Changes in exported key phytoplankton taxa related to a warm anomaly in the Fram Strait inferred from three complementary 18S rRNA gene meta-barcoding primer sets

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Abstract

The Arctic pelagic environment is expected to strongly alter due to global climate change. As a consequence, modification of the unicellular plankton species composition and biomass, with consequences to biogeochemical cycling and pelagic food web, is expected. In this study we used meta-barcoding of the V4 region of the 18S rRNA gene to profile eukaryotic microbial communities exported to deeper water layers at the Long-Term Ecological Research Site HAUSGARTEN in the northeastern Fram Strait. We collected sinking particles at ca. 80 to 300 m depths using long-term deployed sediment traps and analyzed selected samples of spring and summer periods from 2000-2011. Acknowledging the limitations and biases of currently used 18S rRNA gene meta-barcoding primers in detecting certain taxa especially from environmental samples, we developed new primer sets and compared them with those already in use. Using the information generated by three different primer sets, the results of our study suggest decreasing trends in the abundances of large-cell phytoplankton (i.e., diatoms) and increasing pico-phytoplankton (Micromonas sp. and haptophytes) during the warm anomaly of 2005-2007. Phylogenetic analyses further revealed the displacement of cold-adapted with warm-adapted phylotypes of Micromonas and haptophytes, which could be related to the warming event. Ecotype-level changes observed in this study do not only suggest changing structures in community composition and ecosystem functioning but also in the biogeography and distribution of some species. These data provided new insights and information on the potential diversity changes and species displacement brought about by the environmental changes occurring in the Arctic Ocean.

Introduction

It is expected that global warming and the ensuing sea ice melt will strongly alter the Arctic pelagic environment. This could eventually result in a modification in the species composition and biomass of unicellular plankton, changing matter fluxes affecting the entire pelagic and even benthic systems (Wassmann, 2015). Thus, it is necessary to generate information on the temporal occurrences of planktonic species to also better understand their variability and responses towards different environmental conditions. Acknowledging this, in 1999, the Alfred-Wegener-Institute Helmholtz Centre for Polar and Marine Research established the 'Long-Term Ecological Research Site (LTER) HAUSGARTEN to carry out regular observations of the ecosystem in the eastern Fram Strait. This involves an extended sediment trap program based on annual deployments of moored sediment traps at key stations of the observation area (Soltwedel et al. 2005; 2016). Despite uncertainties related to the use of these tools, such as trapping efficiency, validity of results etc. (Butman 1986; Gust et al. 1994; Buesseler et al. 2007), they remain useful in gaining insights of vertical particle flux patterns. Moreover, the deployment of sediment traps facilitates an understanding of plankton dynamics in the upper water column all year-round, even in remote areas such as the Arctic Ocean.

Measurements of bulk parameters like sinking matter and its components combined with light microscopy can provide an estimate of the pelagic eukaryotic microbial community in the catchment area above the traps (Bauerfeind et al., 2009). Unfortunately, these assessments are mainly focused on larger organisms from the micro-plankton fraction, as surveys of small eukaryotic microbial species from the nano- and picoplankton fractions in sediment traps are almost impossible owing to their size and a simple morphology. For example, besides micro-eukaryotic stramenopiles (mainly diatoms), pico- and nano-eukaryotic Mamiellophyceae (mainly *Micromonas*) and haptophyceae (mainly*Phaeocystis* sp.) are key Arctic phytoplankton taxa. They are well known to be major contributors to phytoplankton communities and biomass (Lovejoy and Potvin, 2011; Metfies, von Appen, Kilias, Nicolaus, & Nothig, 2016), although their contributions to vertical flux and particle export are not well understood. Thus, it is particularly important to elucidate this black box, because changes in the abundance of these key phytoplankton taxa have been observed in the area of LTER HAUSGARTEN. These changes have been associated with a warm water anomaly from 2005 to 2007 (Beszczynska-Moller, Fahrbach, Schauer, & Hansen, 2012; Nöthig et al., 2015), while some of them even remained after the warm-water event. During this warm period, higher phytoplankton biomass was observed in the water column, eukaryotic microbial plankton >3 µm changed in composition, and diatoms that dominated the summer period before the warm period significantly decreased (Nöthig et al., 2015). In 2006, *Phaeocystis pouchetii* started to dominate the eukaryotic microbial community and remained prominent in the region since then, while diatom concentrations remained low and small flagellates increased in abundance (Nöthig et al., 2015).

Over the recent years, 18S rRNA gene meta-barcoding using high throughput sequencing (HTS) platforms has become an indispensable tool to generate cultivation-independent and in-depth information on the biodiversity and community composition of eukaryotic microbes, including all size fractions, dominant and rare taxa (de Vargas et al., 2015; Sunagawa et al., 2015). A considerable number of marine surveys have also taken advantage of ribosomal sequence information to broaden our understanding of protist diversity and community structure (e.g., Gescher et al., 2008; Metfies et al., 2010), and revolutionized the field of microbial ecology into a semi quantitative method that allowed testing and modeling of assemblages across time and space (e.g., Vernet et al., 2017), and even determining links between and among communities and/or functions (e.g., Lima-Mendez et al., 2015). The 18S rRNA V4 region is the most frequently used marker for this purpose. It is best suited for HTS-based surveillance of microbial eukaryotes (Dunthorn et al., 2014), as the combination of V4 with the V5 region provides the most detailed phylogenetic information on the 18S rRNA gene (Hugerth et al., 2014). However, different primer-evaluation studies also showed that most of the known primer sets amplifying the V4 region are limited in their potential to provide comprehensive biodiversity information as they discriminate against certain taxa (Bradley, Pinto, & Guest, 2016). Among these, the primers reported by Stoeck et al. (2010) have been widely used in assessing microbial eukaryotic biodiversity in different marine habitats including the Arctic region (e.g., Comeau et al., 2013; Hardge et al., 2017). Although a truthfully 'universal' primer that can amplify all known representative sequences has not yet been described, many efforts have been done to at least minimize or lessen potential PCR biases. Hugerth et al. (2014) for example has systematically designed primers that greatly performed *in silico* but were not tested using mock communities. Their results showed that the majority of primer sets were able to amplify most but not all taxonomic groups. This is important since our capacity to describe and predict patterns in the communities depend on our ability to amplify and detect most members of the community. Such task has become more challenging since many of the existing primers were primarily designed based on available sequences of known species, and accumulating evidence show that many previously unknown taxa are increasingly detected with more environmental surveys (Massana et al., 2011). Thus, there is a need to carefully select for primers to be used depending on the question being asked or the targeted taxonomic groups, or even use complementary primer sets to retrieve realistic information on microbial biodiversity in a sample.

