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AWI

BACHELOR THESIS

Surveillance of Phytoplankton Key Species in the "AWI-HAUSGARTEN" (Fram Strait) 2010-2013 via Quantitative PCR

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STATEMENT OF AUTHORSHIP

I declare that the thesis

Surveillance of Phytoplankton Key Species in the „AWI-HAUSGARTEN“ (Fram Strait) 2010-2013 via Quantitative PCR

has been composed by myself, and describes my own work, unless otherwise acknowledged in the text. It has not been accepted in any previous application for a degree.

Esslingen a. N., July 1st, 2014

Place/date

Sebastian Micheller

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LIST OF ABBREVIATIONS (1 of 2)

Abbreviation	Explanation	Abbreviation	Explanation
Ø	Average	DGGE	Denaturating Gradient Gel Electrophoresis
%	Percent	DMS	Dimethylsulfide
°C	Degree Celsius	DMSO	Dimethyl sulfoxide
µL	Microliter	DNA	Deoxyribonucleic Acid
µm	Micrometer	DOI	Digital Object Identifier
A	Adenosine	dsDNA	Double-strand DNA
AC	Accession Number	e.g.	For example (lat.: *exempli gratia*)
AFLP	Amplified Fragment Length Polymorphism	eds	Editors
ARB	Tree (lat.: arbor) (Software package)	EGC	East Greenland Current
ARK	Arctic cruise of Polarstern	EMSO	European Multidisciplinary Seafloor Observatory
AW	Atlantic Water	ESONET	European Seas Observatory Network
AWI	Alfred-Wegener-Institute	et al.	And others (lat.: *et alii/et aliae*)
A_{XXXnm}	Absorbtion at wavelength X	FEMS	Federation of European Microbiological Societies
BLAST	Basic Local Alignment Search Tool	Fig.	Figure(s)
BMC	BioMed Central	FISH	Fluorescence In Situ Hybridization
bp/bps	Basepair/s	FR	France
BSA	Bovine Serum Albumin	FRET	Fluorescence Resonance Energy Transfer
C	Cytosine	G	Guanine
CCAP	Culture Collection of Algae and Protozoa	GC%	GC-content in percent
CCM	Carbon dioxide concentration mechanism	h	Hour(s)
CCMP	Culture Collection of Marine Phytoplankton	H2O	Water
CO_2	Carbon dioxide	HAB	Harmful Algae Blooms
CT	Threshold cycle	HPLC	High-Performance Liquid Chromatography
CTD	Conductivity, Temperature and Pressure (probe)	i.e.	That is (lat.: id est)
d	Day(s)	ICES	International Council for the Exploration of the Sea
D1	Applied Dilution	ISME	International Society for Microbial Ecology
D2	Searched Dilution	km	Kilometer
ddNTP/s	Dideoxy Nucleoside Triphosphate/s	km^2	Square kilometer
deion.	deionized	km^3	Cubic kilometer

LIST OF ABBREVIATIONS (2 of 2)

Abbreviation	Explanation	Abbreviation	Explanation
L	Liter	qPCR	Quantitative Polymerase Chain Reaction
LM	Light Microscopy	®	Registered Trade Mark
LTER	Long-Term Ecological Research	r^2	Correlation coefficient
m	Meter	RAC	Return Atlantic Current
m. a.	Modified after	RAPD	Random Amplification of Polymorphic DNA
MAST	Marine Stramenopila	RCC	Roscoff Culture Collection
MEGA	Molecular Evolutionary Genetics Analysis (Software)	rDNA	Ribosomal Desoxyribonucleic Acid
$Mg2^+$	Magnesium ion (divalent)	resp.	Respectively
min.	Minute(s)	RFLP	Restriction Fragment Length Polymorphism
mL	Milliliter	Rn	Normalized ratio
mm	Millimeter	RT	Room Temperature
mmol	Millimolar	RuBisCO	Ribulose-1.5-bisphosphate carboxylase/oxygenase
MT	Megatons	s	Second(s)
n. a.	Not available	SAR	Stramenopila, Alveolata, Rhizaria group
n. d.	Not determined	SD	Standard Deviation
n. p.	Not published	SEM	Scanning Electron Microscopy
n. s.	Not specified	sp./spp.	Species (singular/plural)
NAO	North Atlantic Oscilation	SSCP	Single-stranded Conformation Polymorphism
NASA	National Aeronautics and Space Administration	ssDNA	Single-strand DNA
NASC	Norwegian Atlantic Slobe Current	SSU	Small subunit
NCBI	National Center for Biotechnology Information	T	Thymine
NCMA	National Center for Marine Algae and Microbiota	Ta	Annealing temerature
nm	Nanometer	Tm	Melting temperature
nt/nts	Nucleotide/s	™	Unregistered Trade Mark
p	p-value in statistical significance testing	UK	United Kingdom
p.	Page	USA	United States of America
PAL	Photosynthetic Available Light	V	Volt
PCR	Polymerade Chain Reaction	V4	Variable 18S rDNA region number 4
preps	Preperations	WSC	West Spitzbergen Current
PW	Pacific Water	λ	Wavelength

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1. ABSTRACT

Within this thesis, species/phyla specific molecular probe sets were successfully designed and optimized to detect the Arctic/Subarctic phytoplankton key species *Phaeocystis globosa, Phaeocystis pouchetii, Micromonas pusilla* and *Emiliania huxleyi.* Additionally, probe sets specific for *Chaetoceros socialis* and the phylum Dinophyta were tested for their specificity in qPCR application. All probe set binding sites lie next to the hyper variable V4 region of the 18S rDNA, a sequence often used for phylogenetic biodiversity studies in Eukaryota. The optimization process of the probe sets included PCR and qPCR specificity assays, in which DNA of different phytoplankton representatives (laboratory cultures) was amplified. Herein, the probe sets showed amplification of the desired target DNA. Cross-hybridization with non-target DNA was observed for *P. globosa, E. huxleyi* and Dinophyta specific probe sets. These "unspecific" amplifications were found for phytoplankton species, less abundant in the sampling area of further investigation. The probe set for *P. pouchetii* additionally detected *P. globosa* 18S rDNA. Therefore, the two probe sets Pglo1 and Ppou2 have to be used in combination to distinguish between the two *Phaeocystis* spp. In the first approach, the qPCR signal of a designed eukaryotic probe set should have functioned as a reference value, showing the whole abundance of 18S rDNA within an environmental sample. Comparing the qPCR signals of the species/phyla specific probe sets and the eukaryotic probe set, a relative quantification between the examined phytoplankton key species should have been made. This approach was not realized, due to the low amplification efficiency of the Eukaryota specific probe set, compared to the efficiencies of the other probe sets. Therefore, only the relative abundance within a single phytoplankton key species for different sampling sites and sampling years could be determined. On the other side, a deviation of amplification efficiency (Ø 3.5 C_T) was also seen for the species/phyla specific probe sets, when applied in multiple template DNA qPCRs. Despite this, the designed and optimized probe sets were used in qPCR assays, testing environmental samples originated from the deep-sea observatory HAUSGARTEN (Fram Strait) 2010-2013. Herein, *P. pouchetii, E. huxleyi* and Dinophyta were identified. No qPCR signal was obtained for the species *P. globosa, M. pusilla* and *C. socialis.* For the validation of the qPCR data, pyrosequencing data of the same environmental samples were correlated with the obtained qPCR signals. By doing this, only a significant coherence of the data sets for *P. pouchetii* was observed. This finding is questionable due to a low correlation coefficient (0.35). Further optimization processes might be necessary to overcome this deviation and to enable the detection of already reported species such as *M. pusilla* or *C. socialis* (also present in the pyrosequencing data) in the Fram Strait.

Im Rahmen der vorliegenden Bachelorarbeit wurden molekulare Oligionukleotid-Sonden designt und optimiert, die für die spezifische Detektion von arktischen und subarktischen Phytoplanktonspezies *(Phaeocystis globosa, Phaeocystis pouchetii, Micromonas pusilla* und *Emiliania huxley*i) via qPCR anwendbar sind. Zusätzlich wurden für die Algenspezies *Chaeotoceros socialis* sowie für Vertreter des Stammes Dinophyta molekulare Sondensets auf ihre Spezifität und Anwendbarkeit in qPCR-Assays hin untersucht.
Die Hybridizierungsregionen der entwickelten Sonden flankieren die hypervariable V4-Region der 18S rDNA der Zielorganismen. Diese DNA-Sequenz ist in besonderem Maße für phylogenetische Biodiversitätsstudien in Eukaryoten geeignet. Der Optimierungsprozess der Sonden umfasste Spezifitätstests via PCR und qPCR, in denen mögliche Kreuzhybridisierungen mit DNA-Sequenzen von verschiedenen Phytoplanktonvertretern untersucht wurden. Hier konnten jeweils spezifische Amplifikationen der Zielorganismus-DNA beobachtet werden. Für die Sondensets Pglo1, Ehux und Dino wurden Kreuzhybridisierungen mit Phytoplanktonspezies festgestellt, die in arktischen/ subarktischen Gebieten (z. B. Framstraße) jedoch weniger häufig vertreten sind.
Das Sondenset Ppou2, spezifisch für *Phaeocystis pouchetii,* zeigte zudem ein Amplifikationssignal für DNA der Spezies *P. globosa.* Daraus ergibt sich ein kombinatorischer Einsatz der Sondensets Pglo1 und Ppou2 zur Unterscheidung von *P. globosa* und *P. pouchetii* via qPCR innerhalb einer Umweltprobe. Zur Quantifizierung der Abundanz zwischen einzelnen Phytoplanktonspezies in Relation zur Gesamtheit der eukaryotischen 18S rDNA wurde ein Eukaryoten-Sondenset entwickelt. Dieser Ansatz konnte jedoch aufgrund der Unterschiede der Sondensets bezüglich ihrer Amplifikationseffizienz nicht weiter verfolgt werden. Dadurch war es nur möglich, Häufigkeitsschwankungen innerhalb einer Phytoplanktonspezies für verschiedene Probenahmestellen und Jahre via qPCR zu detektieren. Letzlich wurden die Sondensets zur qPCR-Analyse von Umweltproben (Tiefsee-Observatorium HAUSGARTEN, Framstraße, 2010-2013) verwendet.
Hierbei wurden die Phytoplanktonspezies *P. pouchetii, E. huxleyi* und Vertreter des Stamms Dinophyta erfolgreich detektiert. Für die Spezies *P. globosa, M. pusilla* und *C. socialis* konnte kein qPCR-Signal beobachtet werden. Um die Ergebnisse der qPCR-Analyse zu verifizieren, wurden diese mit Pyrosequencing-Daten der Umweltproben verglichen. Eine signifikante Korrelation der beiden Datensets konnte nur bezüglich der Phytoplanktospezies *P. pouchetii* festgestellt werden, wobei dieser Zusammenhang aufgrund eines Korrelationskoeffizienten von 0,35 kritisch betrachtet werden muss. Weitere Optimierungsansätze sind nötig, um den quantitativen Charakter der entwickelten qPCR-Assays zu verbessern, insbesondere für die Detektion von Phytoplanktonspezies wie *M. pusilla* und *C. socialis,* die bereits im Gebiet der Framstraße nachgewiesen wurden (siehe Pyrosequencing-Daten).

2. GENERAL INTRODUCTION

2.1 Arctic Environment

2.1.1 Abiotic Characteristics

The Polar Regions are very important areas of interest in many different studies. This is due to the fact that these regions have unique hydrographical, climatic and biological conditions. To define Polar Regions, different scales can be applied. Geographically, areas within the Arctic and Antarctic Cycles (latitudes 66°33' north and south) are ranked among Polar Regions. This represents 16.5 % (Thomas *et al.*, 2008, p. 1) of the Earth's surface in which various ecosystems are inhabited.

Looking at the Arctic marine ecosystems, many different abiotic factors play a critical role for their diversity. One factor is the amount of light, particularly of photosynthetically available light (PAL; λ = 400 nm – 700 nm), penetrating the Arctic Ocean. Due to the natural turbidity of oceanic waters, caused by inorganic matter and the occurrence of plankton, a nearly exponential coherence of irradiance decrease and water depth can be observed (Kirk, 1994). This leads to approximately 0.1 % of total PAL in a depth of 100 m and marks the border of the euphotic zone (Thomas *et al.* 2008, p. 227), the region were photosynthetic processes can take place. Beside the Polar night period which lasts for 20 h at the Polar Circles till 179 d at the Poles, ice coverage causes light limitation, especially in late February or March. At this time, sea-ice coverage reaches a maximum of 14-16 million km^2 in the Arctic (Thomas *et al.* 2008, p. 5). Here, sea-ice, approximately one to two meters thick, reduces the water penetrating PAL by 90 % compared to ice free areas.

Another critical factor represents the water temperature in the Arctic, which is regulated by a complex heat flux system consisting of winds (cyclones), river inflows (e.g. by the great Siberian rivers) and open sea currents. Thus, the water temperature never falls under -1.9 °C (freezing temperature of full salinity sea water) (Thomas *et al.* 2008, p. 220). The mentioned fluxes also carry nutrients (nitrate, phosphate and silicate) into and out of the Arctic and create turbulences, adjusting nutrition supply in different water layers (Popova *et al.* 2010).

