Mapping the Genetic Diversity of Eukaryotic Protists in the Arctic Ocean

A Dissertation presented

by

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Science is beautiful when it makes simple explanations of phenomena or connections between different observations. Examples include the double helix in biology, and the fundamental equations of physics

Stephen Hawking

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List of Abbreviations

ARISA Automated Ribosomal Intergenic Spacer Analysis

AW Atlantic Water

bp base pair

DNA Deoxyribonucleic Acid

EGC East Greenland Current

HPLC High Performance Liquid Chromatography

ITS Internal Transcribed Spacer

MAST Marine Stramenopile

MDS Multidimensional Scaling

MODIS Moderate Resolution Imaging Spectroradiometer

MW Mixed Water

NAO North Atlantic Oscillation

NPP Net Primary Production

OTU Operational Taxonomic Unit

PCA Principle Component Analysis

PCR Polymerase Chain Reaction

PP Primary Production

PrW Polar Water

PW Pacific Water

RAW Recirculating Atlantic Water

rRNA ribosomal Ribonucleic Acid

sst single strand

SSU Small Subunit

WSC West Spitzbergen Current

1. Summary

This doctoral thesis aimed at the establishment of molecular tools (ARISA and 454-pyrosequencing) for protist diversity assessments in polar regions and at the application of these tools for studying protist diversity in the Fram Strait and in the Central Arctic Ocean. In this thesis, three hypotheses were put forward: i) Molecular surveys of genetic protist diversity, obtained by 454-pyrosequencing, constitute an adequate tool for assessing natural protist diversity ii) Water masses in the Arctic Ocean and in the Fram Strait are characterized by distinct protist communities and iii) Complex hydrographical and environmental situations can be evaluated via genetic information.

A comprehensive study of protist diversity is required, because so far investigations were biased towards big size cells (≥2 μm). A correct identification of smaller cells is almost impossible due to the small size and lack of morphological markers. However, a proper survey of protist diversity demands the inclusion of all size classes. This is of particular relevance, considering the fact that small cells were observed to dominate protist assemblages at certain times, according to abiotic circumstances. The Arctic Ocean constitutes in two ways an important research area: on the one hand, it experiences intense variations in the light regime based on seasonality and sea ice; and on the other hand, it is assumed to be affected more severely by climate change than other world oceans. Since marine microorganisms are highly responsive to environmental forcing, changes will likely impact the protist community structures. Against the background of ongoing environmental changes in the Arctic, a study of protist diversity is further crucial in order to get a baseline for the assessment of future community structure changes.

i) The assay of the 454-pyrosequencing suitability was carried out by using different wide-employed methods for reconciliation, sharing the same (clone library and ARISA) and different drawbacks (light microscopy and HPLC). The use of 18S rRNA clone library sequencing thereby, aimed to compare but also to complement the 454-pyrosequencing data, because of the longer sequence lengths that allow a more comprehensive taxon detailed analysis. However, the clone library approach was not suitable neither for 454-pyrosequencing comparison nor for 454-pyrosequencing complementation. While one approach, comprising the picoplankton fraction, was biased against haptophytes, the other approach, comprising the whole size fraction was biased against diatoms. Moreover, despite the comparable high number of ~140 clones per library, the abundant biosphere of 454-pyrosequencing was not comprehensively recovered. Hence, a consult of previous data

for 454-pyrosequencing gathered by clone library is not advisable or has to be interpreted with caution. The second molecular method, ARISA, reflected community structure shifts that were indeed recovered by 454-pyrosequencing. The indicative limitation of ARISA on sequence length variances however, makes the method more suitable for a preceded sample selection than for a robust 454-pyrosequencing support. The assessment with traditional methods as light microscopy and HPLC presented good analogies. Since light microscopy is biased against small cells and HPLC against heterotrophic protists, the comparison was adapted to the respective limitations. A quantitative comparison of the diatom assemblage showed similar percentages within light microscopy and 454-pyrosequencing (≥10 µm filter), and approved the use of diatoms for the evaluation of 454-pyrosequencing accuracy. The comparison with HPLC was further in accordance with 454-pyrosequencing and agreed in the portions of autotrophic protists. In summary, three out of four evaluation methods presented good analogies with 454-pyrosequencing data and approved the suitability of the molecular method for assessing natural protist diversity.

ii) To address the hypothesis if water masses host specific protist communities, 454-pyrosequencing was tested in different hydrographic environments for different size classes. In the process, the picoplankton community structure was investigated at four stations in the Eastern Fram Strait during the expedition ARK XXIV/2. The community structure of all protists was analyzed at five stations in the Western Fram Strait (ARK XXV/2) and at eight stations in the Central Arctic Ocean (ARK XXVI/3). A relation of water mass, distinguished by abiotic factors such as temperature, salinity and/or nutrients, and protist community structure was observed in all three studies. However, while the water mass regimes in the Fram Strait promoted distinct community structures in protist assemblages of the pico size spectrum and the entire size spectrum, populations of the Central Arctic Ocean showed a less definite association. In the Eastern Fram Strait picoplankton community (0.2-3 µm) in the Atlantic Water was mostly dominated by *Phaeocystis* cells and in the ice-covered station by small dinophytes (e.g. Dinophyte 1). The influence of cold, coastal water at one station however, shifted the community structure from a *Phaeocystis*-based to a *Micromonas*-based protist assemblage. In the Western Fram Strait, the protist community showed a distribution pattern that also corresponded to the different water properties. While Polar Water (EGC) was dominated by diatoms in recently light exposed stations and by dinoflagellates in a station longer exposed to light, the Atlantic Water (WSC) presented a high dominance of *Micromonas* that was not associated with the light availability. The two different

protist communities observed in the Polar Water, however, suggest a high influence of light availability on the protist assemblage by promoting a protist succession. In the Central Arctic Ocean, in contrast, protist communities showed a less pronounced relation to the four water masses (Atlantic Water, Pacific Water, Mixed Water I and II) with no switch of dominant protist community members. All water masses were mainly dominated by dinoflagellates (e.g. Syndiniales 2), and at two stations by *Micromonas*. In principle, different water masses were reflected more significantly by whole community structure changes (ARISA) than by the appearance or disappearance of single protists (454-pyrosequencing) in the abundant biosphere. In particular, protist communities of the so-called Mixed Water I and II were difficult to separate from Atlantic Water and Pacific Water communities. The formation of mixed water, e.g. the mixing of two separate water masses, resulted in a combination of the abiotic characteristics (temperature, salinity, and nutrients) and of the protist communities. Moreover, the high sea-ice concentration and thus low light availability in the Central Arctic Ocean represented a strong selective force that eventually led to a unification of the protist communities.

iii) 454-pyrosequencing revealed an adequate tool not just for investigating the protist diversity but also for reflecting hydrographical situations, as the recirculation of AW in the Fram Strait by protist community structure shifts. The hydrographic system of the Central Arctic Ocean, including Atlantic, Pacific, and mixed water masses, was not that strongly reflected by community structure shifts. The consistent presence of various ice concentrations strongly controlled the community composition and promoted heterotrophic and/or mixotrophic cells. In this regard, the Arctic protist assemblage presented high contributions of dinoflagellates under higher sea-ice concentrations (low light areas) and a higher contribution of diatoms under low sea-ice concentrations (high light areas). The strong response of the Arctic protist assemblage to the changing light conditions finally hampered the identification of water mass associated protist communities and hence, the reflection of the hydrographical situations.

This thesis showed the suitability of 454-pyrosequencing for molecular studies of protist diversity and biogeography, independent of organisms cell size, or organisms nutritional strategy (hetero-, mixo-, or autotrophy). The application of 454-pyrosequencing, to study Arctic protist distribution facilitated to reveal even complex hydrographical situations and indicated the presence of different microbial habitats in polar regions, determined by water

mass properties (T, S, and nutrients) and altered by sea ice concentration. This hypothesizes an improved differentiation of the microbial habitats in the Central Arctic Ocean under continuously sea ice decrease. Moreover, the study of the rare biosphere revealed a constant distribution of taxonomic groups. In contrast to the abundant biosphere, the rare biosphere did not respond to changing nutrient or sea ice concentrations.

2. General Introduction

Protists are defined as complex single-celled organisms, where even the simplest member possesses a nucleus. The evolution can be dated back to the Proterozoic oceans (1.5 billion years). However, the major autotrophic protist groups, as the red algal lineages, evolved just recently in the Mesozoic (251-65 million years). This lineage is characterized by holding a plastid, derived from red algae by secondary endosymbiosis and includes dinoflagellates, coccolithophores, and diatoms (Keeling et al. 2004). The formerly predominant and ancient chlorophytes, in turn, began a long-term decline from the Triassic (Falkowski et al. 2004). Today, protists are important constituents of the marine environment, composing much of the genetic diversity within the eukaryotic domain. Marine autotrophic protists are responsible for about half of the photosynthetic activity on this planet (Field et al. 1998, Falkowski et al. 2004, Simon et al. 2009) and are forming the base of the marine food-web (Priddle et al. 1992). Large cells (e.g. diatoms) are reported to produce vast seasonal blooms under specific hydrographic conditions (Smetacek 2000, Li 2002, Mei et al. 2002), while small cells contribute most of the biomass and production in warm and oligotrophic waters (Agawin et al. 2000).

2.1 Marine Protists

2.1.1 Protist Diversity

Protist systematics has experienced some change after phylogenetic analysis contributed to the discussion on taxonomic classification. The eukaryotic phyto- and protozooplankton fraction is highly divers and hence caused some controversy between the classical (e.g. light microscopy) and the modern (e.g. DNA sequencing) methods. In the historic time course, some revisions have taken place, including the consolidation of photosynthetic and heterotrophic forms that share recent ancestry but differ in nutritional strategy, such as the chloroplast-bearing and heterotrophic crysophytes (Cavalier-Smith & Chao 2006, Caron et al. 2012). Secondary endosymbiotic acquisition of photosynthesis has the potential to hamper the taxonomical classification. In this regard, recent genetic investigations on *Guillardia theta* and *Bigelowiella natan* revealed a genetic and biochemical mosaicism, with host-, endosymbiont-, and foreign algal- derived proteins (Curtis et al. 2012, Gould 2012). Nevertheless, debates on taxonomic relationships are declining and taxonomic affiliations are coalescing. Protists can be subdivided based on their taxonomic affiliation, on their size class (micro-, nano-, and picoplankton) or on their mode of nutrition (autotroph, heterotroph, and mixotroph) (Sieburth et al. 1978). The recent phylogenetic scheme consists of eight

taxonomic supergroups (Figure 2.1). Unikonts consist of two supergroups, such as **opisthoconts** and **amoebozoans** (Baldauf 2008). Opisthoconts comprise marine protists, as choanoflagellates, which are characterized by small cell sizes and heterotrophic nutrition. In polar ecosystems they constitute important bacterivorous species (Throndsen 1970). Amoebozoans in contrast, are mostly known as benthic and less as pelagic contributors (Moran et al. 2007). The cell size is highly variable and can group to the nano- or microplankton, while the nutrition is limited on heterotrophy (phagocytosis).

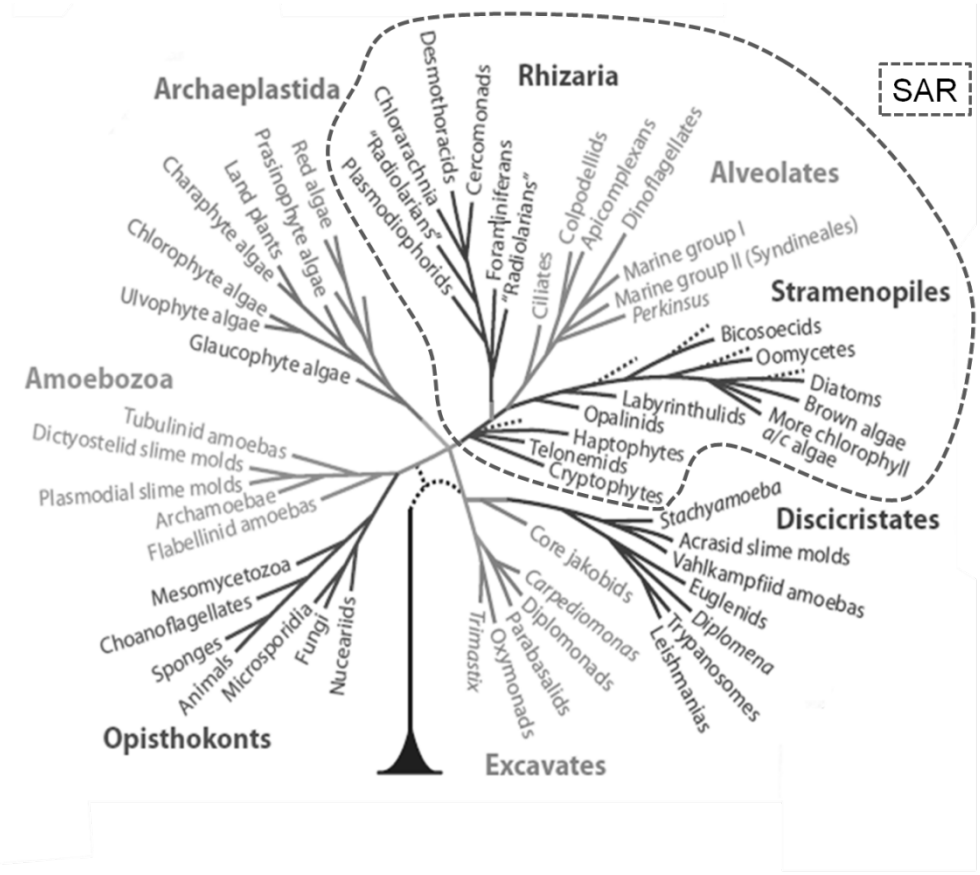

Figure 2.1. Consensus phylogeny of the major protist super groups- modified after Caron et al. 2012.

Another supergroup is formed by the **archeaplastids**, including land plants, chlorophytes, and glaucophytes. Red algae are also included, but just with few single-celled taxa because most red algae are multi-cellular (Caron et al. 2012). Photosynthesis first arose within this supergroup, comprising autotrophic representatives, of all size classes (Adl et al. 2005, Baldauf 2008). Chlorophytes, such as *Micromonas*, constitute important, divers members of the pelagic picoplankton fraction (Guillou et al. 2004). **Rhizaria** are a recent phylogenetic

group and constitute the next supergroup, which inter alia is characterized by a high diversity of free-living unicellular cells (Moreira et al. 2007). The most prominent representatives are the heterotrophic planktonic/benthic foraminifers and the planktonic radiolarians, both covering a wide cell size range. **Alveolates** are subdivided into ciliates and dinoflagellates. Both groups are highly abundant in the marine environment. In particular, dinoflagellates presented in latterly molecular surveys an enormous diversity within the order Syndiniales, containing also parasitic forms (Guillou et al. 2008, Caron et al. 2012). Dinoflagellates present a variety of cell sizes that embrace all three size fractions, while ciliates are mainly represented in the microplankton. Likewise, ciliates are mostly heterotrophic, while dinoflagellates have autotrophic, heterotrophic, mixotrophic, and even parasitic representatives (Hackett et al. 2004). One exception for heterotrophic nutrition in ciliates is the genus *Mesodinium,* which possesses a photosynthetic capacity after a prey on cryptophytes for plastid maintenance (Gustafson et al. 2000, Möller et al. 2011). **Stramenopiles** include diatoms, bloom forming and ecological important members but also marine stramenopiles (MAST), which have been discovered by phylogenetic analysis, just recently (Massana et al. 2004). As dinoflagellates, stramenopiles cover a wide cell size range, starting with small picoplankton cells, such as MAST and *Bolidomonas pacifica* and ending with big microplankton cells, such as *Thalassiosira* sp. and *Fragilariopsis* sp. Moreover, the group comprises autotrophic (e.g. diatoms) as well as heterotrophic (e.g. MAST) representatives. **R**hizaria, **s**tramenopiles, and **a**lveolates are summarized to a broader group, SAR (or RAS) (Burki et al. 2007, Hackett et al. 2007, Baldauf 2008). Haptophytes and cryptophytes are suggested for affiliation into the SAR group as well, however, the evidence is not completely achieved (Burki et al. 2007, Hackett et al. 2007). Prominent haptophytes are classified into the genus *Phaeocystis*, in which *Phaeocystis pouchetii* is particularly abundant in the Northern Hemisphere and represented by small solitary cells and large colonial cell structures. Cryptophytes in contrast, are mainly small celled organisms and primarily found in cold or deep waters (Baldauf 2008). Some haptophytes possess the ability to switch their nutrition from autotrophy to heterotrophy (Medlin 2009), while cryptophytes can be autotrophic, heterotrophic, and mixotrophic. The last two supergroups, **Discicristates** and **Excavates** are unicellular protists, most of them are heterotrophic flagellates. However, one subgroup, euglenozoa, also includes other nutrition modes as phototrophy, heterotrophy, and mixotrophy and is especially common in benthic ecosystems (Buck et al. 2000, Caron et al. 2012). Eukaryotic protists are distributed abundantly and ubiquitously in the world oceans (Caron et al. 2012). The Arctic ecosystem is characterized by protists that are

particularly adapted to the local mostly unfavorable environmental conditions (Sakshaug & Slagstad 1991). Arctic protists possess pelagic as well as sympagic representatives (Poulin et al. 2010) that both contribute significantly to biomass and primary production.

2.1.2 Seasonal Cycles

Protist communities are considered to be controlled by "top-down" processes, when herbivorous grazing mostly limits the standing stock and to be controlled by "bottom-up" processes, when nutrient concentrations and light irradiance are the main limiting factors (Metaxas & Scheibling 1996, Verity & Smetacek 1996, Smith & Lancelot 2004). The top-down/bottom-up hypothesis indicates a strong effect of top-down at the top of the pelagic food web that weakens toward the bottom (McQueen et al. 1989). This prediction bases on the assumption that autotrophic protists are more controlled by resources than by grazing (Benndorf et al. 2002). Grazing, on the other hand, was observed to be an important control mechanism for heterotrophic protists, as for some nanoflagellates (Weisse 1991). Furthermore, small-cell-dominated communities showed a particularly high influence of top-down control, under micrograzer response. The community structure of big cell protists (diatoms or *Phaeocystis*) was more controlled by bottom-up (Smith & Lancelot 2004). Since both regulation processes control protist composition and abundance, they conversely also influence primary production.

Light constitutes the most important factor for primary production and is responsible for the pronounced seasonality of algal growth (phytoplankton and ice algae). In polar waters, the solar angle, sea ice thickness, and snow cover determine the intensity of light (Mundy et al. 2005). In the course of increasing ice melt and the break-up of sea ice, light availability increases and algal blooms start forming (spring/summer). The second important factors limiting primary production are nutrients, such as nitrate (NO_3), phosphate (PO_4), and silicate ($SiOH_4$). Nutrients availability in the ice habitat, is a function of initial nutrient concentration during the freezing process, nutrient supply by advective processes or biological uptake, and finally nutrient concentrations in surface waters (Gradinger & Ikavalko 1998, Gradinger 2009). Nutrient availability in the Arctic pelagic habitat on the other hand, is determined by the maximum penetration of winter mixing and the horizontal exchange with the Pacific and Atlantic basins (Popova et al. 2010).

Figure 2.2 shows the process of the Arctic primary production along a gradient, running from 70°N to 85°N. Sympagic ice algae start to grow within (brine channels) and under the ice in spring, when light conditions become more favorable and sufficient light penetrates the ice.

Ice algae possess a high photoacclimation potential that allows them to grow even under low-light regimes (Kirst & Wiencke 1995, Smetacek & Nicol 2005). The ice algae production varies strongly and ranges between 5-10 g C m^{-2} yr^{-1} (Legendre et al. 1992, Gosselin et al. 1997, Leu et al. 2011). Pelagic phytoplankton, has a growth period that starts in April and ends in early September (Bluhm et al. 2011), whereas the onset shifts according to the longitude or the ice coverage. The annual production of Arctic phytoplankton was referred to range between 12-50 g C m^{-2} yr^{-1} (Legendre et al. 1992, Gosselin et al. 1997, Leu et al. 2011).

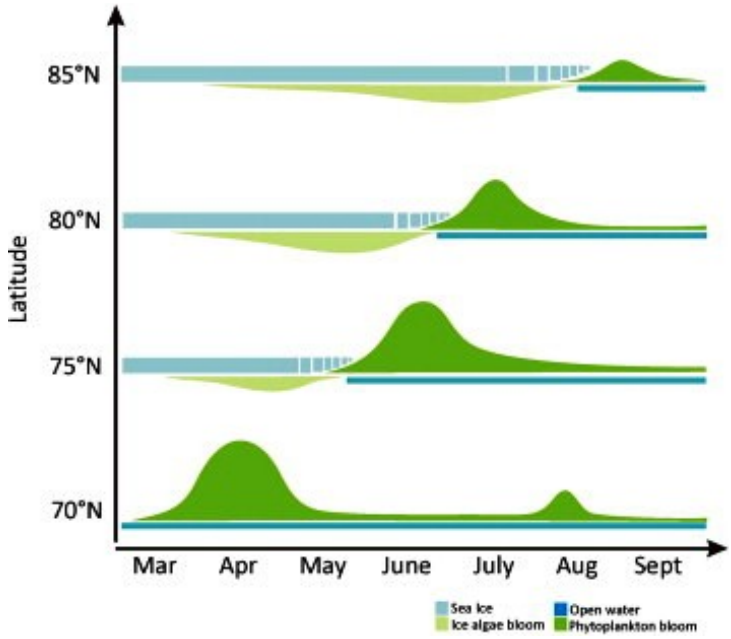

Figure 2.2. Overview of the primary production regimes in the European Arctic (Zenkevich 1963, Leu et al. 2011).

Overall, the Arctic Ocean has been characterized as a region of extremely low primary production, which strongly depends on the presence of sea ice and the length of photosynthetic season (Gosselin et al. 1997).

Two production regimes can be distinguished, the regenerated production system and the new production system (Hill et al. 2005, Li et al. 2009, Tremblay et al. 2009). The regenerative system mainly consists of heterotrophic bacteria, small picophytoplankton, and nanoflagellates (flagellate-based system), and is based on regenerated nutrients like ammonia (Azam et al. 1983, Landry et al. 1997, Ardyna et al. 2011). In contrast, the new production system is mostly composed of larger cells, such as diatoms (diatom-based system) using new nutrients like nitrate (Cushing 1989). However, while the regenerated production system is

associated with strong stratified, nutrient poor waters (25.8- 33.4 PSU), the new production system is usually associated with weaker stratified, nutrient rich waters (29.9-33.3 PSU).

Intense phytoplankton blooms occur under favorable light and nutrient conditions in summer, when the sea ice cover decreases and melt-water increases the stratification of the surface water layer. The decrease in nutrient concentration initiates the decline of the phytoplankton bloom and the numbers of heterotrophic protists increase. The vertical flux of biogenic matter changes thereby, from an initially dominated export of autotrophic cells to an export dominated more of degraded matter (Figure 2.3) (Wassmann & Reigstad 2011). However, in the future, climate change is expected to result in an extension of ice-free periods and in an earlier onset of ice algae and phytoplankton blooms (Perrette et al. 2011). Furthermore, the sea ice retreat is assumed to cause shifts in the protist composition, from ice algae to plankton algae, and to alter the water surface light penetration (Stroeve et al. 2007, Soreide et al. 2010). Since autotrophic protists are intimately linked to the ice cover, as previously referred, timing, quantity, and quality of the primary production and matter flux will consequently be influenced (Arrigo et al. 2008).

In order to assess the influence of less sea ice, a net primary production (NPP) algorithm was calculated for the Arctic Ocean, based on combined satellite Chl *a* data (Sea-WIFS/MODIS) of twelve years. The study reported an increase of 20 % of total annual NPP between 1998 and 2009 that was largest on the nutrient rich continental shelves of the Chukchi, East Siberian, Laptev, and Kara Sea. This gain was attributed to increases in the extent of open water (+27 %) and in the duration of open water seasons (+45 days) (Arrigo & van Dijken 2011). However, for most parts of the Arctic Ocean, the increasing light availability will not result in an increase in algal growth because nutrients, which are essential for algal growth, will not increase. A stable stratification, formed by low-density water of the river discharge and Pacific Water inflow, will impede a vertical nutrient supply by mixing events (Tremblay et al. 2009). As a consequence, nutrients will be depleted faster and heterotrophic processes may last longer. The vertical export of autotrophic and degraded organic matter will behave accordingly (Wassmann & Reigstad 2011). The nutrient depletion may additionally cause shifts in the taxonomic composition of phytoplankton (Tremblay et al. 2009). The whole scenario will favor smaller plankton cells (<2.0 µm) that will in turn provide more strength to the microbial loop (Li et al. 2009, Tremblay et al. 2009, Moran et al. 2010). It follows that irradiance increase should only have a maximum impact on NPP in areas where nutrient supply is sustained, as mostly observed in coastal regions (Ardyna et al. 2011).

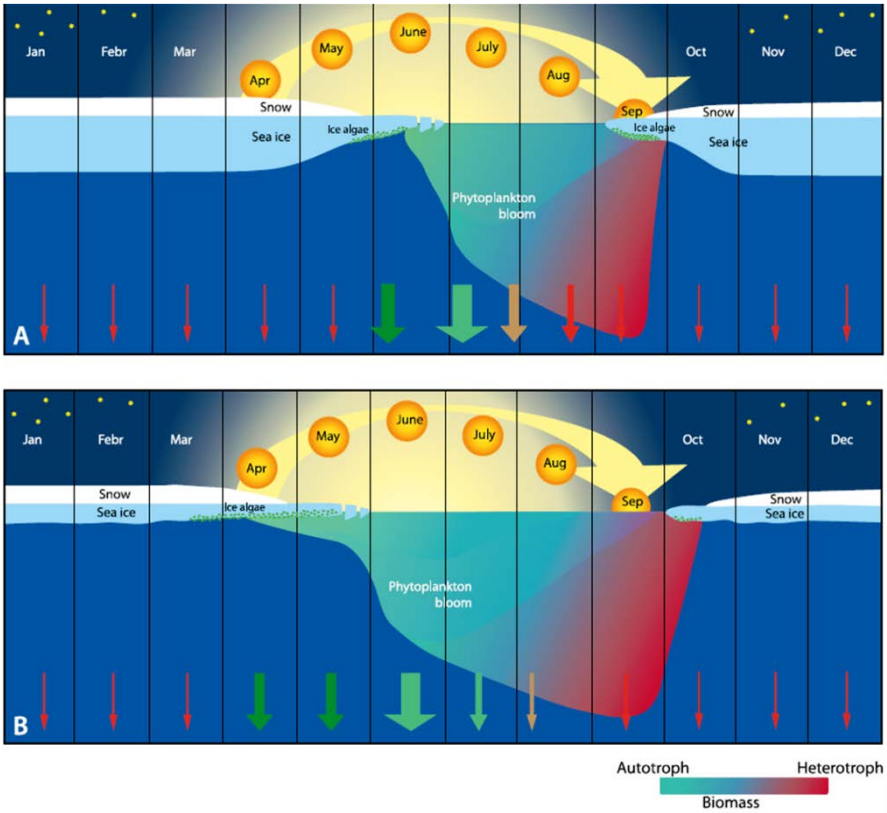

Figure 2.3. Bloom development and carbon export in the Arctic Ocean under A) today's climate and B) future climate (Wassmann & Reigstad 2011).

2.2 Investigation Areas

2.2.1 Fram Strait and "Hausgarten"

The Fram Strait (~500 km wide and 2600 m sill depth) separates the Svalbard archipelago from the northeast Greenland shelf (Forest et al. 2010) and presents the only deep water connection to the Central Arctic Ocean (Rudels et al. 2000, Fahrbach et al. 2001, Langehaug & Falck 2012).

Hydrographically, the Fram Strait is characterized by a two-directional current system, transporting warm and saline Atlantic Water via the West-Spitzbergen Current (WSC) northwards and cold, less saline Polar Water (PrW) via the East Greenland Current (EGC) southwards (Figure 2.4). The boundary between both currents generally occurs at 4-6°E in the upper water layer and exhibits a mesoscale eddy field (Hop et al. 2006). The EGC conveys the export of vast sea ice masses. On the way south, the current is augmented by Recirculating Atlantic Water (RAW) that originates from the WSC and mixes with the outflowing Pacific Water (PW) (Rudels et al. 2012). The intensity of the Atlantic Water (AW) recirculation is variable. In 2010, the RAW e.g. extended over the entire Fram Strait and dominated the upper

water layer of the EGC (Rudels et al. 2012). The WSC is the northernmost extension of the Norwegian Atlantic Current (Aagaard et al. 1987). On the way to the Arctic Ocean at 78-80°N, the WSC splits up in three branches, due to the topographic structure. Thereby, two branches head northwards, one following the shelf edge of Svalbard (Svalbard Branch) and the other following the northwestern slope of the Yermak Plateau (Yermak Branch). The third branch (RAW) recirculates and transports a significant volume of AW back to the Nordic Seas (Schauer et al. 2004).

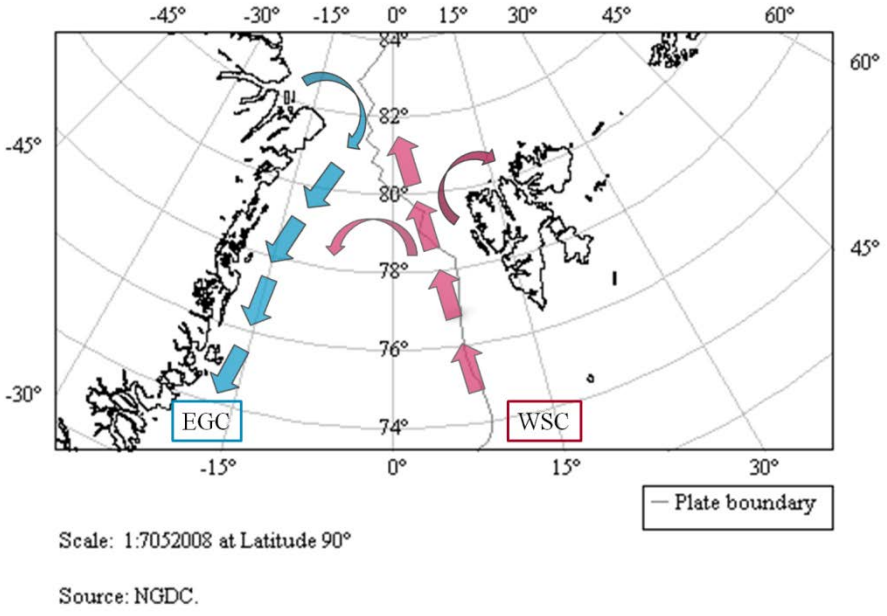

Figure 2.4. Scheme of the hydrographical situation in the Fram Strait.

The Alfred Wegener Institute is running a deep-sea long-term observatory called "Hausgarten" (HG) in the Eastern Fram Strait (WSC), since 1999 (Figure 2.5). The "Hausgarten"-observatory is situated between 78-80°N latitude and between 3-7°E longitude and was established to investigate impacts of environmental changes (Soltwedel et al. 2005). Sixteen stations are orientated in two transects, one running from south to north (eight stations) and one from east to west (eight stations). The central station HGIV connects both transects. Nutrient rich AW, but seasonally varying sea-ice concentrations characterize the "Hausgarten" area (Soltwedel et al. 2005).

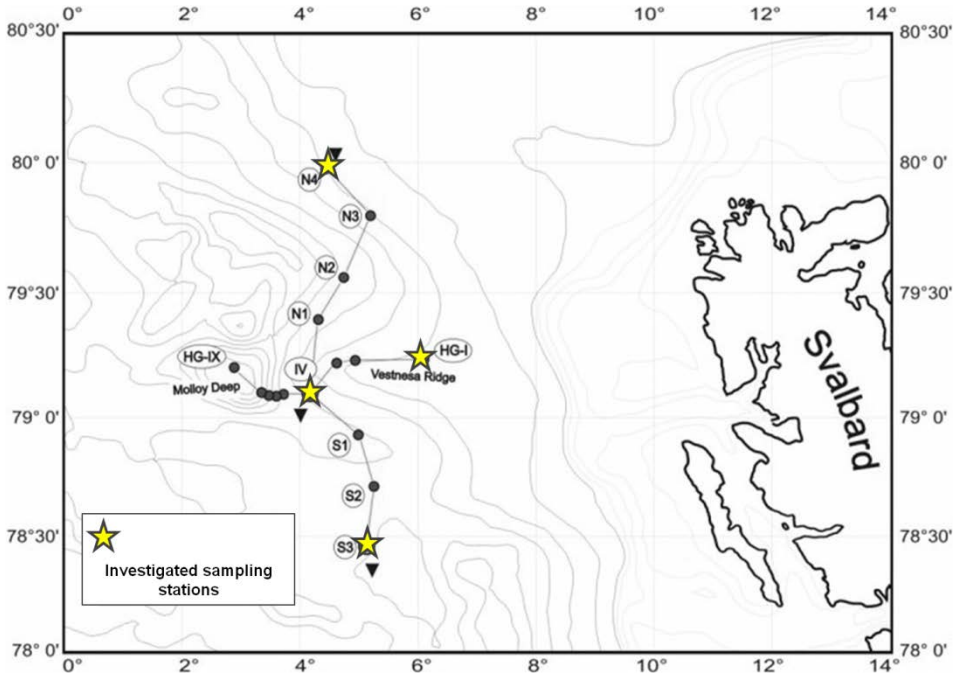

Figure 2.5. Map of the deep-sea long-term observatory "Hausgarten" (www.awi.de/en/research/deep_sea/deep_sea_ecology/deep_sea_long_term_observatory_hausgarten/).

The strength of the WSC, and consequently the inflow of AW into the Arctic Ocean, is, as the sea-ice concentration, also to a great extend variable and differs with the seasons, inter-annualy, and over longer time periods. Seasonal variations include a lower AW inflow during the summer months and a stronger one during the winter months (Beszczynska-Möller et al. 2012). Inter-annual and longtime variations are largely attributed to the North Atlantic Oscillation (NAO). The NAO is defined as a large-scale alternation of atmospheric pressures between the Icelandic low and the Azores high. This climatic phenomenon is most pronounced during winter but affects the North Atlantic climate throughout the year. In this respect, a positive, strong NAO index is correlated with an increased inflow of AW, while in conclusion a negative, weak NAO index is correlated with a lower inflow in the Arctic Ocean. Since 1976, 60 % of the variance in the annual ice flux through the Fram Strait was explained by the winter NAO index (Dickson et al. 2000). The correlation of the AW inflow and the NAO was also confirmed by Schlichtholz & Goszczko (2006), who further pointed that in case of a positive NAO index, strong westerlies advect warmer air masses to the Nordic Seas area, which reduce the local heat loss to the atmosphere and may result in warmer AW, entering the Arctic Ocean. In fact, most of the temperature increase in the Arctic Ocean is

attributed to a strengthening and warming of the WSC, which has the greatest potential to flow far into the Arctic Ocean (Schauer et al. 2004, Beszczynska-Möller et al. 2012).

In summary, the Fram Strait is crucial for understanding interactions of the Arctic and the Atlantic and for estimating changes in sea ice concentration, because 90 % of the heat exchange and 75 % of the mass exchange take place in that area (Wadhams 1983, Hop et al. 2006). Thereby, the WSC is of great importance because recently, warmer AW progressing far towards the Arctic Ocean has been reported (Holliday et al. 2009).

2.2.2 Central Arctic Ocean

The Central Arctic Ocean is an ice covered ocean, surrounded by continental landmasses and thus can be defined as a Mediterranean Sea (Figure 2.6). One third of the Arctic Ocean is characterized by shallow mean depths of about 30 to 50 m (Chukchi, East Siberian, and Laptev Sea) and 400-600 m (Barents and Kara Sea), respectively. Altogether, the Central Arctic Ocean can be subdivided in seven regions namely Laptev Sea, East Siberian Sea, Chukchi Sea, Beaufort Sea, Greenland Sea, Barents Sea, and the Kara Sea. However, only four openings facilitate an exchange with other oceans. In this respect, the Barents Sea, the Canadian Arctic Archipelago, and the Fram Strait connect the Arctic Ocean with the North Atlantic, while the shallow Bering Strait is the connection with the Pacific Ocean (Rudels et al. 1991). The water masses in the Arctic Ocean are formed by the advection of Pacific and Atlantic Water, which are both characterized by different salinities of ~32.5 (PW) and ~35.0 PSU (AW), and by different nutrient signatures. Supplementary, interactions like freezing and melting processes or river run-offs lead to new water mass formations. The surface water layer, Polar Mixed Layer, is distinguished by low temperature and low salinity from a more saline, underlying halocline that ranges between 50 and 250 m. The Atlantic Layer is found at depth of 400 to 600 m and is characterized by higher salinity and temperature (>0 °C) (Rudels et al. 1991). In contrast to AW, the PW is found in shallower depths due to the lower density.

The Arctic Ocean is affected by a large net freshwater input. Freshwater constitutes an important factor, forming a stratification and enabling the formation of sea ice by inhibiting the upward heat transfer, from the subjacent AW, to the sea surface, ice, and atmosphere (Rudels 2010). Input sources comprise river runoffs from the vast Eurasian and North American landmasses, precipitation over the Eurasian shelves (Norwegian coastal current), and ice melt. The annual mean freshwater input thereby, splits up in 38 % river discharge, 30 % inflow through the Bering Strait, and 24 % net precipitation (Serreze et al. 2006). In turn,

freshwater sinks are mainly formed by the outflow through the Canadian Archipelago, the Fram Strait, and by ice formation (Rabe et al. 2011). Most of the low saline water is found in the Beauford Gyre, where the Ekman convergence leads to a storage (Serreze et al. 2006).

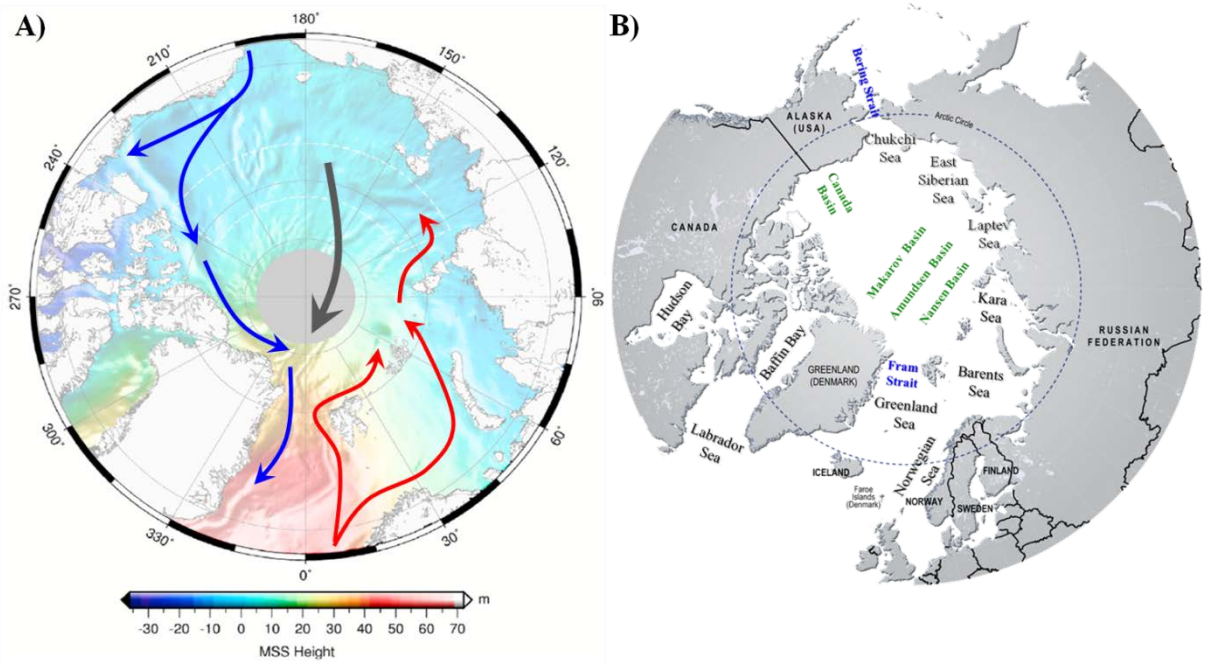

Figure 2.6. The Arctic Ocean. A) Overview of the topography, obtained by mean sea surface model (MSS; scale ranges from -30 to +70 m) and water currents (red: Atlantic Water, blue: Pacific Water and grey: Transpolar Drift) (modified after Farrell et al. 2012) B) Overview of Arctic regions and basins.

The Arctic freshwater content undergoes seasonal as well as interannual variability. However, the interplay of factors, controlling the variability, is still not recovered in full dimension. Prominent seasonality is proven for net precipitation, river run off, Bering Strait inflow, and Fram Strait ice flux. Sea ice is of pivotal importance in the Central Arctic Ocean, because it reduces the effect of wind-driven mixing events and thus contributes to a stable vertical stratification. The ice coverage underlies large seasonal differences in the extent and the thickness, that are smallest in winter and largest at the end of summer (Serreze et al. 2007) (Figure 2.7). However, ice flux is highest during winter and lowest during summer months. Interannual variability is strongly influenced by the amount of sea ice flowing out through the Fram Strait (Vinje 2001, Serreze et al. 2006). The classification of sea ice is based on the age. We distinguish between seasonal sea ice and perennial sea ice, whereas the latter endured at

least one melt period (Perovich & Richter-Menge 2009). An overall downward trend of the ice coverage is evident. In this respect, the ice extent has experienced a series of strong summer minima in the period from 2002 onwards (Stroeve et al. 2012b) (Figure 2.8). Numerous studies reported the decline of the Northern Hemisphere sea ice cover, thickness and the shift from primarily perennial ice to seasonal ice (Anderson et al. 2003, Rigor & Wallace 2004, Lindsay & Zhang 2005, Stroeve et al. 2005, Stroeve et al. 2007, Rothrock et al. 2008, Kwok et al. 2009, Perovich & Richter-Menge 2009, Perovich 2011, Stroeve et al. 2012a, Stroeve et al. 2012b). The sea ice decrease is attributed to thermodynamic and dynamic processes (Serreze et al. 2007). Thermodynamic factors, as for example the increase in air temperature, reduce ice growth and enhance ice melt by extending the melt season (Perovich et al. 2007, Markus et al. 2009). Further, the strengthening and warming of the AW inflow through the Fram Strait and of the PW inflow through the Bering Sea in summer, enhance the ice melt (Schauer et al. 2004, Shimada et al. 2006, Perovich & Richter-Menge 2009).