This study aimed at elucidating changes in the composition of exported pelagic phytoplankton communities in response to a warm anomaly in Fram Strait from 2005-2007. The study is based on using a set of published and newly developed primer-sets considering their complementarity, limitations and advantages

deduced. We explored major taxonomic phytoplankton groups including the Chlorophyta, Haptophyta and Bacillariophyta, and their contribution to the exported eukaryotic microbial communities during the phases of maximum particulate organic carbon (POC) flux in spring and autumn from 2000-2011 in the area of LTER HAUSGARTEN. This study provides new insights on how primer biases could limit or improve our understanding of ecological dynamics of microbial communities and their contribution to carbon transport in the changing oceans, such as the Arctic.

Material and Methods

Primer selection

We designed two new reverse primers, 938iR (5'-GGCAAATGCTTTCGC-3', hereafter referred to as Wolf938) and 964iR (5'-ACTTTCGTTCTTGATYRR-3', hereafter referred to as Wolf964). The primer design put special emphasis on the coverage of Haptophyta, which are important contributors to Arctic eukaryotic microbial communities, and which were known to be underrepresented in previous meta-barcoding studies (Bradley et al., 2016). We combined the resulting new primers with the well-established universal forward primer 528iF (5'-GCGGTAATTCCAGCTCC-3') (Elwood, Olsen, & Sogin, 1985). For the design, we used the primer design function within the ARB software (Ludwig et al., 2004) tested against the SILVA reference database SSU Ref v.119 (Yilmaz et al., 2014). Primers were chosen to have the largest possible coverage across all phyla and to cover an amplicon within 450 bp. We then evaluated these new primers with the widely used primer pairs designed by Stoeck et al. (Stoeck et al., 2010) and Bradley et al. (Bradley et al., 2016) by performing*in silico* PCR using the SILVA TestPrime tool (Klindworth et al., 2013).

Mock community preparation

Twenty-two taxa including the most important planktonic primary producers (i.e., haptophytes, chlorophytes, bacillariophytes) with some focus on those taxa relevant in the Arctic were selected from culture collections (NCMA *ex* -CCMP or RCC). For details, see Supplementary Table S1. Five (5) ml of each culture were grown in 50 ml of fresh K-medium (Keller et al., 1987) for two weeks at 14°C and under light. Cultures were then filtered onto polycarbonate membrane filters (Millipore) with a pore size of 0.2 µm, which were stored at -20°C until extraction.

DNA was extracted with the NucleoSpin Plant II kit (Macherey-Nagel) following the manufacturer's protocol. A fragment of the 18S rRNA gene was amplified using the primer-set 82F (5'-GTGAAACTGCGAATGGCTCAT-3') (Šlapeta, Moreira, & López-García, 2005) and 1200R (5'-GGGCATCACAGACCTG-3') (Wolf, Kilias, & Metfies, 2014). The PCR mixtures contained 1 µl of DNA extract, 1 x HotMaster Taq Buffer containing 2.5 mM Mg^{2+}, 0.8 mM dNTP-mix, 0.2 mM of each Primer and 0.4 U of HotMaster Taq DNA polymerase in a final volume of 20 µl. Reaction conditions were as follows: an initial denaturation at 94°C for 3min, 30 cycles of denaturation at 94°C for 45 sec, annealing at 59°C for 1 min and extension at 72°C for 3 min, and a final extension at 72°C for 10 min. DNA concentration of each PCR product was measured using a Q-bit and converted to copy numbers / µl of PCR product. Equal amounts of 18S rRNA gene copy numbers from each culture were mixed together to form the mock community.

Environmental sample collection

Sinking particles including eukaryotic microbial cells were sampled by modified automatic Kiel sediment traps with a sampling area of 0.5 m^2 and coupled with 20 liquid-tight collector cups (Zeitzschel, Diekmann, & Uhlman, 1978; Kremling, Lentz, Zeitschel, Schulz-Bull, & Duinker, 1996). Here, we present results from the shallowest (˜200-300 m below sea surface) sediment traps at the central station (HG-IV) of the LTER observatory HAUSGARTEN" at ˜79° N, 4° E (water depth 2,550 m) (Figure 1). The trap samples analyzed in this study were collected during phases of maximum POC flux in spring and autumn. An electronic failure in the sediment trap resulted in data gap of data in 2003. The collector cups were filled with filtered sterile North Sea water. Salinity was adjusted with NaCl to 40 psu. The liquid in the collector cups (250 ml or 400 ml, depending on the sediment trap used) was spiked with mercury chloride (0.14% final