2.1.2 Fram Strait, Currents and Deep-Sea Observatory HAUSGARTEN

The physical and chemical conditions such as temperature and salinity in the Arctic Ocean depend mostly on water exchange via open water currents. There are several regions in which these exchanges take place. One of these gateways is the Fram Strait. It is located between the western Svalbard archipelago and the Greenland shelf. With a width of approximately 500 km and a sill depth of 2.600 m it is the only deep water connection to the Central Arctic Ocean (Rudels *et al.* 2000). In this area, the largest water exchange between the Arctic Ocean and adjacent seas is observed. This is due to two main currents, flowing through the Fram Strait: the West Spitzbergen Current (WSC) and the East Greenland Current (EGC) (Fig. 1 A).

The WSC consists of warm (above 3 °C) and relatively saline (> 34.9 salinity) Atlantic Water (AW) that flows northwards into the Arctic Ocean. As an extension of the Norwegian Atlantic Current, one branch passes the western side of the Svalbard archipelago and transports between 60,000 to 250,000 km^3 of AW per year (Thomas *et al.* 2008, p. 12). The strength of the WSC differs seasonally and depends on the severity of the North Atlantic Oscillation (NAO), a large-scale variation of atmospheric pressure between the Iceland low and the Azores high. A strong NAO leads to warmer AW that promotes its inflow in the Arctic Ocean, especially in the winter months (Schlichtholz & Goszczko 2006; Beszczynska-Möller *et al.* 2012).

Flowing along the eastern side of the Greenland archipelago, the EGC transports cold (below 0 °C) and less saline (< 34.4 salinity) Polar Water (PW) southwards. Along with the water amount of 91,000 to 910,000 km^3 $year^{-1}$, the EGC carries 4 million MT of drift ice out of the Arctic Ocean and contributes greatly to the sea-ice export (Thomas *et al.* 2008). A mixing of AW and PW in the Fram Strait is related to the westward recirculation of AW by an offshore branch of the WSC. This forms the recirculation area “Return Atlantic Current” (RAC), which combines the physical and chemical properties of both currents.

Within the WSC and its edge, the deep-sea observatory HAUSGARTEN, consisting of 17 permanent stations is located (Fig. 1 B). Running in crosswise directions from north to south (N1 – N5, S1 – S3) and from west to east (HG1 – HG9), two transects cover the area between 78°N – 80°N latitude and 3°E – 7°E longitude. The central station is recorded as HG4.

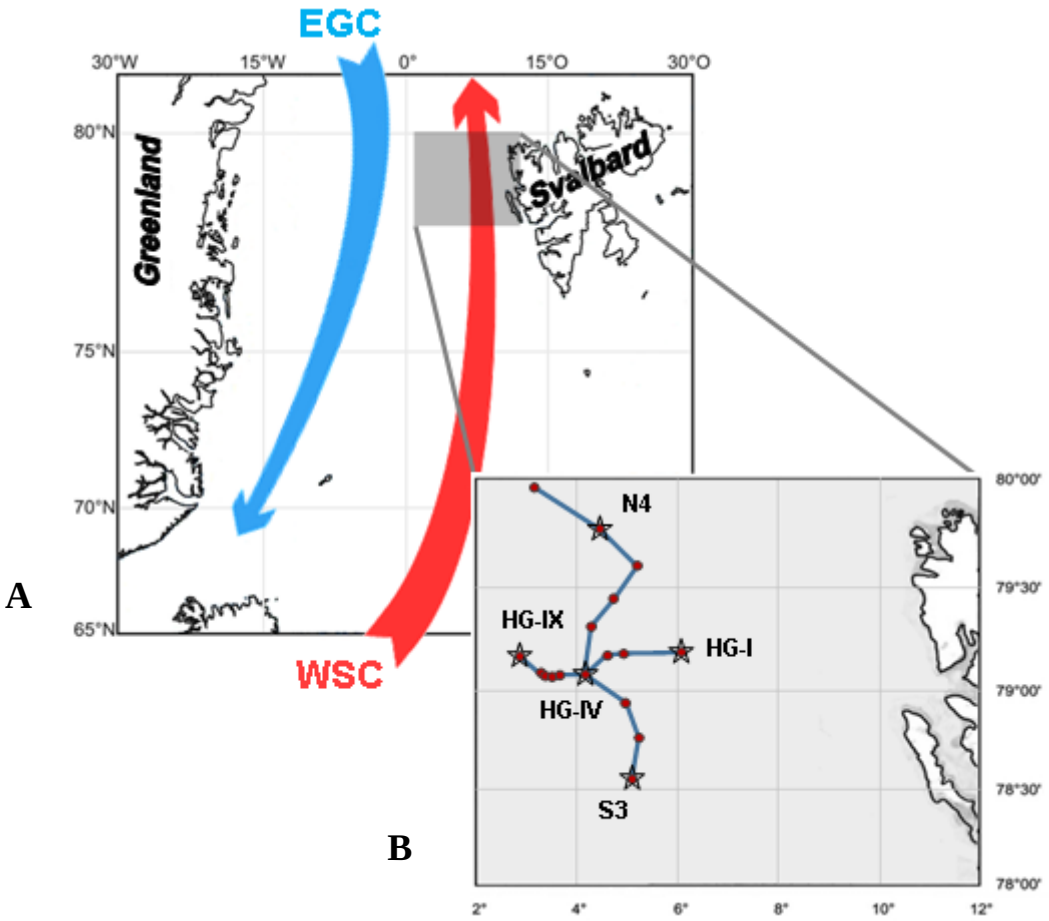


Fig. 1: Scheme of the Fram Strait and the Deep-Sea Observatory HAUSGARTEN [1]

A Current-System, composed of East Greenland Current (EGC; blue arrow) and West Spitzbergen Current (WSC; red arrow).

B Location of the deep-sea observatory HAUSGARTEN. Stations, investigated in this study (HG1, HG4, N4, S3, HG9) are marked by stars.

Since its special location, influenced by AW and seasonally varying sea-ice occurrence (Soltwedel *et al.* 2005), the deep-sea observatory HAUSGARTEN plays an important role in several research networks like the ESONET (European Seas Observatory Network), the infrastructure project EMSO (European Multidisciplinary Seafloor Observatory) and the LTER (Long-Term Ecological Research) Network. Since its establishment in 1999 by the Alfred-Wegener-Institute (AWI), Germany, this research site detects changes of abiotic and biotic parameters in the eastern Fram Strait (Soltwedel *et al.* 2005). These findings help to understand the interaction of Subarctic and Arctic water masses and may be used as an indicator for climatic changes, occurring nowadays.

2.1.3 Arctic Climate Change

Polar Regions serve as an indicator for climatic and biological changes. In the Arctic (as well as in the Antarctic) this is due to the prevalent fragile environment (Dunbar 1973) and ecosystem that changes more rapid than elsewhere. Several intrinsic and extrinsic events characterize the changing climatic state of the Arctic Region. As an extrinsic event, the increase of water temperature in adjacent sea areas like the North Atlantic can be quoted. Here, Beszczynska-Möller *et al.* (2012) observed a positive linear temperature trend of 0.06 °C $year^{-1}$ between 1997 and 2010. In the years 1997/1998 and 2002/2003, two high temperature anomalies occurred in the Norwegian Sea (Orvik and Skagseth, 2005; Skagseth *et al.*, 2008), each taking one to two years to reach the Fram Strait via the WSC and further two years to be spotted in the Eurasian Basin.

As a consequence of the risen AW temperature and warm anomalies pushing forward to the Arctic Ocean, thermodynamic dependent intrinsic factors such as the distribution and thickness of sea-ice are influenced. The maximum extend of Arctic sea-ice in winter 2006 and 2007 was reduced by 1 million km^2 compared to the average maximum extend between 1979 and 2000 (Comiso 2006). Similar observations were made by the NASA in the Arctic Region for the years 2008 to 2014 as well [*]. Furthermore, analyses of sea-ice thickness between Svalbard and the North Pole in the years 1991, 1996, 1998 and 2001 showed a decrease from 3.11 to 2.41 m in average (Haas 2004). These observations point to a declined sea-ice load of the EGC and may engender ice free Arctic seas in summer after the 2050s (Serreze *et al.* 2007). The new sea-ice conditions will also contribute to the positive albedo feedback loop in the Arctic Region, which means an additionally higher absorption of energy imported by solar radiation due to the loss of reflecting sea-ice.

The effects of climate change on the Arctic Region create a new environment with new abiotic conditions, the prevalent ecosystem has to learn to cope with. Observed increases in Arctic water temperatures, associated with a decrease in sea-ice load and salinity, affects organisms of all trophic levels, especially the primary producers such as phytoplankton species. Their bloom`s intensity and period depends on these factors what may bring consequences for the ecological relations, connected with phytoplankton.

[*] http://earthobservatory.nasa.gov/Features/WorldOfChange/sea_ice.php (May 22nd, 2014)

2.2 Arctic Ecosystem

2.2.1 Marine Arctic Food Web

In the last decades, the understanding of the Arctic pelagic food web underwent a change. Former assumptions used a food chain model to describe interactions between different trophic levels. Therein, the members are divided into primary producers (phytoplankton), secondary producers (zooplankton) and tertiary producers (higher organisms like whales). But recent examinations, especially focused on the importance of small sized organisms such as plankton, suggested a more complex coherence, leading to a food web concept.

The model of energy and carbon fluxes in the Arctic marine food web sees walruses, grey whales and seals as part of the main tertiary producers. They feed on benthos, a community of organisms living near or on the seafloor, including macrobenthos (e.g. red algae, brown algae, Crustacean, scallops, Gastropoda, benthic fish and Annelida) as well as microbenthos (e.g. Copepoda, Nematoda, and Foraminifera). The benthos community again depends on the presence of ice algae (Piepenburg, Bluhm 2009). By sedimentation, dead cells or aggregates of ice algae sink down and serve as nutriment for the benthos. Other tertiary producers are sea birds, pelagic fish, and whales (minke, bowhead whales). In contrast to the conceptional main tertiary producers, these animals feed on multicellular zooplankton, whose presence depends on the prevalent unicellular protist community within the euphotic zone. This community of so called primary producers is mainly built of phototrophic phytoplankton, using solar radiation and CO_2 for primary production.

Due to modern climatic changes, causing temperature increase and enhanced ice melting, it is assumed that the dominant primary producers such as ice algae will be more and more replaced by pelagic Protista, e.g. phytoplankton species. This represents a community shift, which may affect higher tropic levels as well. Additionally to the shift within the Arctic ecosystem, there may be an increased migration of Subpolar species because of the new moderate conditions.

2.2.2 Protista Diversity and Ultraplankton

The kingdom of Protista (according to Robert Whittaker's five kingdom classification) includes eukaryotic microorganisms with unicellular, colonial, filamentous or parenchymatous forms of organization. Only for reproduction, vegetative tissue is formed. It is suggested that Protista represent the common ancestor of multicellular organisms. Despite the evidence that the group of Protista is not monophyletic, the term is still used in the systematic of life. Due to the fact that the kingdom of Protista includes distantly related phyla, a wide diversity of nutrition strategies (autotrophy, heterotrophy or mixotrophy) and morphologies (e.g. size classes: micro-, nano- and pico-protista) can be found.

Within the last decades, the taxonomic classification of Protista underlies an extensive modification due to the use of modern molecular phylogenetic analyses (e.g. DNA sequencing). Based on these approaches, a new scheme consisting of eight protist soupergroups is defined (Fig. 2), including Opisthokonta and Amoeboza, Archaeplastida, Stramenopila, Alveolata and Rhizaria, Excavata and Discicristata.

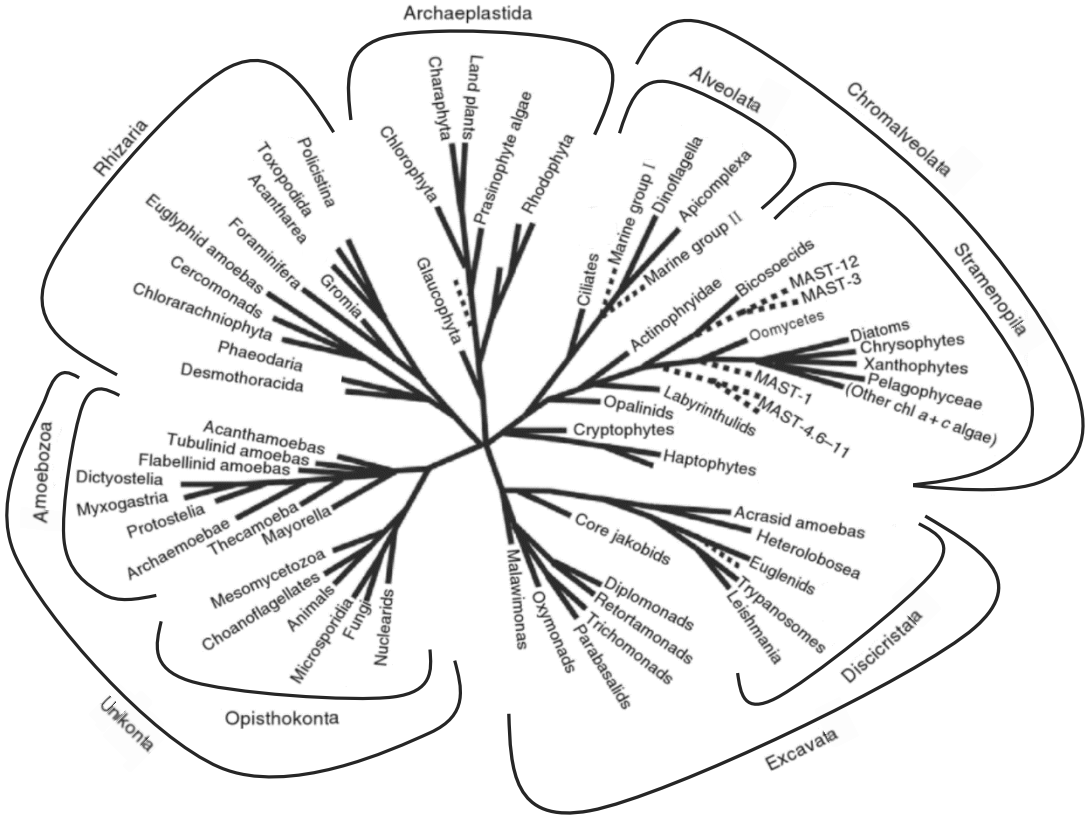


Fig. 2: Phylogenic Characterization of Eukaryotes into Supergroups [2]

The supergroups Opisthokonta and Amoebozoa are often combined using the term Unikonta. For the supergroups Stramenopila, Alveolata and Rhizaria the abbreviation SAR is utilized. Chromalveaolata is a broader term for the groups Stramenopila and Alveolata, additionally including the divisions Haptophyta and Chryptophyta, which both branched off early in the evolution of SAR (Thomas Cavalier-Smith biological classification). The supergroup of Discicristata is often among Excavata.