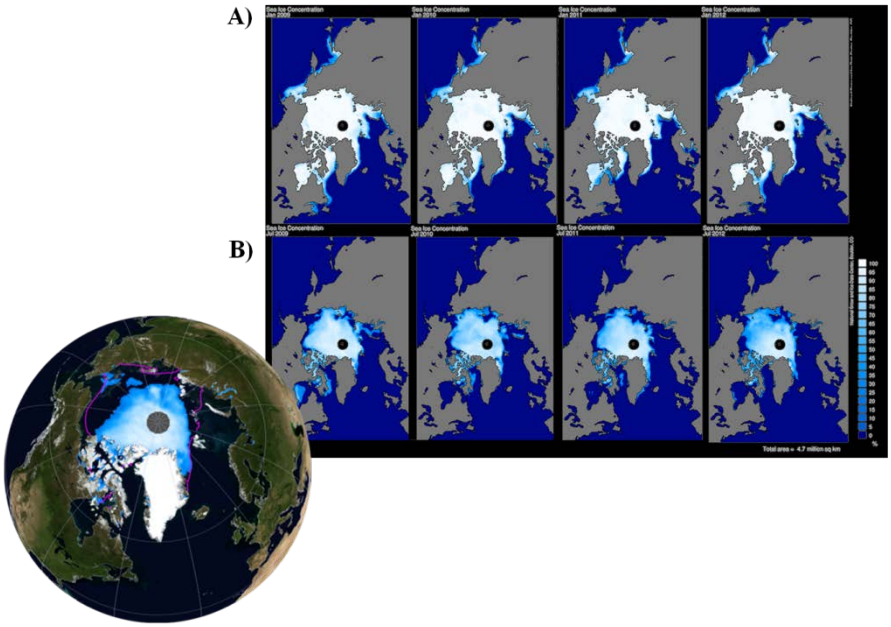

Figure 2.7. Arctic average sea ice extent in the A) Winter maximum (January) and B) Summer minimum (August-September) from 2009 to 2012. Figures derived from the Sea Ice Index courtesy of the National Snow and Ice Data Center. (www.nsidc.org/data/seaice_idex).

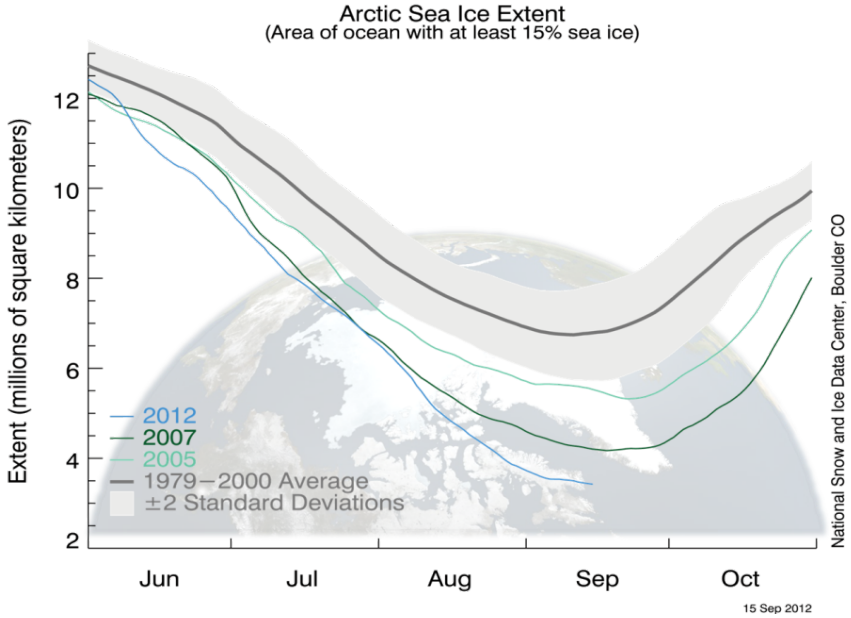

Figure 2.8. Arctic sea ice dynamic during the last years: presented in average for the period 1979 to 2000 and for three summer minima (2005, 2007, and 2012). Data derived from the National Snow & Ice Data Center, from Scanning Multichannel Microwave Radiometer and Special Sensor Microwave/Imager under the implementaion of the NASA Team sea ice algorithm (Meier et al. 2006).

2.3 Molecular Methods

The use of molecular methods has played an important role in our recent understanding of microbial diversity by becoming a key to access the phylogenetic and functional diversity (Pace 1997, Marande et al. 2009). Traditional methods like the observation by light or electron microscopy led to a detailed knowledge concerning the diversity, abundance, and distribution of larger protists that possess important morphological characteristics, which remain intact throughout sampling, preservation, and examination procedures (e.g. cell size, cell shape or skeletal structure) (Caron et al. 1999). Smaller cells lack those morphological features that facilitate the acquisition of accurate identifications and thus cannot be identified by microscopy (Massana & Pedros-Alio 2008) (Figure 2.9).

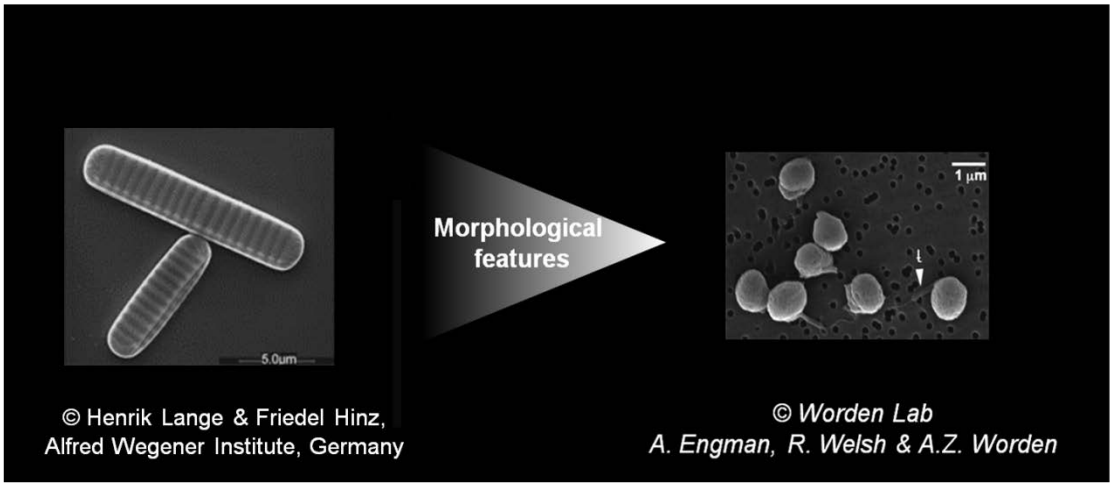

Figure 2.9. Size class dependent resolution of morphological features. Photo of *Fragilariopsis* sp. (Bayer-Giraldi et al. 2011); Photo of *Micromonas* sp. by the Worden Lap (USA).

Here, the molecular methods have a benefit, because they are size independent. Molecular methods used in this study, address two aspects: on the one hand, the DNA fragment length heterogeneities in the ribosomal operon that allow an assessment of microbial community structure (Caron et al. 2004); on the other hand, ribosomal DNA sequences itself (Caron et al. 2012). Hence, they are both based on the extraction of total DNA from an environmental sample and on the amplification of a marker region from the ribosomal operon by PCR. One important marker gene for eukaryotic diversity studies is the nuclear-encoded 18S rRNA gene, coding for the ribosomal small subunit. It has an evolution rate, slow enough to allow phylogenetic investigations, even between distantly related organisms. Further, it consists of well-conserved as well as of fast evolving regions, allowing a classification at different

taxonomic levels (Vaulot et al. 2008). In this study, both general approaches were used, whereas fragment length heterogeneity was analyzed by ARISA (Automated Ribosomal Intergenic Spacer Analysis) and DNA sequence was analyzed by clone library construction and 454-pyrosequencing.

2.3.1 Automated Ribosomal Intergenic Spacer Analysis (ARISA)

ARISA provides a quick snapshot of the community structure and relies on the fragment length of a specific DNA region (Baldwin et al. 1995), the "Internal Transcribed Spacer Region" (ITS). It is located between the 18S and 28S rRNA gene and characterized by a high length heterogeneity that allows discrimination between single species. Until recently, ARISA has been mostly applied to investigate prokaryotic community structures (Smith et al. 2010). Hence, the application on eukaryotic community structure analysis has to our knowledge not been carried out so far.

However, the use of ARISA is not adequate for taxonomic analysis because the method suffers from several biases. In this respect, different taxa can sometimes yield fragments of the same length and hence, cannot provide unambiguous taxonomic information (Caron 2012). Furthermore, not all fragments may be detected, concluding that the sensitivity of ARISA to reflect the total species richness of a community is limited (Bent et al. 2007). Nevertheless, fragment analysis allows a quick and low-cost fingerprint of community structures that can help to reduce the number for further and more explicit investigations.

A brief insight of the process is presented in Figure 2.10. After DNA isolation, the ITS region is amplified under the usage of one fluorescence labeled primer. Fragment lengths are detected by capillary electrophoresis in a sequencer, whereas the obtained electropherogram is subsequently interpreted and transformed to a one-zero-matrix to compare the respective community structures.

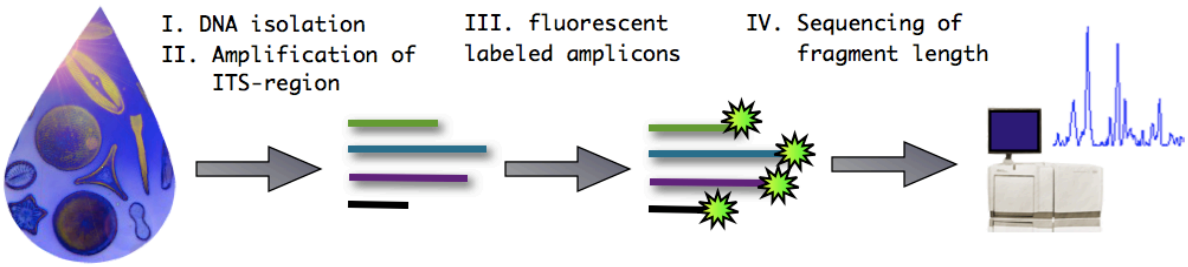

Figure 2.10. Scheme of the ARISA procedure.

2.3.2 Clone Library Analysis

Since sequencing surveys of the small subunit ribosomal RNA (SSU rRNA) regions have been applied to study protist genetic diversity, a huge hidden diversity and new taxa as e.g. picobiliphytes were recorded (Not et al. 2007, Huse et al. 2008, Marande et al. 2009). The SSU 18S rRNA gene is part of the ribosomal functional core and thus all-round exposed to similar selective forces (Moore & Steitz 2002). Therefore, it is the most utilized marker gene in phylogenetic studies (Chenuil 2006).

Clone libraries were considered as gold standard approach for conducting molecular surveys of marine protist diversities (Massana & Pedros-Alio 2008, Not et al. 2009, Palatinszky et al. 2011). Thereby, particularly the obtained sequence lengths of 1000-2000 bp constitute a benefit that allows reliable phylogenetic analysis. In this respect, numerous studies were carried out in the last years to investigate the protist diversity in the Arctic and Southern Ocean (Diez et al. 2001, Lopez-Garcia et al. 2001, Lovejoy et al. 2002, Lovejoy et al. 2006, Lovejoy 2007, Potvin & Lovejoy 2009, Lovejoy & Potvin 2011). However, defiance the great achievement of clone libraries, in analyzing protist diversity within environmental samples, the method suffers from PCR biases that can affect the representation of single species within a protist assemblage due to a reduced amplification of the 18S rRNA gene. In this regard, Wagner et al. (1994) suggested two major ways that lead to PCR bias: PCR selection and PCR drift. The first (selection) comprises primer affinity, which can be different according to the template secondary structure (hampered access) or G+C content (poor denaturation) (Huber et al. 2009). In fact, primers were shown to discriminate for and against certain sequences (Caron et al. 2004, Countway et al. 2005). The second (drift) is assumed to be caused by stochastic variation in the interactions of PCR reagents in the early cycles of the reaction that might be induced by low template concentrations or experimental error and hence, are not reproducible (Polz & Cavanaugh 1998). Other factors as extracellular DNA, multiple rDNA gene copy number (Zhu et al. 2005), chimera, and heteroduplex formation (Huber et al. 2009) have the potential to mask the real diversity as well. Huber et al. (2009) further showed that clone library diversity is highly dependent on the amplicon size, whereas the smallest amplicon presented more diverse community members because of a more readily detection of divergent and lower abundant taxa. The finding was attributed to an easier proceeding of the PCR reaction based on the inability of polymerases to amplify long fragments as efficiently as short fragments and the higher amplification efficiency of the smaller primer set (Suzuki & Giovannoni 1996, Huber et al. 2009). Nevertheless, the strongest bias is formed by the limited throughput (Bent & Forney 2008). In this respect,

rRNA gene libraries of ~100 clones were found to overlap only little in their species lists and thus provide just basic information on the community structure (Stoeck et al. 2006, Cheung et al. 2010).

Figure 2.11 shows a short scheme of the clone library procedure. After the DNA isolation from the environmental sample, 18S rDNA (1800 bp) is amplified and subsequently incorporated in a vector plasmid. Following, each vector is transferred into a *E. coli* cell and plated on a AGAR-medium (+Kanamycin). Only vectors that have successfully inserted the 18S rDNA fragment, provide a Kanamycin resistance and permit the *E.coli* cell to grow on the medium. Before final sequencing, the 18S rDNA is isolated and reamplified.

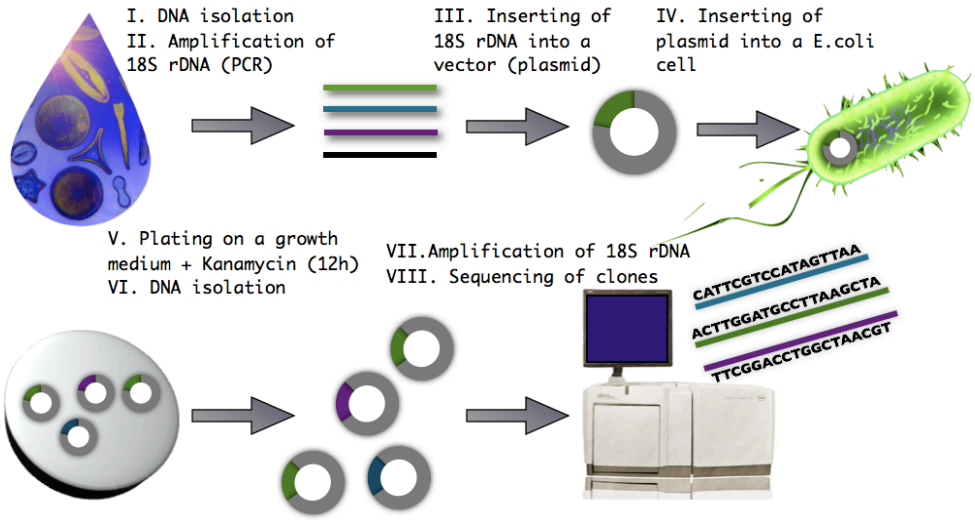

Figure 2.11. Scheme of the clone library procedure.

2.3.3 454-Pyrosequencing

Sogin et al. (2006) first introduced the use of 454-pyrosequencing sequencing of short hypervariable regions (SSU rRNA) to characterize microbial communities. The region V4 is the largest and most complex of the hypervariable regions and ranges from about 230 to >500 bases in eukaryotic protists (Nickrent & Sargent 1991). Initially, several studies tested the utility of 454-pyrosequencing by comparing the results with reads developed using the well-established clone library approach (Krober et al. 2009, Nasidze et al. 2009). All these comparisons highlighted the application of the new method and further described it as a powerful tool, particularly for addressing questions about rare phylotypes (Sogin et al. 2006, Huber et al. 2007). In fact, 454-pyrosequencing is actually more and more replacing traditional Sanger sequencing because of the greater sampling depth (Kunin et al. 2010).

Further advantages are the elimination of cloning bias and the complete sequencing of short reads in a single run, which maximizes the number of sampled organisms, while it minimizes chimera formation (Huse et al. 2008). However, since 454-pyrosequencing comprises an amplification step, the method suffers from the same PCR bias as previously discussed in the clone libraries.

In particular, the small sequence lengths allow no detailed phylogenetic analysis and limits the use of 454-pyrosequencing to a nevertheless, adequate screening tool for protist diversity, due to the sampling depth (Stoeck et al. 2010). The growing database of DNA sequence information further offers the possibility for greatly improving the existing molecular tools (Caron et al. 2009). Altogether, 454-pyrosequencing has revolutionized surveys of microbial diversity because of the delivery of the enormous number of sequence reads in a single experiment (Medinger et al. 2010).

Figure 2.12 shows a brief scheme of the 454-pyrosequencing procedure. First, the target region (V4) is amplified from the environmental DNA sample. Following, a DNA library is prepared where short adaptors are ligated onto the sequence ends, in which one contains a 5'biotin tag that immobilizes the library onto special capture beads. Each bead thereby contains one single strand (sst) DNA sequence. Afterwards, the "bead-bound" library is amplified in a water-in-oil mixture (microreactor). The amplicons (bead-immobilized clonally amplified DNA fragments) are put onto a picotiterplate, where sequence reagents (buffer and nucleotides) are flown over and sstDNA is extended. The incorporation of a nucleotide results in a light signal generating reaction, which is recorded by the instrument.

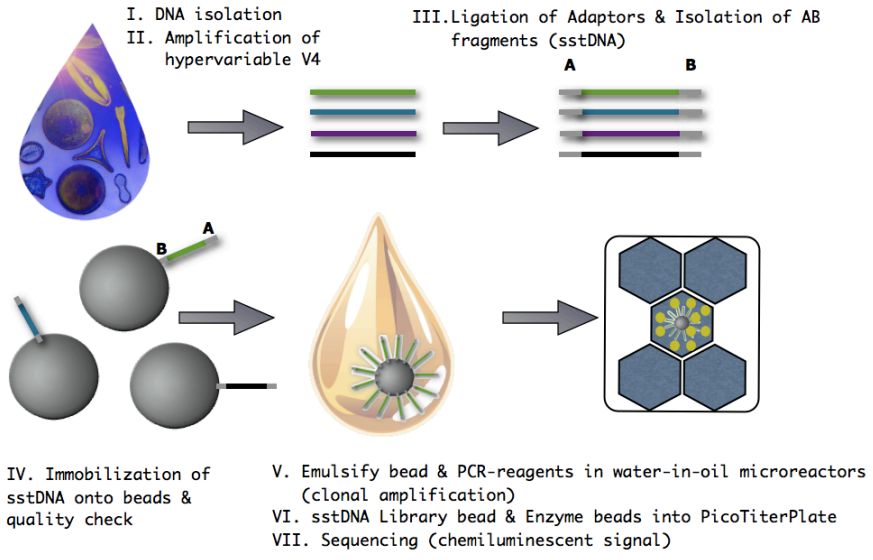

Figure 2.12. Scheme of the 454-pyrosequencing procedure.

2.4 Hypotheses and Outline

2.4.1 Hypotheses

The progress in molecular biology and the development of new molecular tools, led to the allotment of a large quantity of new to date unidentified species that initiated an enormous increase of investigations on protist genetic diversity. These investigations revealed some methodological bias that caused some criticism. Those critics mainly address the problem of multiple rDNA gene copy numbers that might lead to a wrong estimation of the quantitative contribution of a respective taxon to protist communities. Until now, the applicability of molecular tools is still under evaluation, leading to the first objective of this thesis. It addresses the applicability of new molecular tools for protist diversity surveys by comparing 454-pyrosequencing data with different classical approaches such as light microscopy, HPLC, and clone libraries.

Hence, the first hypothesis to be tested is:*"Molecular surveys of genetic protist diversity obtained by 454-pyrosequencing, constitute an adequate tool for assessing natural protist diversity."*

454-pyrosequencing is a recent method not often been used in the past to investigate protist diversity in oceanic regions. Studies concerning the protist assemblage in the Central Arctic Ocean via 454-pyrosequencing are particularly scarce due to the difficult accessibility of the area. Molecular surveys of protist diversity are further lacking in the Fram Strait, despite the better geographical accessibility. However, the protist diversity in the Fram Strait is of particular interest because of the variable environmental conditions that may affect the protist community structure. Hence, another objective was to analyze protist diversity in relation to different water mass distribution, characterized by different abiotic conditions and addressing the second hypothesis:

"Water masses in the Arctic Ocean and in the Fram Strait are characterized by distinct protist communities"

Further questions, such as (1) Which molecular approach is appropriate to elucidate water mass related protist compositions? and (2) Are there organisms that are particularly suitable for tracing water masses? shall be answered.

Based on the previous questions the third hypothesis developed was: "*Complex hydrographical situations can be evaluated via genetic information.*"

In this regard, the investigation of protist diversity and distribution at the entrance of the Central Arctic Ocean, the Fram Strait that constitutes an appropriate model area, because of the variable environmental conditions, is addressing well the third hypothesis. Further questions such as: (1) Can the circulation and recirculation patterns in the Fram Strait be evaluated based on the protist assemblage? and/or (2) Are, based on the findings in the Fram Strait, those approaches still valuable in the Central Arctic Ocean under the coincidental presence of various ice concentrations? will be answered below.

2.4.2 Outline

Publication I

Molecular assessment of marine microbial diversity, derived from sequence analysis, is mainly based on two approaches: clone library and 454-pyrosequencing. Both methods deliver taxon-specific protist surveys, but differ in the cloning bias. So far, more protist sequences were obtained by clone library than by 454-pyrosequencing, due to the longer implementation of the former method. However, the higher throughput and the ongoing improvement of the sequence length of 454-pyrosequencing is likely to increase the application of the method in future. Hence, a comparison of the molecular methods is important in order to assess the comparability of both data sets. In publication I clone library data and 454-pyrosequencing data are compared and address the first hypothesis by evaluating the analogy of protist diversity, obtained by the two different molecular methods. The investigation comprises on the one hand, the entire protist community composition at one station in the Antarctic and on the other hand, the picoplankton community composition at four stations in the Fram Strait. Furthermore, the study addresses the question if clone library is suitable to serve as a backbone for 454-pyrosequencing data, because of the longer sequence lengths and thus higher taxonomic resolution.

Publication II

The purpose of this manuscript was to apply 454-pyrosequencing at four sampling sites in the Eastern Fram Strait, to study the diversity of small picoeukaryotes and to test the second and third hypothesis. Picoplankton is supposed to show a particularly high water mass correlation because of their cell size dependent high buoyancy in relation to water mass density. Moreover, 454-pyrosequencing was put through an internal review process to assess the reproduction of the protist composition after fractionated filtration. Cell breakage is supposed to constitute a major bias, reducing the accuracy of the fractionation process. Estimation of a filtration bias over the three size classes included a detailed investigation of ubiquitous, multiple (micro-/picoplankton and nano-/picoplankton), and unique occurring phylotypes and an adjustment of the major taxonomic group distribution with previous published data. Furthermore, the comparability of protist diversity, obtained by 454-pyrosequencing and classical light microscopy, was assessed by comparing the proportion of diatoms (first hypothesis).

Publication III and IV

Both manuscripts investigate protist diversity of the entire size fraction, using 454-pyrosequencing, and address the second hypothesis, concerning water mass specific communities and the third, concerning the evaluations of complex hydrographical situations. In this respect, **Publication III** studies the protist assemblages of five stations along a transect in the Western Fram Strait that encompass Polar and Atlantic Water. HPLC complemented the study to evaluate the molecular data with pigment data. **Publication IV** focuses on the Central Arctic Ocean and the protist community structure at eight sampling sites, within four distinct water masses. The manuscript further analyzes the influence of the persistent Arctic sea ice cover on the protist composition, with emphasis on the abundant and rare biosphere.

3. Publication

3.1 List of Publications

This doctorial thesis is based on the following publications:

I. CHRISTIAN WOLF, ESTELLE S. KILIAS AND KATJA METFIES
Investigating eukaryotic protist diversity – A comparison of clone library and 454-pyrosequencing data
Journal of Phycology: submitted

II. ESTELLE S. KILIAS, EVA-MARIA NÖTHIG, CHRISTIAN WOLF AND KATJA METFIES
Picoplankton community composition in the Eastern Fram Strait
FEMS Microbiology Ecology: submitted

III. ESTELLE S. KILIAS, CHRISTIAN WOLF, EVA-MARIA NÖTHIG, ILKA PEEKEN AND KATJA METFIES
Protist distribution (the abundant biosphere) in the Western Fram Strait (Arctic Ocean) in summer investigated via molecular techniques
Journal of Phycology: submitted

IV. ESTELLE S. KILIAS, GERHARD KATTNER, CHRISTIAN WOLF, STEFAN FRICKENHAUS AND KATJA METFIES
A molecular survey of protist diversity through the Central Arctic Ocean
Protist: to be submitted (final formatting)

Further contributions:

CHRISTIAN WOLF, ILKA PEEKEN, MIRKO LUNAU, STEPHAN FRICKENHAUS, ESTELLE S. KILIAS AND KATJA METFIES
Oceanographic fronts in the Southern Ocean determine biogeographic differences in eukaryotic protist communities – new insights based on 454-pyrosequencing
Polar Biology: submitted

CHRISTIAN WOLF, ILKA PEEKEN, STEPHAN FRICKENHAUS, ESTELLE S. KILIAS AND KATJA METFIES

Regional variability in eukaryotic protist communities in the Amundsen Sea

Antarctic Science: accepted

STEFAN THIELE, ISABELLE SCHULZ, CHRISTIAN WOLF, BERNHARD M. FUCHS, PHILIPP ASSMY, KATJA METFIES, VICTOR SMETACEK, ESTELLE S. KILIAS AND RUDOLF AMMANN

Comparison of classical methods with modern molecular approaches for the investigation of pico- and nanoplankton assemblages

To be submitted (final correction)

3.2 Statement of Contributions

Publication I

The experiments were planned together with Christian Wolf and Katja Metfies. Sampling was carried out by Katja Metfies. Laboratory work and data analysis was performed by Christian Wolf and me. The publication was written in equally contribution of Christian Wolf and me.

Publication II

The experiments were planned together with Katja Metfies. Sampling was carried out by Katja Metfies. Laboratory work and data analysis was accomplished by me. Molecular data analysis pipeline was developed in collaboration with Christian Wolf. Microscopy was carried out by Eva-Maria Nöthig who further significantly contributed to the discussion and review process. The publication was written by me.

Publication III

The experiments were planned together with Katja Metfies. Molecular experiments and data analysis was performed by me. Molecular data analysis pipeline was developed in collaboration with Christian Wolf. HPLC was conducted by Ilka Peeken. Eva-Maria Nöthig significantly contributed to the discussion and review process. The publication was written by me.

Publication IV

The experiments were planned together with Katja Metfies. Sampling, laboratory work and data analysis was accomplished by me. Nutrients measurements were contributed by Gerhard Kattner. Bioinformatical support was provided by Stephan Frickenhaus. Molecular data analysis pipeline was developed in collaboration with Christian Wolf. The publication was written by me.

Publication I

INVESTIGATING EUKARYOTIC PROTIST DIVERSITY – A COMPARISON OF CLONE LIBRARY AND 454-PYROSEQUENCING DATA

ESTELLE KILIAS, CHRISTIAN WOLF AND KATJA METFIES

3.3 Publication I

INVESTIGATING EUKARYOTIC PROTIST DIVERSITY – A COMPARISON OF CLONE LIBRARY AND 454-PYROSEQUENCING DATA

Christian Wolf [1]* Estelle Kilias[1]* and Katja Metfies*

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[1]First author

Abstract

We compared two molecular approaches, i.e. sequencing of 18S rRNA clone libraries and 454-pyrosequencing, which are commonly used for describing protist diversity. The comparison was conducted with four Arctic water samples, focusing on the picoplankton (0.4-3 µm), and with one Antarctic water sample, examining the whole size spectrum (>0.4 µm). We found different outcomes between the two different methods. Both approaches revealed phylotypes that were not found with the other approach. The abundant biosphere, defined by the 454-pyrosequencing approach, was not fully recovered by the clone library approach. We found a bias of the cloning method against several groups, e.g. haptophytes in the Arctic samples and diatoms in the Antarctic sample. In summary, prior cloning data have to be handled with care, when compared with 454-pyrosequencing data. Additionally, cloning data are only of limited suitability as backbone for phylogenetic analysis of 454-pyrosequencing data.

Key index words

18S rRNA gene, 454-pyrosequencing, Clone library, Eukaryotic protists, Genetic diversity, Phytoplankton

Introduction

Until recently, the majority of phylogenetic investigations of the eukaryotic protist diversity were based on the analysis of 18S rRNA gene clone libraries. Sequencing of 18S rRNA gene clone libraries provides sequence information of the complete gene and allows a reliable phylogenetic characterization (Diez et al. 2001, Lovejoy et al. 2006). These studies contributed significantly to elucidate eukaryotic phytoplankton diversity and community composition in the marine environment. They revealed a huge hidden diversity, especially originating from the picoeukaryotic size fraction. Representatives of all major phytoplankton taxa could be found in the sequence libraries (Diez et al. 2001, Lovejoy et al. 2006, Lovejoy & Potvin 2011). However, it is expected that the real diversity is even higher than observed in the libraries (Diez et al. 2001, Lopez-Garcia et al. 2001, Moon-van der Staay et al. 2001).

In summary, sequencing of the 18S rRNA gene is a reliable approach (Diez et al. 2001, Lovejoy et al. 2006, Cheung et al. 2010, Lovejoy & Potvin 2011). It is used as a gold standard in molecular assessments of phytoplankton diversity. The 18S rRNA gene is such a widely employed and valuable 'bar-code' to assess eukaryotic phytoplankton diversity, because of its slow evolutionary rate and its occurrence in all eukaryotic organisms (Amann & Kuhl 1998, Vaulot et al. 2008). It is sufficiently slow to allow differentiation between organisms at different taxonomic levels (Vaulot et al. 2008).

However, quantitative interpretation of 18S rRNA clone libraries is challenged in various ways, e.g. by the presence of multiple gene copies that may not be identical in all species and thus falsify the diversity and community structure. Furthermore, sequencing of 18S rRNA gene clone libraries has a number of other drawbacks, such as its vulnerability towards a potential bias induced by primer specificity (Farris & Olson 2007) and the production of chimerical sequences (Berney et al. 2004), a disparate incorporation rate within the cloning process and a limited throughput (Bent & Forney 2008). Additionally, the analysis is time-consuming and cost-intensive.

The introduction of the 454-pyrosequencing technology for the assessment of microbial communities is an attractive alternative to the sequencing of clone libraries. It is independent of the cloning step and allows high-resolution sequencing of microbial sequences (Margulies et al. 2005). Furthermore, compared to the analysis of clones, the massively parallel pyrosequencing provides more sequences and uncovers more organisms by less chimera formation and less costs (Huse et al. 2008). In respect of the vast microbial diversity, the greater sampling depth is advantageous and even allows elucidating the diversity of the rare biosphere (Sogin et al. 2006). However, one caveat of the pyrosequencing approach is the

tendency to overestimate the number of rare phylotypes, because of sequencing errors. Such errors will run the risk of inflating the diversity estimates, due to the fact that every single read is considered to represent a community member (Kunin et al. 2010). An additional caveat is the short sequence length of approximately 500 bp, which results in a less robust phylogenetic affiliation. Here, the clone library approach is advantageous over the 454-pyrosequencing approach because it delivers longer sequences that cover the whole 18S rRNA gene and are better suited for phylogenetic analyses (Diez et al. 2001, Lovejoy et al. 2006).

Regarding the advantages and drawbacks of 18S rRNA gene clone libraries and 454-pyrosequencing for the assessment of protist communities, we would like to address three main questions in this study: (i) How do the choice of a molecular method influence our understanding of protist diversity and community structure? (ii) Do clone library data reflect the abundant biosphere? (iii) Are clone library data suitable to serve as backbone for phylogenetic analysis of 454-pyrosequencing data?

To answer these questions, we analyzed four samples from the Arctic Ocean, comprising the picoeukaryotic fraction (0.4-3 µm) and one sample from the Southern Ocean, comprising the whole size fraction (>0.4 µm). We choose the sampling setup to exclude possible bias induced by cell size or geographical location.

Material and Methods

Location and sampling

The study area comprises four stations, located in the Fram Strait (Arctic Ocean), as well as one station from the Southern Ocean (Figure 3.1). All samples are part of other larger studies (Kilias et al. submitted., Wolf et al. submitted). The four Arctic stations extended between 2-6°E longitudes and 78-80°N latitudes and were sampled during the ARK XXIV/2 cruise onboard the *RV Polarstern* in July 2009. The Antarctic station, located at 164.9°W longitude and 69°S latitude, was sampled during the *RV Polarstern* cruise ANT XXVI/3 in February 2010. The Arctic samples were collected at the subsurface maximum chlorophyll layer with a rosette system, fitted with Niskin bottles and appointed with depth, temperature, salinity, and fluorescence profilers. The Antarctic sample was collected using the ship pumping system (membrane pump), located at the bow at 8 m depth below the surface. In both cases, 1.5 l of sea water were successively filtered with a pressure of 200 mbar onto Isopore Membrane Filters (Millipore, USA) with a pore size of 10 µm, 3 µm and finally 0.4 µm. The filters were stored at -80°C until further treatment in the laboratory.

DNA extraction

DNA extraction was carried out with the E.Z.N.A TM SP Plant DNA Kit (Omega Bio-Tek, USA) following the manufacturer's instructions. DNA concentration was determined with a NanoDrop 1000 system (Thermo Fisher Scientific, USA).

Clone library construction

The 18S rRNA gene of the Arctic samples was amplified using the specific primers 82F (5'-GTA AAA CTG CGA ATG GCT CAT-3') and 1528R (5'-TGA TCC TTC TGC AGG TTC ACC TAC-3') and genomic DNA isolated from the 0.4 µm filter as template. The amplification of the Antarctic sample was conducted using the primer combination 300F (5'-AGG GTT CGA TTC CGG AG-3') and 1200R (5'-CAG GTC TGT GAT GCC C-3'), because the former combination resulted in a poor PCR product. Furthermore, the whole protist assemblage was used for the methodological comparison of the Antarctic sample. In this respect, the 18S rRNA gene of each fraction was amplified and equal volumes of each PCR product were pooled before the purification. The PCR reaction mixture contained 1 x HotMaster Taq Buffer containing 2.5 mM Mg^{2+} (5 Prime, USA), 0.4 U of HotMaster Taq polymerase (5 Prime, USA), 10 mg/ml BSA, 10 mM dNTP-mix (Eppendorf, Germany), 10 µM of each Primer and 1 µl of template DNA in a final volume of 20 µl. PCR reactions were carried out in a Mastercycler (Eppendorf, Germany) under the following conditions: an initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 1 min and extension at 72°C for 3 min, and a final extension at 72°C for 10 min. The purification of the resulting PCR fragment was carried out with the Gel Purification Kit (Invitrogen, USA), following the manufacturers protocol. Subsequently, the fragment was cloned into the pDrive Cloning Vector (QIAGEN, Germany) taking advantage of the PCR Cloning Kit (QIAGEN, Germany) and transformed into TOP10 chemo-competent *E.coli* cells (Invitrogen, USA). Positive colonies were screened for similar inserts by performing a restriction fragment length polymorphism (RFLP) analysis, using the multicut enzyme Hae III (New England Biolabs, USA). Clones with a similar RFLP pattern were considered to display the same phylotype and grouped into an OTU (operational taxonomic unit). One to two representatives of each OTU were sequenced using the 300F (see above) and 528F (5'-GCG GTA ATT CCA GCT CCA A-3') primer under the following conditions: an initial denaturation step at 96°C for 1 min, 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s and extension at 60°C for 4 min. The terminal sequencing was carried out on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA).

454-pyrosequencing

The hypervariable V4 region of the 18S rRNA gene was amplified taking advantage of the primer combination 528F (5'-GCG GTA ATT CCA GCT CCA A-3') and 1055R (5'-ACG GCC ATG CAC CAC CAC CCA T-3'). The PCR mixtures were composed as described previously for the clone library construction. Reaction conditions were as following: an initial denaturation at 94°C for 3 min, 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. Subsequently, the amplicons were purified with the Mini Elute PCR Purification Kit (QIAGEN, Germany). In case of the Antarctic sample, an equal volume of PCR reaction of each size fraction was pooled and purified with the MinElute PCR purification kit (Qiagen, Germany) following the manufacturer's instructions. Pyrosequencing was performed on a Genome Sequencer FLX system (Roche, Germany) by GATC Biotech AG (Germany).

Data analysis

The two raw sequences of each sequenced clone were assembled with the software Lasergene 10 (DNASTAR, USA) and a consensus sequence was built. All sequences (clone consensus sequences and 454-pyrosequencing reads) were checked for errors (reads with many unresolved bases) implied by the sequencing process and sequences with more than one uncertain base (N) were removed. Remaining sequences were checked for possible chimera formation by applying the detecting software UCHIME 4.2.40 (Edgar et al. 2011) and all sequences considered being chimeric were excluded from further analysis. Residual sequences were added to the Lasergene 10 software (DNASTAR, USA) and clustered into OTUs at the 97 % similarity level. Subsequently, singletons from the 454-pyrosequencing data were removed. Consensus sequences of OTUs were aligned using the software HMMER 2.3.2 (Eddy 2011). Subsequently, taxonomical affiliation was determined by placing the consensus sequences into a reference tree, consisting of 1200 high quality 18S rRNA gene sequences of Eukarya from the SILVA reference database (SSU Ref 108), using the software pplacer 1.0 (Matsen et al. 2010). The compiled reference database is available on request in ARB-format. Detected non-phytoplankton sequences originating from metazoans and fungi were removed. Rarefaction curves were computed using the freeware program Analytic Rarefaction 1.3. In case of the clone library sequences, a phylogenetic tree was generated using MEGA version 4 (Tamura et al. 2007) on the basis of Maximum Parsimony principles by the application of the Juke Cantor model and 1000 bootstrap restarts. The clone library sequences generated in this study have been deposited at GenBank under Accession No.

JX840877-JX840942. The 454-pyrosequencing reads were deposited at GenBank`s Short Read Archive (SRA) under Accession No. SRA058841 (Arctic samples) and SRA056811 (Antarctic sample).

Results

The five clone libraries resulted in a total of 698 high quality clones (Table 3.1). After the RFLP analysis of 182 (HG1 and HGS3) to 117 (ANT25) clones, a total of 134 Arctic and 64 Antarctic clones were sequenced. Non-target sequences (metazoan and fungi sequences) were only found in the pooled ANT25 clone library (6 %), while chimeras were formed in all clone libraries (6-19 %), except in library HG4 (0 %). Final clustering of the residual sequences resulted in seven (HG4) to 24 (HGS3) different phylotypes.

In total, between 7539 (HG4) and 45772 (ANT25) 454-pyrosequencing reads were obtained. The analytical process revealed 2-6 % of chimeric sequences. The quality filtering reduced the initial read number to a final range of 5220 (HGN4) to 30561 (ANT25) reads, that resulted in 709 (HG4) to 1153 (ANT25) different phylotypes, based on a clustering at the 97 % similarity level.

The clone library and 454-pyrosequencing approach provided different numbers of phylotypes that differ by several levels of magnitude. The rarefaction curves present an estimation of the local species richness based on the respective approach (Figure 3.2). Two Arctic clone libraries (HG1 and HGN4) present a rarefaction curve that is saturated or almost saturated, while the species richness of the residual Arctic and the Antarctic clone library is not totally covered. The 454-pyrosequencing rarefaction curve for sample ANT25 reached the plateau, whereas the curves of the four Arctic samples (HG1, HG4, HGN4 and HGS3) ended in the slope phase.

Comparison of clone library and 454-pyrosequencing data set - Arctic

In total, 47 different phylotypes have been identified in the clone libraries from the Arctic samples (Figure 3.3 and Table 3.2). The number of phylotypes in each sample ranged from seven (HG4) to 24 (HGS3). Most phylotypes (32) grouped within the alveolates, whereas the majority of 30 phylotypes affiliated with dinoflagellates and only two with ciliates. Picobiliphytes, cryptophytes, rhodophytes, and stramenopiles were represented by two, three, one and two phylotypes, respectively. Seven phylotypes affiliated with chlorophytes, of which five were closely related to *Micromonas pusilla*. The majority of the phylotypes (37) were just present at one of the different stations. Only ten phylotypes could be found in more than

one station. Three phylotypes (ARK_3, ARK_5, and ARK_15) were present in three samples. There was no phylotype, which was recovered from all samples. Samples HGN4 and HGS3 showed the highest similarity with five shared phylotypes. The most abundant phylotype in the clone libraries was ARK_15, and affiliated with *Micromonas pusilla* in the phylogenetic tree. This phylotype contributed 77.1 % of the clones in sample HG1, 65.9 % in sample HG4, and 47.5 % in sample HGN4. In sample HGS3, the phylotype was not found at all. In this sample, the phylotypes ARK_12 (Syndiniales clone) and ARK_14 (*Geminigera cryophila*) showed the highest clone abundance with 26.6 % and 23.7 %, respectively.

In total, 709 to 1014 phylotypes were obtained by 454-pyrosequening. The data set was composed of 18.2-51.4 % dinoflagellates, 1-3.4 % ciliates, 16.3-33.1 % haptophytes, 0-0.9 % rhodophytes, 0.5-2.2 % cryptophytes, 3.4-42.2 % chlorophytes, and 14.7-16.8 % stramenopiles. In contrast, the clone libraries did neither contain haptophytes, nor rhodophytes, 0.6-28.1 % cryptophytes, 4.3-82.9 % chlorophytes, 1-2.2 % stramenopiles, 3.5-64.3 % dinoflagellates, and 0.7-9.7 % ciliates.

Thirteen out of the 47 clone library phylotypes (27.7 %) could not be recovered in the 454-pyrosequencing data set. The clone libraries of samples HG1, HG4 and HGS3 each covered 20 % of the abundant phylotypes of the 454 data set (data not shown). The clone library of sample HGN4 covered none of the abundant 454 phylotypes.

Comparison of clone library and 454-pyrosequencing data set - Antarctic

In the clone library generated from the Antarctic sample (ANT25), 19 different phylotypes have been found (Figure 3.4 and Table 3.3). The majority of these phylotypes belonged to alveolates (10). Among the alveolates, eight phylotypes affiliated with dinoflagellates, whereas one belonged to the ciliates and Syndiniales, respectively. There were two phylotypes belonging to the haptophytes, whereas both were close to the genus *Phaeocystis*. Five phylotypes of the library belonged to the stramenopiles, at which two were representatives of diatoms. One cryptophyte, belonging to the genus *Geminingera*, and one picobiliphyte were found.