concentration). After recovery of the moorings, about a year later after deployment, the trap samples were refrigerated until further processing in the laboratory. Samples were split by a wet splitting procedure after manual removal of zooplankton (swimmers) > 0.5 mm under a dissecting microscope at a magnification of 20 and 50. Subsequent molecular analyses were based on 1/32 splits of the original sediment trap sample. We collected aliquot samples for DNA extraction by filtration of a split fraction from the original sample onto a 0.2 µm Isopore GTTP membrane filter (Millipore, Schwalbach, Germany). Filters were washed with sterile North Sea water (˜50 ml) to remove residual mercury chloride from the samples. The sterile sea water was applied and pumped over the filter while it was still kept in the filtration unit. Detailed information on the sediment trap collection, preservation, and sample preparation for DNA isolation have been reported previously (Metfies et al., 2017), while physico-chemical regimes during the sampling period have been reported elsewhere (Bauerfeind et al., 2009; Bauerfeind et al., 2015; Lalande, Bauerfeind, Nöthig, & Beszczynska-Möller, 2013).

DNA extraction, PCR and Sequencing

Isolation of genomic DNA from the samples was carried out using the NucleoSpin Plant Kit (Machery-Nagel, Germany) following the manufacturer's protocol. The resulting DNA-extracts were stored at -20 °C. DNA concentrations were determined using the Quantus Fluorometer (Promega, USA) according to the manufacturer's protocol. A total of 21 samples, which were amplified using the 3 different primer sets (total of 63 rDNA libraries) were processed and compared in this study (Supplementary Table S1). For Illumina Sequencing, a fragment of the 18S rDNA containing the hypervariable V4 region was amplified with the primer sets (i) Reuk454FWD1 (5'-CCAGCASCYGCGGTAATTCC-3') and ReukREV3 (5'- ACTTTCGTTCTTGAT-3') (Stoeck et al., 2010, hereafter referred to as Stoeck), (ii) Wolf938 and (iii) Wolf964. The mock community sample was also amplified with the primer set Reuk454FWD1 and V4r (Bradley et al., 2016).

All PCR reactions had a final volume of 25 µL containing 12.5 µl of KAPA HIFI Mix (Kapa Biosystems, Roche, Germany), 5 µl of each primer [1 µmol/L] and 2.5 µl DNA-template [˜5ng]. The DNA-template was a mix of equal volumes of genomic DNA isolated from the three different filter fractions. PCR amplification was performed in a thermal cycler (Eppendorf, Germany) with an initial denaturation (95 °C, 3 min) followed by 25 cycles of denaturation (95 °C, 30 sec), annealing (55 °C, 30 sec), and extension (72 °C, 30 sec) with a single final extension (72 °C, 5 min). The PCR products were purified from an agarose gel 1% [w/v] with the AMPure XP PCR purification kit (Beckman Coulter, Ing., USA) according to the manufacturer's protocol. Subsequent to purification the DNA concentrations in the samples was determined using the Quantus Fluorometer (Promega, USA). Subsequently, indices and sequencing adapters of the Nextera XT Index Kit (Illumina, USA) were attached in the course of the Index PCR. All PCRs had a final volume of 50 µL and contained 25 µl of KAPA HIFI Mix (Kapa Biosystems, Roche, Germany), 5 µl of each Nextera XT Index Primer [1 µmol/L], 5 µl DNA-template [˜5ng] and 10 µl PCR grade water. PCR amplification was performed in a thermal cycler (Eppendorf, Germany) with an initial denaturation (95 °C, 3 min) followed by 8 cycles of denaturation (95 °C, 30 sec), annealing (55 °C, 30 sec), and extension (72 °C, 30 sec) with a single final extension (72 °C, 5 min). Prior quantification of the PCR products with the Quantus Fluorometer (Promega, USA) for sequencing with the MiSeq-Sequencer (Illumina, USA), the final library was cleaned up using the AMPure XP PCR purification kit (Beckman Coulter, Ing., USA). Sequencing of the DNA-fragments was carried out with the MiSeq Reagent Kit V3 (2 x 300 bp) according to the manufacturer's protocol (Illumina, USA). Sequences generated in this study were deposited at the European Nucleotide Archive (ENA) with the accession number xxx (*accession number will be provided subsequent to acceptance of the manuscript*).

Sequence Processing

Raw sequences had an approximate length of ˜200 bp, which were quality trimmed using Trimmomatic (Bolger, Lohse, & Usadel, 2014) and scanned with a 4-base wide sliding window and cut when the average quality dropped below 15. For merging of paired-end reads, we used the script 'join-paired-ends' within the open-source bioinformatics pipeline Quantitative Insights into Microbial Ecology v.1.8.0 (QIIME; Caporaso et al., 2010) with a minimum read overlap of 20 bases. Further analysis was performed following an in-house developed pipeline (Stecher et al., 2016) also using QIIME v.1.8.0 (Caporaso et al., 2010). Briefly,

reads were quality-filtered according to recommended settings in Bokulich et al. (2013). Only sequences that fully matched the primer sequences at the beginning and end of the sequence, respectively, and which were between 200 and 500 bp in length were further processed. For chimera detection and clustering of sequences into OTUs, we used the QIIME workflow 'usearch.qf', which incorporates UCHIME (Edgar et al., 2011). Pre-clustered sequences were checked for chimeras (*de novo*and with Silva 119 SSU Ref NR). The remaining sequence set was clustered (*de novo*) into OTUs with a similarity threshold of 98%. All OTUs consisting of four or fewer sequences were removed. Quality filtered sequences were then classified using 'Mothur' (Schloss et al., 2009) against the Northern Microbial Eukaryote Database (Lovejoy et al., 2016), with a threshold confidence of (-c) 0.8. All metazoans, bryozoans, fungi and viridiplantae related sequences were removed. The number of reads per library was then rarefied at uniform depth of 9,081 based on the sample with the lowest count. Libraries with less than ca. 5,000 reads in at least one of the samples or primer set were removed in all datasets to ensure comparability.

