, whereas in 2009 the phytoplankton bloom was close to long-term average (Kaitala and Hällfors, 2008, 2009). The increase in phytoplankton blooms and subsequently the dominance of Dinophyceae in our 2008 dataset is possibly related to the warmer than average winter of 2007-2008, and the greater relative abundance of other classes, such as Maxillopoda, due to the smaller phytoplankton bloom in 2009. Because the observed

temporal changes are linked to larger-scale phenomena, the impact is seen equally prominent at both study sites.

In addition to annual changes, season was also a significant factor in explaining community variance in our dataset (Figs 3, 4). As seasonal changes equally affect both sampling sites, this further supports the idea that the large-scale temporal changes, rather spatial heterogeneity are the overdriving factors in shaping the sediment ecosystem structure. The seasonal changes were clearly captured in the relative abundance of different phytoplankton classes. The peak abundance of class Diatomea was typically observed in the spring in both years (Fig. 2), while Dinophyceae was found throughout the year 2008 and mainly during the summer in 2009. This is in accordance with the HELCOM monitoring data, which shows that the spring bloom succession is first dominated by diatoms followed by dinoflagellates (Kaitala and Hällfors 2008, 2009). Other seasonal differences were seen in the species diversity and richness, which were generally higher in summer than in spring and winter (Fig. 3). This is likely to be related to optimum environmental conditions, including high temperature and nutrient and organic matter availability (measured as LOI), yet with sufficient oxygen availability in bottom waters to sustain diverse eukaryotic communities (Supplementary information Table S2). Diversity was exceptionally high in the summer of 2009 at the site Storfjärden, which is also seen in our CCA analyses (Fig. 5) as these samples are clearly separated from the others. The diversity here may have been additionally influenced by an upwelling event, which occurred just before the summer sampling of 2009, and was recorded in the CTD data showing more than 10 degrees lower temperatures and increased salinity compared to sampling in 2008 (Vetterli et al.2015) (Supplementary information Table S2). The community structure in V9 dataset was also significantly influenced by the environmental parameters, DOU and LOI (Fig. 5). Similarly, Vetterli et al. (2015) showed that the bacterial communities, which were sampled simultaneously at the same sites, showed a comparable response to these same parameters.

The effect of seasonal and annual variations on sediment eukaryotic communities is generally understudied in comparison to spatial distribution and heterogeneity. However, as our findings demonstrate, the sediment eukaryote community structure is heavily dependent on sampling time and relatively large changes in the community structure may take place in response to changes in prevailing climatic conditions, i.e. temperature

and ice volume, even within a period of few years. In contrast, the spatial heterogeneity and variation in the community composition was less evident in our dataset and only visible in the V9 dataset (Fig. 5). Therefore, our results support the importance of temporal surveys, contributing to our understanding of prominent environmental changes in any given environment and allowing us to untangle a potential anthropogenic signal from more naturally occurring events. In addition, climatic and anthropogenic factors are known to cause environmental stress that has been documented to manifest as regime shifts in marine environments across the globe (DeYoung et al. 2008). To distinguish these shifts and evaluate their persistence in an environment, long-term temporal surveys are crucially needed.

Metabarcoding approach for environmental biomonitoring: advantages and recommendations

Our results support recent efforts in research (e.g. Chariton et al. 2015, Lejzerowicz et al. 2015, Aylagas et al. 2018), stating that metabarcoding has various assets compared to traditional morphology-based biomonitoring. Firstly, our datasets were dominated by meio- and microfauna, and inclusion of smaller size fraction of eukaryotes typically neglected in biomonitoring surveys increases the potential of detecting the temporal and environmental variations since higher diversity is captured. Compared to macrofaunal species richness, which is typically very low in the Gulf of Finland (e.g. 22 sub-littoral soft-sediment species, Bonsdorff 2006), the species richness estimate based on our metabarcoding approach is approximately 10-fold higher. Metabarcoding of surface sediment samples also enables simultaneous observations of both benthic and pelagic taxa, which allows linking benthic community observations to events occurring in the water column, such as the phytoplankton bloom magnitude and community composition. Therefore, we support the inclusion of non-metazoans in these types of studies as they provide useful information on temporal environmental variation (Lanzén et al. 2016). Additionally, metabarcoding may ensure taxonomic identification. For example, *Biecheleria baltica* co-exists in the Baltic Sea with *Scrippsiella hangoei* and they can only be identified from each other with molecular methods (Kremp et al. 2005). In our data, the majority of the reads in the class Dinophyceae fall into one OTU, similar to *Biecheleria* (Supplementary information Fig. S2), supporting the idea that *B. baltica* plays the major role in the *Scrippsiella*/*Biecheleria* complex in the Gulf of Finland (Sundström et al. 2010).