Within all supergroups of Protista, heterotrophic nutrition modes can be found. Heterotrophic organisms use organic carbon for their growth and draw on light (photoheterotrophic) or inorganic/organic carbon (chemoheterotrophic) as energy source. Heterotrophy is especially characteristic for Unikonta, where **Opisthokonta** groups animals, fungi, choanoflagellates and mesomycetozoa, while the supergroup **Amoebozoa** includes Amoeba, Amoeba-flagellates and smile moulds. The supergroup **Rhizaria** consists of heterotrophic Foraminifera and Radiolaria. The Ciliata subdivision of **Alveolata** is obligate heterotrophic as well. Within the **Stramenopila,** heterotrophic marine Stramenopila (MAST) are present. The supergroups **Discicristata** and **Excavata** are mostly built of heterotrophic flagellates. Heterotrophic modes can also be found in the divisions Haptophyta and Chryptophyta.

Another widespread trophic mode within the Protista is autotrophy. Here, carbon dioxide or other simple compounds are used for the synthesis of complex organic compounds. As energy source, autotrophic organisms utilize either light (photoautotroph) or inorganic substances (chemoautotroph) for their metabolic reactions. The lineages of land plants, Rhodophyta, Chlorophyta (e.g. *Micromonas* sp.) and Glaucophyta, belonging to the supergroup **Archaeplastida,** are mainly characterized by obligate photoautotrophy. Other supergroups of Protista include mostly phototrophic subgroups like Chlorarachniophyta within **Rhizaria**, Dinoflagellata within **Alveolata** and Diatoma within the supergroup **Stramenopila** (e.g. *Chaetoceros* spp.) and many species within the divisions Haptophyta and Chryptophyta. Beside the heterotrophic organisms of the supergroups **Discicristata** and **Excavata,** photoautotrophic representatives can be found (e.g. euglenoids) as well. It has to be cautious, that some organisms of the Protista subgroup Chlorophyta (e.g. *Pyramimonas spec.),* Dinofagellata (e.g. *Ceratium* spp., *Prorocentrum* spp.., *Alexandrium* spp.), Diatoma (e.g. *Odontella* spp., *Thalassiosira* spp.) and the division Chryptophyta have a mixotrophic state, using both, heterotrophic and autotrophic strategies.

Protista and some prokaryotic representatives, gainig energy by sunlight, are summarized to the broader term phytoplankton. In general, plankton is described as free living organisms (viruses, bacteria, microalgae and animals) whose movements are controlled by currents, turbulent processes or even molecular diffusion and not by their own motility. According to Lohmann (1903), plankton, as well as phytoplankton, can be divided by its size into net plankton (> 20 µm) and ultraplankton (< 20 µm). Latter one is moreover seperated into: *femtoplankton* (< 0.2 µm, e.g. viruses, heterotrophic bacteria), *picoplankton* (0.2 – 2.0 µm, e.g. Cyanobacteria, Chlorophyta, heterotrophic Flagellata and Amoeba) and *nanoplankton* (2.0 – 20.0 µm, e.g. Diatoma, Dinoflagellata, Haptophyta, Chlorophyta, heterotrophic Flagellata, Amoeba and Ciliata) (Thomas *et al.* 2008, p. 147). Untill the 1980s, less than 5000 marine phytoplankton species were discovered, in which Diatoma (40 %), Dinoflagellata (40 %), Haptophyta (10 %) and Chlorophyta (6 %) are the most abundant eukaryotes.

The important contribution of ultraplankton to the Arctic marine ecosystem as a main primary producer was noticed in the last decades. Gosselin *et al.* (1997) and Lee & Whitledge (2005) showed in their studies that more than 50 % of the total phytoplankton biomass and productivity are built up of small size fraction cells in the Arctic as well as in the Antarctic. With this, they proofed that the distribution of ultraplankton in the Polar Regions resembles their ubiquitous in other oceanic areas. A reason for its success may be the high volume to surface ratio of small sized cells. This enables a rapid exchange of dissolved nutrients (e.g. nitrate, ammonium, nitrite, phosphate, iron and zinc) (Riebesell and Wolf-Gladrow, in Williams *et al.* 2002), especially in a cold environment with less turbulences.

2.2.3 Phytoplankton Key Species – *Emiliania huxleyi*, *Micromonas pusilla* and *Phaeocystis pouchetii*

Several species of ultraplankton dominate the Arctic and Subarctic plankton community. Thereunder, around 40 species of nine algal classes (Chlorophyceae, Prasinophyceae, Trebouxiophyceae, Prymnesiophyceae, Bolidophyceae, Eustigmatophyceae, Pinguiophyceae, Bacillariophyceae, and Pelagophyceae) are photoautotroph (Vaulot *et al.* 2003).

1.0 µm

Fig. 3: Scannin Electron Microscopy (SEM) Image of *Emiliania huxleyi* [3]

The marine nanoplanktonic species ***Emiliania huxleyi*** (Lohmann) W. W. Hay & H. P. Mohler (3.0 – 5.0 µm) is one representative, indigenous in all world oceans. In Subarctic waters, they can make up to 100 % of the total Coccolithophore community. Belonging to the phylum Haptophyta, algal class Prymnesiophycea, order Isochrysidales, family Noëlaerhabdaceae it is a major provider of carbon for the global carbon cycle. *E. huxleyi* is able to build up organic compounds out of inorganic molecules such as CO_2 and carbonate via photosynthesis and calcification. Latter process is highly dependent of environmental conditions. The availability of light, nutrients, trace metals and CO_2 as well as the ambient temperature has to be mentioned here (Paasche, 2002; Shiraiwa, 2003; Zondervan, 2007). Calcification has several advantages for the species *E. huxleyi.* On the one hand the main product calcium carbonate is released and forms an exoskeleton (coccolith), protecting the cells from viruses, grazers and excessive light irradiation (Raven & Crawfurd, 2012) (Fig. 3). On the other hand, the byproduct CO_2 can be used for the CO_2 concentrating mechanisms (CCM). This mechanism is found in photoautotrophic plankton to improve the assimilation of CO_2 which is limited by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (Giordano *et al.* 2005). Scince its low affinity to the co-substrate CO_2, RuBisCO requires a higher intracellular CO_2 concentration than achievable by atmospheric CO_2. The life cycle of *E. huxleyi* is dominated by a haploid and a diploid stage with heteromorphic cells. While the haploid cells are non-scalled flagellates, the diploid cells are coccolith-bearing without flagella. Rapid increases in the population of algae are called blooms ($\geq 10^6$ cells/L). By bottom up controls (e.g. nutrients, irradiance or stratification) a bloom of *E. huxleyi* is induced, especially in spring. Massive blooms of this algae are observed in the Eastern Bering Sea and Bering Straits (1997) and in the North Atlantic south of Iceland (1991) and can reach dimensions of 200,000 km^2 to 250,000 km^2 (Sukhanova & Flint 1998, Holligan *et al.* 1993). Fortunately, the blooms of *E. huxleyi* do not rank among the harmful algae blooms (HABs), which can produce toxic substances and so no poisoning effects to humans by intoxicated see food products such like fish and oysters can occur (HABs consequences described by Hoagland *et al.* 2002).

However, the massive blooms of *E. huxleyi* can have a big impact on the regional environment. One effect of such an *E. huxleyi* bloom is the increased reflection of solar radiation by the upper water layer due to the higher albedo (Tyrrell *et al.* 1999) and increased turbidity. Here, the trapping of light and heat at the surface is increased, while deeper water layers are exposed to decreased light and heat conditions. Other effects are a changed CO_2 uptake of the ocean (Rost & Riebesell 2000, 2004) and a lifted release of dimethylsulfide (DMS) to the atmosphere by *E. huxleyi* (Steinke *et al.* 2002). Driving cloud condensation, the oxidation products of DMS can have a massive influence on the global climate (Thomas *et al.* 2008, p. 156).

Another phytoplankton key species, inhabiting several oceanic and coastal waters such as the Central California Ocean, Mediterranean and Norwegian Sea, is the Chlorophyta ***Micromonas pusilla*** (Butcher) I. Manton & M. Parke (Throndsen *et al.* 1994, 1969; Thomsen *et al.* 1998) (Fig. 4). It is the only member of the genus *Micromonas* and belongs to the class Prasinophyceae, order Mamiellales, family Mamiellaceae. Other genera, appertaining to this family are *Bathycoccus* and *Ostreococcus.* Unlike other phytoplankton species, *M. pusilla* does not form massive blooms. A strictly Arctic clone (CCMP 2099) of *M. pusilla* was recently described by Lovejoy *et al.* (2007), isolated in the Baffin Bay. By the help of 18S rDNA analyses, different *Micromonas* clones were assigned to five phylogenetic clades (A-E). Therein, the clone CCMP 2099 builds a subclade named clade Ea. The genetically features of this Arctic clone disables its growth at temperatures above 12.5 °C (Lovejoy *et al.* 2007). In 2009, another *M. pusilla* clone (RCC 2306) was isolated from the Beaufort Sea by Vaulot, which can be also affiliated to the Arctic clade (http://roscoff-culture-collection.org/rcc-strain-details/2306, February 20th, 2014). Based on the observation these Arctic clades, the study of Kilias *et al.* (2013) describes the abundance of *Micromonas* spp. in the western Fram Strait using 454-pyrosequencing analysis of several transects. Here, *M. pusilla* builds up to 41 % to 57 % of the Chlorophyta community and 3.6 % to 5.2 % of the total biosphere.

Fig. 4: Lightmicroscopic Image of *Micromonas pusilla* (RCC 114) [4]

A third phytoplanktonic Arctic key species is the algae ***Phaeocystis pouchetii***. Belonging to the phylum Haptophyta, class Coccolithophyceae (subclass Prymnesiophyceae), order Phaeocystales, family Phaeocystacea, it is part of the genus *Phaeocystis* which was introduced by Lagerheim (1893/1896). To date, six species of *Phaeocystis* are known due to small subunit (SSU) rDNA analysis (Medlin 1994, Lange 2002) and morphological characterization: *P. pouchetii* (Hariot) Lagerheim, *P. globosa* Scherffen, *P. antarctica* Karsten, *P. jahnii* Zingone, *P. scrobiculata* Moestrup, *P. cordata* Zingone et Chrétiennot-Dinet. Each of them evolved adaptions to specific environmental conditions which leads to the distribution of *Phaeocystis* spp. from the tropics to the poles. As a cold adapted representative, *P. pouchetii* is only found in Arctic and Subarctic Regions north of 60 °N (Lagerheim 1896) and has its temperature optimum for growth below 5 °C (Schoemann *et al.* 2005). *P. pouchetii* forms massive blooms. During the prä- and post bloom stage (Fig. 5 A), the cells (approx. 5 µm in diameter) of *P. pouchetii* are motile (due to the existence of a flagellum) and scaled (calcium carbonate exoskeleton). While blooming, *P. pouchetii* lose this morphological features and forms colonies (1.5 mm – 2.0 mm in diameter) with cloud-like structures.

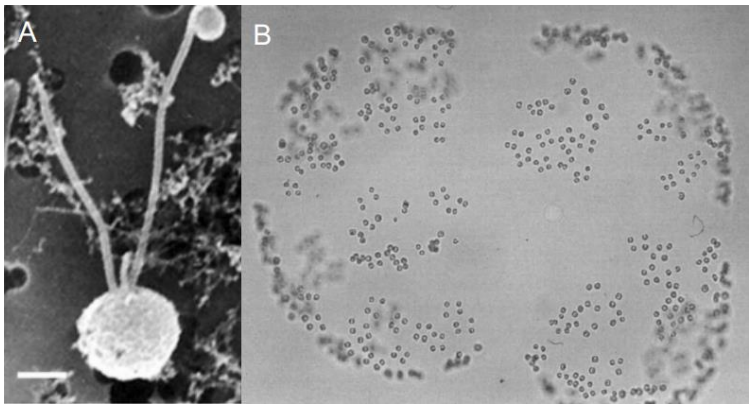


Fig. 5: Morphology of *Phaeocystis* spp. [5]

A Scanning Electron Microscopy (SEM) of *Phaeocystis* spp. flagellate; scale bar = 1 µm.