Phylogenetic placement of short reads

To further compare the primer biases at the sequence level, we used the Rapid Axelerated Maximum Likelihood – Evolutionary Placement Algorithm (RAxML-EPA; Berger, Krompass, & Stamatakis, 2011) approach to ascertain the phylogenetic identities of the three abundant and important phytoplanktonic groups in the Arctic, namely Chlorophyta, Bacillariophyceae and Haptophyceae. To do this, phylogenetic reference trees were generated for each taxonomic group using Randomized Axelerated Maximum Likelihood (RAxML v.8; Stamatakis, 2014) based on literature (Lovejoy et al., 2007; Liu, Aris-Brosou, Probert, & de Vargas, 2010). HTS-generated OTUs were first searched against the NCBI GenBank using BLASTn to identify the best hits. Most of the highly similar sequences (> 98%, e-value 1e-10) were selected and aligned in MUSCLE (Edgar, 2010), with the alignment manually edited and trimmed in MEGA v.7.0 (Kumar, Stecher, & Tamura, 2016), and used to build robust clade-specific reference trees with RAxML v.8 using the GTRGAMMA model, run 1000 times (Stamatakis, 2014). Reference trees were generated separately for each taxonomic group. Short OTUs were then aligned with the reference sequences and mapped back onto the reference trees using the -n option in the EPA-RAxML v.8.2 (Berger et al., 2011). Trees generated from each primer pair were then visualized in Dendroscope v.3.0 (Huson & Scornavacca, 2012) and compared with each other.

Results

In silico primer evaluation and taxonomic biases

Results of *in silico* PCR using the four primer pairs Stoeck (Stoeck et al., 2010), Wolf938 and Wolf964 (this study), and Bradley (Bradley et al., 2016) revealed significant differences in the amplified taxa, indicating potential biases (Supplementary Figure S1). For comparison, we only focused on four major taxonomic groups/phyla that are important in the Arctic realm including Chlorophyta, Haptophyta, Dinoflagellata and Bacillariophyta (Kilias, Wolf, Eva-maria, & Peeken, 2013).

We observed that for Chlorophyta, all four primer pairs targeted between 80% and 90% of all possible chlorophytes included in the reference database. For Haptophyta, only Stoeck covered an average of 1.1%, while the other three primer pairs had over 90% amplification efficiency. For Dinoflagellata, all primer pairs showed a high coverage of over 80%. Lastly, the primer pair Wolf938 only amplified 1% of the bacillariophytes, whereas the Wolf964 covered of about 90%.

Observations *in silico* were evaluated *in situ* by PCR-amplification and subsequent sequencing of a mock community comprised of defined concentrations of 18S PCR fragments of 22 eukaryotic microbial species within 18 genera as a template. The relative abundances of these 18 genera in the mock community are shown in Figure 2. The same figure also shows the relative abundances of genera obtained from sequencing with the four different primer pairs. The primer pairs of Stoeck and Wolf938 showed significant overrepresentations of Dinoflagellata, while the Stoeck primer pair showed additionally a near absence of Haptophyta, and amplification with primer pair Wolf938 resulted in an underrepresentation of Bacillariophyta. The primer pairs Wolf964 and Bradley performed similarly and showed a realistic image of the mock community. Two genera (*Prasinoderma* and *Leptocylindrus*) were not detected by any of the primer pairs. The genera

Chrysochromulina and*Skeletonema* did not appear in the assemblages generated by the primer pairs Bradley and Wolf938.

Seasonal abundance of major phytoplankton groups in Fram Strait

Since the primer pairs of Bradley and Wolf938 performed similarly*in silico* and in mock community tests, we then only used the Wolf938 for subsequent investigations. Using the three primer sets (Wolf964, Stoeck, Wolf938), we generated 18S sequence-based community profiles of eukaryotic microbial communities in Fram Strait collected every spring and/or summer from 2001 to 2011 (except 2003) using sediment traps (Figure 3). Consistent with *in silico* and mock community tests, the three primer sets showed high variability in sequence abundances and composition. Based on relative abundances at higher taxonomic level (Phylum), the most striking differences mainly manifested on major phytoplanktonic groups, the most significant of which was in the Chlorophyta. For example, in both seasons across years, eukaryotic microbial sequence assemblages reflected by Wolf964 primers were mainly dominated by Stramenopiles (ca. 20%), specifically by diatoms, with the chlorophytes only averaging to ca. 2% to 6% in spring and summer, respectively. In contrast, chlorophytes (Mamiellophyceae, Chlorophyceae, Prasinophyceae, Trebouxiophyceae) were the most dominant taxa in the sequence assemblages obtained by both Stoeck and Wolf938, ranging from 35-42% in spring and both 47% in summer. This major difference in chlorophytes among the datasets could have resulted in the heterotrophic-mixotrophic taxa (i.e., Alevolates, Rhizaria) becoming more dominant in the sequence assemblages generated by Wolf964 primers. For example, large Rhizarian taxa was the third most abundant group in the Wolf964 primers especially during summer (29%), while it only ranged from 2-5% for both Stoeck and Wolf938 datasets across seasons. Interestingly, in contrast to results of *in silico* test, all primers were able to amplify sequences from Haptophyceae and with no significant differences in relative abundances (t-test, $p > 0.01$) across years and seasons (2-6%), majority of which were classified belonging to Phaeocystales.