One advantage of metabarcoding, in comparison to traditional monitoring methods, is the small sediment sample that is easy to process and allows for replication. However, recent metabarcoding survey by Nascimento et al. (2018) suggested that the sediment sample size should be approximately 14 g instead of <1 g applied by many benthic surveys, including ours, to achieve sufficient beta diversity. Based on our data, even small (< 1 g) sample sizes are able to capture abundant single-celled eukaryotes comprehensively. However, as mentioned in Nascimento et al. (2018), larger metazoans are likely to have a more heterogenous distribution in sediment, and hence cause some variation between the replicates. This was also seen in our datasets where in 2009 the samples, which were more abundant with metazoan classes such as Maxillopoda, also had a bigger heterogeneity between replicates (Supplementary information, Fig. S3). Therefore, when targeting large metazoans or macrofauna, a bigger sample size may be advisable.

The choice of the targeted region has a potentially significant influence in metabarcoding surveys. The V4 and V9 regions of the 18S gene are two of the most commonly targeted in environmental surveys. V4 has the benefit of being the largest 18S region in eukaryotes with high variability, which makes it well suited to estimate genetic distances (Dunthorn et al. 2012). However, despite being much shorter, V9 has the advantage of capturing virtually all eukaryote phyla (Amaral-Zettler et al. 2009, Pawlowski et al. 2011). This is also seen in our study where estimated species richness was clearly higher in the V9 dataset than in the V4 (Fig. 3). Despite this, the two 18S regions targeted in this study were able to provide a relatively similar overview of the community composition through time (Fig. 2). Nevertheless, a considerable difference was observed in the ability of the V4 region in targeting the class Maxillopoda. The difference was most striking in 2009 summer samples, when the V9 samples indicated that the assemblage was dominated by Maxillopoda and the V4 by Dinophyceae. Furthermore, V9 was also able to identify different Maxillopoda orders (Fig. 2). This is consistent with previous studies, which showed that V9 region is able to better target and resolve the taxonomy of the class Maxillopoda than the V4 region (Wu et al. 2015, Tragin et al. 2018). In addition, the reference database used has potentially a big impact on the obtained eukaryote community. For example, benthic protist diversity is still largely undescribed, and thus it may lead to underestimation of such taxa (Forster et al. 2016). Compared to the V9 dataset, the V4 region gave a higher number of taxa identified as “unclassified”, which may be an issue related to available references in the database. However,

new metabarcoding data is continuously contributing to the existing databases, so the situation is likely to improve in the future. We conclude, that targeting the V4 region instead of V9 may be justifiable due to the larger size and greater variability of this region, which may help to tell closely related taxa apart from one another. However, as our data shows, despite the increasing use of the V4 region, it still fails to identify all eukaryote taxonomic groups. This is especially prominent when targeting the large and abundant class of Maxillopoda. Therefore, in environments where Maxillopoda contributes significantly to the eukaryotic community, the use of V9 target region is advisable.

Conclusions

Here, we demonstrate for the first time that 18S metabarcoding approach can be successfully applied to track temporal changes in sediment eukaryote communities resulting from shifts in regional climate regime or large-scale changes in the environment. These results have important implications for future metabarcoding-based monitoring programs. Firstly, based on the high significance of the seasonal and annual changes, long-term surveys are recommended. Recent metabarcoding studies have focused on spatial variations, providing us only with snap-shot views of environmental status of study locations. To gain a comprehensive perspective of the influence of the prevailing conditions on sediment eukaryotic composition, temporal trends must be taken into account. Secondly, monitoring programs should be carefully designed in respect to sample moment as the sediment eukaryotic communities show large seasonal changes in their composition. Therefore, the timing and frequency of the sampling strategy should reflect the monitoring aims. For example, if the focus is related to impact of coastal eutrophication on sediment community, the sampling should be systematically carried out towards the end of the growth season in order to capture the signal.

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