The 454-pyrosequencing revealed 1153 different phylotypes. The relative abundance of the phylotypes retrieved from the library and the respective relative abundance in the 454-pyrosequencing data set are shown in Table 3.3. Four out of the 19 clone library phylotypes (21.1 %) were not found in the 454 data set. From the 12 abundant phylotypes in the 454 data set (data not shown), only four (33.3 %) were found in the clone library. The phylotype with the highest relative abundance was the same in both data sets (clone ANT_13). The 454-

pyrosequencing data set was composed of 30.8 % haptophytes (39.5 % in the clone library), 1.2 % chlorophytes (0 %), 1.6 % cryptophytes (1 %), 1.1 % rhodophytes (0 %), 22.9 % diatoms (2 %), 9.5 % other stramenopiles (5.8 %), 23.6 % dinoflagellates (42.5 %), 2.5 % Syndiniales (1.9 %), and 6.8 % ciliates (4.8 %).

Both the Arctic and Southern Ocean samples showed that ~20 % of the phylotypes retrieved via the cloning approach were not found in the 454-pyrosequencing data, and only 20-30 % of the abundant phylotypes of the 454-pyrosequencing data were found via the cloning approach.

Discussion

Although, culture-independent methods as traditional clone library and the novel 454-pyrosequencing are often used for screening microbial community structures (Diez et al. 2001, Lovejoy et al. 2006, Cheung et al. 2010), studies that directly compare both approaches are scarce. To our knowledge, those studies primarily focused on the genetic diversity of prokaryotes (Zhang et al. 2011). Here, we present the first comparison of both molecular methods for eukaryotic protists. The comparison has been carried out on picoeukaryotic protists in the Arctic and on the whole protist assemblage (covering all size classes) in the Antarctic. Independent of the size fractionation or geographical location, the two methods showed high discrepancies in distribution as well as in relative abundance.

(I) How does the choice of a molecular method influence our understanding of protist diversity and community structure?

Our data suggest that our understanding of protist diversity and community structure is strongly dependent on the molecular method used. According to the Arctic clone library data, the picoplankton communities were mainly dominated by chlorophytes, while haptophytes were absent. In contrast, the 454-data suggest that haptophytes are dominating in these samples and chlorophytes are less abundant. It can be excluded that it is hard or impossible to clone haptophytes, because numerous *Phaeocystis* sp. clones have been found in the Antarctic library. However, phylotypes that affiliated particularly to small non-colonial haptophytes have been already reported to occur in merely small numbers in clone library data (Lovejoy et al. 2006, Potvin & Lovejoy 2009). Amacher et al. (2011) also proved that it is possible to retrieve *Emiliania huxleyi* (belonging to haptophytes) sequences via cloning. Additionally, we have checked the suitability of the used primers. All primers matched with haptophytes (including *Phaeocystis* and *Emiliania*) in the reference database. Nevertheless, it cannot be

excluded, that primer efficiency could be an explanation for the observed discrepancy. There are several studies showing that different primer sets applied on the same sample resulted in different diversity and abundance patterns (Jeon et al. 2008, Potvin & Lovejoy 2009, Stoeck et al. 2010). Mismatches between the primers and the 18S rRNA genes sequences (Liu et al. 2009), and primer competition (Potvin & Lovejoy 2009) are considered to be responsible for it. However, the primer sets used in this study covered all major taxonomic groups. Nevertheless, we observed that the primer set used for the Arctic samples might be biased against haptophytes and the primer set used for the Antarctic sample against diatoms.

Another explanation for the discrepancies between the clone libraries and the 454 data sets could be the mutual impact of organisms during the PCR or cloning step. Amacher et al. (2011) showed that certain co-occurring organisms bias the recovery of organisms in clone libraries. Our data suggest that *Micromonas* sp. might influence the cloning efficiency of other organisms, especially on *Phaeocystis* sp. *Micromonas* sp. sequences were overrepresented in the Arctic clone libraries and no *Phaeocystis* sequences were retrieved. However, in three of the four 454-pyrosequencing samples, *Phaeocystis* sp. was more abundant than *Micromonas* sp. Thus, if *Micromonas* sp. occurs in sufficient abundance, it might have been favored during the cloning step. In contrast, in the Antarctic clone library, we observed a bias against diatoms. The 454-pyrosequencing revealed a tenfold higher diatom abundance than the clone library. Here, our data suggest that dinoflagellates or *Phaeocystis* had a repressing effect on diatoms during the cloning step.

Although, the picoeukaryotic and the whole protist approach both showed high differences between the clone libraries and the 454-pyrosequencing data, the aberration was higher for picoeukaryotes. Beside the previously discussed bias induced by the use of different primer sets, another possible bias may rely on the interpretation of the RFLP patterns. The interpretation of RFLP patterns may be biased because of incomplete DNA digestion, variable DNA load of the gels, similar cutting sites among species, or limited sensitivity of the detection technique (Wu et al. 2000). In this regard, the whole size community may be represented by more distinct patterns, while patterns of picoeukaryotes could be more similar. This would lead to a reduced picture of picoeukaryotic diversity, because a limited number (1-2) of representatives were sequenced for each interpreted group. Altogether, we have shown that the two different methods revealed different community compositions and the clone library approach even defalcated whole taxonomical groups.

(ii) Do clone library data reflect exclusively the abundant biosphere?

A recent assumption of previous studies (Pedrós-Alió 2006) is that clone libraries cover at least the abundant biosphere of protist communities. However, our results suggest that the clone library data did not cover the whole abundant biosphere of the 454-pyrosequencing data set. All clone libraries missed over 50 % of the abundant biosphere, identified by 454-pyrosequencing. It should be mentioned that of the missing 50 %, most phylotypes were affiliated to *Phaeocystis* (Arctic samples) and to diatoms (Antarctic sample). In addition, we found phylotypes in the clone libraries that were not recovered in the 454-pyrosequencing data. This is most pronounced in the Arctic samples and supported by the 454-pyrosequening rarefaction curves, which showed that the samples were not exhaustively sequenced. Thus, with a higher sequencing effort the additional sequences in the clone libraries might have been recovered by the 454-pyrosequencing. In contrast, the rarefaction curves of the clone libraries simulate that the total diversity was sufficiently recovered, although the 454 approach revealed far more phylotypes. Our data support the common sense that the concept of rarefaction curves is questionable. The calculation is biased by the presence of multiple 18S rRNA gene copies. Additionally, the removal of singletons in the 454 approach during the analytical process consequently leads to a saturated curve (e.g. in the Antarctic sample). The calculation for clone library data is biased by the limited throughput, the low number of phylotypes retrieved, and the overrepresentation of single phylotypes (e.g. *Micromonas* sp. in the Arctic samples).

We have observed that groups, which showed a low abundance in the 454 data (<2 % in total), can also be retrieved via the cloning approach. However, this observation does not apply to all of the clone libraries, suggesting that the recovery of OTUs in clone libraries is random.

(iii) Are clone library data suitable to serve as a backbone for phylogenetic interpretation of 454-pyrosequencing data?

Based on the findings and remarks discussed above, the suitability of clone library data to serve as backbone for 454-pyrosequencing data is only limited, because the recovery of phylotypes extremely differs between the two methods, especially when focusing on picoplankton.

In conclusion, we have shown that our understanding of protist diversity and structure assessed with molecular methods varies strongly depending on the molecular method used.

Furthermore, we suggest that comparisons of new 454-pyrosequencing data with previously published clone library data of protist diversity have to be handled with careand should not be over interpreted. Moreover, the cloning approach seems not to be adequate in general to resolve the abundant biosphere and appears to be of very limited suitability as backbone for a refined phylogenetic analysis of OTUs identified by 454-pyrosequencing data.

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Table 3.1. Summary of recovered clones and 454-pyrosequencing reads.

	HG1	HG4	HGN4	HGS3	ANT25
Clone library:					
High quality clones	175	179	101	139	104
Phylotypes	16	7	13	24	19
454-pyrosequencing:					
Total reads	9830	7539	7938	8786	45772
High quality reads	8154	5434	5220	7020	30561
OTUs (97 %)	754	709	829	1014	1153

Table 3.2. Phylogenetic affiliations of the Arctic clone phylotypes and their relative abundance in the libraries and the 454-pyrosequencing data set.

Phylotype	Closest match (Maximum identity %)	Taxonomic group	Clones (%) / 454 (%)			
			HG1	HG4	HGN4	HGS3
ARK_1	*Bolidomonas pacifica* (92)	Stramenopiles	1.1 / 2.8	- / 0.7	1.0 / 0.2	- / 0.5
ARK_2	Clone EU793918.1	Dinoflagellates	0.6 / 0.8	- / 0.5	- / 0.5	- / 2.6
ARK_3	Clone HM135092.1 (98)	Dinoflagellates	0.6 / 0.4	- / 0.4	4.0 / 0.9	7.2 / 0.2
ARK_4	Clone JF791003.1 (98)	Dinoflagellates	- / -	- / -	5.0 / -	- / -
ARK_5	Clone GU819790.1 (98)	Dinoflagellates	- / 0.24	1.1 / 0.2	3.0 / 0.5	1.4 / 1.7
ARK_6	*Micromonas pusilla* (99)	Chlorophytes	2.9 / 1.4	- / 1.9	- / 0.3	- / 1.3
ARK_11	Clone HQ438132.1 (94)	Dinoflagellates	- / -	- / 0.2	8.9 / 0.7	- / 0.1
ARK_12	Syndiniales EU793925.1 (95)	Dinoflagellates	- / 0.2	- / 0.1	- / 0.8	26.6/ 0.6
ARK_13	*Gyrodinium* AB120001.1	Dinoflagellates	- / -	- / -	4.0 / -	5.0 / -
ARK_14	*Geminigera cryophila* (99)	Cryptophytes	0.6 / 0.4	- / 0.1	- / -	23.7 / 0.1
ARK_15	*Micromonas pusilla* (99)	Chlorophytes	77.1 / 14.4	65.9 / 1.1	47.5 / 0.4	- / 1.1
ARK_16	Clone AY295399.1 (91)	Ciliates	8.0 / 1.2	- / 0.8	- / 0.4	0.7 / 0.2
ARK_17	Clone EU682572.1 (97)	Ciliates	1.7 / 0.3	- / 0.3	- / 0.2	- / 0.1
ARK_20	Clone HQ43812.9 (98)	Dinoflagellates	1.1 / -	- / 0.0	- / -	- / 0.1
ARK_21	Clone JN934892.1 (95)	Picobiliphytes	1.1 / 0.0	- / 0.0	- / 0.0	- / 0.0
ARK_25	*Gyrodinium* sp. (98)	Dinoflagellates	0.6 / 0.4	- / -	- / 0.1	- / -
ARK_26	*Woloszynskia* sp. (99)	Dinoflagellates	0.6 / 0.4	- / 0.0	- / 0.1	- / 0.2
ARK_29	*Micromonas pusilla* (99)	Chlorophytes	0.6 / 3.0	0.6 / 1.1	- / 0.1	- / 0.6
ARK_30	Clone HQ222463.1 (98)	Picobiliphytes	0.6 / -	- / 0.0	- / -	- / 0.0
ARK_31	*Micromonas pusilla* (99)	Chlorophytes	2.3 /	- /	- /	- /
ARK_33	Clone AJ420693.1 (96)	Rhodophytes	0.6 / -	- / -	- / -	- / -

Table 3.2. continued

Phylotype	Closest match (Maximum identity %)	Taxonomic group	Clones (%) / 454 (%)			
			HG1	HG4	HGN4	HGS3
ARK_38	Clone EU682636.1 (97)	Chlorophytes	- / -	1.7 / -	- / -	- / -
ARK_46	*Micromonas pusilla* (91)	Chlorophytes	- / -	0.6 / -	- / -	- / -
ARK_47	Syndiniales EU793375.1 (90)	Dinoflagellates	- / -	29.6 / -	- / -	- / -
ARK_58	Clone EU793946.1 (88)	Dinoflagellates	- / -	- / 0.1	10.9 / -	0.7 / -
ARK_60	Clone EU793957.12 (92)	Dinoflagellates	- / -	- / -	2.0 / -	- / -
ARK_62	Clone EU682577.1 (98)	Dinoflagellates	- / -	- / -	6.9 / -	- / -
ARK_68	Clone EF172940.1 (98)	Dinoflagellates	- / -	- / 0.1	4.0 / 0.1	- / 0.0
ARK_69	Clone JF826365.1 (91)	Dinoflagellates	- / 0.1	- / 0.3	2.0 /0.1	- / 0.1
ARK_70	Clone HQ438143.1 (95)	Dinoflagellates	- / 0.0	- / 0.1	1.0 / 0.1	1.4 / 0.1
ARK_72	Clone EU793201.1 (98)	Dinoflagellates	- / -	- / -	- / -	0.7 / -
ARK_76	Clone EU793383.1 (90)	Dinoflagellates	- / 0.1	- / 0.1	- / -	2.2 / 0.2
ARK_78	Clone EF195735.1 (90)	Cryptophytes	- / 0.3	- / 0.3	- / 0.2	0.7 / 0.1
ARK_82	Clone EU793221.1 (94)	Dinoflagellates	- / -	- / -	- / -	1.4 / 0.0
ARK_83	Clone EU793700.1 (94)	Dinoflagellates	- / 0.2	- / 1.0	- / 1.1	2.2 / 1.3
ARK_86	Clone EU793708.1 (92)	Dinoflagellates	- / 0.1	- / 0.0	- / 0.0	0.7 / 0.1
ARK_87	Clone HM561117.1 (95)	Dinoflagellate	- / -	- / -	- / 0.0	5.0 / -
ARK_90	Clone HQ222399.1 (95)	Dinoflagellates	- / -	- / -	- / -	1.4 / 0.1
ARK_91	Clone FJ537539.1 (92)	Dinoflagellates	- / -	- / -	- / -	2.9 / -
ARK_92	*Bolidomonas pacifica* (95)	Stramenopiles	- / 0.1	- / -	- / 0.1	2.2 / 0.1
ARK_93	*Bathycoccus prasinos* (98)	Chlorophytes	- / 8.0	- / 5.3	- / 1.3	4.3 / 2.2
ARK_97	Clone JF826393.1 (91)	Dinoflagellates	- / -	- / -	- / -	1.4 / -
ARK_100	Clone AF290050.2 (95)	Dinoflagellates	- / 0.5	- / 0.5	- / 0.4	1.4 / 0.4
ARK_102	Clone GU819971.1 (95)	Dinoflagellates	- / -	- / 0.0	- / 0.0	3.6 / -
ARK_103	Clone EU818505.2 (97)	Dinoflagellates	- / -	- / 0.0	- / 0.0	0.7 / -
ARK_104	Clone EU793381.1 (96)	Dinoflagellates	- / 0.2	- / 0.0	- / 0.0	2.2 / 0.4

Table 3.3. Phylogenetic affiliations of the Antarctic clone phylotypes and their relative abundance in the library and the 454-pyrosequencing data set.

Phylotype	Closest match (Maximum identity %)	Taxonomic group	Clones (%) / 454 (%)
ANT_1	clone SGPX577 (98)	Dinoflagellates	2.9 / -
ANT_2	*Gyrodinium fusiforme* (99)	Dinoflagellates	2.9 / -
ANT_3	clone SIF_2C7 (99)	Dinoflagellates	3.9 / <1
ANT_4	clone B16 (98)	Dinoflagellates	1.0 / <1
ANT_5	clone SHAX878 (95)	Dinoflagellates	2.9 / <1
ANT_6	clone CNCIII51_20 (99)	Dinoflagellates	20.2 / 8.7
ANT_7	*Azadinium spinosum* (99)	Dinoflagellates	7.7 / 1.0
ANT_8	*Gyrodinium rubrum* (96)	Dinoflagellates	1.0 / <1
ANT_9	DH147-EKD20 (94)	Syndiniales	1.9 / <1
ANT_10	*Salpingella acuminata* (99)	Ciliates	4.8 / 3.9
ANT_11	clone KRL01E30 (87)	Picobiliphytes	2.9 / <1
ANT_12	*Geminigera cryophila* (99)	Cryptophytes	1.0 / <1
ANT_13	clone B1 (99)	Haptophytes	21.2 / 19.9
ANT_14	clone B1 (99)	Haptophytes	18.3 / 4.3
ANT_15	clone F11N10 (91)	Diatoms	1.0 / <1
ANT_16	*Hemiaulus sinensis* (96)	Diatoms	1.0 / <1
ANT_17	clone RA070625T.073 (96)	Stramenopiles	2.9 / -
ANT_18	clone CNCIII05_73 (93)	Stramenopiles	1.9 / -
ANT_19	clone 14H3Te6QW (95)	Stramenopiles	1.0 / <1

Figure 3.1. Map of the sampling stations located within A) the long-term observatory "Hausgarten" (Fram Strait, Arctic) and B) the Southern Ocean.

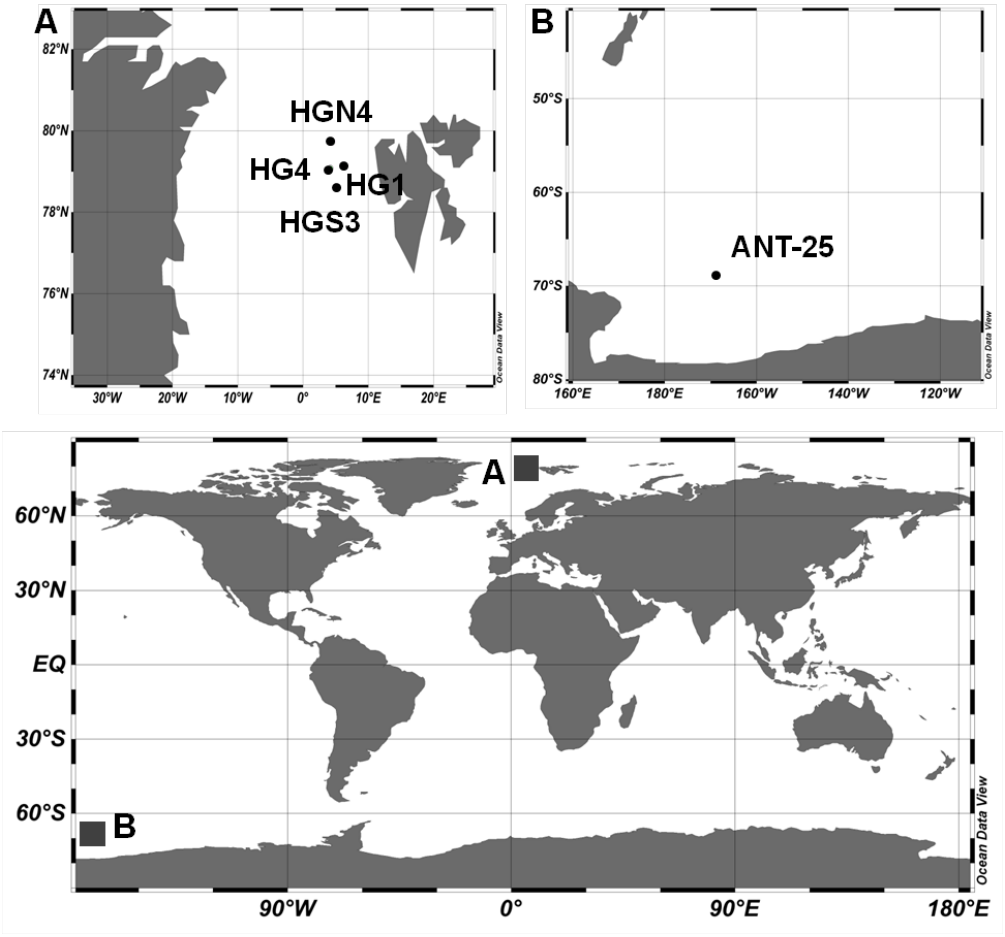


Figure 3.2. Rarefaction curves of A) clone libraries and B) 454-pyrosequencing.

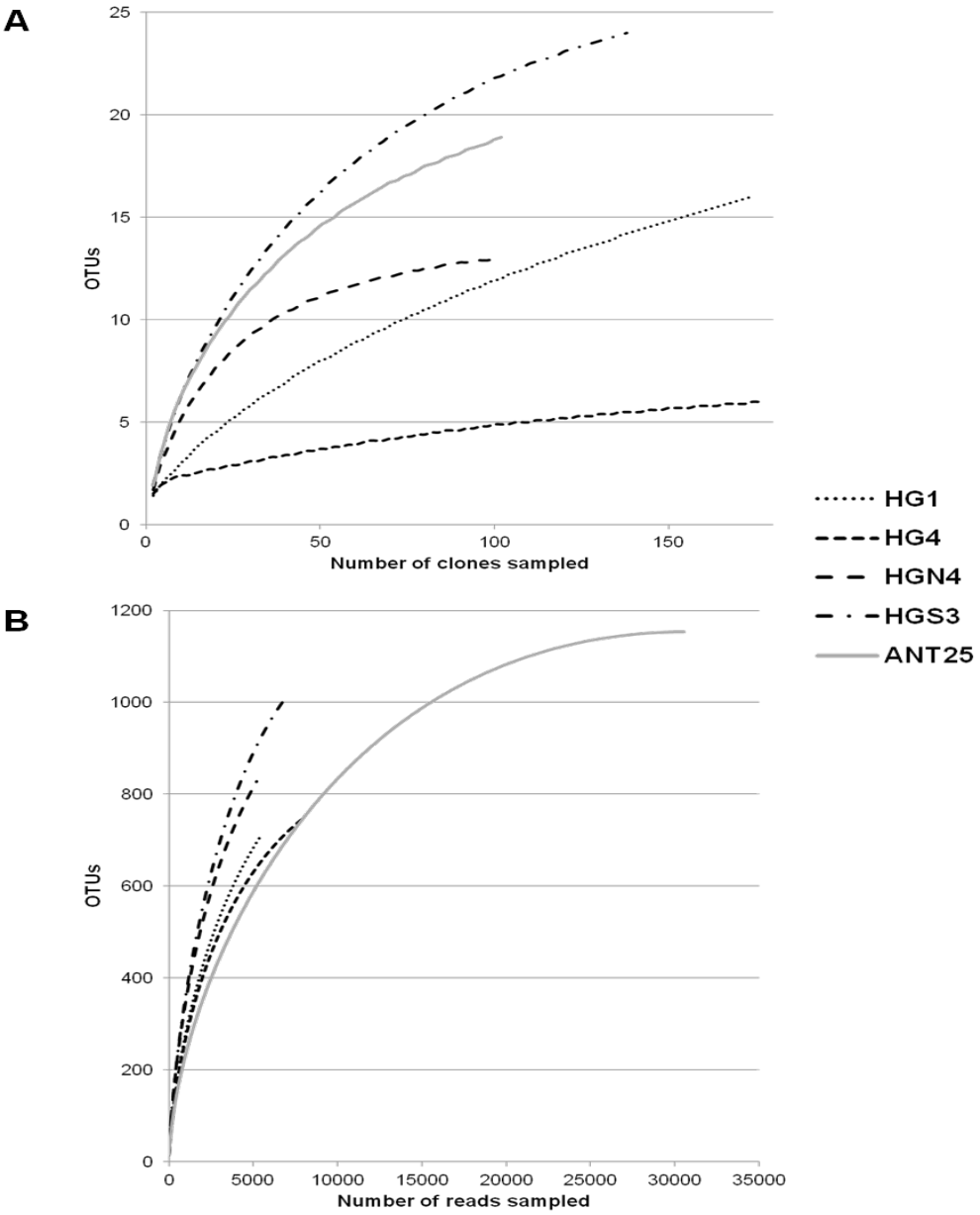


Figure 3.3. ARK24: Phylogenetic tree based on the 18S rRNA gene sequences retrieved from the clone libraries and rooted with *Staurosira* sp. Calculation of the tree has been performed with maximum likelihood under the implementation of the Juke-Cantor model and 1000 bootstraps replications. The symbols are standing for the respective station, where the clone had been found. The triangle stands for HG1, the circle for HGS3, the rhombus for HGN4 and the square for HG4.

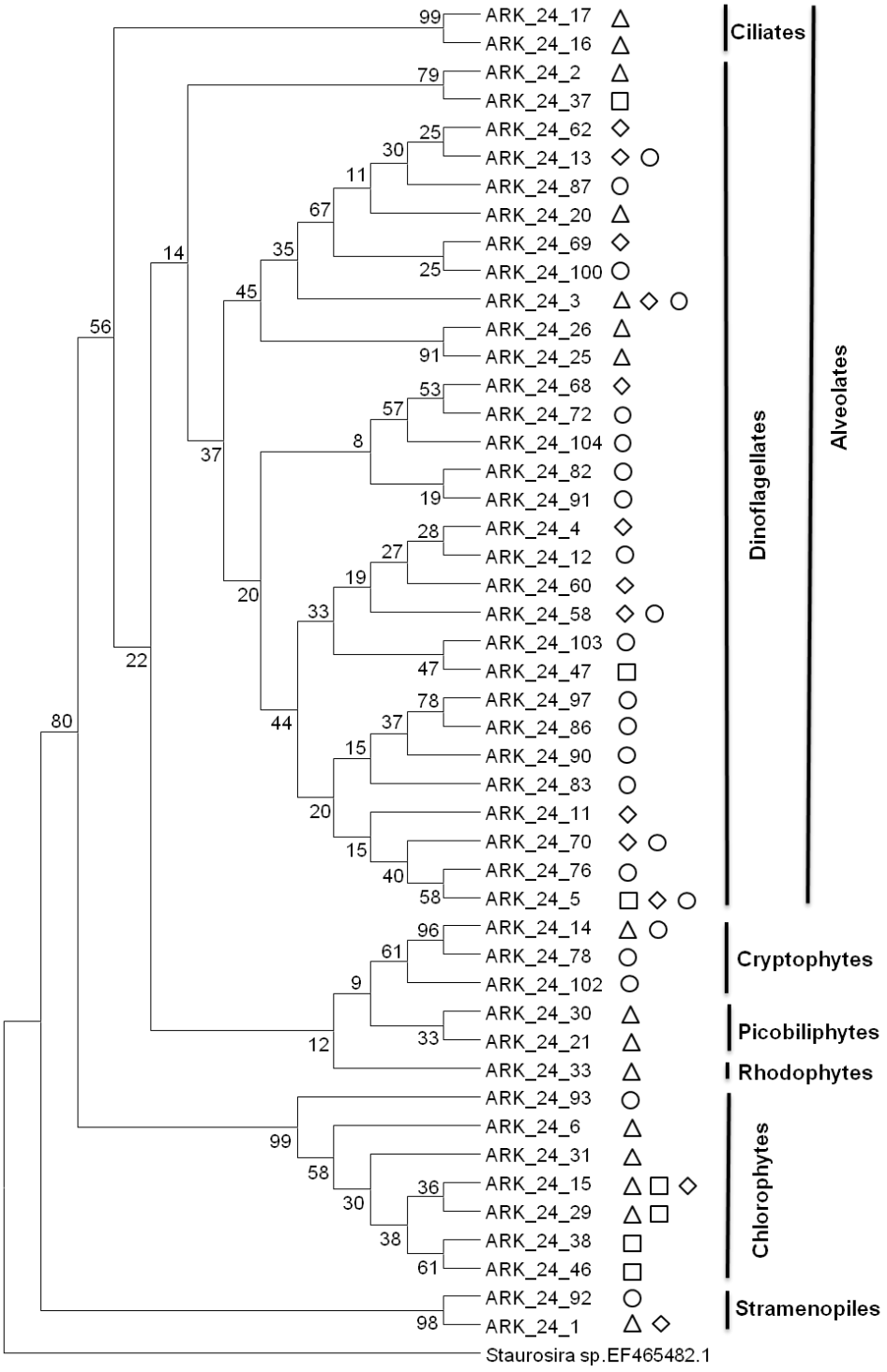
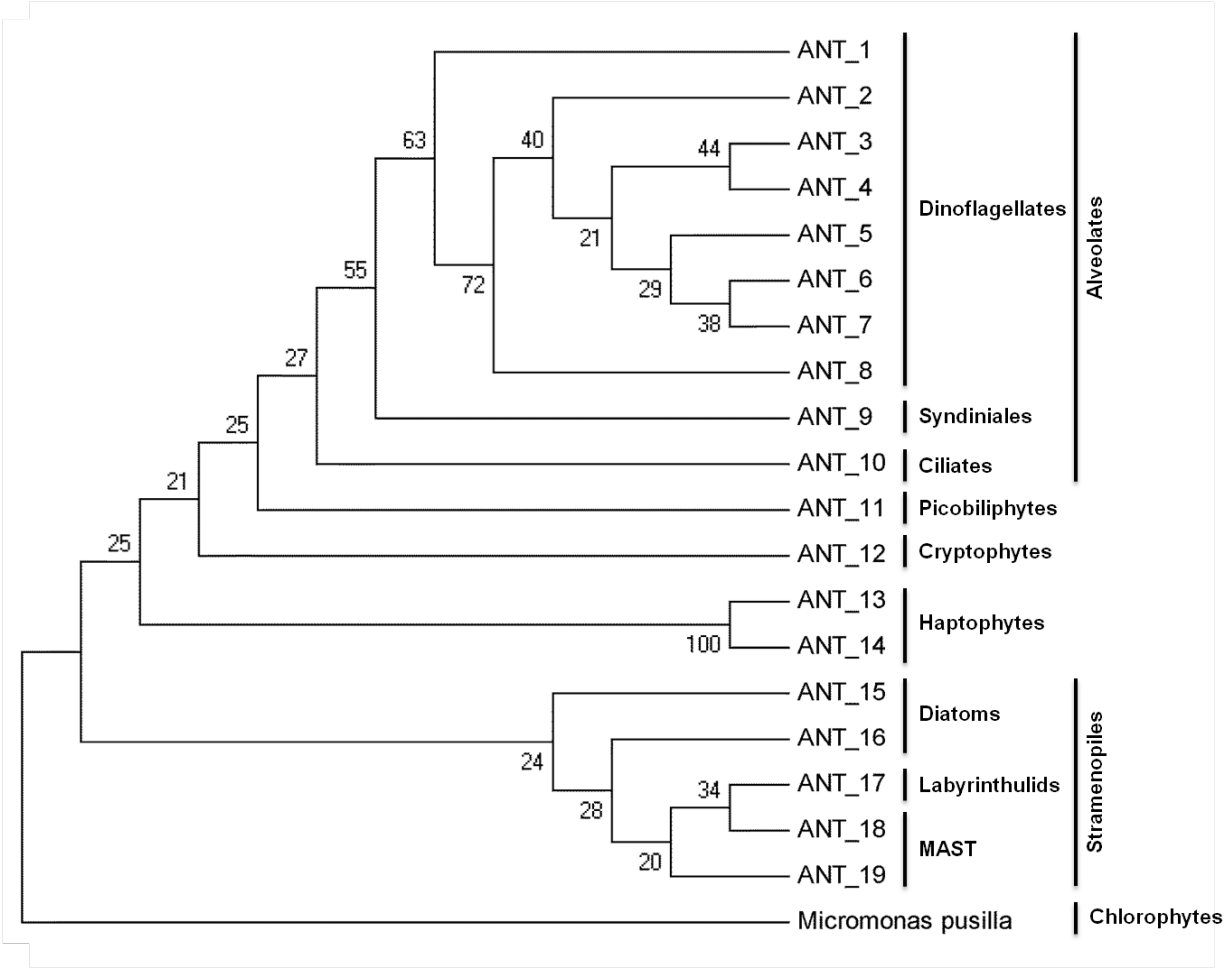


Figure 3.4. ANT25: Phylogenetic tree based on the 18S rRNA gene sequences retrieved from the clone libraries and rooted with *Micromonas pusilla*. Calculation of the tree has been performed with maximum likelihood under the implementation of the Juke-Cantor model and 1000 bootstraps replications.



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Publication II

PICOPLANKTON COMMUNITY COMPOSITION IN THE EASTERN FRAM STRAIT

ESTELLE KILIAS, EVA-MARIA NÖTHIG, CHRISTIAN WOLF AND KATJA METFIES

3.4 Publication II

PICOPLANKTON COMMUNITY COMPOSITION IN THE EASTERN FRAM STRAIT

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Abstract

The investigation of marine eukaryotic picoplankton community composition is limited by missing morphological features for proper identification and requires the application of molecular methods. In this study, we used 454-pyrosequencing to investigate the picoplankton communities at four stations in the Eastern Fram Strait. At one station (HG4), 454-pyrosequencing was put through an evaluation process to assess permeability of fractionated filtration. The resulting data were in accordance with the accepted size distribution of the observed taxa. Only few phylotypes occurred in more than one size fraction, suggesting an adequate filtering procedure. Furthermore, 454-pyrosequencing data were evaluated in a quantitative comparison with microscopy diatom counts at one station. The diatom contribution, revealed by both methods, was in good accordance.

The picoeukaryotic communities were dominated by phylotypes affiliating with *Micromonas pusilla*, *Phaeocystis pouchetii*, and syndiniales in the phylogenetic tree. The picoplankton community was similar at three out of four stations that displayed similar abiotic conditions (T and S). At the fourth station, slightly different abiotic parameter resulted in a significantly different picoeukaryote community composition. This observation is particularly interesting in regard to the ongoing environmental change in the Arctic and highlights the need to fill the gaps of knowledge concerning picoplankton community compositions.

Key index words

454-pyrosequencing, Picoeukaryotes, Diatoms, Arctic Ocean

Introduction

Picoplankton is gaining increasing attention because of its importance for all marine ecosystems in terms of biomass and primary production (Li 1994, Worden et al. 2004). Picoplankton was found to dominate within photosynthetic and heterotrophic processes over much of the world's oceans (Whitman et al. 1998) and is a major contributor to protist assemblages in oligotrophic waters, attaining abundances of 102 to 104 cells ml^{-1} (Ishizaka et al. 1997, Li 2009, Massana 2011). In the Arctic Ocean, the picophytoplankton can constitute significant contributors to primary production (Degerlund & Eilertsen 2010). A large surface-area-to-volume ratio enables an effective nutrient uptake and a concomitant prevention of rapid sinking (Li et al. 2009). Picophytoplankton obtained particular attention after Richardson and Jackson (2007) reported that picophytoplankton can also have an important impact to organic carbon flux to deeper waters. Until then, the general picophytoplankton was assumed to be recycled within the microbial food web (Azam et al. 1983) and to contribute only partially to the carbon export (Michaels & Silver 1988).

A number of molecular surveys reported that the Arctic Ocean picoeukaryotic phytoplankton is dominated by a pan-Arctic ecotype of the mamiellophyte *Micromonas,* which is especially adapted to cold temperatures (Lovejoy et al. 2007, Marin & Melkonian 2010). Other typical picoplankton genera like *Ostreococcus* were further observed to attain high abundances in Arctic waters (>105 cells ml^{-1}) by producing even small "blooms" (Countway & Caron 2006). Recent studies predict increasing abundances of picoplankton under the ongoing warming of the Arctic, also because cell size is suggested to decrease with increasing temperatures (Moran et al. 2010). Therefore, it is crucial to get an overview of current picoplankton community composition and distribution patterns. So far, the knowledge about picoplankton community compositions is scarce, however, representatives can be found in all major taxonomic algal classes (Simon et al. 1994, Veldhuis et al. 1997, 2005). The discovery of phylotypes in the picoplankton community that have until now been associated with bigger size classes were often explained by artifacts of cell breakage or sloppy feeding (Massana et al. 2004b, Romari & Vaulot 2004).

In the past, investigations concentrated on evaluations of the complex diversity of the nano- and picoplankton fraction using different molecular approaches, all based on the analysis of ribosomal genes, such as clone library sequencing, DGGE, and RISA (Diez et al. 2001, López-García et al. 2001, Moon-van der Staay et al. 2001, Hamilton et al. 2008, Not et al. 2008, Potvin & Lovejoy 2009, Vigil et al. 2009). These methods revealed a huge hidden diversity, in particular within the picoeukaryotes, and revolutionized hitherto assumptions of

protist community structure. The slow evolutionary rate and the abundant occurrence in single cells, makes the 18S rDNA a widely employed “bar-code” to assess eukaryotic picoplankton diversity (Amann & Kuhl 1998, Vaulot et al. 2008). Recently, the use of 454-pyrosequencing technology for the investigation of picoplankton communities allows a more detailed survey of the diversity, because it provides higher numbers of ribosomal sequences than clone library sequencing.

In this study, we use the 454-pyrosequencing approach, in order to get a higher resolution insight into picoplankton community composition in the Eastern Fram Strait, where no 454-pyrosequencing data are available so far. This includes an assessment of the permeability of fractionated filtration and a validation of the molecular data with classical light microscopy.

Material and Methods

Sampling area

The sampling was performed during the ARK XXIV/2 expedition, onboard the RV *Polarstern* in July 2009 (07/11/09-07/18/09) at the deep-sea long-term observatory HAUSGARTEN of the Alfred Wegener Institute for Polar and Marine Research. The observatory is located at 78-80°N and 3-7°E close to the coast of Svalbard, in the Fram Strait (Figure 3.1).

Water samples have been taken in or close to the chlorophyll maximum by collecting seawater with Niskin bottles deployed as a rosette sampling system on a CTD (Conductivity Temperature Depth system) frame (Table 3.1). For subsequent filtration, 2 l water subsamples were taken with polycarbonate bottles. Protist cells were filtered into three fractions with Isopore Membran Filters (Millipore, USA) (10 µm, 3 µm, and 0.4 µm) at 200 mbar. Finally, the filters were stored in Eppendorf tubes at -80°C until further processing. The 0.4–3 µm fraction was used for the assessment of picoplankton composition at four stations (HG1, HG4, HGN4, and HGS3). The protist composition, collected on all three filters, was analyzed at HG4.

Microscopy

Protist cells were stored in brown glass bottles before counting. In 50 ml aliquots a minimum of 50-100 cells of the dominant species or groups were counted with an inverted microscope at four different magnifications (100–400x) using phase contrast according to Utermöhl (1958).

DNA isolation

After an initial incubation of the filter in lysis buffer, DNA extraction was carried out with the E.Z.N.A TM SP Plant DNA Kit (Omega Bio-Tek, USA) following the manufacturer's instructions. DNA concentration was determined with a NanoDrop 1000 system (Thermo Fisher Scientific, USA).

Next Generation Sequencing

For subsequent 454-pyrosequencing, the V4 region (~670 bp) of the 18S rDNA was amplified with the primer set 528F (GCG GTA ATT CCA GCT CCA A) and 1055R (ACG GCC ATG CAC CAC CAC CCA T) (Elwood et al. 1985). The PCR reaction mixture contained 1x HotMasterTaq buffer Mg^{2+} 2.5 mM (5'Prime,USA), 0.4 U HotMaster Taq polymerase (5'Prime), 10 mg/ml BSA, 10 mM (each) dNTP (Eppendorf, Germany), 10 µM each Primer, 1 µl of template DNA (~20 ng/µl) in a final volume of 20 µl. PCR amplification was carried out in a MasterCycler (Eppendorf, Germany) under the following conditions: first, an initial denaturation step at 94°C for 3 min succeeded by 35 cycles (denaturation at 94°C for 45 s, annealing at 55°C for 1 min, extension at 72°C for 3 min), and followed by a final extension at 72°C for 10 min. The resulting PCR products were purified with the MinElute PCR purification kit (Qiagen, Germany) following the manufacturer's instructions. Pyrosequencing was performed on a Genome Sequencer FLX system (Roche, Germany) by GATC Biotech AG (Germany).

Data analysis of 454-pyrosequencing

Sequences with a length less than 300 bp were excluded from the analysis to guarantee further analysis of the whole V4 region. Moreover, sequences that exceeded the general amplicon size of >670 bp were also excluded. To avoid the inclusion of chimeras in the following clustering process a chimera-detecting software, UCHIME (Edgar et al. 2011) was applied. The building of artificial operational taxonomic units (OTUs) was achieved by clustering the remaining reads, using the software package Lasergene Seqman Pro (DNAStar, USA). A threshold of 97 % was applied to minimize the danger of overestimating the diversity and to better compare the data with previous analysis, because 97 % similarity has often been utilized in former studies (Kunin et al. 2010, Behnke et al. 2011). All singletons, defined as an OTU composed of one single sequence that only occurs once in the whole analysis were removed to evade possible errors induced by the assembly of sequencing progress (Table 3.2). The consensus sequences were placed into a reference tree build up by 1200 high-quality-

sequences containing representatives of proxys of all main eukaryotic phyla. This involved the use of the pplacer 1.0 software (Matsen et al. 2010). We used a cutoff of 80 % probability instead of a NCBI blast to increase the reliability of the taxonomic affiliation. Sequences that affiliated with non protist phyla in the tree were excluded from further analyses. The remaining reads were aligned with the SILVA aligner (Pruesse et al. 2007) and placed into a reference database tree containing around 50000 eukaryotic sequences from the SILVA reference database (SSU Ref 108).

The analysis of phylotype distribution over the fractionated size classes (Figure 3.2 and 3.3) was conducted according to the previous referred instructions, including a quality trimming and a clustering of the sequences. This has inter alia the advantageous of getting identical descriptions for identical phylotypes. Subsequent, all phylotypes were screened for identical representatives in one of the three size fractions to discriminate between ubiquitous, unique, and multiple (micro-/picoplankton and nano-/picoplankton) occurrences. For the sake of clarity, phylotypes of the respective occurrences were summarized in the context of similar taxonomic affiliation, because of the high phylotype numbers. As the sequencing effort differed strongly over the three size classes the investigation was not based on the OTU numbers but on the relative abundances of the single phylotypes. This is necessary, because higher sequence numbers are assumed to result in higher OTU numbers. The use of the relative abundances qualifies the differences between the three size classes and further displays the allotments of the respective phylotypes in the single size classes. Phylotypes that displayed a percentage of at least 1 % in one of the size classes and thus belonged to the abundant biosphere were listed according to their occurrence in the supplemental table (Table 3.6). The 454-pyrosequencing reads were deposited at GenBanks's Short Read Archive (SRA) under the Accession No. SRA058841.

Results and Discussion

The objective of this study was to investigate picoeukaryote community compositions in the Eastern Fram Strait. This involved 454-pyrosequencing, which has proven to generate reliable environmental surveys of genetic diversity (Sogin et al. 2006, Cheung et al. 2010, Stoeck et al. 2010), an evaluation of the fractionation, and the 454-pyrosequencing data (>10µm fraction) in comparison to microscopic counts, respectively.