Phylogenetic differences in OTU diversity

Since several studies have shown that different ecotypes exist especially in many phytoplankton groups in the Arctic (i.e., Lovejoy et al., 2007; Monier et al., 2013; Onda, Medrinal, Babin, Thaler, & Lovejoy, 2017; Joli et al., 2018), we thoroughly evaluated the OTU diversity amplified from the sediment trap samples by the three different primer pairs. Although all primer pairs were able to amplify sequences belonging to Chlorophyta, Bacillariophyta and Haptophyta, significant differences in OTU diversity were observed at the phylogenetic level using the EPA-RAxML approach.

Among the four classes of Chlorophyta, only Mamiellophyceae was amplified from all samples and dominated the groups across datasets. However, the Wolf964 sequences significantly differed in the phylogenetic placement of its HTS fragments compared to Stoeck and Wolf938 primer sets (Figure 4). Consistent with the comparison at higher taxonomic level, the Wolf964 dataset contained lower numbers of Mamiellophyceae related-OTUs (6 OTUs) compared to Stoeck (33 OTUs) and Wolf938 (47 OTUs) datasets (Figure 4A). Wolf964 also did not amplify the summer ecotype Clade C closest to CCMP1195, which on the other hand was abundant in Stoeck and Wolf938. Placements of some potential Clades B and C sequences in the Wolf964 library were also not well supported in the tree (Figure 4A). All of the three primer pairs however were able to amplify sequences from Clade Ea, most similar to the reference sequence CCMP2099 (*Micromonas polaris*).

For the Haptophyceae, although binned taxonomic abundance did not differ significantly across datasets (Figure 3), the number of OTUs identified and the classification at the lower taxonomic- and sequence-levels however differed significantly. Consistent with the *in silico*tests, Stoeck primers were only able to amplify 2 sub-phyla, including Isochrysidales (*Emiliania*) and Phaeocystales (*Phaeocystis*) totaling to only 8 OTUs, while Wolf964 also amplified sequences belonging to Prymnesiales (mainly *Chrysochomulina*) in addition to the first 2 described genera, also with a total of 8 OTUs. In contrast, the Wolf938 amplified the highest number of different OTUs with 2 from Isochrydales (*Isochrysis* and *Emiliania*), 3 OTUs from Prymnesiales (*Chrysochomulina*), and with 18 OTUs from Phaeocystales (*Phaeocystis*). Further, EPA

approach revealed that the *Phaeocystales-* related sequences even clustered into different clades within the genus, mostly belonging to *P. pouchetii* (Figure 4B).

Another significant difference in the observed amplification biases was on Bacillariophyta. All tested primers were able to amplify sequences from major diatom groups known to occur in the Arctic including the class Coscinodiscophyceae, and mostly from Mediophyceae but with significant variability. Surprisingly, Wolf964 and Wolf938 only amplified a total of 15 and 12 OTUs, respectively while that of Stoeck, which performed poorly in the mock community test detected 26 OTUs. The dominant genus *Chaetoceros* belonged to two phylotypes, namely*C. brevis/debelis* and *C. gelidus/socialis* complexes (Figure 4C).

Changing abundant ecotypes during the warm anomaly period

We further investigated the interannual turnover during the periods of highest POC flux in spring and summer of some of the abundant OTUs and their correlation with the temperature changes observed in Fram Strait during the period 2003 - 2011. One of the interesting patterns that emerged was the alternating abundances between some of the most abundant phytoplankton OTUs. For example, chlorophyte OTUs in the Wolf938 dataset showed that the temperate Clade C represented by OTU 2 had higher read counts (t-test, $p < 0.05$) in both spring and summer from 2005-2008, while the cold adapted Clade Ea represented by OTU 268 dominated the chlorophyte communities in the other years (Figures 5A and 5C). The same general pattern was observed in the OTUs identified from the Stoeck dataset, where Clade C was more dominant among the Chlorophyta in the years 2006-2009 for both seasons (t-test,$p < 0.05$), while Clade Ea was prominent in the other years. Similar observation however was not apparent in any of the OTUs in the Wolf964 dataset.

Interestingly, alternating dominance of some OTUs was also observed in the haptophytes based on the Wolf964, but was not observed in the Stoeck and Wolf964 datasets. Specifically, *Emiliania* sp. (OTU 36) and*Phaeocystis* sp. (OTU 29) followed that of the chlorophytes where the former becoming more prominent in 2006-2008 and the latter in both seasons for the other years (Figure 5B and 5D). No similar pattern was observed for the diatoms at the OTU level. However, their abundances were generally higher before 2004 and after 2008 when haptophytes and chlorophytes were more dominant.

Discussions

Primer biases and implications

Most known primers used in amplifying microbial eukaryotic communities are those reported by Stoeck et al. (2010) and Bradley et al. (2016) but also suffer in their biases, which has implications in inferring ecological insights on microbial communities. We then generated new primer sets (Wolf 938 and Wolf 964) to complement the limitations of the existing ones and evaluated their efficiency *in silico* using known reference sequences, *in vitro* amplification using mock communities, and their applicability *in situ* using environmental samples.