B Light Microscopy of *P.pouchetii* colony with cloud-like structure; scale bar is missing.

This morphological change can be observed for *P. globosa* as well. Due to this and other genetic accordances, it is assumed that *P. pouchetii* evolved out of *P. globosa* (Medlin *et al.* 1994), a strain not found in the Polar Regions (Schoemann *et al.* 2005). A mucilaginous matrix surrounds the colony cells (5.0 µm – 7.0 µm) and thereby forms a cloud-like structure (Fig. 5 B). The function of this mucus is still not clear. It may have a protection role against grazers by acrylate accumulation (Noordkamp *et al.* 2000), pathogens (Jacobsen *et al.* 1996), harmful environmental conditions or functions as a nutrient and energy reservoir (Lasternas *et al.* 2010).

For its biotic environment, the mucus can have toxic effects e. g. for fish and intervenes the propagation of shellfish and macrozooplankton (Lasternas *et al.* 2010, Davidson & Marchant 1992). Beside the influence of *P. pouchetii* to higher organisms, it exerts influence to other phytoplankton species by its colony forming. This ability reduces the grazing events on *P. pouchetii* by organisms of higher trophic levels (like copepods of the species *Acartia*), which was shown by Verity & Smayda (1989). By this means, other phytoplankton organisms come into the focus of grazers, which leads to a reduced competitive nutrient situation for *P. pouchetii.* Several studies showed occurrences of *P. pouchetii* in the European Subarctic Front Zone (Markowski & Wiktor 1998) and the Barents Sea (Ratkova & Wassmann 2002). Schoemann *et al.* (2005) observed recurrent blooms in the Fram Strait where *P. pouchetii* can be found in WSC and EGC water masses.

Other planktonic species are abundant in the Polar and Subpolar Regions as well. Thereunder, representatives of the phylum Stramenopila (e.g. *Chaetoceros sicialis* resp. *Thalassiolsira* spp. (Gradinger & Baumann 1991, Kilias *et al.* 2013) have to be mentioned.

2.3 Methods for Taxonomical Classification of Marine Protista Communities

2.3.1 Traditional Methods

Before the era of molecular genome analysis began, traditional methods such as light, epifluorescence and electron microscopy were applied for the insight into the enormous diversity of marine microbial communities. These techniques employ on the detection of morphological cell features and use them for taxonomical classification. However, studies concentrating on small sized cells (e.g. picoplankton), which are mostly homomorphic in their forms, cannot make recourse of these optical methods for classifications down to species level (Thomsen & Buck 1998, Zingone *et al.* 2006). Another entrenched method of choice for studies, dealing with the detection of picoplankton diversity, is the high-performance liquid chromatography (HPLC), detecting specific cell pigments. Here, the taxonomic identification of community members is possible down to class level, but not below (Guillou *et al.* 1999). This is due to the fact, that most of the phytoplankton pigments, detected by HPLC, are shared between different orders and families (Massana *et al.* 2002). To enable the identification of species not observable via microscopy and HPLC, molecular tools have to be used, detecting nucleic acid sequences.

2.3.2 Genetic Targets – 18S rDNA

As a genetic target, suitable to distinguish between two species, DNA sequences which can be found in all organisms are required. Fulfilling this need, genes of the eukaryotic and prokaryotic expression system (e.g. 18S rDNA, 16S rDNA and their transcripts) represent a possible target for molecular detection methods. This principle was firstly described by Doi & Igarashi (1965) and Dubnau *et al.* (1965). Additionally to their ubiquitous status, ribosomal genes have another advantage for this purpose. They are built up of conserved and variable regions, what enables the design of molecular probes for the separation of genera or lower taxonomic levels (Ebenezer *et al.* 2012). Additionally, they are relatively large in size and no lateral gene transfer was observed between them (Woese 1987).

The most commonly used gene sequence for eukaryotic phylogeny studies is the **18S rDNA** (Chenuil 2006). It encodes for the small subunit (SSU) of the ribosome and is approximately 1800 bps in size, what enables statistically proofed screenings, compared to smaller genes (Sogin *et al.* 1986). Within the 18S rDNA sequence, nine hypervariable regions (V1 – V9) are located, of which V4 is the largest (230 bps – 500 bps) and most complex one (Neefs *et al.* 1993). Applied in taxonomic studies with diatoms (Zimmermann *et al.* 2011) and dinofagellates (Ki 2012), the 18S rDNA found its way into diversity examinations of nanoplanktonic communities.

2.3.3 Molecular Methods – Quantitative Polymerase Chain Reaction

Since molecular probes broadened the possibilities of taxonomical classification, studies on picoplankton diversity have increased in the last decades (Giovannoni *et al.* 1990, López-Garcia *et al.* 2001, Medlin *et al.* 2006). Beside identification of algae species via specific toxins or carbohydrates, methods targeting nucleic acids are preferred. Here, it can be distinguished between hybridization- & polymerase chain reaction (PCR) based approaches. Former includes techniques such as fluorescent in situ hybridization (FISH) (Eller *et al.* 2007), ribonucleic acid biosensors (Metfies *et al.* 2005) and DNA-microarrays (Metfies *et al.* 2004). Latter ones (described by Ebenezer *et al.* 2012) use restriction fragment length polymorphism (RFLP), denaturating gradient gel electrophoresis (DGGE), single-stranded conformation polymorphism (SSCP), random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellites and quantitative PCR (qPCR) .

PCR-based methods imply several advantages such as versatility, sensitivity, specificity and reproducibility in one batch (Saiki *et al.*, 1988; Cha *et al.*, 1993).The principle of PCR is based on the *in vivio* replication (semi-conservative) of DNA. Thereby a mixture of all four deoxynucleoside triphosphates (dNTPs), a single-stranded primer pair (complementary to DNA-target regions) and a thermo stable DNA-polymerase are provided in vitro. A Buffer, containing Mg^{2+} and other enzyme stabilizing reagents establishes the optimal conditions for the catalytic reactions, performed in the PCR. One PCR cycle is characterized by temperature shifts, creating different phases: In the denaturation phase, the double-stranded DNA (dsDNA) target is divided into two single-stranded DNA (ssDNA) molecules by breaking the hydrogen bonds between the complimentary bases at 93 °C – 96 °C. The second phase is the annealing phase. Here the primers (typically 15 – 25 nucleotides (nts) long) bind to complementary target sites, providing free 3'-OH groups for the enzymatic replication. The temperature adjusted for this process has to lie 2 °C – 10 °C beneath the melting temperature (T_m) of the primer pair.

Within the last phase, the primers are elongated by a DNA-polymerase according to complementary base pairing to the DNA-target. For this step, the DNA-polymerase needs Mg^{2+} as co-factor and the optimal temperature (e.g. 75 °C – 80 °C for Taq-polymerase from *Thermus aquaticus).* A standard PCR protocol consists of 35 – 40 of the mentioned cycles and ends up with an enlarged elongation phase (mostly 5 min.). To heighten the success and specificity of the reaction, several additives such as bovine serum albumin (BSA), which binds inhibitors (Woide *et al.* 2010) and dimethyl sulfoxide (DMSO, 1 % - 10 %), decreasing the formation of secondary structures (Mamedov *et al.* 2008) within target DNA and primers, can be added. With every PCR cycle, the DNA-target of the starting point is (theoretically) doubled, what leads to an exponential increase in DNA over the entire PCR.

As a further development of the conventional PCR, the **qPCR** has to be mentioned. Used for quantifying nucleic acids and genotyping, qPCR enables the detection of amplified products by an increase in fluorescence, caused by compounds interacting with the target DNA. By this, the DNA concentration of each cycle of a qPCR can be monitored online, compared to a standard PCR, where only the final DNA concentration can be measured (Heid *et al.* 1996). Beside the more complex and expensive (Giulietti *et al.*, 2001) fluorescent oligonucleotide probes (e.g. molecular beacons, TaqMan®), based on the fluorescence resonance energy transfer (FRET), the intercalating reagent SYBRgreen I can be used.

When bound to the minor groove of dsDNA, SYBRgreen I has a 1000-fold higher fluorescence intensity than as dissolved molecule. SYBRgreen I emits light at a maximum of 520 nm (green light). Therefore, a stimulus with light of 480 nm (blue light) is required (Jin *et al.* 1994). The monitoring of current fluorescence has to be performed in the end of elongation during the qPCR. Advantages of the qPCR compared to the conventional reaction are the wide range of template DNA concentration (Schmittgen *et al.* 2000) and quantification. The main disadvantage of the qPCR (as well es for standard PCR) is the possibility of unspecific primer annealing, followed by the amplification of non-target sequences. To control the amplicon homology, a melting curve at the end of the qPCR is obtained by gradually increasing the temperature up to 95 °C. At the T_m of the target amplicon, which is specific for the amplicon sequence, the fluorescence drops down abruptly because SYBRgreen I is released out of the divided dsDNA. The slope of this curve is mathematically derived and so converted to a peak curve. By the presents of several unspecific amplicons, more than one peak is visible.

For a successful, specific and reproducible qPCR using SYBRgreen I, some requirements have to be fulfilled (Rodríguez-Lázaro & Hernández 2013): The used primers should exhibit a GC-content between 30 % and 80 %, a primer length between 15 bps and 30 bps. Additionally, a maximum amplicon size of 150 bps (minimum 50 bps) should be ensured. To minimize the effects of pipetting errors, a fluorophore, acting as passive reference can be added to the reaction buffer. The signal, gained by the reporter (e.g. SYBRgreen I) is divided by the reference fluorophore signal, resulting in Rn (normalized ratio). This ratio is defined in the first cycles of the qPCR (Rn^-) and at its end (Rn^+). Building the difference between Rn^+ and Rn^- the ΔRn value is obtained. ΔRn, which is proportional to the concentration of DNA during the exponential phase, can be used to describe the magnitude of the generated signal of the prevalent qPCR conditions.

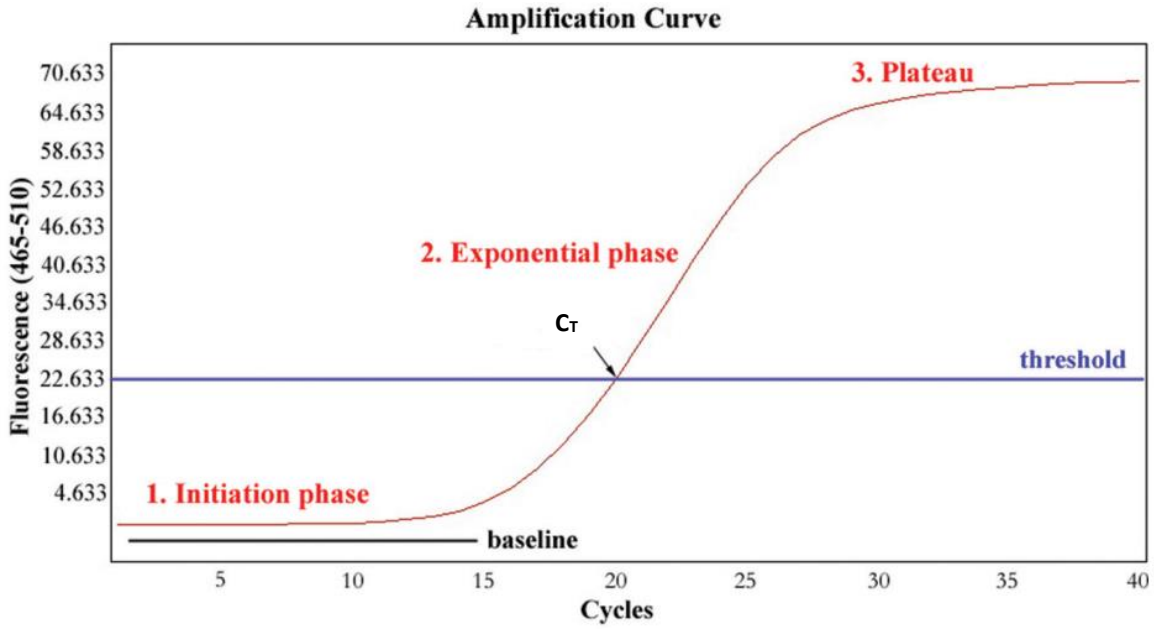


Fig. 6: Course of Positive qPCR`s Amplification Curve [6]
Delineated are the phases of amplification (initiation, exponential and plateau) as well as the baseline, the threshold and the C_T value

The course of a positive qPCR fluorescence curve is shown in Fig. 6. Three different phases can be seen. First, the initiation phase occurring in the early cycles of qPCR, where the fluorescence signal does not contrast from the baseline (the fluorescence, detected within the first cycle). The second phase is characterized by an exponential increase of fluorescence before ending up in the plateau phase (third phase). Only in the exponential phase, a quantification is possible, since the optimal template to reagents ratio is adjusted here, resulting in most efficient amplification.