Microscopy and 454-pyrosequencing (>10 µm fraction)

At station HG4 the contribution of diatoms to the protist community (>10 µm) was assessed with 454-pyrosequencing and light microscopic counts, in order to evaluate the consistency between both methods. We focused on diatoms, because taxonomic surveillance of diatoms with light microscopy is relatively easy and they are reported to be most commonly represented in the size range from 10 to 200 µm (Winder et al. 2009). The relative contribution of diatoms to the protist community (>10 µm) within both approaches were in good agreement. According to 454-pyrosequencing diatoms contributed ~8 % to the protist community (>10 µm), while the contribution was ~6 % according to light microscopic counts. The diatom composition however differed regarding to the applied method (Table 3.3). While *Fragilariopsis* sp. (~5 %) was a major contribution to the diatom assemblage, identified by the light microscopy approach, it was not recovered in the 454-pyrosequencing. In contrast, *Pseudo-nitzschia* sp. (~4 %) and *Actinocyclus* sp. (~3 %) were found to dominate in the 454-pyrosequencing, but were less abundant in the light microscopy analysis. In general, light microscopy and 454-pyrosequencing data coincided well in the relative abundance of diatoms and in the apportionment in pennate and centric diatoms. Differences just emerged after a taxonomical and closer affiliation. The reason for the discrepancy is probably based on the use of different indicative 'markers' within both approaches. In this respect, diatom characterization is delimited by features of the cell wall, which are not always visible under the use of light microscopy. One example comprise representatives of *Pseudo-nitzschia* that are difficult to recognize under the light microscope and demand the implementation of a scanning electron microscope (Morales et al. 2001). The use of 454-pyrosequencing is based on variances in the nucleotide sequence of the hypervariable V4 region. In this study, the V4 region of most recovered diatom species correspond in the nucleotide sequence to 83-89 %. However, *Fragillariopsis* sp. and *Pseudo-nitzschia* sp. presented an identity proportion of ~99 % which was higher than the threshold (97 %) used for OTU generation. Consequently, both species were not differentiated. Deviations for the centric diatom composition and proportion in contrast, are probably a result of different multiple rDNA copy numbers or PCR induced biases (Caron et al. 2004, Countway et al. 2005, Zhu et al. 2005). In this regard, *Thalassiosira* sp. presented the highest discrepancies between both approaches but was at the same time also characterized by a high rDNA copy number (Zhu et al. 2005). Nevertheless, the overall good accordance in diatom proportion suggests that 454-pyrosequencing might be suited to adequately reflect the natural protist composition.

Protist distribution over the size classes

Numerous studies highlight the danger of cell breakage, squeezing of flexible cells through filter pores, and sloppy zooplankton feeding (Massana et al. 2004b, Romari & Vaulot 2004), leading to false interpretations of protist size distributions. Acknowledging this, we assessed the quality of the filtration procedure by comparing species, identified by 454-sequencing in the different size fractions, with the expected size of the species. This involved a detailed comparison of the phylotype composition in the three size classes (>10 µm, 10-3 µm, and 3-0.4 µm) (Figure 3.2). In order to facilitate the illustration of the results, phylotypes were grouped according to their affiliation to major taxonomic groups (e.g. haptophytes, chlorophytes, cryptophytes, stramenopiles, dinoflagellates and ciliates) and to ubiquitous, multiple, and unique occurrences (Figure 3.3 and Table 3.6).

In general, the contribution of the major taxonomic groups to the different size fractions was in good accordance with the size range, expected for a respective taxonomic group (Figure 3.2). This finding supports the accuracy of the filtration process. Phylotypes, assigning to haptophytes, were observed in all size fractions. They were most abundant in the picoplankton fraction (3-0.4 µm), where they contributed 31 %. The contribution within the nano- and microplankton was less and decreased to a share of 17 % and 9 %, respectively. This is consistent with published data that report haptophyte species from all size fractions, such as *Coccolithus pelagicus* or *Emiliania huxleyi*. Both species are important contributors to the nano-plankton communities in North Atlantic Waters (Tyrrell & Taylor 1996, Cubillos et al. 2012). Another important haptophyte, *Phaeocystis pouchetii*, in contrast, pass through a life cycle where the transition from one phase to another comprises small free-living cells of few microns in diameter, grouping to nanoplankton, as well as colonies displaying millimeters in diameter, grouping to microplankton (Rousseau et al. 2007). As we extended the maximum size definition of picoplankton from 2 to 3 µm, small free-living cells also grouped into the picoplanktonic fraction and led to a general high representation of the species in all size fractions. In this study, a phylotype that assigned to *Phaeocystis pouchetii* was ubiquitously observed in all three size classes. It contributed ~6 % of all sequence reads of the micro- and picoplankton fraction, while the contribution to the nanoplankton-fraction was ~14 % (Figure 3.3 and Table 3.6).

Unique haptophyte phylotypes were less abundant, whereas the highest proportions (~3 %) were recovered in the picoplankton fraction, affiliating to the genus *Phaeocystis*. Apart from *Phaeocystis pouchetii*, no other haptophyte was shared between the micro and picoplankton and just a small number between the nano and picoplankton. These observations suggest that

for haptophytes “carry-over” from a larger size fraction to a smaller size fraction is only a minor problem.

Chlorophytes were mainly prominent in the picoplankton (19 %) and showed less percentage in the other size fractions (<0.5 %). This finding is in line with other observations that reported a predominance of chlorophytes in the picoplankton, with prominent representatives of the genera *Bathycoccus*, *Ostreococcus*, and *Micromonas* (Eikrem & Throndsen 1990, Throndsen & Kristiansen 1991, Rodriguez et al. 2005, Lovejoy et al. 2007). Ubiquitous chlorophytes consisted mainly of one phylotype (*Micromonas pusilla*) that accounted for ~6 % in the picoplankton, but for <0.1 % in the bigger size fractions. Unique chlorophyte phylotypes were limited to the picoplankton and mostly characterized as *Micromonas pusilla* or *Bathycoccus prasinos.* Identical phylotypes, recovered in the micro- and picoplankton or in the nano- and picoplankton, respectively were scarcely observed. Chlorophytes almost exclusively occurred in the picoplankton, and the minute proportion of ubiquitous phylotypes in the micro-and nanoplankton suggests only a limited transfer of chlorophytes from the larger size fractions to the picoplankton fraction. We speculate that the occurrence of phylotypes that assigned to Mameliales in the microplankton could be induced by possible attachment of picoplankton on big size cells.

Cryptophytes were the only group that presented no predominance in one of the size fractions, which is likely an artifact of limited sequence numbers. Representatives, such as *Rhodomonas* sp. or *Cryptomonas* sp., are mainly reported within the nanoplankton (Lafarga-De la Cruz et al. 2006). However, the almost absence of cryptophytes at HG4 facilitated no proper comparison of the three size classes, which is also reflected by missing ubiquitous and unique occurring phylotypes. In fact, no cryptophyte phylotype was observed that contributed to more than 1 % to the protist assemblage, regardless the size fraction.

Stramenopiles displayed the highest contributions in the micro- (18 %) and picoplankton (15 %). Stramenopiles consist of species that embrace a wide size range, including picoplankton species (e.g. *Bolidomonas pacifica*) as well as microplankton species (e.g. diatoms). Moreover, marine stramenopiles (MAST), a novel group, were observed to cover a wide size range, ranging from 2 to 20 µm (Lin et al. 2012). In regard to this, for the evaluation of the size fractionation, stramenopiles were subdivided into diatoms, MAST, and undefined stramenopiles. However, the percentages of ubiquitous phylotypes along the three groups were small, not exceeding 1.6 %. The proportion of unique phylotypes in contrast was higher. In this respect, picoplankton presented the highest share of (~8 %) within the undefined stramenopiles and of ~1 % within the MAST, while unique diatoms were primarily limited to

the microplankton. Micro- and picoplankton shared just a few phylotypes within one of the three subgroups. The share between nano- and picoplankton was higher for MAST and undefined stramenopiles, but just composed of several phylotypes of low abundance (~0.1 %). The low abundance of ubiquitous and the higher abundance of unique phylotypes suggest that stramenopiles do not highly bias the picoplankton fraction but cover a wide cell size spectrum.

Dinoflagellates constituted in all three cell size classes an important contributor of the protist assemblage. Maximum proportion of 71 % was found in the nanoplankton size class, followed by smaller shares of 34 and 31 % in the micro- and pico-cell-size-classes, respectively. Indeed, dinoflagellates were often reported to cover a wide cell size spectrum, including all three size fraction, whereas a particular predominance however, was observed in the nano- and microplankton size classes (Silva & Faust 1995, Levinsen et al. 1999). The life cycle of dinoflagellates (e.g. Gymnodiniales) results in the pass through of different cell stages of different cell sizes (Figueroa et al. 2008).

For the more detailed tracing of phylotype distribution we further split the group up and distinguished between dinoflagellates and parasitic syndiniales. Ubiquitously abundant dinoflagellate phylotypes constituted almost half of the dinoflagellate assemblage in the nanoplankton, and just ~15 % for the micro-, and ~5 % for the picoplankton, respectively. On the contrary, the proportion of ubiquitous syndiniales phylotypes was much lower, ranging about ~7 % in all size classes, whereas half of the share in the picoplankton was attributed to four phylotypes. Size class restricted dinoflagellate phylotypes were mostly recovered in the nanoplankton (~9 %) and much less in the micro and picoplankton (~2 %). In contrast, unique syndiniales phylotypes were mainly recovered in the picoplankton fraction, where they were represented by a vast but low abundant diversity. Micro-and picoplankton dinoflagellates shared just a little number of identical phylotypes, while in contrast nano-and picoplankton shared a relatively high proportion. The same aspect was observed for syndiniales phylotypes in the nano-/picoplankton. However, some phylotypes were at least also found to be exclusively represented in the micro- and picoplankton. The presence of single dinoflagellate phylotypes in all size fractions could be either due to broad cell size ranges, including unknown small dinoflagellates or cell breakage or free dissolved DNA (Massana et al. 2004a). So far, the known minimal size of dinoflagellates and also ciliates is 5 to 10 µm (Massana 2011).

Overall, knowledge about picoplankton dinoflagellate representatives is limited and supposed to be mainly composed of parasitic syndiniales phylotypes (Guillou et al. 2008). Within this

study we confirmed this assumption by finding a high dominance of syndiniales phylotypes in the picoplankton fraction. The observation that syndiniales phylotypes were also recovered in the other size fractions suggests that either syndiniales microbes cover a bigger size spectrum than previously assumed or that syndiniales sequences observed in the micro- and nanoplankton size classes actually belong into the picoplankton fraction. In this case, the small parasitic syndiniales cells might be attached to bigger host cells throughout the filtration process and thus were recovered in the respective size fractions. Until today, syndiniales have been reported in a great variety of marine hosts, including dinoflagellates, radiolarians, ciliates, crabs, or copepod eggs (Groisillier et al. 2006).

Ciliates were scarcely observed in the pico and nano size fraction (<2 %) but accounted for 37 % in the microplankton community. Ciliates have been reported to mainly contribute to the microplankton in most studies. Representatives, such as *Strombidium* sp. or *Strobilidium* sp., covered cell sizes of >20 µm (Montagnes 1996, Quevedo & Anadon 2001). The dominance in the microplankton fraction was also confirmed by the primarily occurrence of ubiquitous phylotypes (~12 %) and the exclusively occurrence of unique phylotypes (~20 %) in the >10 µm size fraction. These phylotypes were mainly characterized as tintinnids, whereas some were described in more detailed as *Cymatocylis* sp. No observation was made concerning a multiple occurrence of ciliate phylotypes in the nano- and picoplankton. However, we found a few phylotypes that were solely recovered in the micro- and picoplankton. As previously referred, ciliates are not assumed to contribute to the picoplankton size fraction. Some studies reported the presence of ciliates in picoplankton assemblages (Romari & Vaulot 2004, Medlin et al. 2006, Worden 2006, Cheung et al. 2008), which is indeed most likely an artifact of cell breakage, induced by the fragile nature, but finally does not entirely exclude the existence of undescribed pico-ciliates (Cheung et al. 2008). Nevertheless, the major contribution of ciliates in the microplankton (Perez et al. 2000) was in line with our data.

In summary, the distribution of the major taxonomic groups over the three size classes was in good accordance with the accepted assumption of taxa size distribution and suggest a good representation of protist diversity by 454-pyrosequencing. Information on ubiquitous or multiple (nano-/picoplankton) represented phylotypes further served as an indication for filtration bias on the picoplankton fraction. However, we have to keep in mind that the presence of multiple cell stages during protist life cycles (e.g. dinoflagellates and *Phaeocystis* sp.) and the limited knowledge of real picoeukaryotic representatives hamper declarations on filtration bias. Nevertheless, the number of multiple occurring phylotypes and their proportion was generally low and just higher in case of dinoflagellates and haptophytes. Hence, our

results suggested on one hand, an adequate recovery by the 454-pyrosequencing and on the other hand, an adequate filtering procedure, where cell breakage did not greatly shape the protist fraction and where picoplankton composition in the following is not highly biased by bigger cell sizes. Moreover, we showed that small cells, such as syndiniales, have the potential to bias the nano and microplankton fraction.

Picoplankton diversity

On the basis of the preceded quality check of the filtration procedure we can presume a largely pure picoplankton fraction. The 454-pyrosequencing of the picoplankton resulted in an average read number of 8523 raw reads (Table 3.2). The analytical process of the 454-pyrosequencing data includes surveillance of the sequence quality, which consists of the removal of small reads (<300 bp) to guarantee the investigation of the whole V4 region, a chimera check, a subsequent removal of singletons (OTUs represented but just one sequence) after the clustering, and the exclusion of non target reads (metazoans and fungi). The filtering removed about two third of the initial sequence number and left in average 2280 high quality reads for the picoplankton fraction, while the clustering resulted in ~220 OTUs (operational taxonomic units).

All four stations presented different community structures within the smallest cell size fraction (3-0.4 µm). The distribution of the major taxonomic groups is presented in Figure 3.4, while a more detailed overview of a selection of the abundant biosphere (≥1 % of total reads) is listed in Table 3.5. In fact, not all phylotypes could be taxonomically assigned in detail, whereas the problem accounted for the abundant and rare biosphere to the same extent. The taxonomical assignment of the consensus sequences was conducted by using a cutoff of 80 % probability, whereas reads that presented a smaller percentage were excluded. However, this was generally uncommon. Most common was the task that the consensus sequences did not match to an analogous sequence in the database. In this respect, the consensus sequence was affiliated to the next higher nodal point in the phylogenetic tree. A schedule of the quantitative analysis of phylotype recovery in our reference database, which is in turn based on a selection of the SSU Ref 108 SILVA database, is presented in Table 3.4. In some occasions, several OTUs clustered within one reference species. This could be a result of different species strains or may suggest that different taxonomic groups require different diversity thresholds, because of diverse intraspecific variances. In this respect, a study on the intraspecific diversity of the dinoflagellate *Alexandrium catenella* revealed a high variance of 0-2.9 % (Miranda et al. 2012), suggesting that other species may indeed comprise higher

discrepancies. In average, we were able to characterize 54 % of all picoplankton phylotypes at the four stations, which accounted for ~67 % in the picoplankton assemblages. In contrast, 46 % of the phylotypes were not recovered in our reference database and thus not comprehensively characterized. However, these phylotypes accounted for ~33 % in the picoplankton assemblage. Dictyochophytes and most of the MAST and dinophytes phylotypes showed a good recovery, suggesting a good representation of these groups in our database, while syndiniales phylotypes showed the highest numbers of unrepresented phylotypes and thus were not sufficiently represented. The comparison of represented and unrepresented phylotypes shows that picoplankton diversity is basically not completely retrieved by our reference database. Public databases may contain more picoplankton sequences, however, these sequences are often deposited without taxonomic affiliation. Picoplankton characterization is a difficult task because the cells are difficult to bring in clonal culture, a prerequisite for the proper molecular characterization. However, some picoplankter will probably be unculturable and thus the possessing of the whole insitu diversity in culture is utopian (Massana 2011). By applying our pipeline we were able to reliably assign picoplankton sequences at least to higher taxonomic levels and to assess how they were organized in taxonomic units.

Picoplankton communities and ecology at the four stations

HG1 was located most east and showed lowest salinity, temperature, and no ice coverage. The freshwater supply of the Kongsfjord highly influenced the abiotic characteristics and finally resulted in an alteration of Atlantic Water properties. The picoplankton community at HG1 was characterized by a very high contribution of chlorophytes (60 %), while other taxonomic groups (haptophytes, stramenopiles, and dinophytes) showed less, but almost equal percentages of 11 to 14 % (Figure 3.4). In this respect, haptophytes were mainly represented in the abundant biosphere by *Phaeocystis pouchetii,* stramenopiles by *Bolidomas pacifica,* and dinophytes by Prorocentrales 1 (Table 3.5). However, the abundant biosphere was strongly dominated by *Micromonas pusilla*. *Micromonas* was represented by two phylotypes, in which one (*Micromonas pusilla* 1) was particular dominant (45 %) and grouped to the cold adapted, arctic ecotype (CCMP2099) (Lovejoy et al. 2007). The other phylotype (*Micromonas pusilla* 2), was less abundant (~2 %) and could not be affiliated to a particular ecotype. *Bathycoccus prasinos* was further an abundant contributor, accounting for 9 %. Prasinophytes are reported to contribute in high portions to picophytoplankton in marine waters (Not et al. 2004). The genus *Micromonas* was recently assigned to a new group, mamiellophytes (Marin &

Melkonian 2010) and thus does not belong to the prasinophytes anymore. The order Mamiellales consists of three characteristic representatives such as *Ostreococcus tauri, Micromonas pusilla*, and *Bathycoccus prasinos* (Eikrem & Throndsen 1990, Chretiennotdinet et al. 1995, Lovejoy et al. 2007), in which the latter two were predominant in this study. The dominance of *Micromonas* is in accordance with other studies that observed a more prevalent occurrence in Arctic stations than in Atlantic Water influenced stations (Not et al. 2005, Lovejoy et al. 2007, Brugel et al. 2009). Considering the dominance of *Micromonas* in Pacific Waters, showing lower salinity and temperature and the dominance of *Micromonas* sp. 1 at HG1, we suggest that the freshwater influenced the abiotic conditions, which favored the growth of the cold adapted phylotype. Another aspect that might have had a positive effect on the relative abundance of *Micromonas* at HG1 is the coastal influence. In this regard, a previous study, assessing the potential ecological niche portioning of three *Micromonas pusilla* lineages found particular high cell abundances in two lineages in coastal areas (Foulon et al. 2008).

The northern station, HGN4, was characterized by a relatively warm temperature of ~6° C and a relative high salinity (35.1 PSU), and thus displayed general Atlantic Water properties that are characterized by an average temperature of 3.52°C and salinity of 34.7 PSU, respectively (Montgomery 1958). Moderate ice coverage of ~30-50 % led to a limited light penetration into the water column. HGN4 showed no abundance of *Micromonas* at all, but maximum shares of dinoflagellates (59 %). Stramenopiles and haptophytes both accounted for ~15 %, whereas just the latter group was represented in the abundant biosphere by *Phaeocystis pouchetii* (~14 %). The maximum contribution was attributed to the dinoflagellate Dinophyte 1, accounting for about 22 %. Dinophytes cover a wide spectrum of nutritional strategies, including autotrophy, heterotrophy, and even mixotrophy. In this regard, about half of the species are heterotrophic and show no plastids, while the other half is characterized by the presence of plastids and can be autotrophic or mixotrophic (Gomez 2012). The ice coverage at HGN4 probably suggests a predominance of heterotrophic or at least mixotrophic dinoflagellates.

Dinoflagellates of small cell sizes have already been reported but thereby, the minimal cell size of e.g. *Gyrodinium* sp. ranged around 7 µm (Jakobsen & Hansen 1997) and hence grouped in the nanoplankton. Syndiniales are considered to be picoplankton dinoflagellates. However, because the order is assumed to be exclusively composed by parasitic organisms, that parasite inter alia on other dinoflagellates (Groisillier et al. 2006, Guillou et al. 2008) cells are often not included in dinoflagellate studies. Nevertheless, the knowledge of

picoeukaryotic diversity and in particular of dinoflagellates is scarce, and the existence of possible picoplankton representatives, excluding syndiniales, can be just speculative.

The majority of dinoflagellate phylotypes at HGN4 grouped within the order of syndiniales, in which one half was represented by sequences in our database, while the other half was missing. In principle, syndiniales phylotypes were low abundant and just represented by four phylotypes in the abundant biosphere. Two of them were recovered at HGN4. Yet, the order syndiniales is supposed to be solely marine and to pass through picoplanktonic life stages (Yih & Coats 2000, Guillou et al. 2008). Since we found the highest percentages of syndiniales at stations that further presented the highest shares of dinoflagellates we highly suggest that recovered syndiniales phylotypes were mainly consistent of dinoflagellate parasites. Confirming, HG1 displaying the lowest proportion of dinoflagellates, showed in return no syndiniales phylotype within the abundant biosphere.

HG4, the most westerly located sampling site, showed lower temperature but similar salinity and ice condition as previously measured at HGN4. The western station was dominated by haptophytes and dinophytes, both accounting for ~30 % of the total picoplankton assemblage. The high share of dinoflagellates again leads to the assumption that the ice coverage promoted the growth of heterotrophic microbes. However, dinoflagellates were not represented by abundant phylotypes as Dinophyte 1 or Prorocentrales 1, as observed at HGN4 and HG1, respectively but by a numerous quantity of rare phylotypes (<1 %). Chlorophytes and stramenopiles were less abundant, having 19 and 15 %, respectively. Stramenopiles were represented by Chrysophyte 1, *Bolidomonas pacifica,* and two MAST phylotypes, in which *Bolidomonas* (<2 µm) and MAST (<5 µm) are known to constitute typical picoplankton representatives (Guillou et al. 1999a,b, Massana et al. 2006, Lin et al. 2012). Overall, HG4 showed smaller percentages of chlorophytes within the abundant biosphere, than HG1. In this regard, both phylotypes of *Micromonas pusilla* accounted together for 9 %, while *Bathycoccus prasinos* also presented a smaller contribution of about 5 %. The dominance of haptophytes relied on the abundant allotment of *Phaeocystis pouchetii* (28 %). *Phaeocystis pouchetii* was observed at all stations in abundant proportions, while the picoplankton size fraction is most likely represented by solitary, flagellate cells. In this regard, a dominance of such solitary cells and a minor proportion of colonial cells were found in spring/early summer close to Svalbard Islands (Wassmann et al. 2005). Moreover, *Phaeocystis pouchetii* was reported to contribute in high quantity to the spring bloom in the Atlantic sector of the Arctic Ocean and in the Barents Sea (Degerlund & Eilertsen 2010), whereas the predominance in the Barents Sea was inter alia associated with the high salinity of the Atlantic Water (Rat'kova &

Wassmann 2002, Reigstad et al. 2002). The dominance of *Phaeocystis pouchetii* at HG4 and HGS3 and the low share at HG1 in this study, also suggests the preference of the species to Atlantic Waters.

The southern station, HGS3 was much alike HGN4 in temperature and salinity. However, the station was not ice covered. Here, dinophytes contributed (41 %) to the total picoplankton assemblage. Based on the high abundances of dinoflagellates at the ice influenced stations HGN4 and HG4, a survey of the previous ice situation at HGS3 was done with satellite images, retrieved by MODIS data (Spreen et al. 2008). However, the data showed that HGS3 has been ice-free over the last two months. Hence, considering the high abundance of dinoflagellates at HGN4, HG4, and HGS3 we assume that the high percentages at HGN4 and HG4 were not solely attributed to the local ice concentration but probably also a result of picoplankton advection by the West-Spitzbergen Current (WSC) or natural spring to summer succession. The latter aspect, however, finally cannot be addresses because of the missing nutrient values. Picoplankton in this study was not exclusively dominated by the chlorophyte *Micromonas pusilla,* as for instance reported in the East Canadian Arctic (Lovejoy et al. 2007), but also by dinoflagellates, probably heterotrophic, that constituted a dominant contributor in three out of four stations, profiting inter alia from unfavorable light conditions. The proportion of stramenopiles (14 %), haptophytes (26 %), and chlorophytes (14 %), further showed good analogies with HG4. In this regard, the abundant biosphere of HGS3 was as HG4 mainly characterized by *Phaeocystis pouchetii* and contributed to an analogous percentage of 25 %. Moreover, both stations presented similar proportions of *Micromonas* sp. 1 (~6 %) and 2 (~2 %). The high similarity of picoplankton community structure at HGS3 and HG4, in addition to the fair similarity with HGN4 picoplankton assemblage, suggests a high influence of water mass (WSC). The change of picoplankton diversity and community structure across oceanographic barriers was also reported by Hamilton et al. (2008).

In summary, most recovered species in this study were allocated to the picoplankton size class, which again points to a reliable fractionation during the filtration process. No metazoan or diatom phylotypes were found in the picoplankton dataset. The picoplankton community in the Eastern Fram Strait was dominated by chlorophytes such as *Micromonas pusilla* and haptophytes as *Phaeocystis pouchetii.* Further, high abundances of dinoflagellates in the picoplankton size class were found, suggesting that dinoflagellates, including syndiniales phylotypes, constitute another dominant picoplankton group, beside the chlorophyte *Micromonas pusilla*. The diversity of picoeukaryotes in the Eastern Fram Strait showed, a water mass related distribution which is in line with former studies (Lovejoy et al. 2002,

Hamilton et al. 2008).

In this respect, picoplankton communities displayed higher resemblances at HG4, HGS3, and HGN4, while HG1 affected by different abiotic conditions, displayed variations in the community structure. Our finding that small variations in abiotic conditions (e.g. temperature and salinity) have the potential to affect picoplankton community structure implies that the size class will likely respond with community structure changes to prospective changes in abiotic factors. Nevertheless, big proportions of picoplankton sequences were not recovered in our reference database, but could be affiliated to taxonomic units at least. This implies that more high-throughput sequencing in different spatial and temporal scales has to be carried out on the picoplankton size class in order to close the concurrent gaps in our databases. In the course, the additional construction of clone libraries might shed light by facilitating a more precise insight of the genetic diversity. Moreover, further isolates of picoplankton cells for clonal cultures have to be done to investigate picoplankton not just on the diversity but also on the physiological basis.

Acknowledgment

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Table 3.1. Coordinates and abiotic conditions at the sampling site, taken in a time slot of 7 days in July 2009.

Station-ID	Longitude °E	Latitude °N	Sampling depth [m]	T [°C]	S [PSU]	Ice cover [%]
HG1 (East)	6.102	79.134	15	1.9	34.4	0
HG4 (West)	4.196	79.067	25	4.2	34.9	50
HGN4 (North)	4.478	79.729	30	5.9	35.1	30-50
HGS3 (South)	5.07	78.607	40	6.7	35	0

Table 3.2. 454-pyrosequencing data processing and phylotype (OTU) apportionment in the abundant and rare biosphere.

	HGIV			**HGI**	**HGN4**	**HGS3**
	0.4 µm	3 µm	10 µm	0.4 µm	0.4 µm	0.4 µm
total sequences	9830	22326	27602	7539	7938	8786
final sequences	2744	14031	5948	2533	2407	1438
OTU numbers	301	1073	697	189	233	164
abundant biosphere [≥1 %]	19	9	12	13	11	13
rare biosphere [<1 %]	282	1064	685	176	222	151

Table 3.3. Diatom composition (cell counts) in the light microscopy and 454-pyrosequencing (>10 µm) approach.

	light microscopy [%]	454-pyrosequencing [%]	pennate/ centric
Fragilariopsis sp.	4.5	-	p
Pseudo-nitzschia spp.	1.7	4.2	p
Chaetoceros sp.	0.02	-	c
Thalassiosira spp.	0.2	3.2	c
Actinocyclus sp.	0.03	1.1	c
other protists	93.7	91.6	-

Table 3.4. Quantitative comparison of represented and unrepresented picoplankton phylotypes in the sequence data bank. Represented phylotypes affiliate to a reference sequence in the phylogenetic tree, while unrepresented phylotypes affiliate to the next higher nodal point.

	represented phylotypes				**new (unrepresented) phylotypes**			
	HG1	HG4	HGN4	HGS3	HG1	HG4	HGN4	HGS3
Stramenopiles:								
undef.Stramenopiles	2	4	4	3	3	3	3	2
Diatoms	3	-	1	4	2	1	3	3
MASTs	5	4	5	3	-	1	1	2
Pelagophytes	1	-	1	1	3	1	-	-
Dictyochophytes	4	3	2	2	-	-	-	-
Chrysophytes	4	2	4	2	1	2	2	1
Biosecophytes	1	1	-	1	-	1	-	1
Coscinodiscophytes	-	-	1	-	-	-	-	
Labyrinthulids	-	-	2	1	1	1	-	2
Alveolates:								
undef. Alveolates	1	2	-	-	-	2	3	2
Dinophytes	12	11	9	11	2	4	2	3
Syndiniales	17	22	19	15	15	25	21	23
Ciliates	1	2	1	1	2	-	1	1
Chlorophytes	5	7	2	5	6	5	7	6
Haptophytes	4	4	4	3	5	4	3	1
Cryptophytes	-	1	-	-	4	4	1	2
Rhodophytes	-	-	1	-	2	1	1	3
undef. Eukaryotes	1	3	3	1	1	2	4	-

Table 3.5. Summary of the abundant biosphere (≥1 % of the total reads) along the four sampled stations. Data are presented in percent [%].

	HG1	HG4	HGN4	HGS3
Stramenopiles:				
Chrysophyte 1	1.3	4.0	-	-
Marine Stramenopile (MAST) 1	-	1.2	-	-
MAST 3	-	1.2	-	-
Bolidomonas pacifica	3.1	1.3	-	-
Pelagophyte 1	-	-	-	1.9
Alveolates:				
Dinophyte 1	-	-	22.4	-
Prorocentrales 1	4.1	-	2.1	-
Gymnodiniales 1	-	-	-	1.5
Gymnodiniales 2	-	-	1.6	-
Syndiniales 1	-	1.5	1.2	-
Syndiniales 2	-	2.3	-	-
Syndiniales 3	-	-	1.2	-
Syndiniales 4	-	-	-	1.1
Ciliate 1	2.7	1.9	1.3	-
Ciliate 2	-	6.0	-	-
Haptophytes:				
Phaeocystis pouchetii	9.3	29.6	13.9	25.2
Chlorophytes:				
Micromonas pusilla 1	45.0	6.2	-	6.3
Micromonas pusilla 2	2.3	2.7	-	2.0
Bathycoccus prasinos	9.5	5.4	1.3	3.0
undefined Eukaryotes:				
undef. Eukaryote 1	-	-	1.9	-
undef. Eukaryote 2	-	-	1.1	-
undef. Eukaryote 3	-	-	-	2.2

Figure 3.1. Map of the investigation area: Deep-sea long-term observatory "HAUSGARTEN", indicating sampling stations.

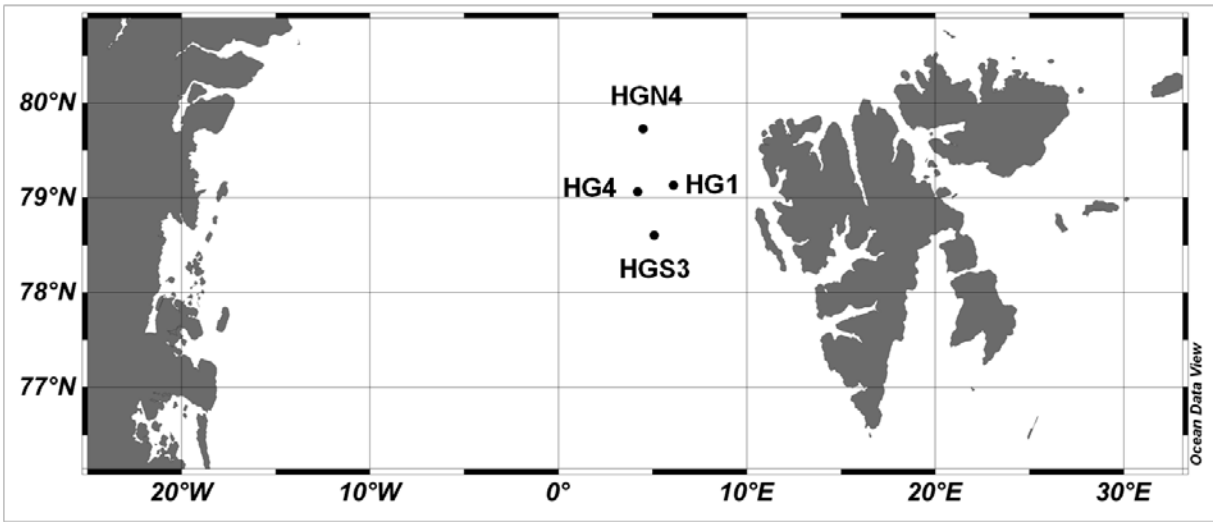

Figure 3.2. Distribution of the major taxonomic groups in the three size classes (>10 µm, 10-3 µm and 3-0.4 µm) at HG4, obtained by 454-pyrosequencing.

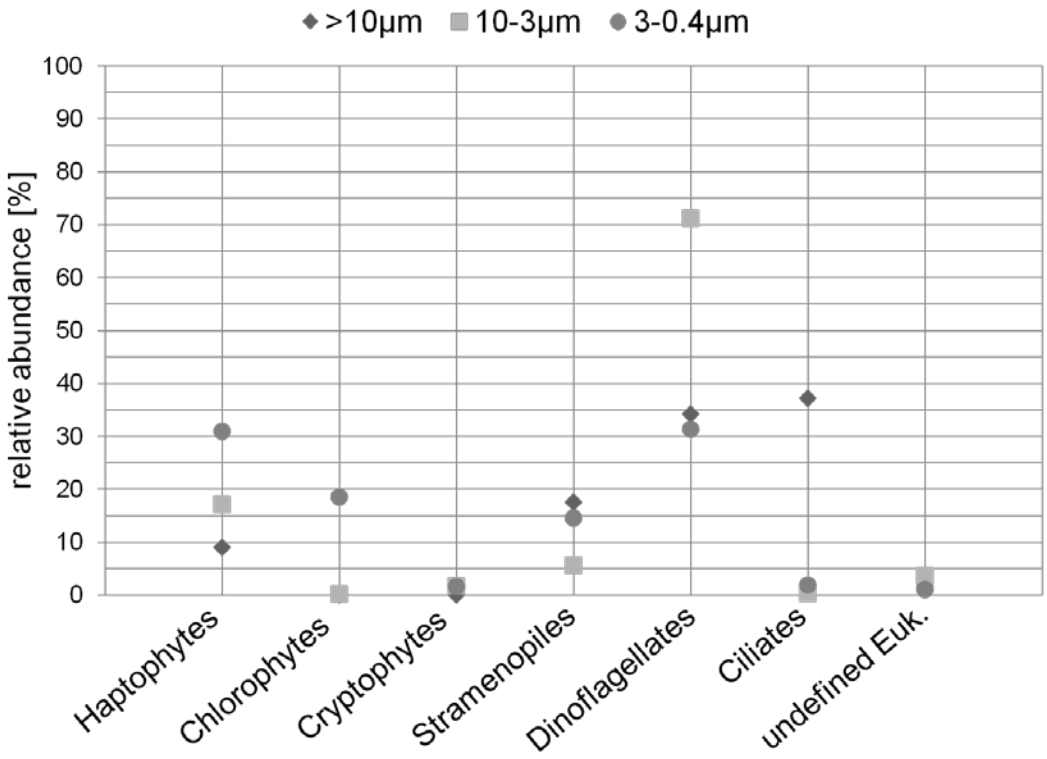

Figure 3.3. Detailed phylotype distribution in the three size-classes. A) ubiquitous, B) unique and multiple occurrences: C) micro-/picoplankton and D) nano-/picoplankton.

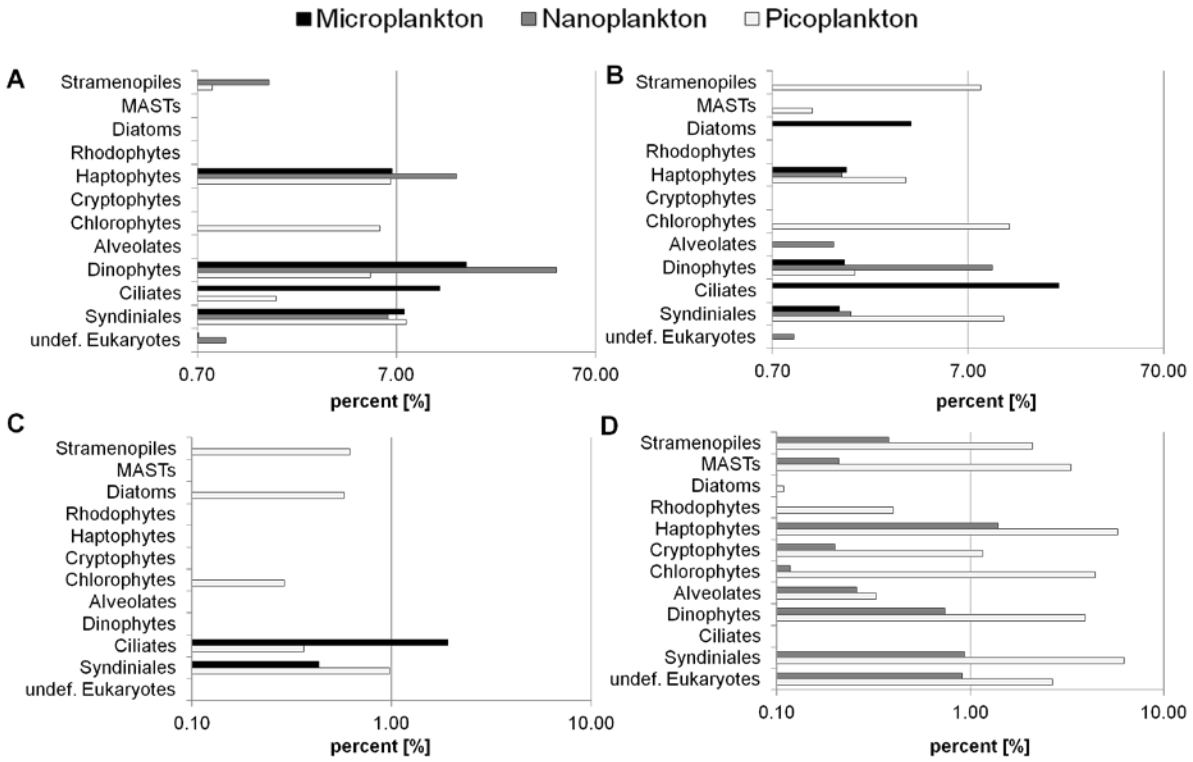

Figure 3.4. Histogram of the major taxonomic group apportionment in the picoeukaryotic fraction over the four sampling sites.

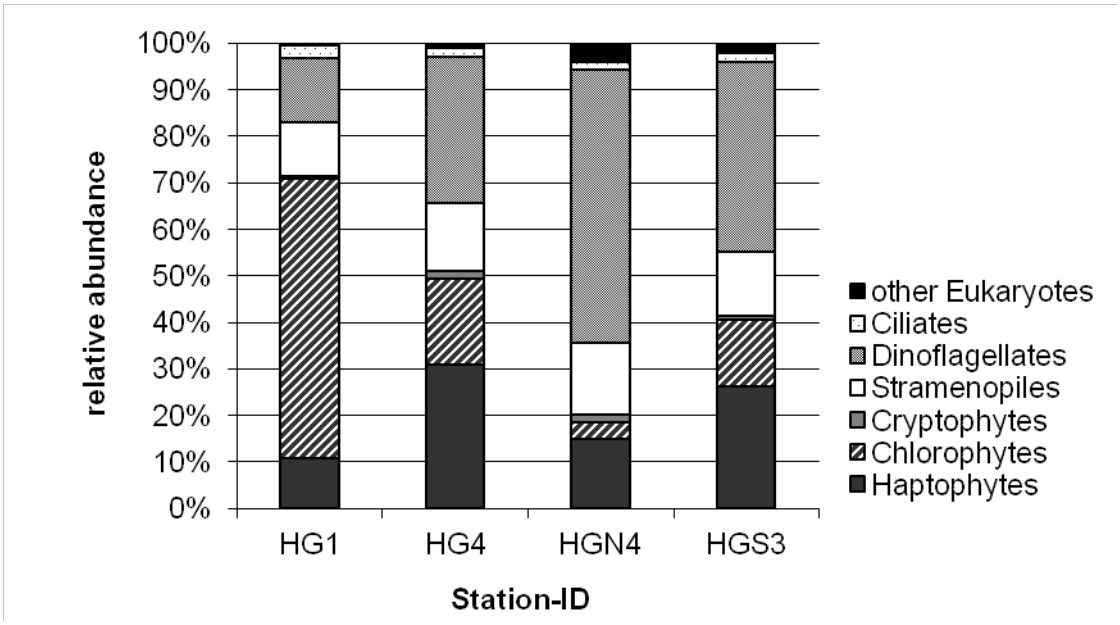

Table 3.6. Schedule of abundant phylotype (>1 %) distribution over the three size classes (>10 µm, 10-3 µm and 3-0.4 µm) and their taxonomic affiliation in the NCBI data base (E-value= 0). Uc.= uncultured; * broad cell size range due to life cycles.