Consistently, the different primer sets we tested (Wolf 938, Stoeck, Wolf 964) were not able to amplify or provide high resolving power in all target taxonomic groups, although all sets amplified some if not all abundant taxa we expected based on microscopy (Lalande et al., 2016; Nöthig et al., 2015). Relative to the three most dominant and significant phytoplankton groups in the Arctic, the Stoeck and Wolf938 primers seemed to be good in detecting all three groups, but differences were found at the sequence level. For example, while primers Stoeck and Wolf938 were able to show ecotype-level variations in chlorophytes, they did not exhibit sensitivity to the haptophytes. In contrast, Wolf964 demonstrated the alternating abundances between *Emiliania* and*Phaeocystis* but did not perform well in detecting the chlorophytes. These results suggest that although we might come close to generating a broader taxonomy-binding primer, a 'universal one' is still not available. Primer usage then will ultimately depend on the questions being asked and the groups being targeted, or a combination of primer sets in case of exploratory work. This is especially true for the marine environment, which possesses one of the highest microbial eukaryotic diversities (see de Vargas et al., 2015).

We further observed disparity between *in silico* and *in situ* tests. For example, Wolf938 did not perform well *in silico*especially in detecting diatoms but was able to amplify sequences and even revealed patterns between *Chaetoceros* phylotypes from environmental samples. This indicates that what works *in silico*might not necessarily readily work in environmental samples and vice-versa. These could be due to efficiency of nucleic acid extraction, priming specificity, PCR biases, and sequencing-related issues, PCR inhibition due to the presence of humic and fulvic acids, preservation of the samples (Lever et al., 2015; Metfies et al., 2017), and competition reaction between known and unknown taxa. It is important to note however that limitations of currently used primers do not render previous and ongoing efforts in investigating eukaryotic microbes wrong or inaccurate. On the contrary, knowing such limitations provide new insights, perspectives, and contexts in analyzing and interpreting HTS-generated data. It is certain however that in some cases, these biases in primers could limit our understanding of natural ecosystems or even provide wrongful conclusions in interpreting data by missing out certain taxa if not considered during analysis.

Changing phytoplankton ecotypes in Fram Strait

After elucidating the limitations, advantages, and the synergistic potential of the primer sets we tested, we then combined the information gathered for each taxonomic group from all primer sets to provide a more holistic overview of how exported eukaryotic microbial communities changed in response to a warm anomaly in Fram Strait (2005-2007).

The Arctic Ocean is one of the most vulnerable regions where effects of the changing climate are very much apparent, mainly demonstrated by the shrinking annual summer ice extent associated with increasing sea surface temperature and an increase of warmer water masses entering from both the Pacific and Atlantic Oceans (Woodgate, Weingartner, & Lindsay, 2010; Beszczynska-Moller et al., 2012). The deep Fram Strait particularly serves as the Arctic gateway to the Arctic Ocean, as its eastern side is influenced by the incoming warmer Northern Warm Atlantic current (NWAC) continuing as the West Spitsbergen Current (WSC) and by the colder East Greenland Current in the west (EGC). Since the physico-chemical conditions of the water masses differ, they have also been reported to harbor distinct plankton communities, including microbial eukaryotes (Kilias, Wolf, Nothig, & Peeken, 2013). The LTER observatory HAUSGARTEN, where the sediments traps used in this study were deployed is situated 120 km west of Spitsbergen (Svalbard) and has been continuously monitored since 1999 (Soltwedel et al., 2016), providing unique long-term data in this particular part of the Arctic Ocean. One remarkable observation was the detection of a warming event of the Atlantic Water in the WSC starting in late 2004, peaking in 2006 and lasting until early 2008, usually referred to as the warm anomaly of 2005-2007 (Beszczynska-Möller et al., 2012). This period was not only accompanied by temperature anomalies but also by decreased ice extent (Lalande et al., 2013). A great number of studies have already been published complimenting the described event including changes in the composition of the community based on microscopy (Kraft et al., 2013; Bauerfeind et al., 2015; Kubiszyn, Piwosz, Wiktor, & Wiktor, 2014), decreased fluxes in biogenic matter (Lalande, Bauerfreind, Nothig, Beszcynska-Moller, 2013), primary productivity (Nöthig et al., 2015), shift in dominant cell size (Vernet, Richardson, Metfies, Nöthig, & Peeken, 2017), and protistan community composition based on gene surveys (Metfies et al., 2017). Although these studies revealed the shift in cell sizes (from large to small) and diversity of dominant taxonomic groups, the methods used in these previous investigations were not powerful enough to reveal potential changes at the level of the ecotypes.

Here, we observed that sequence assemblages of eukaryotic microbial communities based on sequencing were mainly dominated by chlorophytes even before the warm anomaly. However, in addition, we further distinguished that the years before and after the warm anomaly were mainly dominated by OTUs of the cold-ecotype of Mamiellophyceae, particularly closest to CCMP2099 or formerly Clade Ea (Lovejoy et al., 2007) but now known as *Micromonas polaris* (Simon et al., 2017). This ecotype has only been reported in the cold waters of the Arctic Ocean and tend to be the most abundant phytoplankton in the region (Lovejoy et al., 2007; Simon et al., 2017). *M. polaris* thrive even in nutrient-limited conditions, allowing them to outcompete larger phytoplankton due to their surface area to volume ratio advantage (Lovejoy et al., 2007; Li, Mclaughlin, Lovejoy, & Carmack, 2009). In comparison, the warm anomaly years were characterized by the increased