To distinguish between signal and noise (represented by baseline), a threshold has to be set. This happens by multiplying the average standard deviation of Rn for the baseline with an adjustable factor (usually ten) or manually by the operator. Thereby, it is important that the threshold lies in the exponential phase (see reasons above). The cycle of a qPCR, where the amplification-associated fluorescence crosses the threshold for the first time is called threshold cycle (C_T). This point correlates inversely to the DNA concentration at the beginning of the qPCR (Walker 2002). By using either absolute quantification (integrating the target C_T value into a curve of several C_Ts standards with different amounts of DNA) or relative quantification (comparing target C_T value with one standard C_T), the source DNA amount can be obtained.

3. MATERIAL

3.1 Equipment and Consumables

Table 1: Specification of used Equipment i.e. Device Designation, Manufacturer and Serial Number

Equipment	Specifics	Manufacturer	Serial Number
Vortex mixer	Type: *VF2*	Janke & Kunkel [1]	723018
Centrifuges	Heraeus Fresco 17 Centrifuge	Thermo electron [2]	37520
	Centrifuge *5810 R* (Rotor: *A-4-62*)	Eppendorf [3]	5811Z0561522
	Mini-centrifuge *SPROUT*™	Heathrow Scientific [4]	HSA00686
	Rotilabo® -mini-centrifuge (butterfly-rotor)	Roth [5]	028019
Electrophorese chambers	Biozym EasyPhor Maxi	Biozym [4]	BC100719006
	Biozym EasyPhor Mini		MS08011670
Electrophorese power supply	Type: *E122*	Consort [5]	62330
Laboratory balance	Acculab Balance, model: *VIC-212*	Acculab [6]	24803175
pH meter	pH 197	WTW [7]	71184096
Pipettes (Eppendorf Research)	Volume: 100 - 1000 µL	Eppendorf	239393Z
	Volume: 10 - 100 µL		355938Z
	Volume: 0.5 - 10 µL		238757Z
Pump	Type: *NO35_AN.18* (max P = 4 bar)	KNF Neuberger [8]	1217795
qPCR Cycler	7500 Fast Real-Time PCR System	Applied Biosystems - Life Technologies [9]	275014422
Thermo Cyler	Mastercycler gradient	Eppendorf	5331_04456
Thermoshaker	Model: *TS-W*	Schutron Laborgeräte [10]	3298
Sequencer	3130x/ Genetic Analyzer	Applied Biosystems - Life Technologies	18225-006
UV Instrument	UV-transiluminator, model: *TFX36.M* (312 nm, 10600 µW/cm2)	Vilber Lourmat [11]	M034666
	UV-darkroom, model: *CN-TFX.36*		M034693
	CCD camera		V034693

[1] IKA - Janke & Kunkel GmbH & Co. KG; 29219 Staufen, Germany
[2] Thermo electron LED GmbH; 37520 Osteroden, Germany
[3] Eppendorf AG; 22331 Hamburg, Germany
[4] Biozym Scientific GmbH; 31840 Hessisch Oldendorf, Germany
[5] Consort nv; 2300Turnhout, Belgium
[6] Acculab GmbH; 37073 Göttingen, Germany
[7] WTW GmbH; 83262 Weilheim, Germany
[8] KNF Neuberger GmbH, 79122 Freiburg, Germany
[9] Life Technologies Corporation; Foster City, CA 94404, USA
[10] Schutron CLF Laborgeräte GmbH; 69115 Heidelberg, Germany (closed company)
[11] Vilber Lourmat GmbH; 88436 Eberhardzell, Germany

Table 2: Specification of used Consumables i.e. Device Designation, Manufacturer, Reference and Serial Number

Consumables	Specifics	Manufacturer	Reference Number	Lot Number
Cell scraper	16 cm	Sarstedt Inc. [1]	83.1832	3030400
Film applicator	MicroAmp® Adhesive Film Applicator	Applied Biosystems - Life Technologies [2]	4333183	5G06A12-3B
Filter membrane	ISOPORE™ Polycarbonat Membrane Filters Type: 0.4 µm HTTP	Millipore [3]	HTTP04700	R1SA58520
Microtiter plate	MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode (0.1 mL)	Applied Biosystems - Life Technologies	4346906	I3453Q114
Microtiter plate film	MicroAmp® Optical Adhesive Film	Applied Biosystems - Life Technologies	4311971	201207115
Petri dishes	94 x 16 mm (plastic)	Greiner-Bio-One [4]	633180	G130911L
Pipette tips (sterile, aerosol)	Volume: 100 - 1000 µL	VWR [5]	732-0543	10094-137C6-136F
	Volume: 1 - 100 µL		732-0523	0096-036C4-032D
	Volume: 0.1 - 10 µL		732-0516	1065-125C4-125H
Reaction tubes	Safe-Lock Tubes 2.0 mL	Eppendorf [6]	0030120.094	C152354N
	Safe-Lock Tubes 1.5 mL		0030120.086	C151817K
	Safe-Lock Tubes 0.5 mL		0030121.023	C153134O
	PCR Tube Strips 0.2 mL		951010022	C150251G
Serological pipettes	Volume: 50 mL	Sarstedt AG [7]	86.1689.001	13042101
	Volume: 5 mL		86.1253.001	2285 E

[1] Sarstedt Inc.; Newton, NC 28658, USA
[2] Life Technologies Corporation; Foster City, CA 94404, USA
[3] Millipore Corporation; Billerica, MA 01938, USA
[4] Greiner-Bio-One GmbH; 4550 Kremsmünster, Austria
[5] VWR-International LLC.; Radnor, PA 19087, USA
[6] Eppendorf AG; 22331 Hamburg, Germany
[7] Sarstedt AG & Co.; 51588 Nürnbrecht, Germany

3.2 Commercial Kits

Table 3: Specification of used Commercial Kits i.e. used Contents, Manufacturer, Reference and Serial Number

Kit	Used Contents	Manufacturer	Reference Number	Lot Number
NucleoSpin® Gel and PCR Clean-up	Binding Buffer NTI Wash Buffer NT3 (concentrate*) Elution Buffer NE Gel and PCR Clean up Columns (yellow)	Macherey-Nagel [2]	740609.250	1301/005
NucleoSpin® Plant II (250 preps) *[1]	Lysis Buffer PL1 Binding Buffer PC Wash Buffers PW1, PW2 (concentrate*) Elution Buffer PE Rnase A (lyophilized**) Filter Column (violet) Plant II Column (green)	Macherey-Nagel	740770.250	1303/003

Continuation of Table 3:

Kit	Used Contents	Manufacturer	Reference Number	Lot Number
Phusion ® High-Fidelity DNA-Polymerase	Phusion DNA-Polymerase (2000 U/mL) GC-Buffer (5 x) (^) DMSO (100 %) Mg^{2+} (50 mmol/L)	New England Biolabs [3]	M0530S	0051309
REPLI-g® Mini Kit (25) *[2]	REPLI-g Mini Reaction Buffer REPLI-g Mini DNA-Polymerase Reconsituted Buffer DLB Stop solution	Qiagen [4]	150023	145044902
Rneasy® Plant Mini Kit (50)	RNase-Free Water	Qiagen	74904	145041286
7500 Fast Real-Time PCR System	Spectral Calibration Kit I	Applied Biosystems - Life Technologies [1]	4360788	12041616
	Spectral Calibration Kit II		4362201	1204083

[2] NEB - New England Biolabs Inc.; Ipswich, MA 01938, USA

[3] Qiagen GmbH; 40724 Hilden, Germany

[4] Peqlab Biotechnologie GmbH; 91052 Erlangen, Germany

[5] Life Technologies Corporation; Foster City, CA 94404, USA

** Dissolve 15 mg in 1500 µL H_2O

*[1] Carried out protocol: Green algae (not specified)

*[2] Carried out protocol: Amplification of purified DNA

(^) 1.5 mmol/L $MgCl_2$ at the final [1 x] reaction concentration

3.3 Chemicals

Table 4: Specification of used Chemicals i.e. Molecular Formula, Purity, Manufacturer, Reference and Lot Number

Chemicals	Molecular Formula	Purity	Manufacturer	Reference Number	Lot Number
Acetic acid (glacial)	$C_2H_4O_2$	100%	Merck [1]	1.00063.2500	K25686663
Aluminum potassium sulfate dodecahydrate	$AlK(SO_4)_2$ x 12 H_2O	> 98 %	Sigma-Aldrich	237086	n. s.
Ammonium chloride	NH_4Cl	> 99.5 %	Sigma Chemicals [2]	A-4514	76H0605
Ammonium molybdate(VI) tetrahydrate	$(NH_4)_6Mo_7O_{24}$ x 4 H_2O	99.98 %	Aldrich	43,136	04824KK
Biotin (Vitamine H or B7)	$C_{10}H_{16}N_2O_3S$	≈ 99 %	Sigma-Aldrich	B4639	105K16801
Biozym LE Agarose	$C_{24}H_{38}O_{19}$	n. s.	Biozym [3]	840004	120212C274967L
Boric acid (Pufferan®)	H_3Bo_3	99.8 %	Roth [4]	6943.1	39837588
Bromphenol blue	$C_{19}H_{10}Br_4O_5S$	n. s.	Sigma-Aldrich	B-5525	31H-3655
Cacium nitrate tetrahydrate	$Ca(NO_3)_2$ x 4 H_2O	99%	Sigma-Aldrich	237124	n. s.
Cadmium nitrate tetrahydrate	$Cd(NO_3)2$ x 4 H_2O	n. s.	Sigma Chemicals	C-2786	44H100525
Chrome(II) nitrate heptahydrate	$Cr(NO_3)_3$ x 7 H_2O	99%	Sigma-Aldrich	239259	n. s.
Cobalt(II) chloride hexahydrate	$CoCl_2$ x 6 H_2O	n. s.	Sigma Chemicals	C-2911	115H3458
Cobalt(II) nitrate hexahydrate	$Co(NO_3)_2$ x 6 H_2O	> 98 %	Sigma-Aldrich	239267	n. s.
Copper(II) sulfate pentahydrate	$CuSO_4$ x 5 H_2O	≈ 99 %	Sigma Chemicals	C-3036	73H30426
Cyanocobalamin (Viatmine B12)	$C_{63}H_{88}CoN_{14}O_{14}P$	≈ 99 %	Sigma Chemicals	V-2876	69H1328
Dipotassium phosphate	K_2HPO_4	99%	Sigma Chemicals	P-8281	17H1328
Disodium molybdate dihydrate	Na_2MoO_4 x 2 H_2O	n. s.	Sigma Chemicals	S-6646	42H0865
Ethanol	CH_3CH_2OH	> 99.9 %	Th.Geyer [5]	2246.2500	V3L659253
Ethanol (denaturated, 1 % MEK p. A.)	CH_3CH_2OH	99%	AppliChem [6]	A4930,2500	3J004430
Ethylenediaminetetraacetic acid (EDTA)	$C_{10}H_{16}N_2O_8$	n. s.	AppliChem	A5097,0500	5K009423
Ethylenediaminetetraacetic acid disodium salt (Na_2-EDTA; Titriplex(III)®)	Na_2-$C_{10}H_{16}N_2O_8$ x 2 H_2O	> 99 %	Sigma-Aldrich [7]	E5134	095K0204
GelRed (10,000 x in water)	$C_{60}H_{72}I_2N_8O_5$	n. s.	Biotium [8]	41003	13G0306

Continuation of Table 4:

Chemicals	Molecular Formula	Purity	Manufacturer	Reference Number	Lot Number
Glycerol	$C_3H_8O_3$	99%	Sigma-Aldrich	G5516	103K0166
Hydrochloric acid (fuming)	HCl	37%	Merck	1.00317.2500	K29791617138
Iron(III) chloride hexahydrate	$FeCl_3$ x 6 H_2O	> 98 %	Sigma Chemicals	F-2877	126H1423
Magnesium sulfate heptahydrate	$MgSO_4$ x 7 H_2O	n. s.	Sigma-Aldrich	M-7774	86H076325
Mangan(II) sulfate monohydrate	$MnSO_4$ X 1 H_2O	n. s.	Sigma Chemicals	M-7899	115H0998
Mangesium(II) chloride tetrahydrate	$MnCl_2$ x 4 H_2O	> 99.7 %	Merck	1.05927.0100	A317127_141
Nickel sulfate hexahydrate	$NiSO_4$ x 6 H_2O	> 99 %	Merck	1.06727.0100	K27917827_122
Potassium bromide	KBr	> 99%	Sigma-Aldrich	243418	n. s.
Potassium chromate	K_2CrO_4	≈ 99,9 %	Sigma Chemicals	P-0454	38H0561
Potassium iodide	KI	> 99%	Sigma-Aldrich	P-4286	n. s.
Selenious acid	H_2SeO_3	≈ 98 %	Aldrich Chemical [9]	21,117-6	n. s.
Sodim metavanadate	$NaVO_3$	≈ 90 %	Aldrich Chemical	28,936-1	14720CR
Sodium bicarbonate	$NaHCO_3$	99.7 %	Riedel-de Haën	31437	92720
Sodium dihydrogen phosphate dihydrate	NaH_2PO_4 x 2 H_2O	n. s.	Sigma-Aldrich	S5012	35H2512
Sodium hydroxide solution	NaOH	> 98 %	Sigma-Aldrich	S-5881	122K0166
Sodium nitrate	$NaNO_3$	n. s.	Sigma-Aldrich	S5022	094K1210
Sodium silicate nonahydrate	Na_2SiO_3 x 9 H_2O	> 98 %	Sigma-Aldrich	S4392	055K0643
Sodium tungstate dihydrate	Na_2WO_4 x 2 H_2O	99%	Sigma-Aldrich	223336	n. s.
Thiamin (Vitamine B1)	$C_{12}H_{17}N_4OS$	n. s.	Sigma Chemicals	T-4625	59F0758
TRIS (Trizma® Hydrochloride)	$C_4H_{11}NO_3$	> 99 %	Sigma-Aldrich	T3253	025K5409
Vanadyl sulfate dihydrate	$VOSO_4$ x 2 H_2O	97%	Sigma-Aldrich	233706	n. s.
Zinc sulfate heptahydrate	$ZnSO_4$ x 7 H_2O	n. s.	Sigma Chemicals	Z-4750	52H0706