Occurrence	Description	10 µm [%]	10-3 µm [%]	3-0.4 µm [%]	Accession	max. identity	general size division	Sample-ID
ubiquitous	*Cymatocylis sp.*	9.50	0.00	0.00	JQ924046.1	99	Microplankton	ArkXXIV2_C721
	Dinoflagellate	0.40	1.00	0.30	AF290050.2	99	no information	ArkXXIV2_C769
	Dinoflagellate	0.10	1.90	0.50	FJ431597.1	99	no information	ArkXXIV2_C1791
	Gymnodiniales	10.80	32.70	2.90	FJ431812.1	99	Micro-/Nano-/Picoplankton*	ArkXXIV2_C59
	Gymnodiniales	6.90	4.60	0.40	FJ431836.1	99	Micro-/Nano-/Picoplankton*	ArkXXIV2_C571
	Gymnodiniales	3.20	4.80	0.60	JQ639761.1	98	Micro-/Nano-/Picoplankton*	ArkXXIV2_C271
	Gymnodiniales	0.40	1.20	0.00	FJ431807.1	99	Micro-/Nano-/Picoplankton*	ArkXXIV2_C1790
	Gymnodinium sp.	0.20	1.90	0.10	AF022196.1	99	Micro-/Nano-/Picoplankton*	ArkXXIV2_C371
	Syndiniales	0.10	0.30	1.20	EU793554.1	99	Picoplankton	ArkXXIV2_C58
	Syndiniales	0.10	0.00	2.00	FJ431860.1	97	Picoplankton	ArkXXIV2_C1806
	Syndiniales	0.10	0.10	1.40	EU793772.1	94	Picoplankton	ArkXXIV2_C309
	Phaeocystis pouchetii	6.30	12.50	6.10	AF182114.1	99	Micro-/Nano-/Picoplankton*	ArkXXIV2_C180
	Bolidomonas sp.	0.20	1.20	0.40	HQ912557.1	99	Nano-/Picoplankton	ArkXXIV2_C1362
	Micromonas pusilla	0.10	0.10	5.80	JF794057.1	99	Picoplankton	ArkXXIV2_C38
multiple	*Tintinnopsis* sp.	1.20	-	0.00	AB640670.1	98	Microplankton	ArkXXIV2_C1375
	Cercozoa	-	0.10	1.30	JF698748.1	99	Micro-/Nano-/Picoplankton	ArkXXIV2_C966
	Alveolate	-	0.00	3.10	HM561124.1	99	no information	ArkXXIV2_C1349
	Stramenopile	-	0.00	1.60	FJ431721.1	98	no information	ArkXXIV2_C1159
	Micromonas pusilla	-	0.10	2.80	AY954993.1	99	Picoplankton	ArkXXIV2_C185
	Micromonas pusilla	-	0.00	1.60	AY954993.1	98	Picoplankton	ArkXXIV2_C186
	Phaeocystis pouchetii	-	0.00	3.70	AF182114.1	98	Micro-/Nano-/Picoplankton*	ArkXXIV2_C1909
	Phaeocystis pouchetii	-	0.00	1.20	AF182114.1	98	Micro-/Nano-/Picoplankton*	ArkXXIV2_C1729
	Uc. marine eukaryote	-	0.00	1.70	HQ869207.1	98	no information	ArkXXIV2_C1852

Table 3.6. continued

Occurrence	Description	≥ 10 µm [%]	10-3 µm [%]	3-0.4 µm [%]	Accession	max. identity	general size division	Sample-ID
unique	Tintinnid	6.73	-	-	JX567398.1	99	Microplankton	ArkXXIV2_C1302
	Cymatocylis sp.	3.27	-	-	JQ924046.1	98	Microplankton	ArkXXIV2_C1802
	Cymatocylis sp.	1.64	-	-	JQ924046.1	99	Microplankton	ArkXXIV2_C437
	Coccolithus sp.	1.02	-	-	AJ544117.1	99	Micro-/Nanoplankton	ArkXXIV2_C843
	Uc. marine eukaryote	1.36	-	-	HM581790.1	95	no information	ArkXXIV2_C439
	Phaeocystis pouchetii	-	-	1.38	AF182114.1	98	Micro-/Nano-/Picoplankton*	ArkXXIV2_C756
	Micromonas pusilla	-	-	1.99	DQ025753.1	98	Picoplankton	ArkXXIV2_C37
	Bathycoccus prasinos	-	-	3.05	FO082268.1	99	Picoplankton	ArkXXIV2_C13
	Bathycoccus prasinos	-	-	4.02	FO082268.1	98	Picoplankton	ArkXXIV2_C221
	Uc. marine eukaryote	-	-	1.41	HQ867227.1	99	no information	ArkXXIV2_C1717

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Publication III

PROTIST DISTRIBUTION (THE ABUNDANT BIOSPHERE) IN THE WESTERN FRAM STRAIT (ARCTIC OCEAN) IN SUMMER INVESTIGATED VIA MOLECULAR TECHNIQUES

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3.5 Publication III

PROTIST DISTRIBUTION (THE ABUNDANT BIOSPHERE) IN THE WESTERN FRAM STRAIT (ARCTIC OCEAN) IN SUMMER INVESTIGATED VIA MOLECULAR TECHNIQUES

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Abstract

For the first time, diversity and distribution of the whole protist community (micro-, nano-, and picoeukaryotes) was analyzed comprehensively by using 454-pyrosequencing and high-pressure liquid chromatography (HPLC), at five stations in the Western Fram Strait during summer 2010. Three stations (T1; T5; T7) were located in cold Arctic water with lower salinity (<33 PSU) and different extents of ice concentrations. One station (T6) was located in cold modified Atlantic water with intermediate salinity (~33 PSU) and high ice-concentrations, and one station was located in warm Atlantic water with high salinity (~35 PSU) and no ice-coverage at all (T9). General trends in community structure, according to prevailing environmental settings, observed with both methods coincide well. At two stations (T1 and T7), located in Arctic waters and characterized by lower ice concentrations, diatoms (*Fragilariopsis* sp., *Porosira* sp., *Thalassiosira* spp.) dominated the protist community. The third station in Arctic waters (T5) was ice-covered, but has been ice-free for ~4 weeks prior to sampling. At this station, dinoflagellates (Dinophyceae 1 and *Woloszynskia* sp.) were dominant, reflecting a post-bloom situation. In Atlantic waters, the protist communities were dominated by picoeukaryotes, e.g. *Micromonas* spp. irrespective of ice-concentration or temperature. Based on our results, 454-pyrosequencing has proven to be an adequate tool to provide comprehensive information on the composition of Arctic protist communities. Furthermore, we show that a few, but well-chosen samples can be sufficient to detect community structure patterns in a dynamic environment.

Key index words

454-pyrosequencing, Biogeography, Genetic diversity, HPLC, Phytoplankton

Introduction

Global warming is transforming ecosystems on an extraordinary scale. Changes in the Arctic are more intense than in other regions, e.g. the Arctic Ocean has been facing a drastic decrease of sea ice cover during the past decades (IPCC 2007). The ongoing environmental change requires evaluations of its impact on pelagic systems. These impacts could include species expansions into new areas with more tolerable abiotic conditions, intermingling of formerly non-overlapping species or even species extinctions. All these events have in common that they cause changes of biodiversity and thus affect the marine ecosystems, as well as biogeochemical cycling in the Arctic (Wassmann et al. 2011).

Marine phytoplankton forms the basis of the food-web and displays a major contributor to pelagic systems. The taxonomic composition as well as the biomass of phytoplankton influences the Arctic marine food-web, including its trophic interactions and the fluxes of essential nutrients into the euphotic zone (Falkowski et al. 1998, Wassmann et al. 2011). In this respect, a shift in the autotrophic community from a diatom to a flagellate-based system could result in less POC (particulate organic carbon) export to the benthos and enhanced recycling in the water column (Moran et al. 2012). In order to evaluate consequences of environmental change at the base of the Arctic food-web, it is necessary to gain information on the temporal dynamics of phytoplankton compositions and their variability in relation to changing environmental conditions (Wassmann et al. 2011).

Phytoplankton occurs in a broad size spectrum, ranging from single cells with a size <0.5 µm to long chains of cells with sizes >200 µm. Within this broad size range, cells with a size of >20 µm are described as microplankton, those ranging from 2-20 µm are described as nanoplankton, while the term picoplankton describes cells in the size range from 0.2-2 µm (Sieburth et al. 1978). The size distribution has a big influence on the pelagic food-web and thus has the potential to affect the rate of POC export to deep water (Legendre & Le Fèvre 1991). Larger phytoplankton cells from the microplankton produced seasonal blooms under specific hydrographic conditions (Legendre et al. 1993, Li 2002, Mei et al. 2002), and significantly contribute to carbon flux (Michaels & Silver 1988). Periods outside the microphytoplankton bloom can be dominated by small flagellates, e.g. picophytoplankton that also account during this time for a major part of the prevalent chlorophyll biomass (Rat'kova & Wassmann 2002, Gescher et al. 2008). Thus, picoeukaryotic cells are considered as fundamental component of marine ecosystems (Sherr & Sherr 2000). In the past, however, a common theory was that picoeukaryotes were recycled within the microbial loop in the upper water layers (Azam et al. 1983). This assumption changed recently after it was shown that

picoplankton was incorporated into large aggregates that were able to sink rapidly into deep water layers (Richardson & Jackson 2007, Tremblay et al. 2009).

Hitherto, a number of publications described evaluations of the complex diversity of the protist assemblages. Studies have focused on either the microplankton fraction (Booth & Horner 1997, Tremblay et al. 2006, Hegseth & Sundfjord 2008) or on the small size fraction e.g. nano- and picoplankton (Diez et al. 2001, Lopez-Garcia et al. 2001, Moon-van der Staay et al. 2001, Lovejoy et al. 2006, 2007). To our knowledge, studies that include protists from all size classes are scarce and lacking. Based on the different impact of the respective size fractions on the marine ecosystem, information on the whole protist community structure is needed, because environmental changes are expected to cause shifts in size class. Recent investigations in the area of the North Atlantic indicate that rising temperatures promote a shift in the phytoplankton community towards small cells (Daufresne et al. 2009, Moran et al. 2010), suggesting a higher relevance of this size class and an urgent need to include those cells in phytoplankton studies.

In the past, a considerable number of marine surveys took advantage on ribosomal sequence information, which contributed to broaden our understanding of phytoplankton diversity and community structure, including all size fractions (Medlin et al. 2006, Not et al. 2008). Cloning and sequencing of genes, coding for the small- or large subunit, is a widely applied approach to gain insight into protist phylogeny, diversity, and community structure. However, characterization of microbial communities by sequencing of ribosomal sequences is labor-, time-, and cost-intensive. Recently, the 454-sequencing high throughput approach allows to assess microbial communities with less effort, but high resolution based on sufficient deep taxon sampling (Margulies et al. 2005, Stoeck et al. 2010).

In order to elucidate the impact of changing environmental factors on Arctic phytoplankton communities, sea ice dynamics, salinity, temperature, and currents need to be considered, as particularly eukaryotic picoplankton community structure in a marine habitat is strongly impacted by shifts in circulation patterns and changing hydrographic conditions (Greene & Pershing 2007). The variable hydrographical and sea ice conditions in the Fram Strait present an excellent observation area to analyze the polar eukaryotic phytoplankton communities in the presence of changing abiotic factors. With depths up to 5000 m, the Fram Strait represents the only deep connection between the central Arctic Ocean and the Nordic Seas (Rudels et al. 2000, Fahrbach et al. 2001). The hydrography of the Fram Strait is characterized by the inflow of warm and saline Atlantic Water (AW), via the West-Spitzbergen Current (WSC), which constitutes the major heat advection towards the Arctic Ocean. In contrast, the East

Greenland Current transports cold and less saline Polar Water (PW) out of the central Arctic Ocean along the east Greenland site. A significant amount of the AW recirculates directly in the Fram Strait, partly mixing with the colder water and also returning southwards (Rudels et al. 2005).

Considering the sensitivity of the Arctic Ocean to global warming and the expected shift in protist size fraction, this study aims to provide information on the genetic diversity and the distribution of eukaryotic protists within the Fram Strait by taking advantage of new molecular methods that for the first time facilitate to analyze the whole size spectrum, detecting even small cell classes. By achieving this, the present work also relates the corresponding protist composition to prevailing environmental conditions for a better understanding of impacts from future changes in the Arctic Ocean. In this study, High Performance Liquid Chromatography (HPLC) was applied to provide information on the distribution of the main autotrophic phyla by using the CHEMTAX® program (Mackey et al. 1996, Higgins et al. 2011). To complement and to provide more detailed information on the local protist diversity, the 454-pyrosequencing approach was used.

Material and Methods

Sampling area

The sampling was performed during the ARK XXV/2 expedition aboard the RV Polarstern in July 2010, on a transect navigated from 11°58.362' - 0°30.498'W longitude and at 78°50' N latitude (Figure 3.1). Water samples were taken in the euphotic zone by collecting seawater with 12 l Niskin bottles deployed on a CTD (conductivity, temperature, depth system) (Table 3.1). In total, five samples were taken in the upper 15 m water depth and used for further molecular analysis. The sampling sites covered diverse environmental conditions, such as difference in ice coverage, ice melt, salinity, and temperature. For subsequent filtration, 2 l water subsamples were transferred into polycarbonate bottles. Protist cells were collected by fractionated filtration, through Isopore Membran Filters (Millipore) with three different sizes (10 µm, 3 µm, and 0.4 µm) at 200 mbar low pressure. Finally, the filters were transferred into Eppendorf tubes and stored at -80°C until further processing.

Chlorophyll a from satellite data

In order to get an overview of the phytoplankton developmental stage during the investigation, the area-averaged chlorophyll *a* concentrations from remote sensing observations of the Moderate Resolution Imaging Spectrodiometer (MODIS), provided by

the Goddard Earth Science Data and Information Services Center (GES DISC) (Acker & Leptoukh 2007), were depicted from the month of April through August 2010 (Figure 3.2).

HPLC

For HPLC-pigment analyses, 1-2 l seawater was filtered on GF/F filters, immediately frozen in liquid nitrogen, and stored at -80°C until further analysis in the laboratory. The pigment analysis was carried out taking advantage of a Waters HPLC-system. This system was equipped with an auto sampler (717 plus), a pump (600), a Photodiodearray detector (2996), a fluorescence detector (2475), and finally the EMPOWER software. The filters were homogenized for 20 sec with 50 µl internal standard (canthaxanthin), 1.5 ml acetone, and small glass beads in a Precellys® tissue homogenizer. Subsequently, a centrifugation was performed, in which the supernatant liquid was kept and filtered through a 0.2 µm PTFE filter (Rotilabo). An aliquot of 100 µl was transferred to the auto sampler (4°C), and mixed with 1 M ammonium acetate solution (ratio: 1:1). Subsequently, the liquid was injected into the HPLC-system. The analysis of the pigments was conducted by reverse-phase HPLC, by the utilization of a VARIAN Microsorb-MV3 C8 column (4.6x100 mm), and a HPLC-grade solvent (Merck). The mixture of solvent A was built up of 70 % methanol and 30 % 1 M ammonium acetate whereas solvent B contained 100 % methanol (gradient modified after Barlow et al. 1997). Eluting pigments were detected by absorbance (440 nm), and fluorescence (Ex: 410 nm; Em: >600 nm). Retention times served to identify the pigments by comparing them with the retention times of pure algal extracts, and pure standards. To assure the identity of each pigment diode, array absorbance spectrum (390-750 nm) were compared with the library from the injected standards. Pigment concentration was quantified, based on the peak areas of external standards. Concentrations of external standards were spectrophotometrically determined using extinction coefficients of Bidigare (1991), and Jeffrey et al. (1997). A normalization of the pigment concentrations to the internal standard (canthaxanthin) was finally achieved to counteract possible bias by volume change and experimental losses. The taxonomic structure of the phytoplankton classes was calculated from marker pigment ratios using the CHEMTAX® program (Mackey et al. 1996). Microscopic examination of representative samples was used to identify the various taxa and to constrain the pigment ratio as suggested by (Higgins et al. 2011). The resulting phytoplankton group composition was expressed in chlorophyll *a* concentrations to determine their relative contribution to the total phytoplankton biomass.

DNA isolation

DNA extraction was carried out with the E.Z.N.A TM SP Plant DNA Kit Dry Specimen Protocol (Omega Bio-Tek), following the manufacturer. To assure a maximum of DNA concentration, the elution step was adjusted by an additional transfer of the 60 µm eluted DNA solution into the binding column.

Next Generation Sequencing

For subsequent 454 sequencing, the V4 region of the 18S rRNA gene was amplified with the primer set 528F (GCG GTA ATT CCA GCT CCA A), and 1055R (ACG GCC ATG CAC CAC CAC CCA T) (Elwood et al. 1985). The PCR reaction mixture contained 1x HotMasterTaq buffer Mg^{2+} 2.5 mM (5'Prime), 0.4 U HotMaster Taq polymerase (5'Prime), 10 mg/ml BSA, 10mM (each) dNTP (Eppendorf), 10 µM of each Primer, 1 µl of template DNA (~20 ng/µl) in a final volume of 20 µl. PCR amplification was carried out in a MasterCycler (Eppendorf) under the following conditions: first, an initial denaturation step at 94°C for 3 min, succeeded by 35 cycles (denaturation at 94°C for 45 s, annealing at 55°C for 1 min, extension at 72°C for 3 min), and followed by a final extension at 72°C for 10 min. The resulting PCR products were purified taking advantage of the Mini Elute PCR Purification Kit (QIAgen). The purified amplicon was sequenced by GATC Biotech GmbH (Germany) taking advantage of a 454 GS FLX sequencer (Roche).

Data analysis of 454-pyrosequening

Sequences, shorter than 300 bp were excluded from the analysis to guarantee further analysis of the whole V4 region. Chimeric sequences were detected using the chimera-detecting software UCHIME 4.2 (Edgar et al. 2011), and excluded from further analysis. Operational taxonomic units (OTUs) were generated by aligning the remaining reads using the software package Lasergene Seqman Pro (DNAStar). A threshold of 97 %, and a match size of 50 bp was applied to minimize the danger of overestimating the diversity, and to allow a comparison of the current data set with other published data that used the 97 % similarity threshold (Kunin et al. 2010). All singletons (defined as an OTU composed of one single sequence, i.e. that only occurs once in the whole analysis) were removed to evade possible errors induced during the sequencing process. The consensus sequences of the OTUs were placed into a reference tree, build up by 1200 high-quality sequences, containing representatives of all main eukaryotic phyla. This involved the use of the pplacer software 1.0 (Matsen et al. 2010). Sequences that affiliated with non-protist phyla in the tree were

excluded from further analyses. The remaining reads were aligned with the SILVA aligner (Pruesse et al. 2007), and placed into the ARB SSU reference database tree containing around 50 000 eukaryotic sequences (Ludwig et al. 2004). The 454-pyrosequencing sequences were deposited at GenBank`s Short Read Archive (SRA) under Accession No. SRA061498.

Results

Environmental characteristics

The investigated transect in the Western Fram Strait was located in a hydrodynamic zone composed of two water masses that could be designated oceanographically (temperature and salinity) as the warm West Spitzbergen Current (WSC) in the East and the cold East Greenland Current (EGC) in the West (Figure 3.1). In the westerly section of the transect (T1 to T7), the temperatures were around the freezing point ranging from -1.5 to 0.7°C. In the more eastern section of the transect the temperatures were higher and reached up to 4.9°C at T9. The salinity was lowest in the western section of the transect (30.7–32.8 PSU) and increased towards east. T6 showed higher salinity (>33 PSU) than T1, T5, and T7, however maximum salinity (34.2 PSU) was observed at T9. These data suggest that the stations T1, T5, and T7 were located in Arctic waters of the EGC and T6 and T9 were located in Atlantic water of the recirculating branch of the WSC.

The ice conditions during the sampling period were not evenly distributed along the transect. A large polynya with patchy ice-coverage stretched out along the east Greenland coastline. This influenced three of the sampling sites (T1, T5, and T6) and resulted in variable ice coverages, in which the ice concentration was highest in the East (T6; 80 %) and declined towards the West (T1: 30 %) (Figure 3.1). In contrast, station T7 and T9 were virtually not ice covered. However, while T7 was directly located at the ice edge and thus still affected by the prevailing ice cover, T9 was located in moderate distance and served as an example for a sampling site, located in the open ocean with no ice coverage at all during the sampling period.

Chlorophyll a biomass

The area-averaged chlorophyll *a* concentrations obtained by remote sensing observations showed similar values over the whole transect, ranging around ~0.4 mg m^{-3} within our investigation period in July. Concerning the entire growth period in the area of the transect, a peak in biomass (chlorophyll a >1.5 mg m^{-3}) was observed in June at the more eastern stations T6 and T9 (5°W-0°E). At the western stations, the chlorophyll *a* values remained low

throughout the whole growth season with an almost linear increase from April to August 2010.

Diversity analysis

HPLC

The results of the HPLC analysis are presented in Figure 3.3. To facilitate the comparison with the 454-pyrosequencing reads, the HPLC data set was adjusted by removing the chlorophyll *a* values originating from cyanobacteria. The residual pigment patterns were used to identify main phytoplankton divisions such as haptophytes, chlorophytes, cryptophytes, stramenopiles, and dinoflagellates. In summary, the majority of the samples were dominated by stramenopiles, dinoflagellates, and chlorophytes, while cryptophytes contributed less in the phytoplankton community, never exceeding 11 % (T9). The relative contribution of the three dominating groups to the phytoplankton community varied at the different sampling sites. Stramenopiles accounted for up to the half of the autotrophic assemblage at station T1 (50 %), T5 (54 %), and T7 (52 %), while they accounted to a lesser extent to the protist assemblages at the other two stations. With the exception of station T6, dinoflagellates were observed with a proportion of 20 to 43 % at all remaining sampling sites. The highest contribution of dinoflagellates was observed at the western station T5 (43 %). Chlorophytes exhibited the lowest proportion, counting for 4 % and 1 % at station T1 and T5, respectively. However, they displayed a high contribution at T6 (59 %) and were strongly represented at T9 (24 %).

To achieve a better insight of the community shifts along the transect, each station was analyzed successively. Starting at the western station, T1, we found a community structure that was dominated by stramenopiles and dinoflagellates, accounting for 82 % of the total community. The residual fraction of 18 % was primarily composed by haptophytes (13 %), and some chlorophytes (4 %). A similar community structure was observed at T5, where stramenopiles and dinoflagellates cumulated abundance up to 97 %. At station T6, phytoplankton composition changed. Here, we observed a dominance of chlorophytes (59 %), a smaller proportion of stramenopiles (28 %), and an absence of dinoflagellates. In comparison to the other stations, cryptophytes (5 %) and haptophytes (7 %) accounted for a higher share to the protist community at T6 however, the overall contribution remained minor. At T7, one half of the protist community consisted of stramenopiles and the other half was composed by dinoflagellates (24 %), chlorophytes (15 %), and haptophytes (8 %). Finally, protist community composition was the most balanced in the open ocean reference station, T9. Haptophytes as well as chlorophytes constituted approximately a quarter of the total

assemblage, while dinoflagellates (20 %), stramenopiles (18 %), and cryptophytes (11 %) were sharing the remaining assemblage.

454-pyrosequencing

The sequencing of the five water samples resulted in 10141-44713 raw sequences. Quality filtering, including chimera check and removal of short reads (<300 bp), reduced the number of reads to 5706 (T6)–23034 (T9) quality checked sequences (Table 3.2). Subsequent clustering of the quality checked sequences resulted in 526 (T1)-1108 OTUs (T9) (Figure 3.4). The OTUs were composed on one hand, of few OTUs that were represented by many reads (abundant taxa) and on the other hand, of many OTUs that were represented by just a few reads (rare taxa).

A quantitative comparison of the abundant biosphere (>1 %) along the stations, didn't reveal major differences in the OTU numbers. It ranged between a small spectrum of five OTUs at T5 to ten OTUs at T9. Both stations, presented a protist community that was dominated by stramenopiles (83 and 87 %), haptophytes (11 and 7 %), and dinoflagellates (~6 %). In contrast, major contributors to phytoplankton community at T6 and T9 were chlorophytes, consisting 72 % of the reads at T6 and 52 % at T9. The residual assemblage was composed of alveolates, of which ciliates presented a higher share than dinoflagellates. Ciliates contributed up to 15 % of the reads at T6, and 28 % of the reads at T9, while only 6 and 7 % of the reads were associated to dinoflagellates. In comparison to the previous stations (T1 and T7), haptophytes represented a small portion of 5 % (T6) and 8 % (T9) of the reads. Furthermore, stramenopiles contributed only for 1 % and 4 %, respectively to the whole protist community. T5 displayed a complete different protist composition. Dinoflagellates dominated the read assemblage with a proportion of 90 %, while haptophytes (2 %), stramenopiles (2 %), and ciliates (3 %) were less represented in the protist community. The qualitative species composition of the abundant biosphere (Table 3.3) was very similar at T1 and T7, but differences were observed for the quantitative composition of the protist communities. The portion of *Phaeocystis* sp. and *Prorosira* sp. was two-fold higher at T1 than at T7. The genus *Thalassiosira* was the most abundant in both stations, represented by four different phylotypes: *Thalassiosira* sp.1 dominated at T1 (23 %), but accounted for 7 % of the reads at station T7. The most abundant phylotype at T1 (9 %) and T7 (10 %) was assigned to *Thalassiosira* sp. 3. Furthermore, an OTU closely related to *Gyrodinium* sp. was also observed to account for similar proportions at T1 (3 %) and T7 (2 %). In contrast, *Fragilariopsis* sp. was present in the abundant biosphere of T1 (3 %), but contributed to the

rare biosphere at T7 (<1 %). The qualitative resemblance of the abundant biosphere of T6 and T9 was also much alike. In this respect, the protist community at T6 and T9 was dominated by chlorophytes that were represented by two phylotypes of the genus *Micromonas,* and one of the genus *Bathycoccus*. *Bathycoccus* sp. was in both stations a minor contributor (~2 %), while *Micromonas* accounted for 57 % (T6) and 41 % (T9) of the sequence reads. One phylotype, *Micromonas* sp. 1, contributed 90 % of the total OTUs, while the other one, *Micromonas* sp. 2, made only a small contribution to the protist assemblage. *Phaeocystis* sp. accounted for similar proportion of the protist community as previously observed at T1 and T7, by showing 3 % at T6 and 6 % at T9, respectively. OTUs assigned to dinoflagellates made only a minor contribution to the protist community at T6 (2 %) and T9 (3 %). Stramenopiles, e.g. diatoms, were neither present in the abundant biosphere at T6, nor at T9. Ciliates contributed to the abundant community at T9, but just one phylotype was shared with T6 (*Ciliophora* 1; 3 %). In summary, ciliates composed for 13 % at T9, in which the proportion of the single phylotypes ranged between 2 and 4 %. Station T5, showed a different picture of the protist community. The abundant biosphere of T5 was constituted exclusively by dinoflagellates, which were represented by five different phylotypes accounting for 74 % of the total reads. Two phylotypes, Dinophyceae 1 and *Woloszynskia* sp. contributed a higher proportion of the total read abundance (32 % and 27 %, respectively), while the residual phylotypes accounted for 1 to 9 %. The majority of phylotypes that were abundant in one of the stations could at least be found within the rare biosphere of one of the other stations. Exceptions were mainly restricted to station T1 and T7.

Discussion

The purpose of this study was to investigate protist assemblages in the physically complex environment of the Western Fram Strait, taking advantage of 454-pyrosequencing, in order to provide a baseline for similar future investigations in the fast changing Arctic Ocean. Prior to an interpretation of our results, in the light of the respective environmental conditions, we start the discussion with a comparison between the 454-pyrosequencing and the HPLC data. On one hand, we analyzed our samples with both techniques to proof the significance of using molecular tools in polar plankton ecology and on the other hand, to point out some pros and cons for the use of both methods, in combination, for assessments of plankton ecology.

454-pyrosequencing and HPLC

In the last years, nucleic acid based screening tools have experienced a rapid development,

enabling deep taxon sequencing, which allowed the consideration of even the rare biosphere (Sogin et al. 2006). In this publication, 454-pyrosequencing was applied to analyze the genetic diversity of the whole protist size spectrum in the Western Fram Strait, while HPLC was used as an evaluation of the 454-pyrosequencing results. To our knowledge, an evaluation of 454-pyrosequencing data with an established pigment-based method like HPLC has not been exercised so far. The first thing to consider is that both methods rely on different markers, the 18S rDNA gene (Vaulot et al. 2008) and photosynthetic pigments (Wright et al. 1991), respectively. Thus, 454-pyrosequencing detects all, autotrophic, mixotrophic, and heterotrophic protists, while the detection of protists with HPLC is restricted to autotrophic cells. Consequently, an appropriate evaluation of 454-pyrosequencing data with HPLC demands the consideration of these methodological differences. In this study, five samples from different locations in the Western Fram Strait were analyzed with both methods, which results give good agreement considering the main protist shifts. In this respect, the contribution of haptophytes and chlorophytes to the protist communities at the different sampling sites coincided well. Both taxa have mostly or exclusively (chlorophytes) autotrophic contributors (Baldauf 2008). Thus, HPLC and 454-pyrosequencing detect the same group of species (autotrophs) and consequently result in similar protist observations. Furthermore, HPLC and 454-pyrosequencing were in strong accordance for cryptophytes. Only two stations (T6 and T9) showed slight elevated numbers in the HPLC data. The main differences between the results of HPLC and 454-pyrosequencing sets were observed for the dinoflagellates and stramenopiles. In this case, particularly station T1, T5, and T7 were affected. In comparison to 454-pyrosequencing, HPLC is overestimating the proportion of dinoflagellates at T1 and T7, while the proportion is underestimated at T6. In contrast, stramenopiles are underestimated at T1 and T7, while they appear as overestimated at T6. If the proportions of dinoflagellates and stramenopiles are summed up, HPLC and 454-pyrosequencing give similar relative amounts of the two taxa to the protist community. The underestimation of dinoflagellates at T6 could be explained by the fact that HPLC is limited on autotrophs, while 454-pyrosequencing also detects heterotrophic protists that are present in both taxa. Apart from that, HPLC has the tendency to underestimate the abundance of dinophytes, because a diatom marker pigment (fucoxanthin), has been reported to occur to some extent within several dinoflagellates, and may lead to an overestimation of diatoms (stramenopiles) (Irigoien et al. 2004). The critical point in retrieving a pigment based phytoplankton composition is the input ratio of the marker pigments (reviewed in: (Higgins et al. 2011)). In particular, dinoflagellates are in this regard underestimated (Higgins et al.

2011). Indeed, the effect of symbiosis may influence the interpretation of pigment signatures, as dinoflagellates have been reported with characteristics specific for chrysophytes (Jeffrey et al. 1975), haptophytes (Bjørnland and Liaaen-Jensen, 1989), and green algae (Watanabe et al. 1987, 1990).

In contrast, the presence of several copies of the rRNA operon that are not identical for the different protist taxa could explain the underestimation of dinoflagellates at T1 and T7 by 454-pyrosequencing. Diatoms, and especially the genus *Thalassiosira*, were found to hold a high number of rRNA copies (Zhu et al. 2005). This effect is more essential for the big sized compared to the small sized cells, because latter have a smaller genome size and therefore limited rRNA copy numbers (Vaulot et al. 2008). As the cell size plays a critical role, diatoms could be overrepresented in case of the simultaneous presence of small dinoflagellates.

In summary, based on the evaluation of HPLC data with autotrophic taxa, 454-pyrosequencing proved to be a reliable molecular tool in polar plankton ecology that facilitates a profound overview of the whole protist assemblage, and a quick compass of community structure shifts, including information on the taxa involved.

Protist distribution in an environmental context

Future investigations of climate change consequences on the diversity and distribution of Arctic protist require first a characterization of the "standing stock" and the respective environmental factors, influencing geographical expansion (Lovejoy et al. 2007, Hamilton et al. 2008). A variety of different approaches is suitable to assess protist composition. Most of them are based on microscopic analysis. However, common light and epifluorescence microscopic approaches are not well suited for taxonomic characterization of the very small size classes, of partly nano- and mainly picoplankton. Picoeukaryotes are too small to be recognized, and informative morphological features to differentiate them from another one are missing (Massana & Pedrós-Alió 2008). Consequently, an investigation of this planktonic fraction requires the use of molecular methods, like 454-pyrosequencing, which is size independent. The present work is one of the first studies that took advantage of molecular methods to analyze the whole size range of protists in the hydrodynamic zone of the Western Fram Strait, including picoeukaryotic cells.

The investigated transect passed through the confluence zone of the West Spitzbergen Current (WSC) and East Greenland Current (EGC). The study area comprised both Atlantic and Polar Water and was characterized by a dynamic sea ice condition. Our five stations were selected under the aspect of characteristic physical factors. In this respect, two stations were chosen in

the Atlantic Water, showing total ice coverage (T6) and no ice coverage (T9), and three were chosen in the EGC, situated in the marginal ice zone of east Greenland, showing moderate ice coverages (T1, T5, and T7). The latter water samples were all located in the upper water layer (15 m) of the same water mass. Since the ice condition can highly influence the biomass and the diversity of protists, a survey of the previous ice situation (four weeks) was done with satellite images, retrieved by MODIS data (Spreen et al. 2008). These satellite images showed a pack-ice tongue that had shifted over the geographical position of T5. Until then, T5 had been ice-free for at least six weeks before the surveys, while T1 and T7 showed high ice coverages that recently had started to melt. Chlorophyll *a* concentration, that also derived from the MODIS data, concur with our biomass measurements and showed at all five stations low Chl *a* concentrations, suggesting no bloom event during the sampling period.

Stations T1 and T7 were dominated by centric diatoms that grouped into the genera *Thalassiosira* and *Porosira* and by a pennate diatom of the genus *Fragilariopsis*. All three genera have been previously reported in that area during main biomass increase (Booth & Smith 1997, vonQuillfeldt 1997). The similar abundant species composition at T1 and T7 is likely to be caused by the ongoing ice melt at both stations. In this respect, the ice may constitute a possible source of the abundant protists assemblage by releasing protist cells into the water column, as first reported by Garrison et al. (1987). Moreover, the formation of a stabilizing melt water lens facilitated the cells to persist for a longer period in the light exposed water surface. Another aspect might be the initial stock of protists at the sampling stations. Since both stations were located in the same water mass a similar source-population could have been prevalent. In fact, water masses were previously reported to be associated with the distribution of protist and even bacterial assemblages (Lovejoy et al. 2002, Galand et al. 2009).

The third station (T5) was located in the same water mass but displayed in contrast, a completely different community that was exclusively dominated by dinoflagellates. As previously discussed, in certain species assemblages, 454-pyrosequencing might overestimate the dinoflagellates relative abundance. As a consequence, dinoflagellates could be less abundant, however, still dominant. In this respect, the most abundant phylotypes encountered were Dinophyceae 1, *Woloszynskia* sp., and *Gyrodinium* sp. These observations are in accordance with previous findings that report small forms of *Gyrodinium* spp. to be abundant during periods characterized by low phytoplankton biomass (Hansen 1991). Furthermore, heterotrophic dinoflagellates are active phytoplankton grazers, and thus good indicators for post bloom events (Strom & Strom 1996, Levinsen et al. 2000, Poulsen & Reuss 2002).

Hence, the presence of many dinoflagellates might indicate a more advanced protist succession at T5, where the diatoms have already been grazed or sunk into deeper water layers. In retrospect, the ice conditions, in which T5 had been ice-free for a relatively long time, support our assumption. Beyond any doubt, the classification of abundant diatom or dinoflagellate sequences to the genus level was beneficial for these ecological interpretations.

The benefit for ecological interpretation was also observed at the stations T6 and T9, located in Atlantic Water of the recirculating WSC. Even though T6 was ice-covered, while T9 was not ice-covered at all, both stations presented a similar protist community that was very different from the ones previously discussed and situated in the Polar Water. In the warmer, more saline Atlantic Water at both stations, picoeukaryotes such as *Micromonas* spp. and *Bathycoccus* sp. were found to be abundant. The most abundant phylotype, *Micromonas* sp. 1 could be assigned to a *Micromonas* strain (CCMP 2099) already sampled in the Baffin Bay (Slapeta et al. 2006) as well as in the North Water Polynya between Ellesmere Island and Greenland (Lovejoy et al. 2007). Those studies identified in general five *Micromonas* clades (A-E), in which one (Ea) included exclusively Arctic phylotypes (Slapeta et al. 2006, Lovejoy et al. 2007). *Micromonas* sp. 1 is part of the Arctic Ea clade, that contains strains of Micromonas sp., growing faster under low light and low temperature (Lovejoy et al. 2007). The other abundant strain, *Micromonas* sp. 2, could not be assigned in detail, but resembled more the residual four identified clades A-C that contain strains with worldwide distributions (Slapeta et al. 2006), which might be an indication of Atlantic Water inflow.

Ciliates were another dominant group at T6 and T9, showing higher diversity at T9. This group occurs in higher abundance in the presence of small-celled protists and in the open ocean (Hansen 1991, Nielsen & Kiorboe 1994). Although, we cannot make any statements on the prevalent nutrient conditions, which would finally approve the post bloom stage, we can refer to previous studies that analyzed the local nutrient concentration. These studies observed low and almost depleted nitrate concentrations apart from a limited protist biomass in late summer (Lara et al. 1994, Pesant et al. 1996, Booth & Smith 1997, Kattner & Budeus 1997). This supports our hypothesis of the post bloom stage at T6 and T9, and points to a shift from a productive to a regenerative system, which is composed of regenerated nutrients and mainly characterized by small size protists and an active microbial loop (Cushing 1989, Landry et al. 1997, Falkowski et al. 1998). In this scenario, the recirculating West Spitzbergen Current could have transported the protist communities of stations T6 and T9, in which the higher ice coverage at T6 further constrained the autotrophic biomass.

In summary, the protist distribution observed on the transect in the Western Fram Strait could be particularly related to the previous and the prevailing ice coverage. The protist community in long-light exposed areas was composed mainly of dinoflagellates, while diatoms dominated in areas with ongoing sea ice melt and, finally picoeukaryotes (*Micromonas*) displayed highest abundances in highly ice-covered and open ocean areas. However, water mass characteristics such as salinity and temperature further influenced the community structure and supported a high abundance of small cells (*Micromonas* and *Bathycoccus*) in the warm and saline Atlantic Water. In conclusion, our data suggest that less ice coverage, higher temperatures, and higher salinity promote the abundance of picoeukaryotes. Our observations in combination with predictions of sea ice decline and increasing water temperatures in the Arctic, suggest that small picoeukaryotic cells might become more abundant and further distributed, if abiotic conditions in the Arctic Ocean become more favorable for them. This hypothesis is in agreement with other studies that predicted a shift towards smaller cells in the presence of higher water temperatures (Li et al. 2009). The molecular technique, 454-pyrosequencing, proved to be a valuable and appropriate tool for retrieving protist community structure shifts, including all size fractions in a highly dynamic system like the Western Fram Strait. In this respect, this study has shown that a limited number of five samples with an appropriate preselection based on a set of distinct environmental conditions can be sufficient to interpret ecological features.

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Table 3.1. Coordinates, chlorophyll *a*, and ice content of surface water samples along the transect.

Station-ID	CTD	Date	Longitude W	Latitude N	Sampling depth [m]	Chl *a* [ng/l]	Ice coverage [%]
T1	237	23/07/2010	11°58.362‘	78°50.418‘	15	357	30
T5	231	23/07/2010	8°0.361‘	78°49.92‘	5	368	40
T6	223	22/07/2010	4°55.932‘	78°50.088‘	15	321	80
T7	220	21/07/2010	3°53,688‘	78°49.992‘	5	1399	20
T9	204	18/07/2010	0°30,498‘	78°49.998‘	15	692	0

Table 3.2. 454-pyrosequencing- Summary of the read quantities during the analysis process.

	T1	**T5**	**T6**	**T7**	**T9**
raw reads number	44 713	18 163	10 141	38 126	36 691
quality filtering	33 511	12 457	4435	26 522	13 657
final reads number	11 202	17 125	5706	11 604	23 034
OTU (threshold: 97 %)	526	795	531	745	1108

Table 3.3. Summary of the phylotypes representing the abundant biosphere (≥1 %), r refers to the occurrence of the phylotype in the rare biosphere (<1 %).

Phylotype	**T1**	**[%]**	**T5**	**[%]**	**T6**	**[%]**	**T7**	**[%]**	**T9**	**[%]**
Phaeocystis sp.	1177	10.5	r	r	160	2.8	504	4.3	1336	5.8
Micromonas sp. 1	r	r	r	r	2936	51.5	r	r	8680	37.7
Micromonas sp. 2	r	r	r	r	298	5.2	-	-	839	3.6
Bathycoccus sp.	-	-	r	r	117	2.1	-	-	405	1.8
Prorosira sp.	1239	11.1	r	r	r	r	744	6.4	r	r
Thalassiosira sp. 1	2597	23.2	r	r	r	r	828	7.1	r	r
Thalassiosira sp. 2	1134	10.1	r	r	r	r	672	5.8	r	r
Thalassiosira sp. 3	957	8.8	r	r	r	r	1134	9.8	r	r
Thalassiosira sp. 4	194	1.7	r	r	r	r	132	1.1	r	r
Fragilariopsis sp.	284	2.5	r	r	-	-	r	r	r	r
Gyrodinium sp.	323	2.9	1514	8.8	r	r	248	2.1	345	1.5
Dinophyceae 1	r	r	5518	32.2	102	1.8	r	r	603	2.6
Woloszynskia sp.	r	r	4643	27.1	r	r	r	r	r	r
Dinophyceae 3	r	r	864	5.0	r	r	r	r	r	r
Symbiodinium sp.	r	r	191	1.1	r	r	r	r	-	-
Ciliophora 1	r	r	r	r	146	2.6	-	-	477	2.1
Ciliophora 2	r	r	r	r	r	r	r	r	833	3.6
Ciliophora 3	r	r	r	r	r	r	r	r	604	2.6
Ciliophora 4	-	-	r	r	r	r	r	r	999	4.3

Figure 3.1. A) Map and ice coverage (MODIS) of the transect running from 11°58.362’ to 0°30.498’W longitude along 78°50’N latitude, taken in July 2010 (ARKXXV/2); B) Temperature [°C] and salinity [PSU] profile. Water samples have been taken within the chlorophyll maximum layer (exception: T1).

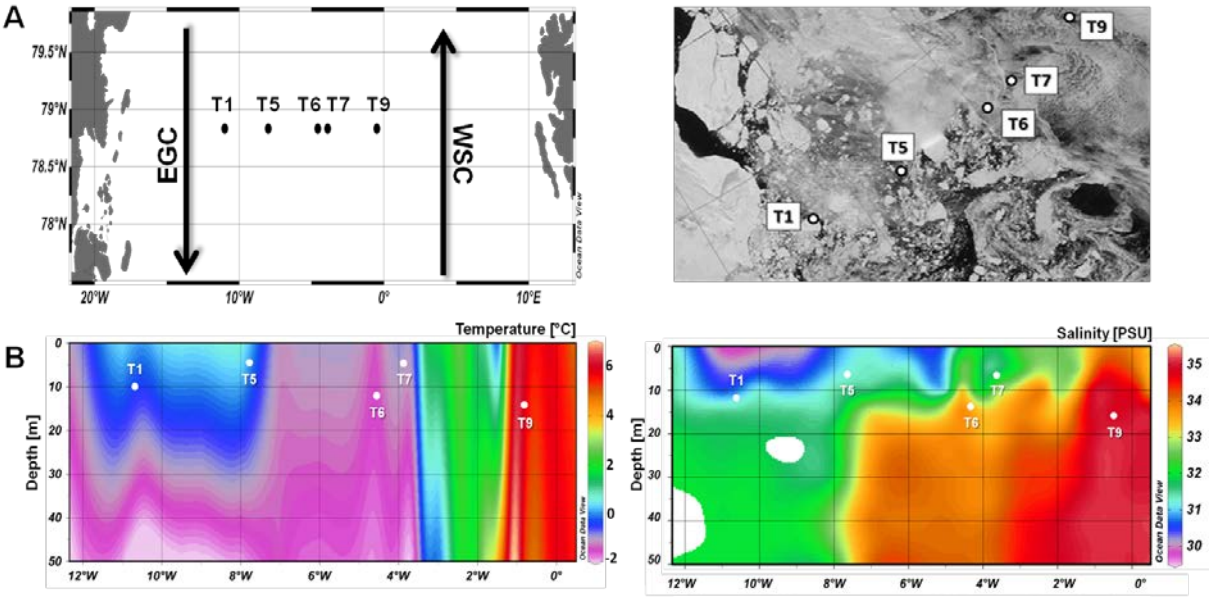


Figure 3.2. Seasonal development of area-averaged chlorophyll *a* concentration obtained by remote sensing observations. Two different areas have been selected: 12°W - 4°W, 78°N - 80° N, solid line, representing the stations T1, T5, T7, and 5°W - 0°E, 78°N - 80° N, scattered line, representing T6 and T9, respectively. The period of our investigation was in July. Concentrations are derived from MODIS data.