abundance of the warm ecotype Clade C (CCMP1195), now known as*Micromonas commoda* commonly found in temperate and tropical regions (Simon et al., 2017). Recently, Hoppe, Flintrop, and Rost (2018) showed through laboratory experiments that the *Micromonas pusilla*isolated from Kongsfjorden, Svalbard benefited from warming and acidification with increased growth rate and biomass buildup. The strain they tested however might actually be more associated with the warm ecotype of *M. commoda* (Clade C), which we found abundant in this study, rather than the Arctic resident *M. polaris* (Clade Ea). This could partially explain why chlorophytes (Clade C) were also abundant during the warm anomaly period. The alternating patterns between *M. commoda* and *M. polaris* have significantly contributed to the sustained overall high abundance of chlorophytes despite changes in the conditions, which might have implications to ecosystem resilience in the study area. To our knowledge, the same alternating pattern in ecotypes has not been reported elsewhere in the Arctic and could be unique to Fram Strait and specifically to Atlantic-influenced waters of the Arctic Ocean. The mechanisms of sinking of the small *Micromonas* however that allowed them to be transported to around ˜300 m in this study remain unclear. Nevertheless, the sensitivity and patterns shown by*Micromonas* species make them sentinels of the changing environment (Demory et al., 2019).

We also observed alternating abundances in the dominant OTUs of the coccolithophore *Emiliania huxleyi* and colony-forming*Phaeocystis pouchetii* , with the former being higher during the warm anomaly period. Although the seasonal succession of*Emiliania* and *Phaeocystis* from May until July has been reported in one short-term mooring in the Fram Strait in 2003 (Lalande, Bauerfeind, & Nöthig, 2011), their inter-annual variability has not yet been fully explored. *Phaeocystis* is a major bloom former in the Eurasian side of the Arctic, making up much of the blooms in Svalbard fjords (Hegseth and Tverberg, 2013; Marquadt, Vader, Stubner, Reigstad, & Gabrielsen, 2016), Fram Strait (Smith, Baumann, Wilson, & Aletsee, 1987; Gradinger and Baumann, 1991; Fadeev et al., 2018), Barents Sea (Hovland et al., 2014), Dutch waters (Veldhuist et al., 1986), Greenland (Arendt, Nielsen, Rysgaard, & Tönnesson, 2010; Waniek et al., 2005), Norwegian coasts, Barents Sea (Degerlund and Eilertsen 2010), Southern Ocean (Arrigo et al., 1999) and even under ice blooms (Assmy et al., 2017), and has been implicated in carbon transport in most regions of the central Arctic Ocean (Lalande et al., 2014, Wollenburg et al. 2018). In comparison, *E. huxleyi* was also reported in Fram Strait and North of Svalbard but in lower abundance and biomass (Hegseth and Sundfjord, 2008; Lalande et al., 2014). The high sequence abundance of the coccolithophore during the warm anomaly, known to contain carbonaceous cell walls, could partially explain the relatively sustained $CaCO_3$ transport for the same period (Bauerfeind et al., 2009). This is consistent with the report of Neukermans, Oziel, & Babin (2018), where using satellite-derived data showed that blooms of *E. huxleyi* has been increasing and further moving northward in the Arctic. Interestingly, despite the decreased abundance of diatoms, POC flux was observed to peak in some years in Fram Strait, coinciding with peak abundance of *Phaeocystis* , suggesting their potential role in the transport of particulate organic carbon. Intriguingly however, some studies argued that*Phaeocystis* colonies do not readily sink (Passow and Wassmann, 1994; Wolf, Kilias, & Metfies, 2015). Recent evidence however suggests that their lysis and disintegration either through grazing, apoptosis, and viral infections could induce the formation of transparent exopolymer (TEP) that allows production of *Phaeocystis* -derived aggregates (Engel et al., 2017), which then efficiently sink (Schoemann et al., 2005; Verity et al., 2007). Further, mineral ballasting particularly that of gypsum among *P. pouchetii* cells in the Arctic has been shown to enhance the vertical transport of carbon under the ice (Wollenburg et al., 2018), emphasizing the increasing role of the haptophytes in the biogeochemical cycles in the polar regions.

Compared to haptophytes and chlorophytes, which are small and difficult to identify using conventional microscopy techniques, identity and abundance of diatoms are easier to track and detect due to their larger size and distinct rigid silicate tests. Previous studies based on microscopy reported that the diatoms abundant before the warm anomaly were mostly *Thallasiosira* spp., *Chaetoceros* spp.,*Fragilariopsis* spp., *Navicula* spp., *Achnanthes*spp., and *Fossula arctica* (Kubiszyn et al., 2014; Nöthig et al., 2015; Vernet et al., 2017). In contrast, findings in this study revealed that diatom reads in all libraries generated by all primer sets were dominated by *Chaetoceros* , either indicating biases towards this group or inefficiency in amplifying other species. In addition, the RAxML-EPA approach was also able to identify the sequences down to the species level with some belonging to the *C. brevis/debilis* and majority from *C. gelidus/socialis* complexes, which