[1] Merck KGaA; 64271 Darmstadt, Germany
[2] Sigma Chemicals Co.; St. Louis, MO 63178, USA
[3] Biozym Scientific GmbH; 31833 Oldenburg, Germany
[4] Roth GmbH & Co. KG; 64271 Darmstadt, Germany
[5] Th.Geyer GmbH & Co. KG; 71272 Renningen
[6] AppliChem GmbH; 64271 Darmstadt, Germany
[7] Sigma-Aldrich Inc.; St. Louis, MO 63178, USA
[8] Biotium Inc.; Hayward, CA 94545, USA
[9] Aldrich Chemical Co., Inc.; Milwaukee, WI 53233, USA
n. s.: not specified

3.4 Buffers and Stock Solutions

Table 5: Composition of used Buffers and Stock Solutions

Buffers and Stock Solutions	Composition (final concentration)
Agarose gel electrophoresis	
Loading Dye	75 mmol/L EDTA; 33 % glycerol; 15 µmol/L bromphenol blue
TAE Buffer	40 mmol/L TRIS; 20 mmol/L acetic acid; 1 mmol/L EDTA; pH 8.0
Media	
Concentrate (K)	880 mmol/L $NaNO_3$; 36 mmol/L NaH_2PO_4 x 1 H_2O; 500 mmol/L Na_2SiO_3 x 9 H_2O; 50 mmol/L NH_4Cl; 1 mol/L TRIS; pH 7.2
Trace metal presolution (K)*	900 mmol/L $MnCl_2$ x 4 H_2O; 80 mmol/L $ZnSO_4$ x 7 H_2O; 30 mmol/L Na_2MoO_4 x 2 H_2O; 50 mmol/L $CoCl_2$ x 6 H_2O; 10 mmol/L $CuSO_4$ x 5 H_2O; 10 mmol/L H_2SeO_3
Vitamine presolution (°) (K, L1, F/2)	370 pmol/L cyanocobalamin; 2 nmol/L biotin
Concentrate (L1, F/2)	880 mmol/L $NaNO_3$; 36 mmol/L NaH_2PO_4 x 1 H_2O; 100 mmol/L Na_2SiO_3 x 9 H_2O
Trace metal presolution (L1)**	900 mmol/L $MnCl_2$ x 4 H_2O; 80 mmol/L $ZnSO_4$ x 7 H_2O; 80 mmol/L Na_2MoO_4 x 2 H_2O; 50 mmol/L $CoCl_2$ x 6 H_2O; 10 mmol/L $CuSO_4$ x 5 H_2O; 10 mmol/L $NiSO_4$ x 6 H_2O; 10 mmol/L K_2CrO_4; 10 mmol/L $NaVO_3$;10 mmol/L H_2SeO_3

Continuation of Table 5:

Buffers and Stock Solutions	Composition (final concentration)
Trace metal presolution (F/2)**	900 mmol/L $MnCl_2$ x 4 H_2O; 77 mmol/L $ZnSO_4$ x 7 H_2O; 26 mmol/L Na_2MoO_4 x 2 H_2O; 42 mmol/L $CoCl_2$ x 6 H_2O; 40 mmol/L $CuSO_4$ x 5 H_2O
Concentrate (Zehnder)	5 mmol/L $Ca(NO_3)_2$ x 4 H_2O; 110 mmol/L $NaNO_3$; 2 mmol/L $MgSO_4$ x 7 H_2O
Gaffron trace element solution (Zender)	50 mmol/L H_3BO_3; 9 µmol/L Na_2WO_4 x 2 H_2O; 1 mmol/L KBr; 1 mmol/L $ZnSO_4$ x 7 H_2O; 500 µmol/L $Co(NO_3)_2$ x 6 H_2O; 525 µmol/L $(NH_4)Ni(SO_4)_2$ x 6 H_2O; 100 µmol $VOSO_4$ x 2 H_2O; 10 mmol/L $MnSO_4$ x 2 H_2O; 710 µmol/L $(NH_4)_6Mo_7O_{24}$ x 4 H_2O; 500 µmol/L KI; 500 µmol/L $Cd(NO_3)_2$ x 4 H_2O; 500 µmol/L $CuSO_4$ x 5 H_2O; 100 µmol/L $Cr(NO_3)_3$ x 7 H_2O; 770 µmol/L $AlK(SO_4)_2$ x 12 H_2O
Iron-presolution [^] (x) (Zehnder)	33 mmol/L $FeCl_3$ x 6 H_2O
EDTA-presolution (x) (Zehnder)	56 mmol/L TitriplexIII
NaHCO3 solution (Zehnder)	100 mmol/L $NaHCO_3$
K2HPO4 solution (Zehnder)	90 mmol/L K_2HPO_4
Vitamin stock solution (Zehnder)	38 nmol/L thiamin; 7.4 nmol/L cyanocobalamin; 39 nmol/L biotin

* Add 37.42 g TitriplexIII and 3.16 g $FeCl_3$ x 6 H_2O to 1.0 L trace metal presolution for trace matel stock solution

** Add 4.38 g TitriplexIII and 3.16 g $FeCl_3$ x 6 H_2O to 1.0 L Trace metal presolution for trace metal stock solution

(°) Add 99,83 mg thiamin to 1.0 L vitamine presolution for vitamine stock solution

[^] Dissolve 9.0 g $FeCl_3$ x 6 H_2O in 1.0 L 0.1 N HCl

(x) Add 5 mL of Iron-presolution and 5 mL of EDTA-presolution up to 500 mL deion. H_2O to generate Iron-EDTA stock solution

3.5 Media

Table 6: Composition of used Media

Media	Stock Solutions	Volume of Stock Stock Solution for 1.0 L Media
K	Concentrate (K)	1.00 mL
	Trace element stock solution (K)	1.00 mL
	Vitamine stock solution (K)	1.00 mL
L1	Concentrate (L1)	1.00 mL
	Trace element stock solution (L1)	1.00 mL
	Vitamine stock solution (L1)	1.00 mL
F/2	Concentrate (F/2)	1.00 mL
	Trace element stock solution (F/2)	1.00 mL
	Vitamine stock solution (F/2)	1.00 mL
Zehnder	Concentrate (Zehnder)	50.00 mL
	Gaffron trace element solution (Zehnder)	0.50 mL
	Iron-EDTA stock solution	10.00 mL
	Vitamine stock solution (Zehnder)	0.50 mL
	NaHCO3 solution (Zehnder)	2.00 mL
	K2HPO4 solution (Zehnder)	2.00 mL

The concentrates as well as the vitamin stock solutions for the media K, L1, F/2 and Zehnder were sterile-filtered (0.2 µm) and added to the autoclaved (20 min, 121 °C, 2 bar) trace element stock solutions. The required pH was adjusted before sterilization process via HCl (10 %) and NaOH (10 %).

3.6 Microorganisms

The laboratory cultures were obtained from the National Center for Marine Algae and Microbiota (NCMA), formerly known as National Culture Collection of Marine Phytoplankton (CCMP), East Boothbay (Maine, USA) as well as the Roscoff Culture Collection (RCC), Roscoff (FR) and the Culture Collection of Algae and Protozoa (CCAP), Oban (UK). The explicit origin of these cultures is shown in Table 7.

Table 7: Used Eukaryotic and Prokaryotic Microorganisms, specified towards Origin, Strain Number and Collection Area

Phytoplankton Species	Culture Collection	Strain Number	Collection Area
Alexandrium minutum	CCAP	1119/48	Offshore East coast Scotland (Atlantic)
Bathycoccus prasinos	RCC	2486	English Channel (Atlantic)
Ceratium longipes	CCMP	1770	Gulf of Maine (North Atlantic)
Chaetoceros mülleri	CCMP	1316	Hawaii (North Pacific)
Chaetoceros socialis	CCMP	1579	North Sea (Atlantic)
Chrysochromulina ericina	CCMP	281	North Pacific (Pacific)
Emiliania huxleyi	RCC	1225	North Sea (Atlantic)
Microcystis aeruginosa	n. a.	n. a.	n. a. (freshwater organism)
Micromonas pusilla	CCMP	2306	Baffin Bay (Arctic)
Odontella aurita	CCMP	595	Caribbean Sea (North Atlantic)
Phaeocystis globosa	CCMP	1524	Indian Sea (Pacific)
Prorocentrum micans	RCC	3046	English Channel (Atlantic)
Pyramimonas parkeae	CCMP	724	Santa Catalina Island (North Pacific)
Thalassiosira weissflogii	CCMP	1010	Gulf Stream (North Atlantic)

In the following, only the strain numbers of the phytoplankton species *E. huxleyi*, *M. pusilla* and *P. globosa* are used as reference. The other species are only reffered to Table 7, using the taxonomic species classification.

Table 8 shows the taxonomic affiliation of the used strains.

Table 8: Taxonomic Classification of used Microorganisms

Taxonomy				
Kingdom	**(Phylum) Class**	**Order**	**Family**	**Strain**
Eukaryota	(Haptophyta) Prymnesiophycea	Isochrysidales	Noëlaerhabdaceae	*Emiliania huxleyi*
		Phaeocystales	Phaeocystaceae	*Phaeocystis globosa*
				Phaeocystis pouchetii
		Prymnesiales	Chrysochromulinaceae	*Chrysochromulina ericina*
	(Chlorophyta) Prasinophyceae	Mammiellales	Mammiellaceae	*Micromonas pusilla*
			Bathycoccaceae	*Bathycoccus prasinos*
		Pyramimonadales	Pyramimonadaceae	*Pyramimonas parkeae*
	(Dinophyta) Dinophycae	Gonyaulacales	Goniodomataceae	*Alexandrium minutum*
			Ceratiaceae	*Ceratium longipes*
		Prorocentrales	Prorocentraceae	*Prorocentrum micans*
	(Straminoplila) Coscinodiscophyceae	Chaetocerotales	Chaetocerotaceae	*Chaetoceros socialis*
				Chaetoceros mülleri
		Triceratiales	Triceratiaceae	*Odontella aurita*
		Thalassiosirales	Thalassiosiraceae	*Thalassiosira weissflogii*
Prokaryota	(Cyanobacteria) Cyanophyceae	Chroococcales	Microcystaceae	*Microcystis aeruginosa*

3.7 Samples of Nucleic Acid – Retrospective Treatment

Nucleic Acid from *P. pouchetii* Cultures

Cultures of the algae *P. pouchetii* were isolated from Dr. Steffi Gäbler-Schwarz during former Polar expeditions in the years 2010 and 2012. The explicit isolation sites (representing different water masses) are shown in Table 9. A map of the sampling region is shown in the appendix (Fig. 7).

Table 9: Culture Notation, Culture Number, Collection Site and Collection Year for *P. pouchetii* Isolates

Culture Notation	Culture Number	Collection Site	Collection Year
S018-03-D	2314	75.014 N; 11.276 W (EGC)	2010
Station 1-09	2977	65.039 N; 5.214 E (NASC)	2010
PS78/70_1	3039	78.835 N; 5.994 E (WSC)	2012
S194-01-B	2621	78.500 N; 0.234 E (RAC)	2010

EGC = East Greenland Current
WSC = West Spitzbergen Current
NASC = Norwegian Atlantic Slope Current
RAC = Return Atlantic Current

The cells were grown in 200 mL ARK-GP5 media (composition not shown) at 1 °C under a light intensity of 18 to 33 µEin m^{-2} s^{-1} (day-night cycle 12 h : 12 h). The culturing time is not available. Following a protocol of Dr. Steffi Gäbler-Schwarz, the cells of *P. pouchetii* were harvested. Subsequently the DNA was isolated following the protocol of the E.Z.N.A.® Plant DNA Kit (Omega Bio-Tek). The DNA samples of the four *P. pouchetii* representatives were stored at -20 °C.

Nucleic Acid from Environmental Samples

Cells of planktonic organisms were obtained during Arctic research cruises in 2010, 2011, 2012 and 2013. Therefore, water samples were taken at four to five stations (HG1, HG4, N4, S3 and HG9) in the depth of chlorophyll maximum (measured online by CTD). Samples were filtered through polycarbonate membrane filters (Millipore) with three different pore sizes (10.0 µm, 3.0 µm, 0.4 µm) applying a pressure of 200 mbar. For explicit sample annotation see Table 10.