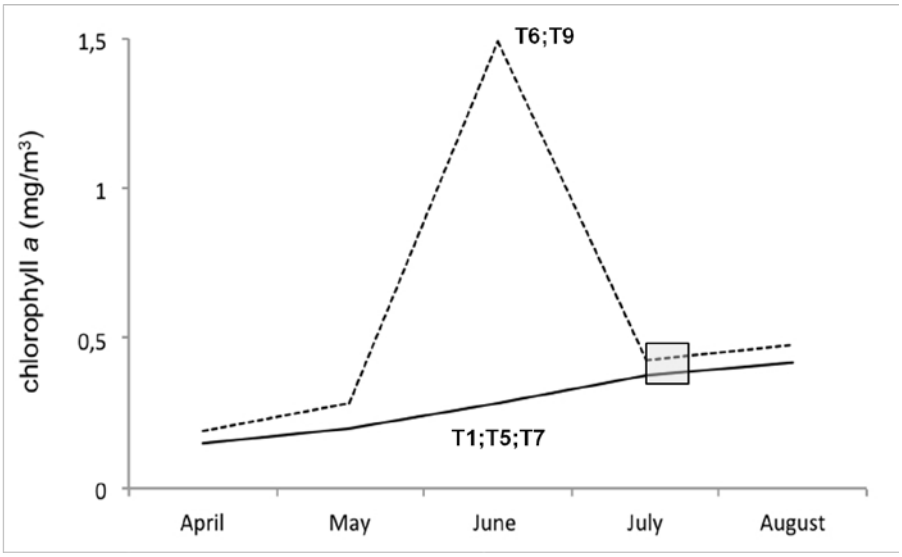


Figure 3.3. Histogram of the main taxonomic groups in the surface water samples A) High Performance Liquid Chromatography (HPLC) and B) 454-pyrosequencing.

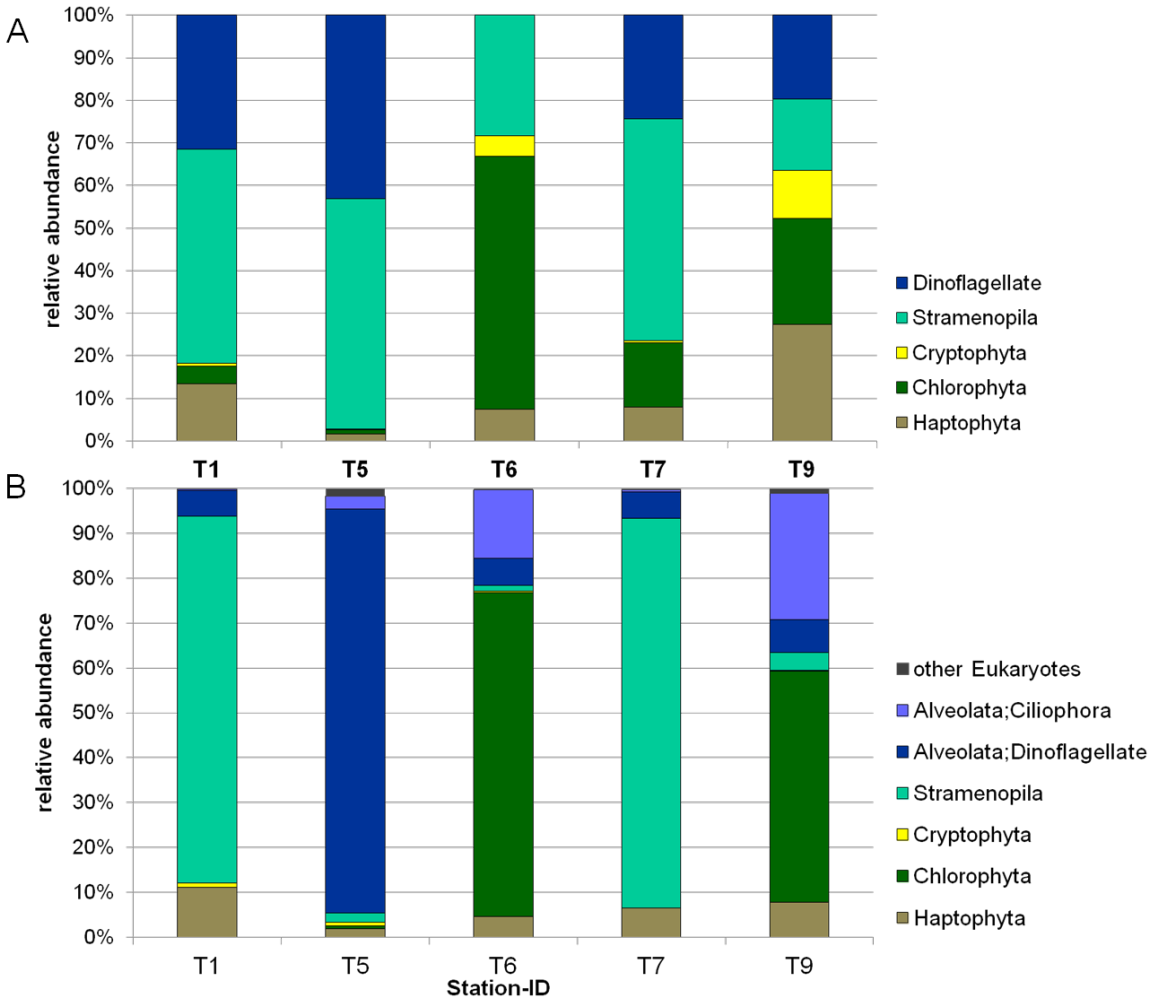
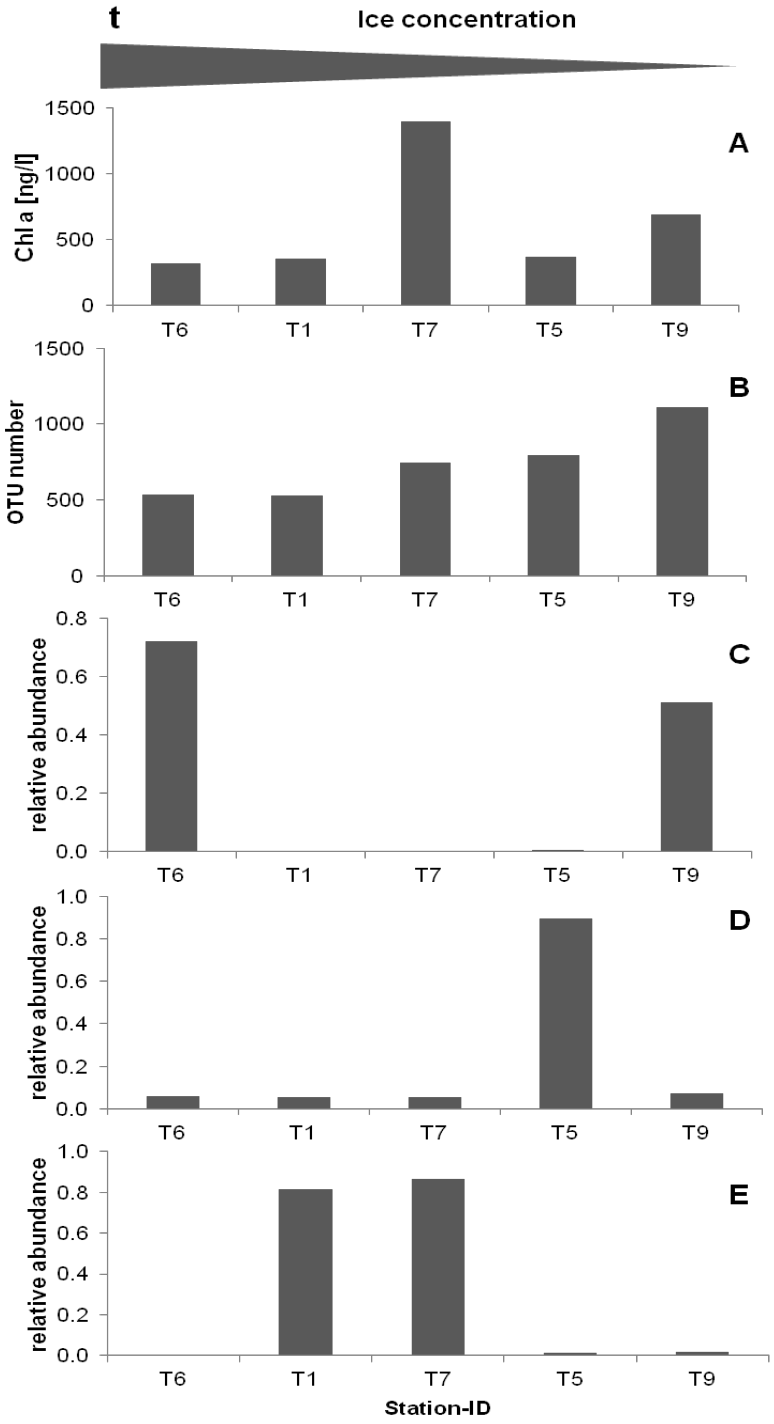


Figure 3.4. Biomass, species richness (OTU number) and main protist groups along the decreasing ice concentration. A) biomass (chlorophyll *a*: Chl *a* ng/l), B) species richness (OTU number), C) chlororphytes [%], D) dinophytes [%] and E) diatoms [%]. T1, T5, and T7 are situated in cold and less saline polar water of the East Greenland Current, T6 and T9 belong to the warmer and saltier water of the recirculation branch of the West Spitzbergen Current.



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Publication IV

A MOLECULAR SURVEY OF PROTIST DIVERSITY THROUGH THE CENTRAL ARCTIC OCEAN

ESTELLE KILIAS, GERHARD KATTNER, CHRISTIAN WOLF, STEFAN FRICKENHAUS AND KATJA METFIES

3.6 Publication IV

A MOLECULAR SURVEY OF PROTIST DIVERSITY THROUGH THE CENTRAL ARCTIC OCEAN

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Abstract

In the Central Arctic Ocean surveys of the eukaryotic protist assemblage are scarce although they are the major primary producers. In late summer 2011, samples were collected, during the ARK XXVI/3 expedition of the *RV* Polarstern to study Arctic protist community structures, including the whole size fraction by implementation of ARISA and 454-pyrosequencing. Protist assemblages were related to water masses and the environmental factors, temperature, salinity, ice coverage, nitrate, phosphate, and silicate. The diversity analysis was focused on the abundant (≥1 %) and rare biosphere (<1 %) to reveal mutual relationships. Dinoflagellates and chlorophytes (*Micromonas* sp. 1) were dominant at all stations. A relation between the protist community structure and water masses was highly supported by ARISA and partially by 454-pyrosequencing. However, sea ice showed a stronger influence on the local community structure than nutrients, making statements on the water mass influence more difficult. No significant correlation was found between the abundant and rare biosphere. However, relative contributions of major taxonomic groups revealed an unexpected stable community structure within the rare biosphere, indicating that this biosphere not only serves as a protist reservoir, but further provide these reservoirs in constant taxonomic relations.

Key index words

454-pyrosequencing, ARISA, Water masses, Ice coverage

Introduction

Eukaryotic protists are the major primary producers in the Central Arctic Ocean and constitute the base of the Arctic marine food-web (Caron et al. 2012). The Arctic Ocean promotes the occurrence of species that are especially adapted to the harsh environment (Sakshaug & Slagstad 1991). Local conditions, such as multiyear ice vs. annual sea ice or limitations of light and/or nutrient have the potential to alter the phytoplankton composition (Li et al. 2009, Tremblay et al. 2009). In particular, small cells, defined as picoeukaryotes, (0.2-2.0 µm) benefit from the oligotrophic conditions in the Arctic Ocean because of higher rates of nutrient uptake (Hein et al. 1995) and reduced metabolic requirements (Grover 1991). Numerous studies already demonstrated the importance of picoeukaryotes in terms of biomass, production, and diversity (Li 1994, Diez et al. 2001, Lopez-Garcia et al. 2001, Moon-van der Staay et al. 2001, Worden et al. 2004, Lovejoy et al. 2006, 2007), particularly in oligotrophic habitats. Micro- (>20 µm) and nanoplanktonic (20-2 µm) fractions however, are also significant for the Arctic ecosystem because of their potential to build up high biomasses during bloom periods and their high relevance for the carbon and nutrient flux to the deep ocean (Tremblay et al. 1997, Brown & Landry 2001a, b, Le Borgne et al. 2002).

Arctic surface waters are mainly sourced by Atlantic Water (AW), entering through Fram Strait and Barents Sea, and Pacific Water (PW), entering through Bering Strait (Rudels et al. 1991, Jones et al. 1998). Both water masses are characterized by specific nutrient signatures. The Arctic surface water is often nutrient limited because of a strong vertical stratification that impedes an upward supply from deeper water layers (Tremblay et al. 2009) and consequently restrains the inhabiting protist community. Different water masses have already been reported to host different protist assemblages (Lovejoy et al. 2002), nevertheless, so far no molecular study has been carried out in the Central Arctic Ocean.

Sea ice retreat, thinning of multiyear ice or even a replacement of multiyear ice by annual sea ice (Stroeve et al. 2007) constitute environmental conditions that strongly influence the light regime in the Arctic surface water. Since autotrophic protists are dependent on a good light and nutrient regime for photosynthesis, the prevailing limitations lead to the general description of the Arctic Ocean as a region of low phytoplankton productivity (15 g C $m^{-2}yr^{-1}$) (Gosselin et al. 1997), where the timing of primary production is more affected by light irradiance, whereas the new production is more determined by nutrient supply (Carmack et al. 2006).

In principal, studies regarding the diversity and biogeography of microbial eukaryotic plankton are scarce for the Central Arctic Ocean because of the limited accessibility. Thus,

investigations of eukaryotic protist communities are patchy with only little spatio-temporal resolution. Moreover, protist communities in those areas have not been analyzed consistently, due to the application of different tools. Most previous investigations were based on microscopy or flow cytometry. However, these methods are limited in respect to comprehensive assessments of the whole phytoplankton community. Microscopy is well suited for species specific analyses of the microplankton fraction, but microscopy of the nano- and picoplankton fraction is challenging because of the small sizes and insufficient morphological features. In contrast, molecular methods, such as molecular fingerprinting techniques like ARISA (automated ribosomal intergenic spacer analysis) and 454-pyrosequencing, are independent of size and morphological features because of their focus on length and sequence heterogeneity (Caron et al. 2012). Thus, they include all size fractions in surveys of protist communities. Previous studies have shown the power of ribosomal genes (rRNA-genes) analysis for comprehensive protists assessments (Ebenezer et al. 2012). Genes, coding for the rRNA are particularly well suited for molecular investigations of microbial diversity. The ubiquitous presence in eukaryotic organisms and the low evolutionary rate make the 18S rDNA a good marker (Amann & Kuhl 1998, Vaulot et al. 2008). Molecular fingerprints, as ARISA, have frequently been used for quick comparisons of microbial communities and bases on the comparison of the specific fragment lengths of the intergenic spacer region (ITS), localized between the 18S rDNA and 28S rDNA (Caron et al. 2012). However, most studies, taking advantage of ARISA, focused on the investigation of prokaryotes (Smith et al. 2010) while in our study the method was applied for a primary screening of eukaryotes.

454-pyrosequencing allows assessing microbial communities with less effort, but with high resolution based on sufficient deep taxon sampling (Margulies et al. 2005, Stoeck et al. 2010). This new approach allows including the rare biosphere in investigations of microbial communities, which has been previously missed by the classical approaches (Sogin et al. 2006). The rare biosphere contains most of the protist diversity (Pedrós-Alió 2006) and is suggested to play a key role in ecological buffering (Caron & Countway 2009, Caron et al. 2012). However, the vast majority of rare phylotypes (bacteria) was never detected to become abundant so far (Galand et al. 2009a, Kirchman et al. 2010) and information about distribution and influence of abiotic parameters remains scarce.

Considering the ongoing changes in the Arctic Ocean by global warming and its implications, it is crucial to understand the joint role of environmental factors on the protist distribution and to provide detailed data on the prevalent taxa. Hence, the objective of this study is to address

the questions: (i) Are water masses in the Central Arctic Ocean characterized by distinct protist communities? (ii) Is ice-concentration a driving factor that shapes the protist community in the water column? (iii) What is the contribution of the rare biosphere to the protist communities in the Central Arctic Ocean?

Material and Methods

Study area and sampling program

The samples were collected from 5 August to 7 October 2011 during the ARK XXVI/3 expedition of the RV Polarstern to the Central Arctic Ocean (Figure 3.1). Twenty-four stations were sampled for the determination of the local protist community structure as well as for temperature, salinity (S), chlorophyll *a* (Chl *a*), and nutrients (Table 3.1). Meltpond quantity, floe size, ice concentration, ice thickness, and snow thickness were further categorized to assess the light penetrability (doi:10.1594/PANGEA.803312). Ice thickness was embraced in first year ice category I (0.3-0.7 m), II (0.7-1.2 m), III (>1.2 m), and multiyear ice (>2 m).

Water samples have been taken in the upper water layer (1-200 m) by a rosette sampler equipped with 24 Niskin bottles and a CTD probe. Two l subsamples were taken in PVC bottles and filtered on GF/F (Whatman) filters for the chl *a* and on Isopore Membran Filters (Millipore) for the eukaryotic protist determination. Protist cells were collected by fractionated filtration through three different sizes (10 µm, 3 µm, 0.4 µm) at 200 mbar. The fractionated filtration facilitates a separated amplification in the subsequent PCR step and thus minimizes the danger of under-amplifying picoeukaryotes, due to the limited gene copy number. Filters were stored in Eppendorf tubes at -80°C until further processing. The samples were subjected to ARISA analysis for a quick community structure overview and a subset of eight samples was analyzed by 454-pyrosequencing for a more comprehensive diversity insight.

Measurement of chlorophyll a and dissolved inorganic nutrients

Chlorophyll *a* concentrations were determined by filtering 0.5-2 l of seawater through Whatman GF/F glass fibre filters and stored at -20°C. The filters were extracted in 90 % acetone and analyzed with a Turner-Design fluorometer according the method described in Edler (1979) and Evans & O'Reily (1983). Calibration of the fluorometer was carried out with standard solutions of chlorophyll *a* (Sigma, Germany). To quantify the concentration of dissolved inorganic nutrients (nitrate, nitrite, ammonium, phosphate, silicate) samples were

measured directly on board and analyzed according to standard methods (Kattner & Becker 1991, Kerouel & Aminot 1997) with a nutrient analyzer (Evolution III, Alliance Instruments).

DNA isolation

DNA extraction was carried out with E.Z.N.A TM SP Plant DNA Kit Dry Specimen Protocol (Omega Bio-Tek, USA) following the manufacturer's protocol. Subsequently, the extracts were stored at -20°C until analysis. A NanoDrop 1000 (Thermo Fisher Scientific, USA) was used to measure the isolated DNA concentration.

ARISA (Automated Ribosomal Intergenic Spacer Analysis)

Initially, identical DNA volumes of each size class (10 µm, 3 µm, and 0.4 µm) of each sample were pooled. The amplification of the eukaryotic ITS region from the protist samples was carried out with the fluorescently (dye 6-FAM; 6-Carboxyfluorescein) labeled primer, 1528F (GTA GGT GAA CCT GCA GAA GGA TCA), modified after Medlin et al. (1988) and the primer ITS 2 (GCT GCG TTC TTC ATC GAT GC) (White 1990). The PCR reaction mixture contained 1 x HotMasterTaq buffer Mg^{2+} 2.5 mM (5'Prime, USA), 0.4 U HotMaster Taq polymerase (5'Prime, USA), 10 mg/ml BSA, 10 mM (each) dNTP (Eppendorf, Germany), 10 µM each Primer, 1 µl of template DNA (~20 ng/µl) in a final volume of 20 µl. The PCR amplification was carried out in a MasterCycler (Eppendorf, Germany) under the following conditions: first an initial denaturation step for 3 min at 94°C succeeded by 35 cycles (denaturation at 94°C for 45 s, annealing at 55°C for 1 min, extension at 72°C for 3 min) and followed by a final extension at 72°C for 10 min. The PCR reaction in the analysis was carried out in triplicate for each of the samples. Finally, the size of the PCR fragments was determined by capillary electrophoresis with an ABI 310 Prism Genetic Analyzer (Applied Biosystems, USA).

Data processing of ARISA

The analysis of the electropherograms was carried out with the GeneMapper v4.0 software (Applied Biosystems, USA). To exclude fragments originating from primers or primer dimmers a threshold of 50 bp for peaks was applied.

A binning was carried out in R (Ramette, 2009; R Development Core Team, 2008). The resulting data were converted to a presence/absence matrix. Differences in the phytoplankton community structure represented by differences in the respective ARISA profiles were determined by calculating the Jaccard index with an ordination of 10000 restarts under the

implementation of the R package Vegan (Oksanen 2011). MetaMDS plots were calculated and possible clusters were identified using the hclust function of the same R package. An ANOSIM was conducted to test the significance of the clustering, while a Mantel test (10000 permutations) was used to test the correlation of the protist community structure distance matrix and the environmental distance matrix. For the Mantel test and for the subsequently performed PCA the ade4 R package was applied (Dray & Dufour 2007).

Next Generation Sequencing

For subsequent 454-pyrosequencing, the V4 region of the 18S rDNA was amplified with the primer set 528F (GCG GTA ATT CCA GCT CCA A) and 1055R (ACG GCC ATG CAC CAC CAC CCA T) (Elwood et al. 1985). In total, eight samples were analyzed (Table 3.2). The PCR was carried out according to the PCR protocol for ARISA. The resulting PCR products were purified with the Mini Elute PCR Purification Kit (QIAgen, Germany) and sent to GATC Biotech AG (Germany) where the final pyrosequencing was conducted with a 454 Genome Sequencer FLX system (Roche, Germany).

Quality control and data processing of 454-pyrosequening

Sequences with a length less than 300 bp were excluded from the analysis to guarantee the analysis of the whole V4 region. Furthermore, sequences with a length longer than the amplified fragment (~670 bp) were removed as well. Chimeras were detected by the use of the chimera-detecting software, UCHIME 4.2.40 (Edgar et al. 2011) and also excluded. Artificial operational taxonomic units (OTUs) were generated, aligning the remaining high quality reads using the software package Lasergene 10 Seqman Pro (DNAStar, USA). A threshold of 97 % was applied to minimize the danger of overestimating the diversity. The application of the 97 % similarity threshold further insures a comparison of our data set with previously published data, using the same threshold. Moreover, it has been shown that the 97 % threshold is capable of removing most of the sequencing errors (Kunin et al. 2010) and displaying the original protist diversity (Behnke et al. 2011). All singletons, defined as an OTU composed of one single sequence that only occurs once in the whole analysis, were removed to evade possible errors induced by the assembly of the sequencing progress. The consensus sequences were placed into a reference tree based on 1200 high-quality sequences containing representatives of proxys of all main eukaryotic phyla, originating from the SSU Ref 108 SILVA database. This involved the use of the pplacer 1.0 software (Matsen et al. 2010). Sequences that affiliated with non protist phyla in the tree under a threshold of 99 %

were excluded from further analyses. The remaining reads were aligned with the SILVA aligner (Pruesse et al. 2007) and placed into the ARB reference database tree containing around 50000 eukaryotic sequences (Ludwig et al. 2004). Based on the limited sequence length obtained by 454-pyrosequencing, we identified phylotypes to the genus but not to the species level. Multiple phylotypes that clustered to the same genus but differed in at least 3 % were numbered. Rarefaction curves were calculated using the freeware program Analytic Rarefaction 1.3.

Results

Physical and chemical environment

Twenty-four stations were sampled in the Central Arctic Ocean representing four regions with different water masses: Atlantic Water (AW), Mixed Water I (MWI), Pacific Water (PW), and Mixed Water II (MWII) (Table 3.1). Based on the combination of temperature, salinity, and nutrients, stations 202 to 218 were allocated to the AW, stations 220 to 227 to the MWI, stations 233 to 250 to the PW, and stations 257 to 290 to MWII. AW stations were on average characterized by lowest temperature (~ -1.69°C), highest salinity (~32.95), nitrate (2.99 µM), and silicate (~5.65 µM), while stations in the PW mass displayed lower salinity (~30.51), nitrate (~1.33 µM), and silicate (~4.45 µM). Properties of the MWI were in between the ones of the AW and PW or similar to either of them, whereas the MWII displayed a clearer classification and was characterized by lowest salinity (30.29), nitrate (~0.48 µM), silicate (~3.09 µM), but highest temperature (~ -0.16°C).

The ice concentration was at least 70 % at the stations in AW, MWI, PW and at half of the stations located in MWII. At one station in MWII the ice concentration was ~10 % and three stations were ice-free. The ice-thickness in the area of AW and MWI was 1.2–2 m at the majority of stations. In contrast, in the area of PW and MWII the ice-thickness was less than 1.2 m at most of the stations. The floe size in AW and PW was >100 m at most of the stations, while the flow size at stations located in MWI and MWII was mostly <100 m. High numbers of meltponds were observed in the AW (~50 %) and MWI (~40 %). The number declined towards the last Pacific stations and was further reduced in the MWII.

The chlorophyll *a* (chl *a*) concentrations were generally low (0.04–0.85 µg/l) and appeared not always as a distinct peak. The highest mean concentration of chl *a* was observed in MWII (~0.36 µg/l), while the concentrations in AW (~0.19 µg/l) and MWI (~0.18 µg/l) were significantly lower and lowest in PW (~0.08 µg/l).

ARISA

Twenty-three stations were used for the ARISA, including one station (222) with two depths, to control the applicability of the method. The analysis resulted in 260 different fragments of the ribosomal ITS-region that ranged between 50 to 444 bp in size. The average fragment number in a sample was 76, in which the maximum number (444) was found at station 239 (PW) and the minimum (50) at station 202 (AW). In total, 54 fragments were unique, occurring just once in the analysis, while four fragments were ubiquitously found in all samples. The similarity between the ARISA profiles of all samples was calculated by the Jaccard index and presented in a metaMDS plot (Figure 3.2a).

Subsequent to an *à priori* grouping the samples segregated into five different clusters (A-E), in which some were located in proximity. An ANOSIM analysis to test the significance of *à priori* grouping resulted in an R-value of 0.44 and p-value of 0.001, suggesting significant differences between the groups. Cluster A included samples that originated from the AW (205-218) and from the MWI (220-227) water mass. Cluster B consisted of just one AW sample (202) that presented a highly different ARISA profile and hence was considered as an outlier. Cluster C included exclusively samples from PW, while the Cluster D (257 to 276) and Cluster E (280 and 285) contained samples from MWII.

The clustering was in good accordance with the water mass properties at the sampling stations. In the PCA (Figure 3.2b) the *à priori* clusters were together with their correlations with the abiotic factors (temperature (T), ice thickness (it), floe size (fl), nitrate (NO3), phosphate (PO4), and silicate (Si)). The physical factors (T, it and fl) explained most of the differences in the protist community structures, while the impact of salinity and nutrients was limited. Cluster D and E were separated from the other clusters by lower nutrient concentrations and the majority of samples in cluster C originated from locations with lower nutrient concentrations. However, cluster A and B contained samples from stations with significant differences in nutrient concentrations, even though the protist communities were highly similar.

454-pyrosequencing

A subset of eight samples was chosen for a detailed analysis of the protist composition based on the ARISA results. Each water mass was represented by two samples and each cluster by at least one sample. In total, 454-pyrosequencing resulted in an average of 42366 raw reads per sample. Quality filtering left on average 21585 high quality reads that clustered in 156 (sample 202) to 3372 (sample 245) OTU's. The rarefaction curve (Figure 3.3) presents the

local species richness that was yielded by the pyrosequencing and serves as a reference of the covered diversity in the samples. The curves show no saturation, suggesting that the diversity was not completely covered at all stations, regardless of the final read number. However, the curve of station 245 was close to the plateau phase while station 202 and 218 already ended in the early slope phase.

Diversity of the whole and abundant biosphere

The relative abundance of the major taxonomic groups is presented in Figure 3.4. The variability in the protist composition was assessed by calculating the standard deviation of the different taxon contributions. The whole (A) and the abundant (C) biosphere showed a high similarity in the taxonomical apportionment which was around 9 % for haptophytes, 15 % for chlorophytes, 2 % for cryptophytes, 1 % for rhodophytes, 7 % for stramenopiles, 15 % for dinoflagellates, and 3 % for ciliates.

Alveolates were the prominent taxonomic group at all stations except of 272. Dinoflagellates were far more abundant than ciliates and contributed between 41 and 62 % to the protist community, in which the abundant biosphere was well represented with a minimum share of 8 % (MWII; 272) and a maximum share of 49 % (AW; 212). Ciliates never exceeded 10 % of the protist community and were in general not found within the abundant biosphere. Chlorophytes presented high read abundances at all sampling sites with highest proportions in the MWII (272; 44 %) and in the AW (202; 43 %), which were almost exclusively composed of abundant phylotypes. The proportions of chlorophytes at the other stations varied around ~3-15 %. The so far observed high analogy of both biospheres did not apply to the stramenopiles. In this regard, the share of the whole biosphere ranged between 2-13 %, with two maxima of ~20 % (227 and 280), while the abundant biosphere showed no stramenopiles in the AW and at station 272 in the MWII. Similarly, cryptophytes and rhodophytes contribution to the whole biosphere (maximum: 212 (~5 %)) were not reflected by the abundant biosphere which presented rhodophytes in just two (212 and 280; ~1 %) and cryptophytes in just one station (212; 3 %). Haptophytes, in turn, again coincided well in both biospheres, having a minimum at 212 (~0 %) and a maximum at 272 (~26 %).

In detail, the abundant biosphere counted in total 39 phylotypes at eight stations. The AW (202 and 212) showed the smallest diversity with seven phylotypes and the highest number of 21 to 22 missing phylotypes. In the MWI (218 and 227), the diversity increased to 10 and 11 phylotypes, whereas the number of missing abundant phylotypes decreased to 11 phylotypes. PW (235 and 245) showed a similar diversity as the MWI, with 10 and 12 phylotypes. The

amount of absent phylotypes further decreased from six to three at station 245. The diversity of the abundant biosphere in the MWII was very variable and showed on the one hand, a low diversity of seven abundant phylotypes (272), but on the other hand, the highest diversity of 14 phylotypes (280). Missing phylotypes ranged between 11 (272) and 6 (280).

Only two phylotypes were found in the abundant biosphere of all stations, as *Micromonas* sp. 1 and Syndiniales 2. The remaining abundant phylotypes were found in the rare biosphere or were absent in at least one of the samples. *Micromonas* sp.1 was found at all stations in high read abundances, although the percentages differed strongly. A high proportion was observed in the AW (202; 41 %), while in the MWI the proportion decreased to 2 % at station 218. The contribution of *Micromonas* sp. 1 in the PW was moderate (13 and 16 %), but subsequently increased to a maximum proportion of 42 % in the MWII (272). The second ubiquitously abundant phylotype, Syndiniales 2, was found in maximum proportion in the AW at station 212 (44 %) and in similar high proportions in the MWI (~31 %). The percentages decreased in the PW (~17 %), reaching a minimum of ~11 % in the MWII.

Phaeocystis sp. 2 and *Gyrodinium* sp. 1 were also widely distributed, even though far less abundant. *Phaeocystis* sp. 2 presented the highest shares of 9 and 12 % in the MWI (218) and PW (235), respectively. However, its contribution to MWII was low (~3 %) and even rare (< 1 %) at the AW station 212. In contrast to *Phaeocystis* sp. 2, *Phaeocystis* sp. 1 was only abundant in the transition zone of MWI and PW, showing a maximum proportion of 10 % at station 227 (MWI). *Chrysochromulina* sp., represented by two phylotypes, showed similar characteristics as *Phaeocystis* sp., in which one phylotype (*Chrysochromulina* sp.1) was more widely distributed than the other (*Chrysochromulina* sp.2). The latter was just abundant in the MWII, contributing 23 % (272) and 6 % (280). *Micromonas* sp. 3 (6 %) and *Pyramimonas* sp. (2 %) were limited to the MWI (227). Picobiliphytes were only found in the AW (212) and marine stramenopiles (MAST) phylotypes only in the PW (245). D*ictyocha* sp. was abundant at one station in the MWII (280; 10 %). *Fragilariopsis* sp. constituted the only diatom that was partially found in the abundant biosphere. The highest values (218; 5 % and 227; 6 %) were obtained in the MWI. Syndiniales were the most diverse group represented by nine phylotypes however, this group was mostly limited to the PW with the exception of two phylotypes (Syndiniales 1 and 9).

Diversity of the rare biosphere

The rare biosphere (<1 %) accounted in general for 29 % of the whole biosphere. The variability of the taxonomical contributions was, for the majority of the taxa, smaller than for

the whole or abundant biosphere. Haptophytes contributed 3 %, chlorophytes 3 %, cryptophytes 2 %, rhodophytes 1 %, stramenopiles 5 %, dinoflagellates 9 % and ciliates 8 %.

In general, the composition reflected the dominant taxonomical structure of the previous described biospheres. Reads of alveolates and particularly of dinoflagellates were most abundant, where the contribution of dinoflagellates ranged between 29 (MWII; 272) and 59 % (MWI; 218), and the one of ciliates between 4 (AW; 212) and 28 % (MWII; 272). Chlorophytes were most abundant at station 202 (AW; 10 %) and 272 (MWII; 9 %) and showed the lowest contribution to the rare biosphere at station 235 (PW; 2 %). Stramenopiles varied between 12 (MWI; 218) and 25 % (MWI; 212). Cryptophytes and rhodophytes were present at most of the stations and were more important contributors of the rare than of the abundant biosphere. The relative contribution of haptophytes varied between 8 (MWI; 218) and 11 % (MWII 280).

The detailed analysis of the rare biosphere was difficult because most phylotypes could not be exactly assigned to the genus level. A section of precisely identified phylotypes that ranged between 0.1 and 0.99 % in abundance is given in Table 3.3. Within the section, not a single phylotype was found exclusively in rare portions, because in at least one station they were abundant or absent. In this respect, flagellates as *Telonema* sp. or dinoflagellates as *Pelagodinium* sp. and *Prorocentrum* sp. were rare at all stations except at 202 and 212, respectively, both located in the AW.

MAST's were represented by three phylotypes (1, 2 and 3). While MAST 1 and 2 also contributed to the abundant biosphere (245), MAST 3 was exclusively rare. The proportions of MAST 1 and 2 were variable and often did not exceed 0.1 %. However, exceptions were found in the AW (212) and MWII (280), where both phylotypes accounted for 0.2 to 0.8 %. In contrast, MAST 3 was completely missing in the AW and at the first station of the MWI (218) but in general presented higher portions than MAST 1 and 2. Maximum portions of MAST 3 were observed in the PW (235; 0.8 % and 245; 0.7 %).

Diatoms further displayed a higher diversity within the rare biosphere. In addition to *Fragilariopsis* sp., two phylotypes were found that affiliated to *Florella* sp. and *Diadesmis* sp. While *Diadesmis* sp. occurred in the transition zone of the AW (212) and MWI (218), *Florella* sp. was completely missing in the AW water mass and presented the highest portion in the PW (235; 0.4 %) and MWII (272; 0.3 %).

Discussion

Protist community structure and water masses

Protist communities are transported by the different water masses, which contain distinct assemblages (Lovejoy et al. 2002, Galand et al. 2009a). Thus, hydrographical structures can be considered as tracer for protist distribution. However, water mass influence might be less in the Central Arctic Ocean because microbial communities are not just constrained by density and nutrient availability but also by the variability of sea ice coverage. To investigate the importance of water mass influence and to obtain a first insight in the community structures the ARISA approach was employed. The results revealed water mass specific community structures because samples clustered according to the respective water masses, in which the AW and MWI communities clustered together, while PW communities formed one and MWII, two separate clusters. The findings, obtained by ARISA, suggest that the protist composition is indeed linked to hydrographic regimes, which is in line with the correlation between water mass and archaea, bacteria, and protist distribution (Gradinger & Baumann 1991, Hamilton et al. 2008, Galand et al. 2009b,c). However, differences between the clusters were in general small, suggesting an additional factor that influences surface water communities in the Arctic Ocean.

A correlation between water masses and the 454-pyrosequencing analyses was also partially observed but less pronounced than with the ARISA analysis. One reason for the weaker recovered relationship of water mass and the taxonomical protist community structure is probably due to the limited sample number, in which six out of the eight samples clustered closely in the ARISA approach and hence showed no big differences. We extended our investigation therefore from a preliminary taxonomic group based analysis to a comprehensive phylotype specific analysis of the abundant and rare biosphere.

Ubiquitous abundant phylotypes constitute a problematic issue because they address environmental changes just in terms of abundance shifts. In this regard, *Phaeocystis* sp.2, *Gyrodinium* sp.1, and Syndiniales 2 showed no clear water mass association and only varied in relative contributions. *Micromonas* sp.1 further presented no distinct biogeography. Dominant contributions of *Micromonas* sp.1, as observed at station 202 and 272, were likely shaped by a previous protist succession that returned to the picoplankton dominated oligotrophic state. This assumption is confirmed by the local low nutrient values. However, the observation that minimum portions of *Micromonas* were limited to MWI and that similar portions of *Micromonas* were observed in the PW, suggests an influence of water mass on the distribution. *Micromonas* was often reported to contribute significantly to Arctic protist

assemblages (Lovejoy et al. 2002, 2006, 2007, Potvin & Lovejoy 2007, 2009, Lovejoy & Potvin 2011). Phylogenetic analysis revealed the existence of distinct phylotypes, in which one (CCMP2099) affiliated to *Micromonas* sp. 1 and has been found predominantly in the Arctic Ocean (Slapeta et al. 2006, Lovejoy et al. 2007).

Other phylotypes displayed more distinct water mass relations based on presence/absence patterns, such as *Pyramimonas* sp., missing in the AW and being abundant in the MWI, or Stramenopile 1 and *Dictyocha* sp. that were also absent in the AW. *Fragilariopsis* sp. was rare in the AW but displayed the highest contribution in the MWI, where AW and PW mixed and provided an adequate nutrient regime. *Fragilariopsis* constituted another significant Arctic protist, with *F. cylindrus* as the most significant cold water diatom of the polar oceans (von Quillfeldt 2004), dominant in sea ice and water column (Mock et al. 2006).

Statements on the distribution of rare phylotypes were difficult to make because most phylotypes could not be characterized in detail. Most sequences were not recorded in the database because rare phylotypes are not cultivable, a prerequisite for a proper identification. However, recorded phylotypes, as e.g. MASTs showed distribution profiles. MASTs are reported to be important contributors to the rare biosphere (Massana et al. 2006b). From the three phylotypes none was recovered at station 202 (AW), just as *Pelagodinium* sp., which presented a significant contribution in the MWI.

The implementation of ARISA point to different, water mass related protist community structures, however, the distribution of major taxonomic groups, obtained by 454-pyrosequencing at eight samples, did not approve statements according to water mass specificity thoroughly. Nevertheless, a more detailed insight within the abundant and rare biosphere revealed some distinct distribution patterns, which lead to the suggestion that a coupling of water mass and protist diversity is also common in the Central Arctic Ocean but less pronounced.

Protist community structure and ice coverage

Two physical factors, ice thickness and floe size, explained most of the variances between the community structures of the abundant biosphere. However, some community structure differences (e.g. 218 and 235) were also attributed to nutrient concentrations. An influence of the ice coverage was not observed on the community structure of the rare biosphere. General assumptions that physical factors, particularly light irradiance controlling factors, are mostly influencing protist community structure have previously been referred (Mundy et al. 2005); however, light irradiance was not directly measured in this study and just assessed indirectly

by ice conditions.

Most of the eight stations (except 272 and 280) showed an ice concentration of at least 80 %. The analysis of the major taxonomic groups had a relatively stable distribution, pointing to a strong controlling effect of sea ice concentration on the protist assemblage. Dinoflagellates dominated the protists community structure throughout all sampling sites. The prominence of heterotrophic dinoflagellates in the Central Arctic Ocean has previously been reported, contributing 30 to 40 % to the total biomass (Sherr et al. 1997, Rat'kova & Wassmann 2002, Richardson et al. 2005). Such flagellate-based systems are mainly consistent of small picophytoplankton and nanoflagellates that are supported by low regenerated nutrient concentration (Azam et al. 1983, Landry et al. 1997, Ardyna et al. 2011), as found during our sampling period.

Two stations (202; AW and 272; MWI), that were not characterized by a dominance of dinoflagellates but by chlorophytes, showed a high abundance of small size cells. The high abundance of picoplankton was found to be positively correlated with increasing sea ice concentration (Booth & Horner 1997). This finding confirms the relatively high contribution of chlorophytes, observed at our stations. However, the maximum portion was found at station 272, which was merely ice covered (10 %). Based on the enhanced light availability over a longer period, it is reasonable to presume that station 272 was characterized by a post-bloom scenario. Periods outside short blooming events are often dominated by small cells as nano-and picophytoplankton (Not et al. 2005) and the generally low nutrient concentrations at this station further confirm this assumption.

Stramenopiles constitute another important taxonomical group in the Arctic Ocean (Lovejoy et al. 2006). The group includes light dependent and light independent representatives as autotrophic diatoms and heterotrophic MAST, respectively, and ought to respond to changing ice concentrations. The highest contribution of diatoms was in fact observed at the completely ice free station 280 (MWII) but also at station 227 with a high ice concentration of small ice floes (~20-100 m) and many meltponds. The high abundance of meltponds is suggested to affect the light climate and to consequently favor algal growth, (Gradinger 1996, Perovich et al. 1998, Mundy et al. 2009). Station 227 showed the highest contributions of the ice algae, *Fragilariopsis* sp., which has been released into the water column presumably by sea ice melt. The diatom was observed to possess a high tolerance to salinity changes (Sogaard et al. 2011), which inter alia facilitates an active growth in the surface water after sea ice release. In contrast, heterotrophic MAST were found in maximum percentages at station 245, which was characterized by the worst light conditions due to big floe sizes (100 % ice concentration)

with only few meltponds. In situ experiments have shown that MAST increase in cell abundance, even under no light treatments (Massana et al. 2006a) which is in particular advantageous in the central, permanent ice-covered Arctic Ocean.