are almost not distinguishable based on light microscopy. The more abundant '*C. socialis* ' species complex is described to be cosmopolitan, occurring in the colder polar waters to the warmer Mediterranean and Asian waters (Hasle & Syvertsen, 1997; Degerlund, Huseby, Zingone, Sarno, & Landfald, 2010; Kooistra et al., 2010). Careful morphological and phylogenetic re-examination of the representatives of the *C. socialis* complex however revealed the presence of a new cold-adapted clade *C. gelidus* sp. nov. and the warm-associated clade *C. socialis* (Chamnansinp, Li, Lundholm, & Moestrup, 2013). Interestingly, the most abundant OTU in the Stoeck dataset (OTU2) belonged to *C. gelidus* (Figure 4C). To our knowledge, this would be the first time that this species will be reported abundant in this region. OTUs belonging to this species have also been reported in the Arctic waters on the Canadian side of Baffin Bay (Joli et al., 2018) and abundance of cells in Beaufort Sea (Balzano et al., 2017), indicating their potential widespread occurrence and distribution but underappreciated role in the Arctic Ocean. In addition, *C. brevis / debilis* species have also been found in northern temperate areas and Arctic waters but seemed to be a more important diatom in the Antarctic (Trimborn et al., 2017). Nevertheless, the presence of these cold-adapted species signifies favorable conditions for their proliferation and in turn, their absence during the warm anomaly period indicates significant changes that filtered them out from the environment. Most of these diatoms are bloom- and colony-forming species, making them important drivers of carbon and silica fluxes, and their decreased abundance significantly affected the vertical transport of silicate in Fram Strait (Lalande et al., 2014; 2016). Even after the warm anomaly, the relative abundances of these species did not return to their before-state, indicating that long-term changes in the community occurred, which would have significant implications to the pelagic-benthic coupling of Arctic ecosystems.

Species displacement and diversity loss

Ecotype-level changes observed in this study did not only suggest changing structures in community composition and ecosystem functioning but also in the biogeography and distribution of some species, such as the northward advancement of the warm-adapted chlorophytes and *E. huxleyi* . These data provided new insights and information on the potential loss in diversity and species displacement brought about by the changes occurring in the polar region. It is not clear however if the change in the abundant ecotype was more associated with the increased transport of the warm water mass or the absence or loss of ice, or both. Neukermans et al. (2018) showed a strong correlation between the blooms of *E. huxleyi* in the Arctic and the increasing sea surface temperature. Such observations are consistent with the 'Atlantification' event, where physico-chemical conditions in the Eurasian Arctic are becoming more similar with that of the Atlantic (i.e., Polyakov et al., 2017; Lind, Ingvaldsen, & Furevik, 2018). Changes in physico-chemical regimes would also mean more favorable conditions for Atlantic-type species to thrive in the Arctic region. This has been documented for many macroorganisms including amphipods (Kraft et al., 2013), the avian black legged kittiwakes (*Rissa tridactyla;* Vihtakari et al., 2018), boreal fishes (Fossheim et al., 2015) but rarely reported for microorganisms (Neukermans et al., 2018).

In the long term, these changes in microbial communities would have profound implications to ecosystem functionality and services since cold- or ice-associated communities have been estimated to contribute to as high as 60% in high Arctic primary productivity (Fernández-Gómez, Montserrat Sala, & Pedrós-Alió, 2014). Functionally, it is interesting to note that the replacing taxa or those proliferating more during warm anomaly has similar biogenic composition as those they replaced (e.g.,*M. polaris* with *M. commoda*). This has significant implications on our understanding of the biogenic matter fluxes in the Arctic in the wake of the changing climate. Recent ice loss has also been implicated with increased frequencies of 'fall blooms' based on satellite images (Ardyna et al., 2014; Renaut, Devred, & Babin, 2018). However, these studies were not able to identify the blooming species, which would be important when organic matter transport is being considered. For example, smaller cells are thought to be less efficient in sinking, and thus, would be highly retained in the upper trophic waters where they are regenerated resulting in less particulate transport. Interestingly, in this study, we found high abundance of chlorophytes and haptophytes in the sediment traps, indicating that cells associated with these taxa could actually sink at least to the depths of 200 to 300 m. The underlying mechanisms for such transport however remain little explored, and roles of trophic upgrading through biotic interactions are still unknown.

Since the phytoplankton serve as the foundation of food webs especially in oceanic systems such as the Arctic Ocean, understanding of their diversity, distribution, and biogeography are critical to gaining insights into their roles and responses to the changing environment. Here, we demonstrated that HTS-generated data could actually contribute significant information not available through conventional means. However, we also highlighted the importance of primer efficiency and sensitivity in exploring and realizing such goals and further emphasized to take caution in interpreting environmental data from gene-based surveys.

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Figures

Figure 1. Map of the stations in the LTER HAUSGARTEN at the West Spitsbergen Current in the Fram Strait. The sediment traps used in this study were deployed at station HG-IV.

Figure 2. Summary of the different community profiles collected from the sediments traps and generated using different primer sets but the same mock community.

Figure 3. The summer and spring eukaryotic microbial communities collected from the sediment traps deployed at 200 to 300 m in HAUSGARTEN, Fram Strait, from 2000-2011, and amplified using the Stoeck, Wolf938 and Wolf964 primer sets.

Figure 4. Results of the EPA-RAxML placement of the OTUs generated by the Stoeck, Wolf938 and Wolf964 primer sets back onto the reference trees for (A) chlorophytes, (B) haptophytes, and (C) diatoms. The black circles correspond to the placement of the OTUs in the tree, the size represent the number of OTUs placed in a particular node relative to each primer dataset for each species.

Figure 5 . Dominant OTUs exhibited alternating patterns before, during and after the warm anomaly years 2005-2007 in both spring and summer periods, including the (A, C) *Micromonas* OTUs 2 and 268, which represent warm-adapted (Clade C) and cold-adapted (Clade Ea), and (B, D) the warm-associated *Emiliania* and Arctic resident*Phaeocystis* from the haptophyte group.

Supplementary Figure 1S . Result of the *in silico* test to evaluate the sensitivity of the different primer sets to the major phytoplankton groups in the Arctic including the chlorophytes, haptophytes, dinoflagellates and diatoms.

Supplementary Table S1. Summary of the cultures and strains used to generate the mock community that was used to evaluate the biases and limitations of the primer sets.

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