Table 10: Used Environmental Samples with Expedition Annotations and ID Numbers

Expedition		Sampling Site		ID Number
Year	Annotation	Station	Specification	
2010	ARK25/2	HG1	PS76/132	100706-132-**15**-*X*
		HG4	PS76/173	100712-173-**10**-*X*
		N4	PS76/179	100714-179-**10**-*X*
		S3	PS76/124	100705-124-**15**-*X*
		HG9	PS76/170	100712-170-**45**-*X*
2011	ARK26/2	HG1	PS78/140	110714-140-**28**-*X*
		HG4	PS78/177	110729-177-**20**-*X*
		N4	PS78/162	110723-162-**18**-*X*
		S3	PS78/174	110727-174-**20**-*X*
		HG9	PS78/145	110717-145-**35**-*X*
2012	ARK27/2	HG1	PS80/168	120717-168-**20**-*X*
		HG4	PS80/165	120716-165-**20**-*X*
		N4	PS80/185	120723-185-**25**-*X*
		S3	PS80/176	120720-176-**20**-*X*
		HG9	PS80/184	120722-184-**20**-*X*
2013	ARK28/2	HG1	PS82/425	130626-425-**12**-*X*
		HG4	PS82/452	130704-452-**30**-*X*
		N4	PS82/432	130628-432-**28**-*X*
		S3	PS82/439	130701-439-**25**-*X*

ID Number: Date(yymmdd)-Sample Site Specification Number-**Collection Depth in m**-*X;* the *X* can be replaced by the filter fractions 10.0 µm, 3.0 µm and 0.4 µm for each sample

The DNA of the sampling years 2010 and 2011 were isolated using the E.Z.N.A.® Plant DNA Kit (Omega Bio-Tek), while the DNA isolations of 2012 and 2013 were carried out with NucleoSpin® Plant II Kit (Machery-Nagel).

Nucleic Acid from *Micromonas spp.* (clone library, clones 170 and 179)

A clone library including the clones 170 and 179 of *Micromonas* spp. was generated with the filtered fractions (3.0 µm and 0.4 µm), sampled during Polarstern cruise ARK28/2 in 2009 (Wolf *et al.* 2014). Therefore, the DNA of the two fractions was isolated, following the protocol of the E.Z.N.A. ™ SP Plant DNA Kit (Omega Bio-Tek). The 18S rDNA amplification was realized by PCR. Subsequently the amplicons were purified, using the Gel Purification Kit (Invitrogen, USA), following manufacturer`s protocol. Gained sequences were cloned into the pDrive Cloning Vector (QIAGEN) by the help of the PCR Cloning Kit (QIAGEN). Subsequently, the vector was transformed (via heat shock, QIAGEN) into TOP10 chemo-competent *Escherichia coli* cells (Invitrogen) and the plasmids were isolated. The DNA was stored at -20 °C.

Nucleic Acid Sequences

To check the specificity of the probes and for their modification in MEGA, several environmental sequences (partial published) of phytoplanktonic organisms (referred to as "contigs") gained from former Polar cruises were used (Table 11 and Table 12). Additionally, 18S rDNA sequences of the nucleotide database GenBank of NCBI were utilized.

Table 11: Used Nucleic Acid Sequences *(Micromonas* spp.) from former Expeditions and NCBI GenBank

***Micromonas* spp. Contigs and NCBI GenBank sequences**

Sequence Identification	Source	Sequence Identification	Source
AY954993_CCMP1195_M_pusilla_18S_rDNA		AO2012_MP_AB_Contig_15468	
AY954994_CCMP1545_M_pusilla_18S_rDNA		AO2012_MP_AB_Contig_15568	
AY954995_CCMP1646_M_pusilla_18S_rDNA		AO2012_MP_AB_Contig_15929	Dr. Katja Metfies, n. p.
AY954998_CCMP1764_M_pusilla_18S_rDNA		AO2012_MP_AB_Contig_16700	
AY955003_CCMP490A_Ms_pusilla_18S_rDNA		AO2012_MP_AB_Contig_19310	
AY955006.1_CCMP492_M_pusilla_18S_rDNA	NCBI	AO2012_MP_AB_Contig_21456	
AY955008_CCMP494_M_pusilla_18S_rDNA		Hausgarten_MP_AB_Contig_88	
AY955010_CS222_M_pusilla_18S_rDNA		Hausgarten_MP_AB_Contig_1487	Dr. Estelle Kilias, n. p.
DQ02575_CCMP2099_Ms_pusilla_18S_rDNA		Hausgarten_MP_AB_Contig_1661	
HM191693.1_M_sp._RCC299_18S_rDNA		Hausgarten_MP_AB_Contig_2011	
JF794057.1_M_pusilla_RCC2306_18S_rDNA		Transekt_MP_AB_Contig_534	
AO2011_MP_AB_Contig_5726	Kilias et al. 2014	Transekt_MP_AB_Contig_569	
AO2011_MP_AB_Contig_81		Transekt_MP_AB_Contig_590	
AO2012_MP_AB_Contig_609		Transekt_MP_AB_Contig_816	Kilias et al. 2013
AO2012_MP_AB_Contig_4627	Dr. Katja Metfies, n. p.	Transekt_MP_AB_Contig_2270	
AO2012_MP_AB_Contig_7179		Transekt_MP_AB_Contig_2737	
AO2012_MP_AB_Contig_12181		Transekt_MP_AB_Contig_2761	

n. p. = not published

Table 12: Used Nucleic Acid Sequences *(Phaeocystis* spp.) from former Expeditions and NCBI GenBank

***Phaeocystis* spp. Contigs and NCBI GenBank sequences**

Sequence Identification	Source	Sequence Identification	Source
AJ278036.1_P_pouchetii_P361_18S_rDNA	NCBI	AO2012_P_AB_Contig_20207	Dr. Katja Metfies, n. p.
EF100712.1_P.globosa_robertsonii_18S_rDNA		AO2012_P_AB_Contig_20615	
AO2011_P_AB_Contig_701	Kilias et al. 2014	AO2012_P_AB_Contig_20955	
AO2011_P_AB_Contig_1224		AO2012_P_AB_Contig_3419	
AO2011_P_AB_Contig_1370		AO2012_P_AB_Contig_3494	
AO2012_P_AB_Contig_306	Dr. Katja Metfies, n. p.	AO2012_P_AB_Contig_3675	
AO2012_P_AB_Contig_1288		AO2012_P_AB_Contig_3985	
AO2012_P_AB_Contig_10131		AO2012_P_AB_Contig_5085	
AO2012_P_AB_Contig_10429		AO2012_P_AB_Contig_7522	
AO2012_P_AB_Contig_13901		HG_Phaeo_AB_Contig_744	Dr. Estelle Kilias, n. p.
AO2012_P_AB_Contig_14024		HG_Phaeo_AB_Contig_1930	
AO2012_P_AB_Contig_15059		Transekt_P_AB_Contig259	Kilias et al. 2013
AO2012_P_AB_Contig_15266		Transekt_P_AB_Contig359	
AO2012_P_AB_Contig_15496		Transekt_P_AB_Contig408	
AO2012_P_AB_Contig_17290		Transekt_P_AB_Contig1235	

n. p. = not published

3.8 Primer and Probes

Table 13: Used Primer for PCR, qPCR and Sanger-Sequencing, ordered by Specificity

Primer	Sequence (5'->3')	Length (bp)	GC (%)	T_m (°C)	Source
Eukaryota specific primer					
EukR18Fn	TCTGGTTAATTCCGATAACGAACG	24	42	60.2	m. a. Hardy et al. (2011)
EukR18Rn	CGTTCGTTATCGGAATTAACCAGA	24	42	60.2	m. a. Hardy et al. (2011)
82F	GTGAAACTGCGAATGGCTCAT	21	48	58.5	Lopez-Garcia et al. (2001)
300F	AGGGTTCGATTCCGGAG	17	59	54.5	Elwood et al. (1985)
300R	TCTCCGGAATCGAACCCTAATTCTCC	26	58	66.0	Elwood et al. (1985)
528F	GCGGTAATTCCAGCTCCAA	19	53	57.4	m. a. Elwood et al. (1985)
1055Fn	GGTGGTGCATGGCCGT	16	69	58.3	m. a. Elwood et al. (1985)
1055R	ACGGCCATGCACCACCACCCAT	22	67	71.1	Elwood et al. (1985)
1200R	GGGCATCACAGACCTG	16	63	48.9	Giovannoni et al. (1988)
1200Rn	ATCTAAGGGCATCACAGACCTGTTAT	26	42	59.3	m. a. Giovannoni et al. (1988)
1528R	TGATCCTTCTGCAGGTTCACCTAC	24	50	60.5	m. a. Elwood et al. (1985)
Chaetoceros socialis specific primer					
Chae soc. F	AGAGGATCCGGTCCGACCTTTTGGT	25	56	68.3	m. a. * Wollschläger et al. (2014)
Chae soc. R	CGCTCCGACAACCTAATGCCAGAAC	25	56	67.4	Dr. Christian Wolf (n. p.)
Dinophyta specific primer					
Dino 18SF1N	AAGGGTTGTGTTYATTAGNTACAGAACC	28	36 - 43	57.5 - 61.8	m. a. Senjie et al. (2006)
Dino E-12 ReverseN	GTCGGAAGCTGATAGGTCAGAAAC	24	50	59.3	m. a. Medlin et al. (2006)
Emiliania huxleyi specific primer					
EHux F-745n	GTTTCAAGCAGGCAGTCGCT	20	55	59.2	m. a. Nejstgaard et al. (2003)
EHux R-803n	AGCACCAGAGTCCTATTTCATTATCC	26	42	59.0	m. a. Nejstgaard et al. (2003)
Phaeocystis spp. Specific primer					
P4A	GCGCCAAACAGTGCATACCCA	21	57	64.8	This study
P4G	GCGCCAAGCAGTGCATACCCA	21	62	67.1	This study
P1n	CGGGCGAACCCGAGAAGGTT	20	65	66.2	This study
P1np	CGGGCGGACCCGAGATGGTT	20	70	69.1	This study
Micromonas pusilla specific primer					
Primer A	CGGTCGCTCTTAACCGAAATC	21	52	59.7	This study
Primer B	GGCCGTTCTCAACCGAAATC	20	55	58.0	This study
Primer C	CGCGCCCTCTTAACCAAAA	19	53	59.8	This study
Primer D	GGCCGCTCTCAACCGAAA	18	61	60.8	This study

n. p. = not published m. a. = modified after * reverse complimentary sequence

3.9 Used Software

ARB – Version 5.5-org-9167: ARB is a freely available software package from the "Department of Microbiology – Technical University of Munich", Germany (© 1993-2012). Downloaded via: *http*://*www.arb-home.de* (January 8th, 2014)

MEGA – Version 6.0.5 MEGA (Molecular Evolution Genetics Analysis) is a freely available software from Tamura, K.; Stecher, G.; Peterson, D. and Kumar, S. (© 1993-2014). Downloaded via: *http://www.megasoftware.net/* (January 9th, 2014)

SILVA: This tool is an online resource for quality checked and aligned ribosomal RNA sequence data by Ribocon GmbH (Bremen, Germany) in cooperation with the working group "Microbial Genomic" at the Max-Planck-Institute for Marine Microbiology (Bremen, Germany). SILVA represents the official database of the software package ARB. The SILVA database SSURef_111 was downloaded via *http://www.arb-silva.de/* (January 9th, 2014) and used to build an ARB PT-Server. Therefore the sequences of bacteria and archaea were removed from SSURef_111.

Primer Express® – Version 3.0.1.: Primer Express is licensed software (Applied Biosystems – Life Technologies) for the design of probes and primers used in qPCR.

7500 Software® – Version 2.0.6: Analyzing software of 7500 Fast Real-Time PCR System (Applied Biosystems – Life Technologies).

Foundation Data Collection V.3.0.: Sequencing data collection (Applied Biosystems – Life Technologies, © 2004)

Lasergene® SeqMan Pro™: Software suit for sequence analysis (DNAStar Inc.)

PhyloAssigner: Bioinformatic pipeline by Vergin *et al.* 2013

R 3.1.0 "Spring Dance": R project for statistical computing. Downlaoded via *http://www.r-project.org/* (May 5th, 2014)

4. METHODS

4.1 Bioinformatical Methods

4.1.1 Molecular Probe Design

Specifications to be fulfilled by Molecular Probes

For qPCR applicability, molecular probes have to fulfill several specifications. First, the probe sequence itself has to adhere between 30 % and 80 % GC-content within a sequence length of around 20 nts. Second, the melting temperature (T_m) has to range between 58 °C and 60 °C. This temperature range represents the optimal elongation condition for the Taq-Polymerase that was used within the qPCR master mix. However, the Taq-Polymerase is able to catalyze the elongation process up to 70 °C. Third, the used primer set (consisting of forward and reverse primer) has to be selected to produce amplicons with 50 bp to 150 bp. This represents the optimal length to relate nucleic acid amplification to the fluorescence signal, obtained by intercalating SYBRgreen molecules.