The contribution of the rare biosphere to the protist communities

Many studies focus on the diversity, distribution, and function of the rare biosphere (Pedrós-Alió 2006, 2007, Sogin et al. 2006, Caron et al. 2009, Galand et al. 2009a). In particular, the distribution of the rare biosphere has led to many discussions, in which a cosmopolitan distribution is suggested due to the high dispersal and low loss rates. A biogeographical distribution is proposed, where rare phylotypes inhabit an area according to ecological mechanisms equivalent to those that account for the abundant representatives (Martiny et al. 2006, Kirchman et al. 2009). In our study, the rare biosphere showed a high genetic diversity, however, only a limited number of phylotype could be identified in detail due to the limited representation in data bases. Prerequisite of increasing the hit rate in databases is the generation of clonal cultures, however, clonal cultures of the rare biosphere are extremely difficult to establish. In our samples, the rare biosphere was characterized by a more diverse diatom and dinoflagellate assemblage, where diatoms were increased by e.g. a low-saline water diatom *Diadesmis* sp. and a marine diatom *Florella* sp. (Navarro 2002, Antoniades et al. 2005), while dinoflagellates were increased by *Pelagodinium* sp. and *Prorocentrum* sp. A comparison with the abundant biosphere revealed that rare phylotypes were not exclusively rare but occasional abundant at stations, characterized by specific environmental conditions. The pattern of the rare biosphere showed a constant distribution of the major taxonomic groups across the Central Arctic Ocean that strengthened the assumption of a role as backup community. The ecological role of the rare biosphere as a backup or seed reservoir, enhancing the biological buffer capacity to environmental changes, has been suggested by several studies (Sogin et al. 2006, Caron & Countway 2009). The observed constant taxonomical distribution, however, has not been referred so far. One explanation of the stable distribution may rely on the assumption that the low abundance of rare phylotypes provides a perfect refuge from grazing mortality (Fenchel & Finlay 1983, Pernthaler 2005, Pedrós-Alió 2006).

Summary and Conclusion

Water mass associated protist distribution patterns in the Central Arctic Ocean were generally difficult to investigate because factors such as sea ice coverage additionally influence the community composition. Physical environmental factors, like light ice-coverage and light

availability, were more important for the community structure than nutrient concentrations. Ice coverage favored heterotrophic and small size protists, e.g. dinoflagellates and chlorophytes, instead of stramenopiles, e.g. diatoms. Nevertheless, community structures (ARISA) and several abundant phylotypes like *Pyramimonas* sp., *Dictyocha* sp., and *Fragilariopsis* sp. as well as rare phylotypes like *Pelagodinium* sp. and *Diadesmis* sp. showed water mass associated distributions. The rare biosphere in the Arctic Ocean is an autonomous system without a significant correlation with the abundant biosphere or the different water masses. The relative contribution of the major taxonomic groups within the rare biosphere was unexpectedly uniform, and we support the hypothesis of a seed-reservoir function. We further suggest that the rare biosphere not just provides backup protists in case of environmental changing conditions (Sogin et al. 2006), but also provides these protists in constant taxonomic relations.

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Table 3.1. Hydrological and biological properties encountered in the chlorophyll maximum (Abbreviations: Ic= ice concentration; It= ice thickness; fl= floe size; Mp= melt pond). Nutrients are given in µM. Bold-marked station were further used for 454-pyrosequencing.

Station-ID	Date (m/d/yr)	Longitude	Latitude	Depth [m]	S	NO3	PO4	Si	T [°C]	Ic [%]	It [m]	fl [m]	Mp [%]	Chl a [ng/l]	
202	08/14/2011	59°55.91'E	85°48.19'N	25	33.48	3.61	0.29	1.8	-1.72	90	0.7-1.2	100-500	50	0.32	Atlantic Water
205	08/15/2011	59°15.00‘E	86°19.64‘N	10	33.28	0.88	0.13	0.98	-1.71	80	> 1.2	> 2000	30	0.81	
207	08/16/2011	61°14.23‘E	86°42.5‘N	25	33.69	2.3	0.26	2.42	-1.68	100	> 1.2	100-500	50	0.31	
209	08/17/2011	58°29.30‘E	86°59.25‘N	14	32.84	1.2	0.23	2.82	-1.73	90	> 1.2	20-100	30	0.17	
212	08/19/2011	59°57.42'E	88°1.10'N	10	32.80	2.65	0.45	5.07	-1.69	100	0.7-1.2	> 2000	40	0.06	
216	08/21/2011	60°42.18‘E	89°35.98‘N	20	32.16	4.78	0.96	13.29	-1.67	90	> 1.2	20-100	50	0.24	
218	08/22/2011	148°06.72'E	89°57.86‘'N	20	32.42	5.55	0.99	13.2	-1.69	90	> 1.2	100-500	50	0.19	Mixed Water I
220	08/24/2011	116°42 ‘W	89°14.9‘N	18	32.18	3.09	0.65	8.48	-1.64	90	> 1.2	100-500	20	0.19	
222	08/26/2011	128°15‘W	88°44.2‘N	10	30.49	0.53	0.55	6.3	-1.62	100	> 1.2	20-100	40	0.24	
222	08/26/2011	128°15‘W	88°44.2‘N	25	31.92	1.13	0.42	5.12	-1.59	100	> 1.2	20-100	40	0.17	
227	08/29/2011	155°02.72'W	86°51.64'N	10	31.02	0.3	0.34	4.12	-1.59	80	> 1.2	20-100	40	0.10	
233	09/02/2011	132°21.55‘W	83°55.95‘N	25	29.95	0.02	0.67	1.58	-1.31	90	0.7-1.2	500-2000	40	0.04	Pacific Water
235	09/03/2011	130°02.34'W	83°1.80'N	50	30.75	3.42	0.96	7.28	-1.47	100	0.7-1.2	500-2000	40	0.11	
239	09/05/2011	164°12.36‘W	84°04.41‘N	25	30.12	0.58	0.26	3.83	-1.46	100	> 1.2	500-2000	20	0.15	
245	09/09/2011	166°24.86'E	84°47.67'N	18	30.32	1.3	0.26	5.57	-1.6	100	0.7-1.2	500-2000	10	0.04	
250	09/11/2011	139°54.35‘E	84°23.24‘N	25	31.44	1.33	0.26	3.97	-1.54	80	0.3-0.7	100-500	40	0.08	
257	09/13/2011	124°54.20‘E	83°19.97‘N	10	30.47	0.61	0.19	2.35	-1.65	90	0.3-0.7	20-100	20	0.23	Mixed Water II
259	09/14/2011	117°56.78‘E	83°8.92‘N	10	30.95	1.04	0.24	3.0	-1.69	70	0.3-0.7	20-100	20	0.85	
260	09/14/2011	114°39.51‘E	82°59.65‘N	10	31.72	-	-	-	-1.74	70	0.7-1.2	100-500	50	-	
272	09/19/2011	119°58.14'E	81°46.50'N	10	31.60	0.11	0.14	1.55	-1.64	10	0.3-0.7	< 20	0	0.27	
276	09/20/2011	121°19.8‘E	80°38.6‘N	10	30.83	0.29	0.18	3.16	-0.09	0	0	0	0	0.19	
280	09/21/2011	124°07.59'E	79°8.84'N	10	30.54	0.55	0.21	3.66	0.87	0	0	0	0	0.35	
285	09/22/2011	125°48.11‘E	78°29.59‘N	10	30.55	0.08	0.16	4.85	1.58	0	0	0	0	0.39	
290	09/23/2011	128°09.40‘E	77°07.60‘N	10	25.66	-	-	-	3.05	0	0	0	0	0.26	

Table 3.2. Analytical process and quality control of the 454-pyrosequencing data.

Station-ID	total reads	reads ≥ 300bp	chimeras	singletons	non target	final reads	OTUs
202	8898	7645	3260	1275	1250	1860	156
212	24981	23022	8662	4493	2841	7026	713
218	8557	6016	1770	1428	1	2817	385
227	26161	23033	1354	4115	930	16634	804
235	70490	65477	11060	13766	5568	35083	1823
245	140918	124036	11988	28166	3189	80693	3372
272	23079	19065	4476	3707	1168	11952	640
280	35850	31236	2840	8438	3340	16618	1356

Table 3.3. Phylotypes, relative contribution in percent to the A) abundant biosphere (≥1 %) and B) rare biosphere (<1 %) at all stations, **r** refers to the occurrence in the rare biosphere, **a** to the abundant biosphere and **x** to no occurrence at all.

A

	Station-ID							
	202	**212**	**218**	**227**	**235**	**245**	**272**	**280**
Micromonas sp.1	40.91	18.52	1.50	7.60	15.60	13.06	42.10	11.40
Micromonas sp. 3	r	x	r	6.19	r	r	r	r
Pyramimonas sp.	x	x	x	2.45	r	r	r	r
Phaeocystis sp. 1	r	x	r	9.91	2.40	1.34	r	r
Phaeocystis sp. 2	2.96	r	8.64	2.82	12.19	1.05	1.75	3.63
Chrysochromulina sp. 1	4.35	x	6.65	2.49	1.60	r	r	1.02
Chrysochromulina sp. 2	x	x	x	r	x	r	23.12	5.70
Picobilliphyta 1	x	2.18	x	x	x	x	x	x
Picobilliphyta 2	x	1.14	x	r	r	r	x	r
Picobilliphyta 3	x	1.08	x	r	r	r	r	x
Stramenopile 1	x	x	r	6.97	r	r	r	r
MAST 1	x	r	r	r	r	3.12	r	r
MAST 2	x	r	r	r	r	1.57	x	r
Dictyocha sp.	x	x	r	r	r	r	r	9.89
Fragilariopsis sp.	r	r	4.98	6.08	1.25	r	r	1.43
Dinoflagellate 1	x	x	x	x	x	x	r	1.17
Gyrodinium sp. 1	4.19	3.15	4.70	2.57	1.47	r	1.74	2.35
Gyrodinium sp. 2	r	r	1.15	r	r	r	r	1.55
Gyrodinium sp. 3	x	r	1.36	r	r	r	r	x
Woloszynskia sp. 1	r	x	x	r	r	r	r	1.44
Woloszynskia sp. 2	1.08	r	x	r	r	r	r	r
Syndiniales 1	r	1.15	r	r	r	r	r	r
Syndiniales 2	29.19	44.29	35.11	26.16	22.99	10.79	6.07	16.02
Syndiniales 3	r	x	r	r	3.94	r	r	r
Syndiniales 4	x	r	r	r	r	12.77	x	r
Syndiniales 5	x	x	r	r	x	6.70	x	r
Syndiniales 6	x	x	r	x	1.38	r	x	r
Syndiniales 7	x	x	r	x	r	1.02	x	r
Syndiniales 8	x	x	x	x	r	1.70	x	r
Syndiniales 9	r	x	r	r	r	x	r	1.10
Strombidium sp.1	r	r	1.60	r	r	r	r	r
Strombidium sp.2	x	x	x	r	r	r	1.26	x
Ciliophora 1	x	r	1.57	r	r	r	r	r
Ciliophora 3	r	x	r	r	r	r	r	1.20
Ciliophora 4	x	x	x	r	x	r	1.00	x
Ciliophora 5	x	x	r	r	r	1.98	x	1.89
Ciliophora 2	1.29	x	r	r	r	r	r	r
Eukarya	x	x	r	2.33	x	r	x	x
Eukarya	x	r	r	r	2.38	3.11	x	r

B

	Station-ID							
	202	**212**	**218**	**227**	**235**	**245**	**272**	**280**
Pyramimonas sp.	x	x	x	a	< 0.1	0.24	0.14	0.34
Stramenopile 1	x	x	0.38	0.62	a	0.81	0.35	< 0.1
MAST 1	x	0.47	< 0.1	0.24	< 0.1	a	< 0.1	0.79
MAST 2	x	0.83	0.1	< 0.1	< 0.1	a	< 0.1	0.2
MAST 3	x	x	x	0.14	0.76	0.74	x	0.57
Florella sp.	x	x	0.1	0.16	0.39	0.25	0.32	0.16
Dictyocha sp.	x	x	0.91	0.16	0.57	< 0.1	0.2	a
Diadesmis sp.	x	0.17	0.14	x	x	x	x	x
Fragilariopsis sp.	0.11	0.43	a	a	a	0.12	< 0.1	a
Telonema sp.	0.32	x	0.98	0.51	0.63	0.83	< 0.1	0.59
Woloszynskia sp. 1	0.16	x	x	0.31	< 0.1	< 0.1	0.27	a
Woloszynskia sp. 2	a	0.21	x	< 0.1	< 0.1	< 0.1	< 0.1	0.42
Pelagodinium sp.	x	< 0.1	0.91	0.65	< 0.1	< 0.1	0.11	< 0.1
Prorocentrum sp.	x	0.4	0.56	0.35	0.35	0.2	< 0.1	0.28

Figure 3.1. Map of the investigated stations in the Central Arctic Ocean during a expedition of the RV Polarstern (ARK-XXVI/3) from August to October 2011.

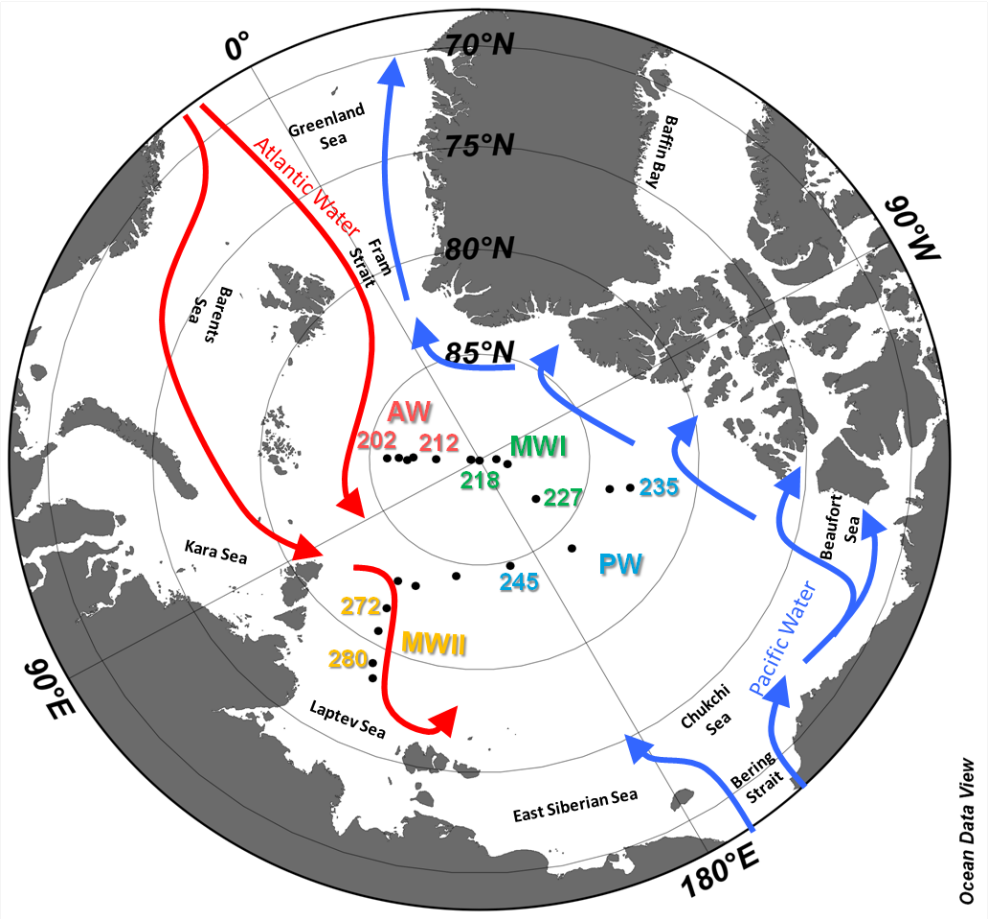


Figure 3.2. A) metaMDS plot of the community structure relations along the stations (red: Atlantic Water; green: Mixed Water I; blue: Pacific Water; yellow: Mixed Water II); B) PCA of the environmental factors and the ARISA grouping.

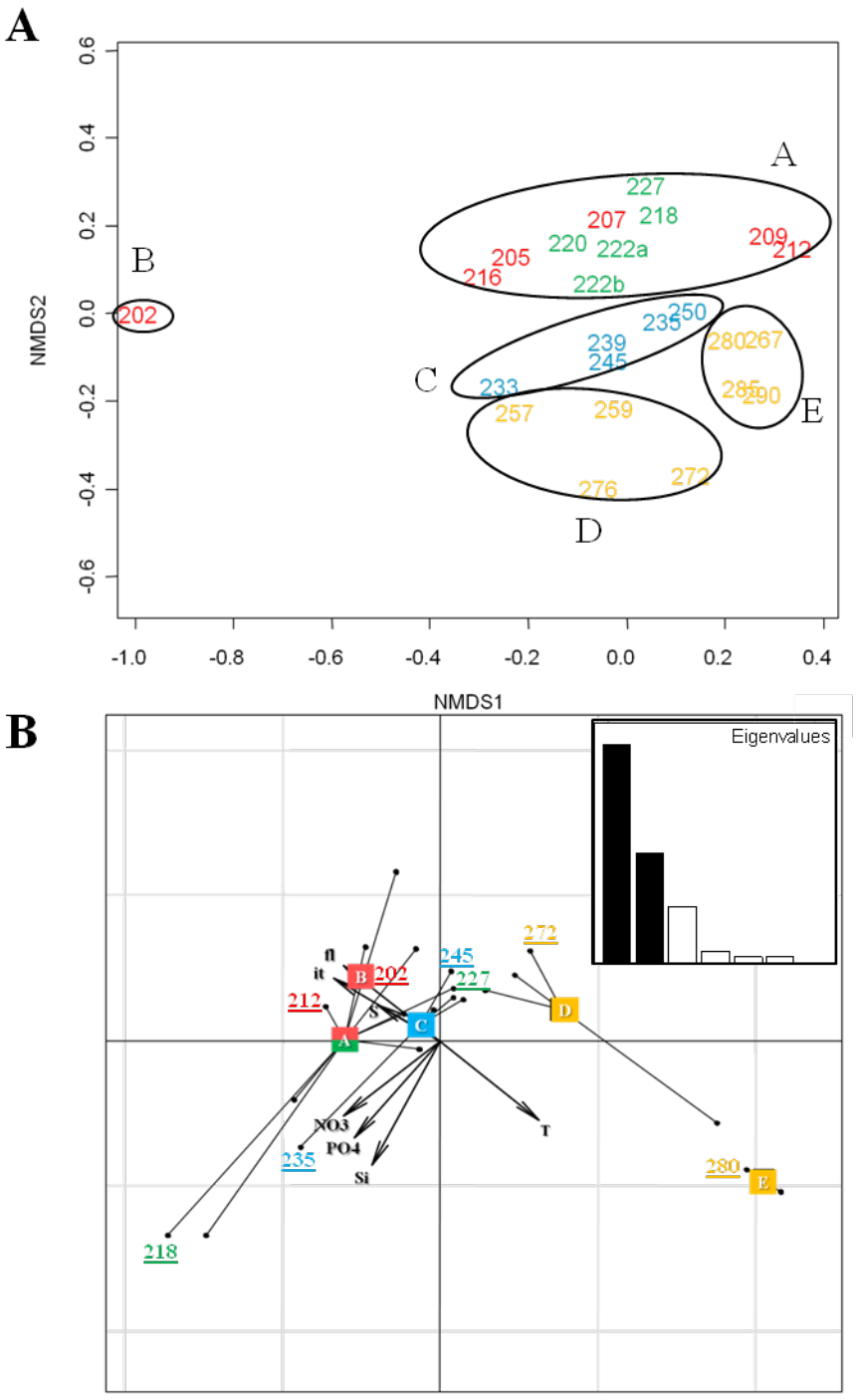


Figure 3.3. Rarefaction curve of the 454-pyrosequencing data after quality control (red: Atlantic Water; green: Mixed Water I; blue: Pacific Water; yellow: Mixed Water II).

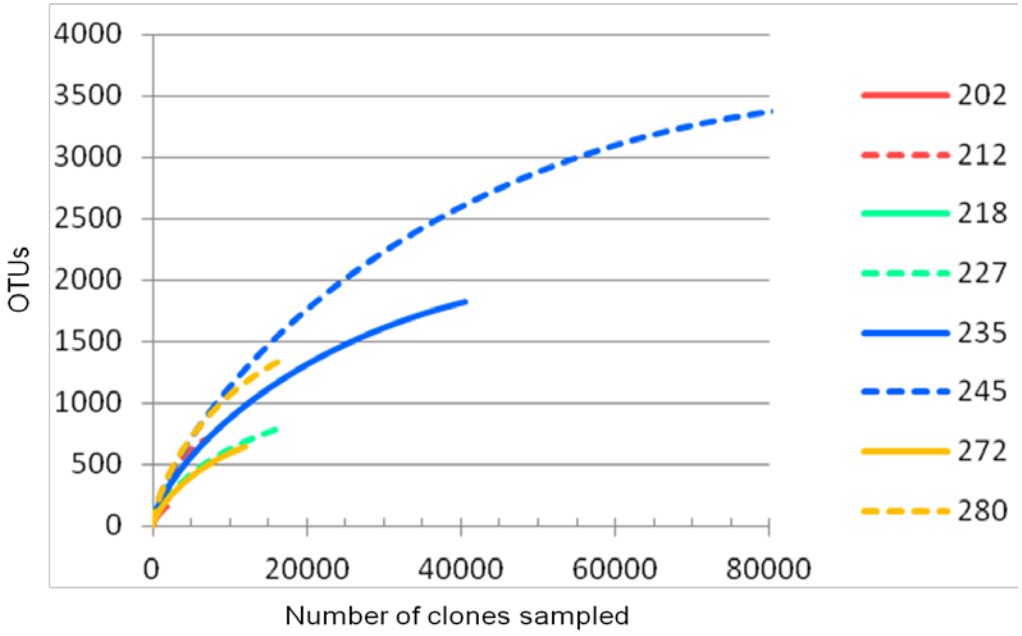
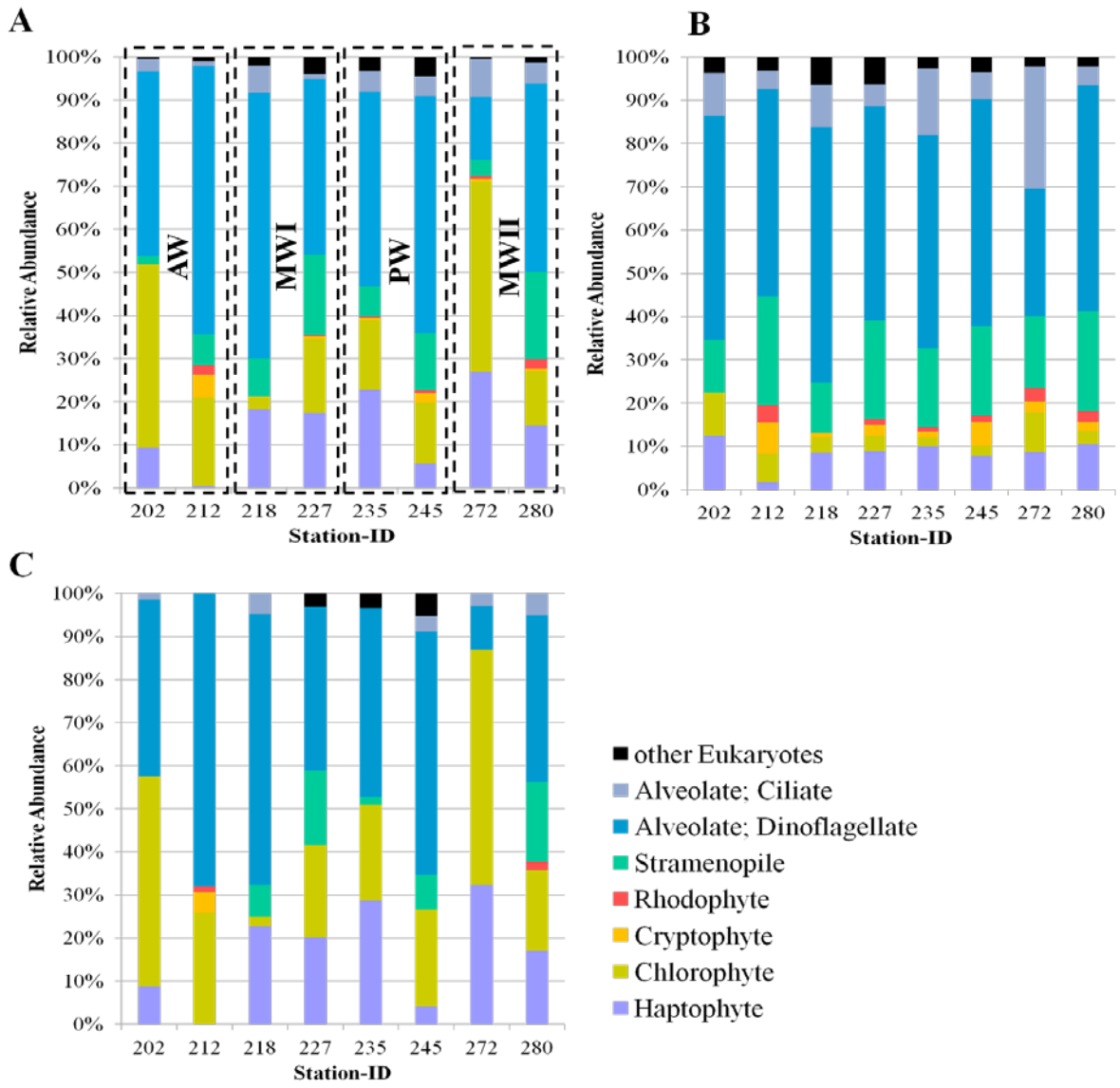


Figure 3.4. Distribution of the main protist groups in the Arctic Ocean A) for the whole biosphere, B) for the rare biosphere (<1%), C) for the abundant biosphere (≥1%). The dashed border alludes to the single water masses (AW, MWI, PW, and MWII).



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4. Synthesis

The Arctic Ocean is a permanent ice covered ocean, which comprises of shallow shelf seas and two deep basins: the Eurasian and Canadian Basin (Rudels et al. 1991, Meincke et al. 1997). During the last decades, environmental changes have affected the Arctic Ocean more severely than other world oceans (ACIA 2005, IPCC et al. 2007). In this regard, the sea ice cover constitutes a sensitive indicator of climate change (Serreze & Francis 2006), whereas the latest record low was just reported during the summer of 2012. Different sea ice conditions, such as seasonal sea ice, annual sea ice, and multiyear ice, together with nutrient and light limitations, constitute harsh environments for the local microorganisms.

Arctic protist communities, experience an interaction of environmental factors that present a wide range of fluctuations, constrain their biomass, and promote the occurrence of special adaptations (Sakshaug & Slagstad 1991, Sherr et al. 2003, Carmack et al. 2004, Sakshaug 2004, Greene & Pershing 2007). These adaptations, however, might be of disadvantage in case of rapid changing environmental conditions. In this respect, recent changes in the Arctic Ocean are exposing the local protist community to additional environmental pressure and have the potential to result in regime shifts of protist communities. Tracing those shifts from the start is crucial and demands a comprehensive sampling, including all marine protists.

The implementation of molecular methods facilitated a detailed insight into the complex protist diversity and shared the advantage of size-independence and of ecotype characterization. In order to deliver detailed information on the Arctic protist taxonomy, an investigation procedure was established that is based on molecular methods, such as ARISA and 454-pyrosequencing, and the respective data processing. Hence, the two main objectives of the thesis are to evaluate the analyses pipeline under the additional use of classical methods (light microscopy and HPLC), and to apply the new pipeline for getting an overview of the Arctic protist diversity. In this context, three hypotheses were put forward:

Molecular surveys of genetic protist diversity obtained by 454-pyrosequencing, constitute an adequate tool for assessing natural protist diversity

In the last decades, molecular biology has provided powerful sets of alternative diversity approaches to the classical microscopy, such as clone library sequencing and 454-pyrosequencing (Caron & Gast 1995, Stoeck et al. 2010). The general principle, the sequencing of indicative marker genes for the assignment of taxonomical relationships within protists, is shared by both molecular approaches. One marker gene, the 18S rRNA gene, has

frequently been used for diversity studies in which the whole 18S rRNA gene (clone library) or a part, e.g. the V4 region (454-pyrosequencing), was analyzed. These studies have deepened our understanding of protist diversity by the inclusion of small planktonic cells (picoplankton) and rare distributed cells (rare biosphere) (Diez et al. 2001, Diez et al. 2004, Lovejoy et al. 2006, Stoeck et al. 2006, Lovejoy et al. 2007, Stoeck et al. 2010, Lovejoy & Potvin 2011). However, there have been some concerns on the use of 454-pyrosequencing as single approach to investigate protist assemblages because of the limited sequence length and thus limited depth of taxon characterization (Wommack et al. 2008). Further, the PCR step generates some bias that can hamper the interpretation of sequence based molecular tools. Therefore, an obvious suggestion to test the applicability of 454-pyrosequencing, is the comparison with other indicative methods of protist diversity, such as clone libraries, ARISA, HPLC, and light microscopy.

Clone library sequencing differs from 454-pyrosequencing in the need of a cloning step of a template DNA, into a bacterial vector. Alternatively, 454-pyrosequencing generates clonal representatives of the template DNA and thus sidesteps a cloning step (Margulies et al. 2005). The first manuscript (**Publication I**), focused on the comparison of both molecular approaches that use the diversity in ribosomal RNA genes (rRNA) as categorizing marker. The comparison was applied once for the picoplankton and once for the whole plankton size community, in order to further assess, how an extensive or limited comprehension of the protist assemblage changes the outcome. The method comparison presented high discrepancies of protist diversity in both size treatments that were dependent on the respective method and that even did not coincided for the abundant biosphere. In this regard, clone library sequences, were strongly biased against haptophytes (picoplankton) and diatoms (whole size fraction). The underrepresentation of haptophytes in clone library surveys has also previously been reported (Liu et al. 2009, Marie et al. 2010, Sorensen et al. 2012). In contrast, other groups such as chlorophytes (picoplankton) were highly overrepresented.

Primer specificity might be one explanation for the bias against haptophytes and diatoms. The test of both primer sets, concerning possible sequence mismatches, however, indicates no binding error. Furthermore, the underrepresentation of haptophytes and diatoms can be due to a disparate incorporation rate during the cloning process, where some groups were ligated more preferentially than others (Bent & Forney 2008). The protist composition constitutes under this aspect an important factor that can influence the data outcome. This would explain the differences in haptophyte recovery in both size treatments as well as the overrepresentation of *Micromonas* in the picoplankton library. Altogether, the results lead to

the conclusion that 454-pyrosequencing is a more suitable approach to generate protist diversity surveys, because of the higher throughput and thus smaller danger of missing important phylotypes and taxonomic groups. Inferring on that conclusion, the use of clone library data to complement diversity surveys of 454-pyrosequencing with regard to a better taxonomical resolution is not advisable and at most applicable for a section of abundant protists (e.g. *Micromonas pusilla*). Furthermore, the results support no use of previously generated clone library data to compare or to complete the diversity picture obtained by 454-pyrosequencing.

Another molecular tool that can be used for assessing community structure differences and for sustaining 454-pyrosequencing data is ARISA. In contrast to 454-pyrosequencing, where the nucleotide sequence is the indicative marker, ARISA is based on the fragment length of the small subunit rRNA, located between the 18S and 28S rRNA. The applicability of this method was tested in **Publication IV**, because of the large data set. Large data sets are a prerequisite for the ordination of ARISA data because the number is associated with the stress values, an indicator for the robustness of the positioning in the diagram. Small data sets lead to false positive (<0.1) stress values, and thence to misinterpretations. The employment of ARISA showed reliable results and distinguished between closely related and unrelated community structures. This observation was also confirmed by the 454-pyrosequencing approach that further defined the driving key players, for community shifts. However, since ARISA does not provide detailed information on the taxonomic composition, it is more suitable to serve as a preceded tool for filtering interesting samples and minimizing the sample number than to serve as a comparison method. Moreover, the presence of species that may possess equal fragment lengths (Ranjard et al. 2001) and thus cannot be separated, further constrains the applicability of ARISA.

HPLC is a pigment based approach for studying autotrophic protist diversity and relies on the presence of characteristic photosynthetic pigments for characteristic groups. As a result, a comparison with 454-pyrosequencing data is likely to differ in the proportion of taxonomic groups, comprising heterotrophic protists. **Publication III** investigated the protist assemblage in the Western Fram Strait by using both methods. The data were in good accordance for principal autotrophic groups such as chlorophytes, haptophytes, and cryptophytes. In contrast, main differences were observed inter alia in the dinoflagellate relations. Dinoflagellates comprise several nutrition strategies that include, besides autotrophy, also heterotrophy and mixotrophy. Moreover, HPLC has the tendency to underrate dinoflagellate proportions as a result of misleading pigments (Irigoien et al. 2004, Higgins et al. 2011), whereas symbiosis

also showed to influence the pigment signature and thus led to further underrepresentation (Jeffrey et al. 1975, Watanabe et al. 1987, Bjørnland & Liaaen-Jensen 1989, Watanabe et al. 1990). The suffering of 454-pyrosequencing from different, cell size associated, multiple rRNA gene copies that are particularly pronounced for diatoms and dinoflagellates, caused more discrepancies (Zhu et al. 2005). Cell size, however, also constitutes an important issue for the HPLC analysis because concentrations of some accessory pigments are dependent on protist cell size and on environmental conditions as light and nutrient availability (Latasa 1995, Goericke & Montoya 1998, Schluter et al. 2000). Studies on pigment concentration observed an increase of light protecting pigments and a decrease of light harvesting pigments during high light and low nutrient periods (Latasa 1995, Schluter et al. 2000, Schluter et al. 2006). In summary, HPLC and 454-pyrosequencing suffer from a cell size dependent bias, which magnitude is not entirely assessable. Nevertheless, both methods coincided well in autotrophic protists proportions, approving the suitability of 454-pyrosequencing for generating protist surveys. Moreover, the fact that pigment concentrations are responsive to environmental conditions and that a presetting of pigment ratio is required for accurate protist diversity representation, urge for caution in the use of HPLC. Furthermore, the constant adjustment of the pigment ratio is not practicable and still needs an adaptation for the Arctic Ocean. These drawbacks makes the application of 454-pyrosequencing for studying the Arctic protist diversity more suitable.

Light microscopy has been the standard method for assessing protist diversity over many years and is still very suitable for analyzing the micro- and nanoplankton fraction. This method depends on phenotypic, characteristic markers. However, some species lack those morphological features and thus cannot be characterized properly. Cell size further constitutes a limiting factor because small cells as picoplankton are not detected. Based on the limitations, a comparison with 454-pyrosequencing is only advisable for the >10 µm filter, which also guarantees a proper identification by microscopy. **Publication II** compared the percentages of diatoms obtained by microscopy to the one obtained by 454-pyrosequencing. Diatoms were chosen as a reference group because of their size dimension, ranging between 10 and 200 µm (Winder et al. 2009). This size range facilitated on the one hand an almost complete recovery on one size filter (454-pyrosequencing) and on the other hand, a proper microscopic quantification. The results of both methods were in good accordance and coincided in the general diatom proportion and in the more detailed comparison of pennate and centric diatom percentages. Differences were observed on the genus level, where microscopy counts referred to high cell numbers of *Fragilariopsis* and 454-pyrosequencing

analysis to higher read numbers of *Pseudo-nitzschia*, *Actinocyclus,* and *Thalassiosira*. The missing of *Fragilariopsis* in the 454-pyrosequencing is based on the 99 % similarity of the V4 region sequence of *Fragilariopsis* and *Pseudo-nitzschia*. Since we used a threshold of 97 %, both diatoms were not differentiated. The genus *Thalassiosira* is reported to hold high rRNA copy numbers, which leads to a general overrepresentation in molecular data sets (Zhu et al. 2005). Overall, the well coincidence of diatom proportion in both methods, supports the molecular data and the use of microscopy for 454-pyrosequencing evaluation. However, we have to keep in mind that, in contrast to 454-pyrosequencing, microscopy analysis may be subjective (Schluter et al. 2000, Havskum et al. 2004). The dependence on the taxonomic skills of the operator, which is particularly relevant for the classification of ambiguous algal taxa, constitutes just one error source. Furthermore, the non-random distribution of protists within the counting chambers, the influence of the detrital load on the counting efficiency, and the high numerical variability of different taxa in a protist community represent other methodological biases (Willén 1976, Kalff & Knoechel 1978, Sandgren & Robinson 1984, Duarte et al. 1990, First & Drake 2012). Hence, under the perspective of getting a quick overview of the whole protist diversity, including all size fractions, the use of 454-pyrosequencing is more appropriate.

Concluding, all methods that are used for protist monitoring have limitations that bias the picture of the natural protist diversity. However, a monitoring of protist diversity has to include all protists regardless of cell size, nutrition or abundance. In this respect, the basic limitations of light microscopy, leads to difficulties in the investigation of small cell communities, while the drawbacks of HPLC restrict the diversity analysis of autotrophs in new regions that are characterized by new pigment ratio. All molecular methods suffer from a PCR bias. The usage of molecular methods for 454-pyrosequencing evaluation however, is despite the same bias, qualified because additional biases and the use of different primer sets reduces the danger of artificial similarities in molecular data. Since ARISA presents community structure shifts and supports, no information on taxonomical differentiation the molecular method can just strengthen the major changes in protist composition. Clone library, in contrast, delivers a comprehensive taxonomical resolution of the protist diversity, which is not limited by cell size or nutrition. The uneven cloning bias and the small throughput, however, confine the application for diversity analysis. Overall, the good accordance in protist diversity of 454-pyrosequencing and particularly the non-molecular methods proved the suitability of the molecular approach to generate reliable protist surveys. Furthermore, 454-pyrosequencing has the great advantage of being independent of protist cell size,

nutrition, and abundance, important aspects for comprehensive protist diversity studies. The limited sequence length and the problem of multiple rRNA gene copy number urge, at present, for some attention and demands the need of a second method to support the molecular data, however developments to qualify these biases are promising (e.g. Real-Time PCR).

Water masses in the Arctic Ocean and in the Fram Strait are characterized by distinct protist communities

Distinct water masses are characterizing the hydrodynamic properties of the ocean and are defined by distinct temperature profile, salinity profile, and the chemical composition. Differences in density between water masses constitute physical boundaries that can affect the dispersion of microbes because of the small cell size and the planktonic lifestyle (Galand et al. 2009). Consequently, the reduced cell size of nanoplankton and in particular of picoplankton impedes an active dispersion (Smetacek 2002, Durham et al. 2009). Many oligotrophic oceanic regions present a significant contribution of nano- and picoplankton to the microbial community, suggesting a similar significant influence of water mass on their distribution (Ishizaka et al. 1997, Li et al. 2009, Massana 2011). Previous molecular surveys reported a large influence of water mass on microbial communities in the Arctic Ocean (Lovejoy et al. 2006, Hamilton et al. 2008, Terrado et al. 2009, Lovejoy & Potvin 2011). However, these studies were mostly limited to few oceanic regions (e.g. Frankling Bay, Baffin Bay and Beaufort Sea), where they observed a predominance of small alveolates and picoeukaryotes (Lovejoy et al. 2007, Terrado et al. 2009). If the theory of water mass correlated, protist distribution is still valuable for the Central Arctic Ocean or for the Fram Strait has not been tested yet and is part of the following analysis.

So far, protist surveys in the Fram Strait were conducted by classical approaches as light microscopy and HPLC. **Publication II** and **Publication III** deepened the investigation of the protist assemblage by using 454-pyrosequencing. The evidence for water mass influence was interpreted based on presence/absence patterns and/or changes in protist abundance.

Publication II focused on the distribution of picoplankton in the Eastern Fram Strait, at the deep-sea long-term observatory “Hausgarten”. A total of four stations, located most south, east, north, and in the center of the observatory, displayed a high association of the picoplankton community structure and the water mass properties. All stations were located in the West Spitzbergen Current (WSC), transporting AW northwards. Three out of four stations

were characterized by similar temperature and salinity regimes and resembled in the community structures. However, the variation of abiotic environmental factors in the fourth station (HG1), induced by the mixing of freshwater (Kongsfjord) and the associated protist community, resulted in a different community structure. **Publication III** concentrated on the whole size fraction of protists in the Western Fram Strait, in order to assess if the water mass association of the picoplankton also applies for the whole size community. In fact, the comparatively higher momentum of microplankton, relative to the water viscosity, keeps larger cells longer moving and thus might result in different distribution patterns (Berg & Purcell 1977, Purcell 1977, Mann 2009). Overall, five stations have been analyzed along a transect running through the confluence zone of the West Spitzbergen Current (WSC) and the East Greenland Current (EGC). Along this transect, two stations were sampled in the AW once with high, once with no ice coverage and three stations were sampled in the Polar Water (PrW), covering different ice concentrations. The investigation of the entire protist community confirmed the high influence of water mass on the protist distribution and showed a dominance of chlorophytes in the AW and a dominance of stramenopiles and dinoflagellates in the PrW. However, apart from water mass attributes (temperature and salinity), sea-ice concentration also influences protist community structure by promoting the development of periodical cycles. The melting of sea-ice forms a stratified water layer and induces favorable conditions for the formation of phytoplankton blooms (Bauerfeind et al. 2009), which are grazed by heterotrophic and mixotrophic protists. The bloom forming and periodical changing protist assemblage includes several diatom species (e.g. *Thalassiosira* sp., *Pseudo-nitzschia* sp., *Chaetoceros* sp. and *Fragilariopsis* sp.), while grazers are often represented by heterotrophic dinoflagellates and ciliates, indicators for post bloom events (Strom & Strom 1996, Levinsen et al. 2000, Poulsen & Reuss 2002). The dominance of stramenopiles and dinoflagellates in the PrW, under different sea-ice concentrations, suggests such a protist succession. However, the overall low chl *a* concentration finally, does not support a bloom scenario before or during the study period. Nevertheless, the bloom forming diatom, *Thalassiosira* sp., was observed in abundant proportions at the melt water influenced stations T1 and T7, while consumers of bloom forming diatoms, such as *Gyrodinium* sp. (Saito et al. 2006, Sherr & Sherr 2007) were found in abundant proportions at the ice free station T5, suggesting a high influence of sea-ice concentration on protist community structure. In summary, **Publications II** and **III** showed a correlation of protist communities and water masses in the Fram Strait that was not dependent on protist size class but partly influenced by sea-ice concentration.

Publication IV addressed the question of the water mass influence on protist composition in the Central Arctic Ocean. Protist community structure changes were analyzed, across twenty-three stations in the central Arctic, by the application of ARISA, while protist diversity was investigated in a selection of eight stations by the use of 454-pyrosequencing.

ARISA resulted in a clustering of the protist communities according to the distinct water masses (Atlantic Water, Pacific Water, Mixed Water I, and Mixed Water II) and facilitated to distinguish between ice-free and ice-affected sampling sites. However, the differences between the clusters were small. The ordination of the data in a principal component analysis (PCA) indicated a stronger influence of sea ice concentration on protist community structure than of water mass determining factors (T, S, and nutrients). The effect of water mass on the protist composition was also observed in the 454-pyrosequencing analysis, just not as much pronounced. Strong evidences of missing phylotypes were less observed than small indices of changing phylotype proportions. One reason for the minor recovered relationship of water mass and protist community structure is probably due to the limited number of samples. 454-pyrosequencing analysis comprised two samples of each water mass, whereas six out of eight stations clustered close in the metaMDS plot (ARISA). These stations were mostly dominated by dinoflagellates, whereas other taxonomic groups showed no big variances as well. The other two stations, in contrast, were dominated by chlorophytes and displayed highly different community structures in the ARISA. A detailed insight in the abundant (≥1 % relative abundance) and rare biosphere (<1 % relative abundance) revealed small indices of water mass coupled biogeography. In this respect, abundant phylotypes as *Pyramimonas* sp. and *Dictyocha* sp. were completely missing in the AW, while *Micromonas* sp.1 and *Fragilariopsis* sp. presented the lowest and highest proportion in the MWI, respectively. Rare phylotypes, as *Pelagodinium* sp. and all MAST representatives, showed a water mass influence by being absent at the first AW station.

In summary, the influence of large-scale water masses on protist distribution was confirmed for the Fram Strait and, in parts, for the Central Arctic Ocean. The answer on the question concerning the most suitable method to elucidate water mass related protist structures, is positive for both adopted molecular methods: ARISA and 454-pyrosequencing. Depending on the objective of the study, the use of ARISA is suitable for tracing general shifts in community structure, while 454-pyrosequencing is suggested when investigating the diversity of communities. A combination of both tools, however, provides a powerful tool that allows the investigation of numerous samples and the filtering of interesting samples for further taxonomic analysis.

The question regarding possible suitable indicator organisms for different water masses, is more difficult to address. Water masses are characterized by the holding of different temperatures, salinities, and nutrient signatures. Potential indicative species for water mass are rare and have been found more within the zooplankton than within the phytoplankton, as for example *Themisto compressa*, an AW species that was found to spread further north (Kraft et al. 2011) or *Calanus finmarchicus*, a characteristic for North Atlantic Water (Hirche & Kosobokova 2007). Although, single species such as *Coccolithus pelagicus* or single diatoms species (e.g. *Fragilariopsis cylindrus)* show preferences in salinity and temperature regimes, the use for water mass indication is limited. In fact, potential indicative species should fulfill various requirements. First, they need to be sentinel to water mass defining attributes, in order to correspond to changes with presence or abundace shifts, second they should not display an ubiquitous distribution, and third possible indicative protists should at least not undergo blooming successions, because variabilities in abundance could hence not be entirely related to water mass properties.

In order to confine the search for indicative organisms, it is appropriate to decide at first, which group, the abundant or the rare biosphere, is more convenient and might contain potential marker species. The rare biosphere is characterized by a high diversity, which is assumed to serve as a seed reservoir, enhancing the biological buffer capacity (Sogin et al. 2006, Caron & Countway 2009). Under this aspect, the rare biosphere comprehends a variety of most diverse protists to provide a high number of specialists for different marine habitats or changing environmental conditions. **Publication IV** studied the distribution of the rare biosphere in the Central Arctic Ocean. The taxonomic group composition did not differ throughout the entire course plot, suggesting a likewise inappropriate composition of single protists in the other investigated water masses. Furthermore, the limited insight in the diversity makes the rare biosphere deficient to search for water mass indicators. Hence, marker species have to be searched within the abundant biosphere. The search of indicator species can be further confined, by choosing a size class that covers most of the aforementioned requirements. Picoplankton has the advantage that the distribution is exclusively controlled by water mass circulation due to the small size and the resulting density constrains. Moreover, picoplankton species are not forming blooms and the abundance was reported to be positively correlated with temperature increase and nutrient decrease (Li et al. 2009, Moran et al. 2010). This can be particularly suitable for tracing the circulation of warm and oligotrophic water masses. However, the distribution of picoplankton is still a subject of discussion, where one side suggests an ubiquitous distribution (“ubiquity

model"), while the other side argues against it ("moderate endemicity model") (Finlay et al. 1996, Foissner 1999, Fenchel & Finlay 2004, Lachance 2004, Richards et al. 2005, Finlay et al. 2006, Foissner 2006, 2008, Galand et al. 2009). This and the limited knowledge about the diversity constrain the use of picoplankton species for water mass identification in the end.

Publication II, **III**, and **IV**, showed no clear evidence of suitable indicator species in the abundant biosphere. However, a general trend was observed in the distribution of chlorophytes, which were mainly represented by the ubiquitously occurring picoeukaryote *Micromonas pusilla*. In this study, the species showed a preference for warmer and/or nutrient limited areas and presented high proportions in AW stations. Moreover, changes in the abundance of *Micromonas*, in the Central Arctic Ocean, were often coupled with different water masses. In this respect, Micromonas showed in average the highest proportions in the AW and MWII, lowest in the MWI and moderate percentages in the PW. However, the similar proportions of *Micromonas* in two water masses that were characterized by distinct properties, finally questions the application of the species as water mass indicator because of the limited sensitivity. Overall, the use of the whole protist community to trace water mass shifts is more suitable, because of the higher validity, induced by the combination of different protists.

Complex hydrographical situations can be evaluated via genetic information

Different hydrographical situations were observed in the Fram Strait, a passage that connects the North Atlantic with the Arctic Ocean. Beside the characteristic two-directional boundary currents (WSC and EGC) that flow on each side of the passage, the circulation is further affected by the bottom topography (Rudels et al. 2000). As a result an intense mesoscale eddy field forms on top of the Molloy Ridge and leads to the recirculation of AW to the North Atlantic (Schlichtholz & Houssais 2002). The formation of short period and flexible eddies further complicates the circulation structure in that area and causes diffusions of the AW (WSC) and the PrW (EGC) (Johannessen et al. 1987).

Publication III investigated the protist community structure along a transect that passed through both current systems. Data obtained by 454-pyrosequencing were very suitable to distinguish between the community structures of the EGC and the WSC. Moreover, they were able to trace more complex situations as the recirculation of the AW. In this regard, the station located in the recirculating WSC branch resembled in the protist community structure the one, located in the principle current. The similar structure was characterized by a high proportion

of *Micromonas pusilla* that would probably have been missed by the single application of light microscopy. However, as most clearly observed in **Publication III** and, to a lesser degree in **Publication II**, protist communities in the Fram Strait were affected by the hydrodynamic situation and in addition by the prevalent sea ice concentration. The presence of sea ice influenced in particular the diatom/dinoflagellate relation and showed a diatom-based system under no/low ice concentrations and a dinoflagellate-based system in combination with high ice concentrations. The high abundance of diatoms in areas of no or little ice concentration is due to the nutrition strategy because diatoms are autotrophic and hence require a good light regime. Dinoflagellates, in contrast, also contain heterotrophic and mixotrophic representatives and are thus capable to dominate low light areas of high ice concentration.

Based on the influence of sea ice on the protist assemblage, the question arises if hydrodynamic situations can also be recovered in the Central Arctic Ocean, where a perennial sea ice coverage influences the local protist communities. **Publication IV** investigated the relation of community structure and water mass at twenty-three stations in the Central Arctic Ocean, where most stations were characterized by a sea ice concentration of >80 %. The application of ARISA pointed to a small response of protist community structure to local hydrography and to a strong influence of ice thickness and floe size on community structure shifts. The use of 454-pyrosequencing, on a selection of eight stations, revealed a stable distribution of the major taxonomic groups at almost all stations that was characterized by a high dominance of dinoflagellates. The high abundance of heterotrophic dinoflagellates and small flagellates has also been observed in the Central Arctic Ocean by previous studies (Sherr et al. 1997, Rat'kova & Wassmann 2002, Sherr et al. 2003, Richardson et al. 2005, Terrado et al. 2008). The similar observations lead to the suggestion that the perennial ice coverage promotes the growth of particular groups that mask the influence of hydrography. For example, as previously observed in the Fram Strait, the Arctic protist assemblage presented high contributions of dinoflagellates in low light areas and higher contributions of diatoms in high light areas, where the sea ice was characterized by low thickness or small floe sizes. Previous studies of Sherr et al. (2003) and Terrado et al. (2008) also reported the significance of light irradiance for protist distribution.

In summary, ARISA and 454-pyrosequencing were appropriate to recover complex hydrographical systems in marine habitats. Shifts in the protist community structure were detected for the EGC as well as for the WSC. Moreover, molecular data were suitable to trace even weaker structures as the recirculation branch of the WSC. However, tracing those

hydrographical situations was constrained by the coincidental presence of sea ice, which masked the protist community structure by fostering small flagellates and dinoflagellates, and thus decreased the range of indicative variations. Consequently, hydrographical induced community shifts displayed a higher resolution in no or low ice covered areas, as the Fram Strait, and a lower resolution in high ice covered areas, as the Central Arctic Ocean.

5. Outlook

A wealth of literature describes the biodiversity, biogeography, and biogeochemical role of nano- and microplankton in many oceanic regions. Most of these investigations were carried out in coastal areas. Less attention has been paid to open oceans, while high-productive areas were investigated more exhaustively than areas of lower productivity. Overall, just a few studies were carried out in the permanent ice covered Central Arctic Ocean and hence, information on marine ecosystem function in that area is scarce. Comprehensive investigations, however, are of particular interest with regard to the actuality that environmental conditions in the Arctic Ocean are changing fast and profoundly. At present, we assume that the temperature increase promote the occurrence of picoeukaryotic cells which will affect the food-web structure by enforcing the microbial loop. Furthermore, the sea ice decrease and the better light climate in surface water are not expected to increase the average protist biomass in the Central Arctic Ocean because offshore nutrient availability will not change accordingly.

However, in order to understand the response of the Arctic marine ecosystem more comprehensively, more information concerning the protist diversity, distribution, physiology and interactions is needed. At the present time, we are far away from capturing the natural protist diversity and we have not yet understood the forces that are driving the protist composition and distribution in full detail. Therefore, we have to address the gaps of knowledge concerning the protist diversity, including for instance detailed information on key species, on pelagic/sympagic-associated protists and on intraspecific diversities, in order to assess the potential of species to adapt to a spectrum of different abiotic factors. Moreover, information on autotrophic, mixotrophic, and heterotrophic representatives is needed to fully understand protist interactions, their role in the food-web, and in biogeochemical pathways. To achieve all this, more sampling has to be carried out in different Arctic regions that encompass water column and sea-ice communities. Analyzing the effects of different abiotic and biotic factors on protist behavior further demands the combination of field research and laboratory research. Field research includes protist surveys at harsh environmental conditions, as e.g. the polar night period, and a monitoring of protist diversity and community structure. Such a monitoring allows thereby investigations on seasonal successions, on invasive species, and on consequences of temperature increase and sea ice decrease. Laboratory research includes in-situ experiments of protist cultures and environmental samples that are performed under different abiotic and biotic factors and that study the specific response of single protists (e.g. key species), the competition for nutrients and light, the predator-prey relations, the

succession, and the relations of autotrophic, mixotrophic, and heterotrophic species within the protist community. Microcosm experiments are crucial because they provide information on the physiology of protists, which are important for estimating the ecophysiological response to environmental change.

The actual lack of knowledge concerning Arctic protist diversity, distribution, physiology, processes, and food-web interactions also constitutes an important drawback for modeling studies. Hence, filling these gaps will greatly improve predictions concerning the influence of environmental change on the Arctic marine ecosystem.

One method that facilitates an increase of knowledge in protist diversity and distribution is 454-pyrosequencing because it includes even small and rare species. The field of 454-pyrosequencing development is a fast-moving area of research, where latest inventions enhance the read length of DNA from actual 500 bp to up to 1000 bp (Ebenezer et al. 2012). The increase in DNA read length will improve assessments of protist diversity and distribution in future. Moreover, apart from a comprehensive diversity analysis on inter- and intraspecific level, 454-pyrosequencing can be applied to study the expression of genes (e.g. transporter genes) that are associated with protist responses on environmental stress. Hence, the ability of 454-pyrosequencing to investigate the influence of environmental changes on protist communities by providing information on the protist diversity, distribution, and on the protist stress level makes the method a particularly suitable tool for protist studies and will increase the application in future.

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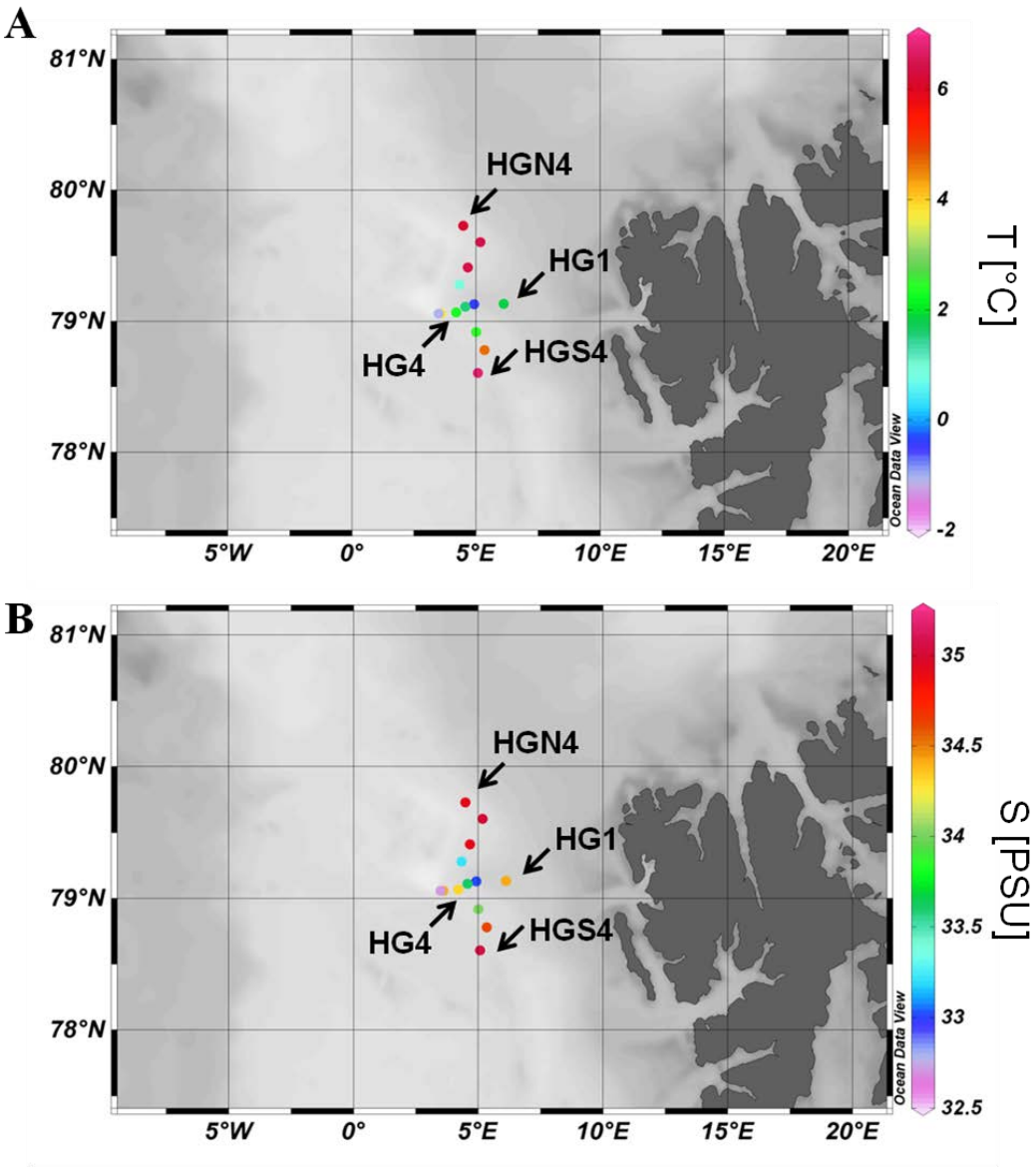
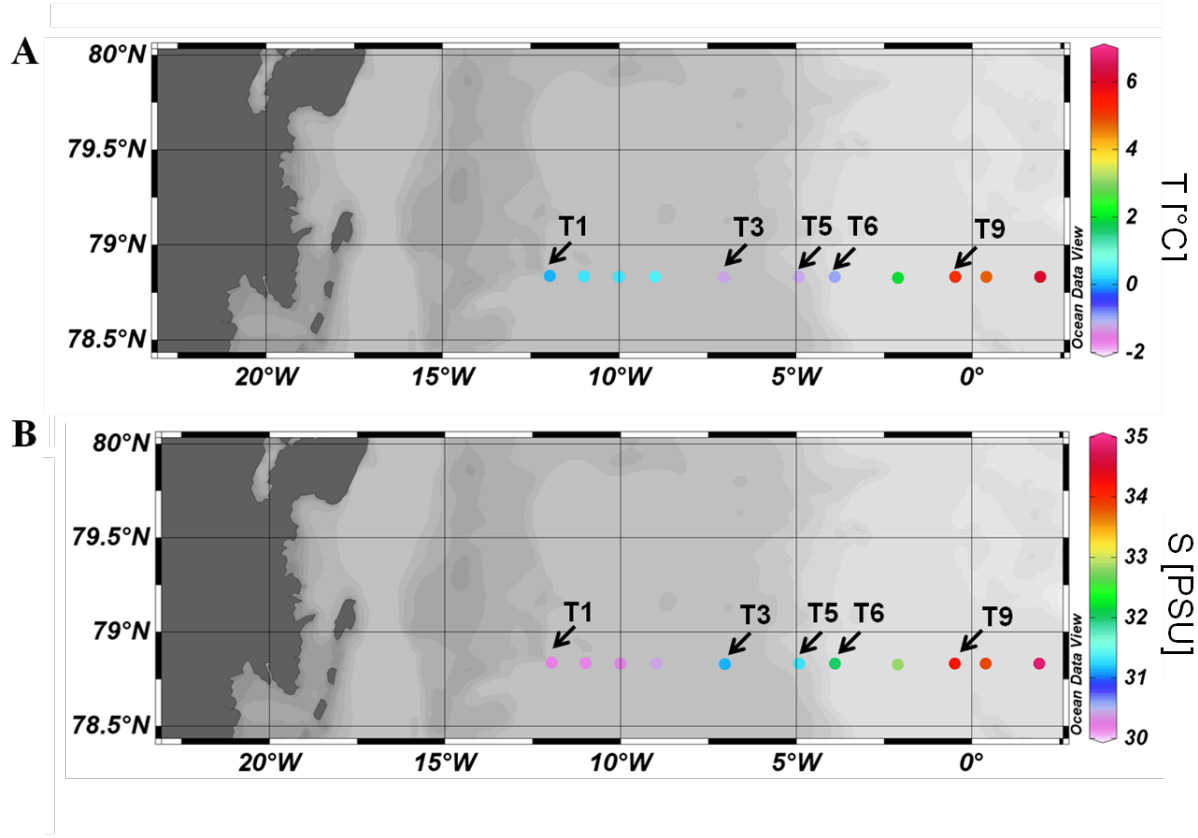
7. Appendix

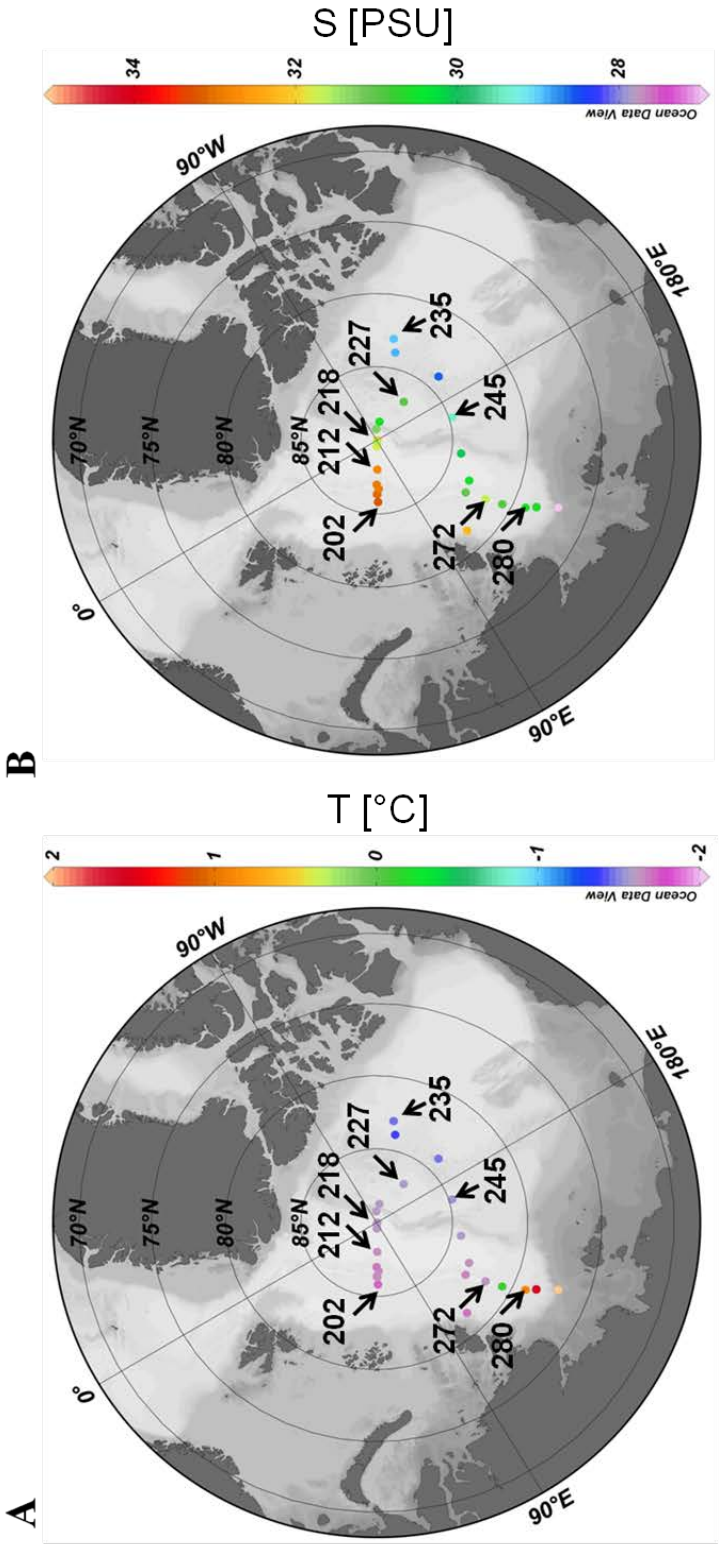
Appendix 1. A) Temperature and B) Salinity distribution of the "Hausgarten" in summer 2009 (ARKXXIV/2). The distribution of both parameters is presented for the surface water layer. Stations (HGN4, HG4, HG1, and HGS3) have been sampled between the 11th and 18th July.



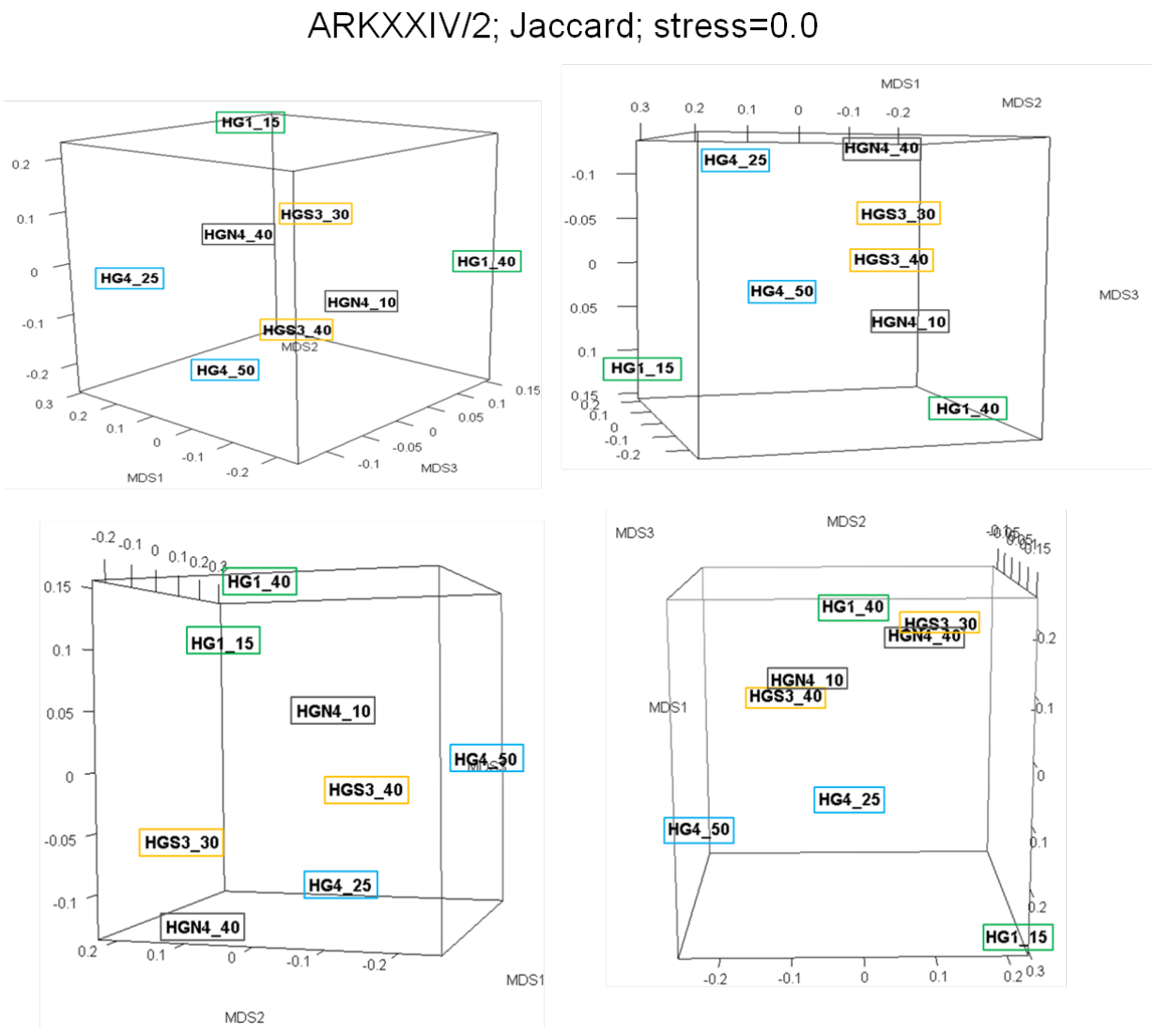
Appendix 2. A) Temperature and B) Salinity distribution of the Western Fram Strait in summer 2010 (ARKXXV/2). The distribution of both parameters is presented for the surface water layer. Stations (T1-T9) have been sampled between the 18th and 23rd July.



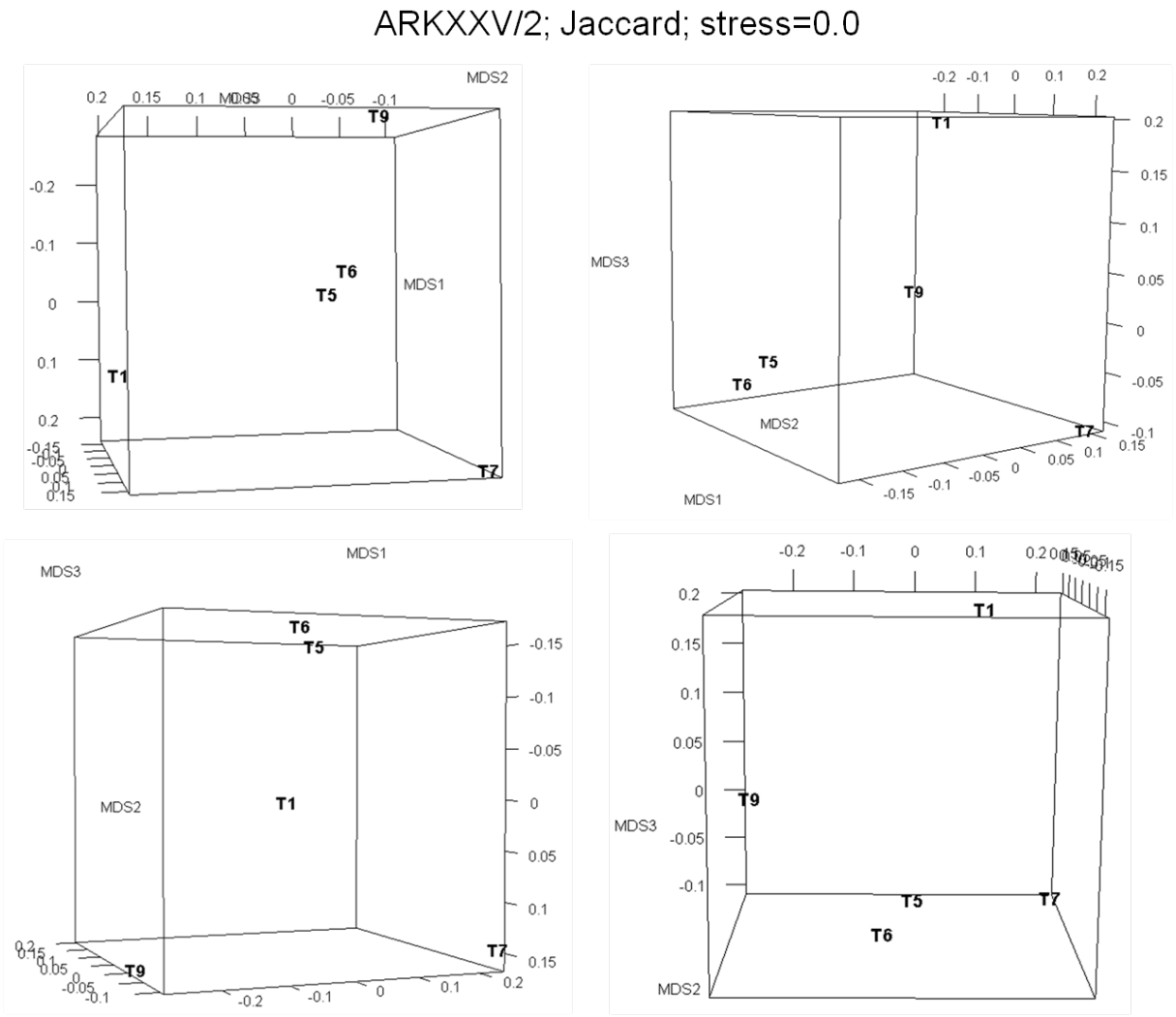
Appendix 3. A) Temperature and B) Salinity distribution of the Central Arctic Ocean in late summer/fall 2011 (ARKXXVI/3). The distribution of both parameters is presented for the surface water layer. Stations (202-280) have been sampled between the 14th August and 21st September.



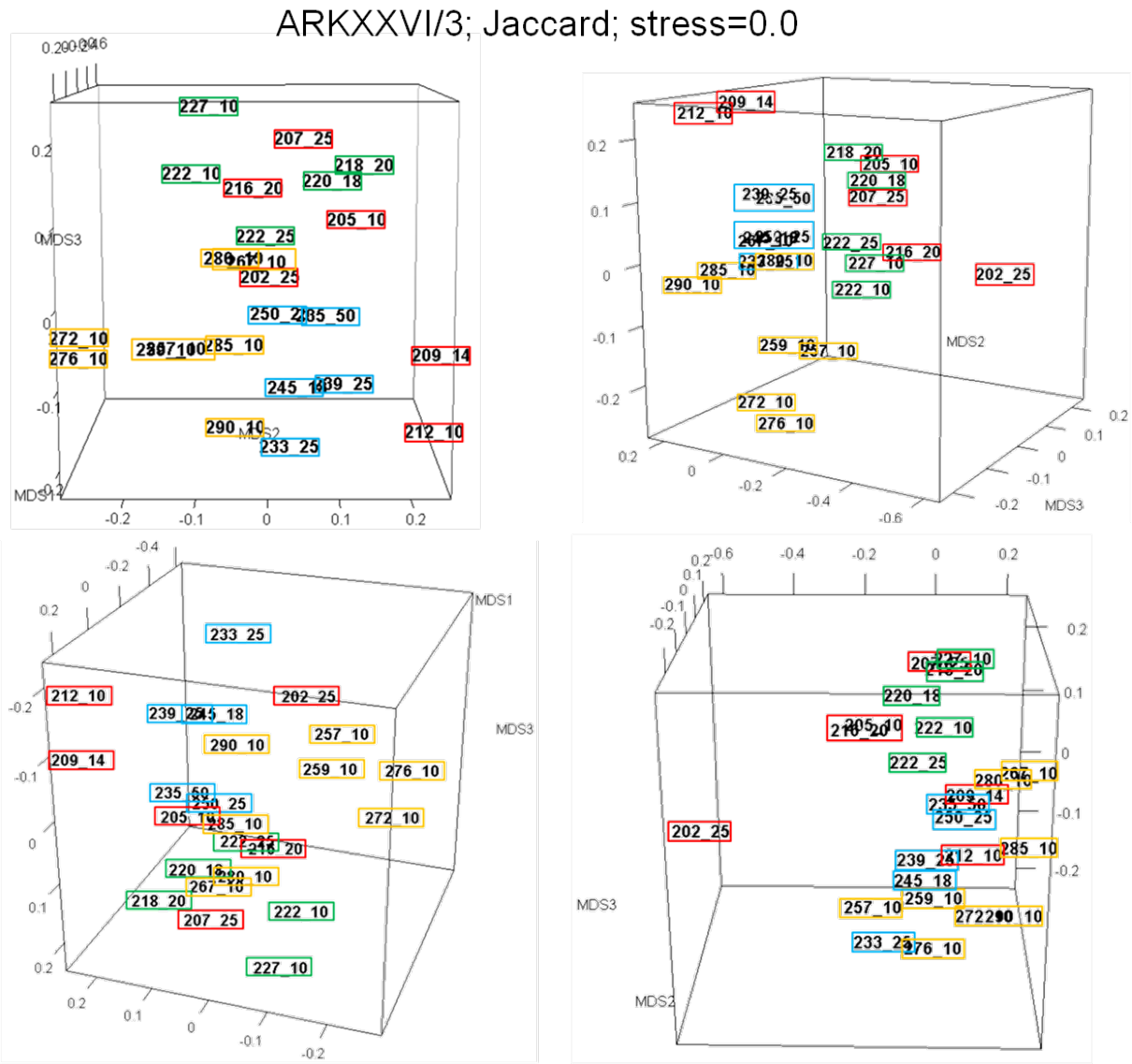
Appendix 4. 3D metaMDS plot of picoplankton community structure in the "Hausgarten". Community structure changes were calculated by the implementation of the Jaccard-index and put into a multidimensional graph with no axis unit. The stress value refers to the reliability of the positioning. Each station is represented by two depths and marked in different colors (grey: HGN4, blue: HG4, green: HG1 and yellow: HGS3).



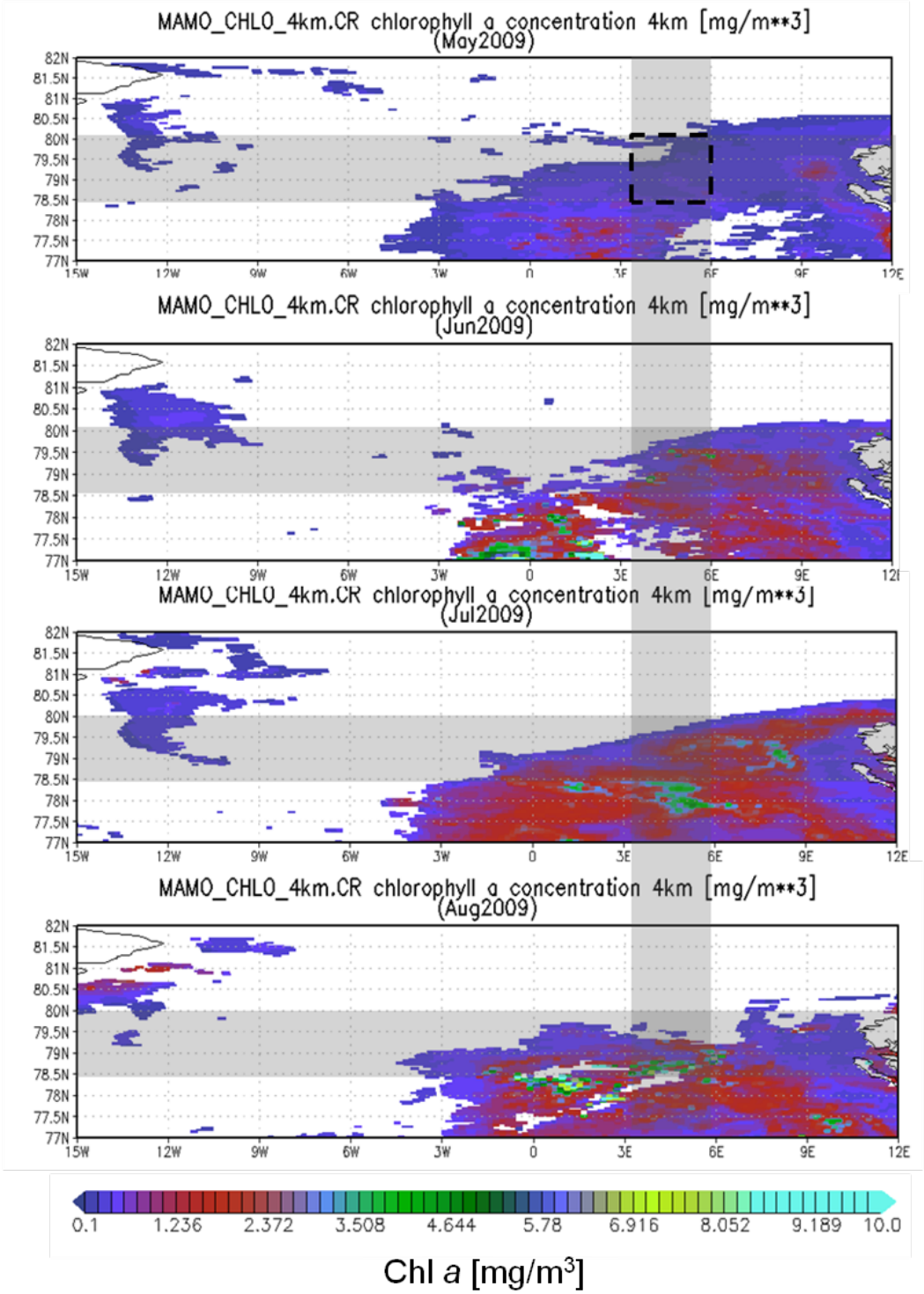
Appendix 5. 3D metaMDS plot of the protist community structure in the Western Fram Strait. Community structure changes were calculated by the implementation of the Jaccard-index and put into a multidimensional graph with no axis unit. The stress value refers to the reliability of the positioning. The stations (T1, T5, T6, T7 and T9) were taken between 5 and 15 m depth.



Appendix 6. 3D metaMDS plot of the protist community structure in the Central Arctic Ocean. Community structure changes were calculated by the implementation of the Jaccard-index and put into a multidimensional graph with no axis unit. The stress value refers to the reliability of the positioning. The color code refers to stations of the same water masse (red: Atlantic Water, green: Mixed Water I, blue: Pacific Water and yellow: Mixed water II), while the first number presents the station number and the second the sampling depth.



Appendix 7. Seasonal development of the area-averaged chl *a* concentration (MODIS) in the "Hausgarten", obtained by remote sensing observation.



Appendix 8. Seasonal development of the area-averaged chl *a* concentration (MODIS) in the Western Fram Strait, obtained by remote sensing observation.

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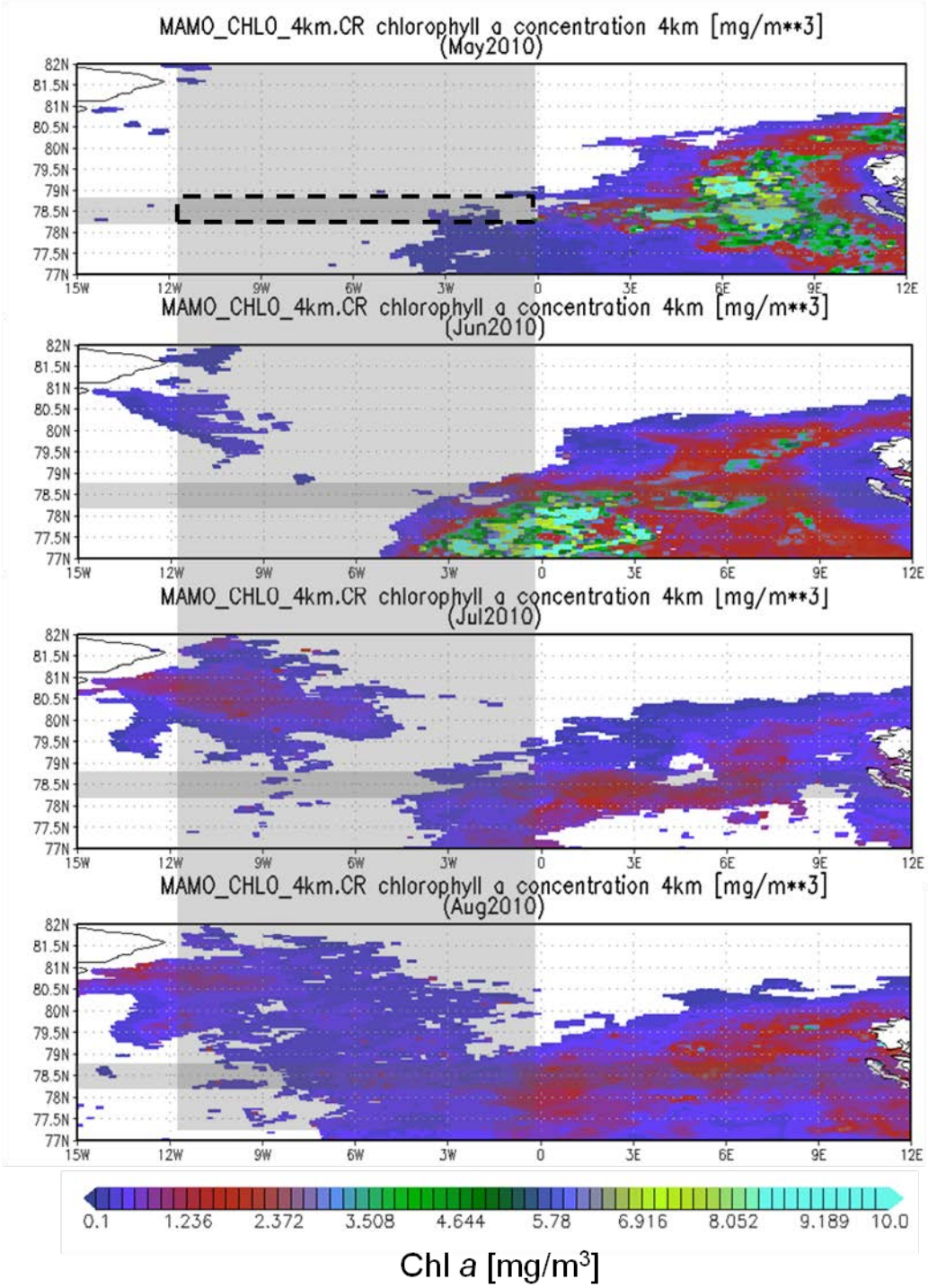

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