General Approach

To design taxon-specific primer (hereafter referred to as probes) for the Arctic phytoplankton key species *Phaeocystis* spp. and *Micromonas pusilla*, the software package ARB was used in combination with Primer Express® and MEGA. The SILVA database SSURef_111 was used to build up a PT-server, which represents the central search index for ARB. Additionally, sequences of environmental samples of *Phaeocystis* spp. and *Micromonas* spp. as well as 18S rDNA sequences obtained from NCBI`s "GenBank" (Tables 11 and 12) were added to the PT-server. By this, the designed probes were checked to cover sequences, found in the Arctic environment. Using the ARB tool "Probe Design" specific probes for the organisms *P. globosa, P. pouchetii M. pusilla* (this study) and *C. socialis* (Dr. Christian Wolf, n. p.) were designed. Additionally, probes for the kingdom of eukaryotes (this study) and for Dinophyta (Dr. Christian Wolf, n. p.) were designed using this ARB tool. Applied settings see below:

"Marked Organism":	Name of organism (see above)
"Length of Probe":	25
"Temperature":	30.0 – 100.0 °C
"GC-content":	50.0 – 100.0 %
"*E. coli* Position":	0 – 100000
"Max Non Group Hits":	0
"Min Group Hits2.	50 %

Subsequently, the probes were tested for their GC-content and especially their T_m (melting temperature) using the Primer Express® software. This step is necessary, since the chemical conditions within the qPCR master mix are not known by the user but are taken into account by Primer Express®. If the probe check came to the conclusion that the designed probes were not applicable in qPCR, the probe sequence was adjusted using the software MEGA. In MEGA, several 18S rDNA sequences (see Tables 11 and 12) of the organisms mentioned above were aligned ("Clustal W") and the target sequences of the designed probes were searched within the alignment. The flanking parts of the found target sequences were used to elongate the probe sequences to fulfill the qPCR requirements. The probes, specific for Dinophyta, *Emiliania huxleyi*, *Chaetoceros socialis* and Eukaryota have have their origin in previous publications (or are not published yet). For their use the fulfillment of the qPCR requires was tested via Primer Express®. The tested probes were modified and their specificity was determined bioinformatically.

4.1.2 Probe Specificity Test

The specificity test for the designed probes was carried out *in silico* using the SILVA online tool "TestProbe". The settings were chosen as follows:

"Silva Database":	SSU r117
"Sequence Collection":	REFNR
"Maximum Mismatches":	Varying from 0 to 2 mismatches
"Consider x occurrences of N as match":	0 N's
"Additional Options":	Reversed and complement probes

By doing this, the specificity of the probes within the groups Stramenopila, Alveolata, Rhizaria, Haptophyta, Archaeplastida, Amoebozoa, Opisthoconta, Chryptophyceae, Excavata as well as within *Incertae Sedis* (taxonomic group with with unknown/undefined relationships) were checked.

4.1.3 Preparation of Pyrosequencing Data

Pyrosequencing data, obtained from Dr. Estelle Kilias (not published) were bioinformatically prepared for further use. The samples, were taken during former Polarstern cruises (2010, 2011 and 2012) in the area of the deep-sea observatory HAUSGARTEN at the chlorophyll maximum (same water depth than the environmental samples that were used for further qPCR-analyses) of the stations HG1, HG4, N4, S3 and HG9.

The sequences, obtained from pyrosequencing, were assembled with Lasergene® SeqMan Pro™ (DNAStar Inc). Subsequently, they were aligned and taxonomically assigned to a reference database (Silva111 SSU Ref) using PhyloAssigner (Vergin, *et al.* 2013).

The previous data preparation included a sub-sampling process in which a defined number of sequences were chosen randomly from each sample. Subsequently, these sequences were screened for chimera and homopolymers (hexamers and above) of nucleotides. Chimeric and homopholymeric sequences were removed. Additionally, sequences of the phyla Bacteria und Archeae were extinguished as well as sequences which occurred only once (singletons). Singletons are assumed to arise from PCR or sequencing errors. The remaining sequences were used to determine the relative abundance of different phytoplankton phyla/species. Therefore, the searching keywords "Dino" (Dinophyta), "Phaeocystis" (*Phaeocystis* spp.), "Mamiellales" (*Micromonas* spp.), "Coscinodiscophyceae" (*Chaetoceros* spp.) and "Isochrysidales" (*Emiliania* spp.) were used. The percentage of each query for each station within the sampling years 2010-2012 was calculated. To blur the true results of this data processing, the percentages of each species/phyum were divided by the smallest percentage value of all stations over the three sampling years. These dimensionless values were compared to qPCR analysis results of the environmental samples (Kilias *et al.*).

4.2 Microbiological Methods

4.2.1 Cultivation Conditions

For non-binding assays in standard PCR as well as for further applications in qPCR, various cultures of different phytoplanktonic phyla were cultured (see Table 7). For the specific cultivation conditions see Table 14.

Table 14: Cultivation Conditions (Media, Temperature) of used Eukaryotic and Prokaryotic Microorganisms

Phytoplankton Species	Media	Temperature (°C)
Alexandrium minutum	L1	15.0
Bathycoccus prasinos	K	15.0
Ceratium longipes	F/2	15.0
Chaetoceros mülleri	F/2	22.0
Chaetoceros socialis	F/2	15.0
Chrysochromulina ericina	K	15.0
Emiliania huxleyi	K	15.0
Microcystis aeruginosa	Zehnder	RT
Micromonas pusilla	K	4.0
Odontella aurita	F/2	15.0
Phaeocystis globosa	K	22.0
Prorocentrum micans	F/2	15.0
Pyramimonas parkeae	F/2	15.0
Thalassiosira weissflogii	F/2	15.0

The algae strains were grown for two weeks at 4.0 °C or 15 °C, one week at 22°C or one month at RT (room temperature) in their specific media (50 mL of L1, F/2, K or Zehnder). For the inoculation, 2 mL to 5 mL (depending on the cell density, visible to the naked eye) of the precultures, obtained by culture collections were used. The intensity of light was approximately 100 µEin m^{-2} s^{-1} under a day-night cycle of 12 h : 12 h.

4.2.2 Cell Harvest

The algae cells were harvested using a "KNF Neuberger" pump and a polycarbonate Isopore™ filter membrane (Millipore) with a pore size of 0.4 µm. The applied pressure was 200 mbar. Beside the cultures of *A. minutum* and *P. micans*, for which 100 mL culture volumes were used, 50 mL were filtered for each harvest. The resulting cell pellets were stored at -20 °C in petri dishes.

4.3 Molecular Biological Methods

4.3.1 DNA Extraction

The DNA extraction of the harvested cells was realized by employing the NucleoSpin® Plant II Kit (Machery-Nagel). For this, the collected cells were resuspended in 400 µl Buffer PL1 using a cellscraper. Following the manufacturer`s protocol "Green algae (not specified)", 100 µl of DNA were eluted out of each culture. The obtained DNA solutions are stored at -20 °C. For further use, the DNA solutions were diluted (1 : 2, 1 : 5, 1 : 10, and 1 : 20) with qPCR-H_2O. The concentrations of the DNA solutions were determined using a NanoDrop (ND-1000 Spectrophotometer, serial numer: 7141; Peqlab Biotechnology GmbH, 91052 Erlangen, Germany). The concentrations lied between 5-30 ng/µL with A_{260}/A_{280} ratios ≥ 1.7.

4.3.2 Amplification of purified genomic DNA using REPLI-g® Mini Kit

Prior to the specificity tests via qPCR, genomic DNA of *P. globosa* 1524, *E. huxleyi* 1225 and the four strains of *P. pouchetii* (2314, 2621, 2977, 3039) was amplified using the REPLI-g® Mini Kit, following the manufacturer`s protocol. The resulting DNA solutions were diluted 1 : 6 with qPCR-H_2O to meet a DNA concentration between 20-30 ng/uL. To be used in qPCR, another dilution (1 : 2, 1 : 5 and 1 : 10) with qPCR-H_2O was carried out. During all qPCR assays, in which DNA of *P. globosa* 1524, *E. huxleyi* 1225 and the four *P. pouchetii* isolates were tested, the REPLI-g® treated DNA solutions were used.

The same procedure was applied to the environmental samples. For this, 3.0 µL of the three filtrated fractions (0.4 µm, 3.0 µm and 10.0 µm) of each sample were firstly pooled. The fraction mixture was then used in the REPLI-g® Mini Kit, following the protocol. The obtained 50 µL were diluted 1 : 20 with qPCR-H_2O (following the protocol) and subsequently used for qPCR.

4.3.3 Standard PCR

PCR - Specificity Assays

To examine the probe specificities, PCR assays were carried out. The assay consists of DNA solutions isolated in 3.3.1 (this study), DNA solutions of *P. pouchetii* provided by Dr. Steffi Gäbler-Schwarz and DNA solutions of the *Micromonas spp.* clones 170 and 179 provided by Dr. Estelle Killias (Kilias *et al.* 2013). In the first assay, the probe sets for *P. globosa* were tested.

The pipetting scheme of the PCR master mix is shown in Table 15.

Table 15: Pipetting Scheme (Components, Volumes and Final Concentrations) of the PCR Master Mix for a Single Reaction

Component	Volume	Final Concentration
5 x Phusion GC Buffer (7,5 mmol/L MgCl2))	4.00 µL	1 x
Phusion® High-Fidelity DNA Polymerase (2 U/µL)	0.08 µL	0.16 U/20 µL
dNTPs (10 mmol/L)	0.40 µL	0.2 mmol/L
MgCl2 (50 mmol/L) *	0.00/ 0.41 / 0.95 µL	1.5 / 2.5 / 3.8 mmol/L
DMSO (100 %) *	0.00 / 1.00 µL	0 / 5 %
Forward Primer (10 pmol/µL)	0.40 µL	0.2 µmol/L
Reverse Primer (10 pmol/µL)	0.40 µL	0.2 µmol/L
DNA Template	1.00 µL	n. a.
deion. H2O / qPCR H2O	*ad* 20.00 µL	/ / /
Σ	20.00 µL	

* optional, n. a. = not available

The annealing temperature (T_a) of the molecular probe sets for *Phaeocystis globosa* was assumed between 60 °C (optimal qPCR setting) and 70 °C (optimum of Taq-Polymerase). Due to this, a temperature gradient PCR (60 °C to 66 °C) was performed to determine the optimal T_a of the probe sets (PCR program see Table 16).

Table 16: PCR Program (Annealing Temperature Gradient)

PCR Step	Temperature	Duration	Number of Cycles
Lid	94 °C	/ / /	
Initial Denaturation	94 °C	3 min	
Denaturation	94 °C	30 s	
Annealing	53.2 - 65.8 °C *	20 s	35
Elongation	68 °C	20 s	
Final Elongation	68 °C	5 min	

* Ø temperature steps: 1.6 °C

The optimal T_a, determined by this gradient PCR was applied to the following PCRs (pipetting scheme see Table 15). All remaining settings (temperature, duration and number of cycles) were kept constant for the following PCRs, testing for specificity of the probe sets Pglo1, Pglo2, Mpus1-Mpus4, Ehux and Euk under different conditions (Mg^{2+}, DMSO concentration). For the probe sets Dino and Csoc, a T_a of 60 °C was applied in each PCR.

PCR – Sanger-Sequencing

To prepare samples (isolated DNA of *P. pouchetii* 2977, *P. pouchetii* 3039 and *Microcystis aeruginosa)* for Sanger-Sequencing, two PCRs have to be carried out in advance. The first was used to amplify a DNA fragment of the 18S rDNA between the primer binding sites of primer 82F and 1528R. The used pipetting scheme is shown in Table 15, while the applied PCR program is shown in Table 17.

Table 17: PCR Amplification Program of 18S rDNA Fragment, flanked by the Primer 82F and 1200R

PCR Step	Temperature	Duration	Number of Cycles
Lid	94 °C	/ / /	
Initial Denaturation	94 °C	3 min	
Denaturation	94 °C	45 s	
Annealing	55 °C	1 min	35
Elongation	72 °C	3 min	
Final Elongation	72 °C	10 min	

The resulting amplicons were purified using the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel) resulting in 30 µL of DNA solution for each sample. Therefore, the enclosed PCR clean-up protocol was used, whereby Buffer NTI was diluted 1 : 6 with deion. H_2O before use, the centrifugation times were changed to 1 min and the recommended second washing step was carried out.

Subsequently, two PCRs (Dye-PCRs) were carried out for the amplicons, using the primers 300F and 528F. Here, fluorescence marked ddNTPs were used beside non-marked dNTPs to enable the posterior sequencing of the amplicons via Sanger-Sequencing (3130xI Genetic Analyzer). The applied PCR pipetting scheme and program are shown in the Tables 18 and 19.

Table 18: Pipetting Scheme for Dye-PCR

Component	Volume	Final Concentration
5 x Sequencing buffer	1.5 µL	1 x
BigDye® Terminator v3.1 Ready Reaction Mix *	1.0 µL	n. a.
Primer (10 pmol/µL)	1.0 µL	0.5 µmol/L
DNA Template	1.0 µL	5 - 50 ng/µL per reaction.
qPCR H2O	5.5 µL	/ / /
Σ	10.0 µL	

* including buffer, MgCl2, dNTPs, ddNTPs (fluorescence marked) and polymerase (concentration not known = n. a.)

Table 19: PCR Program of Dye-PCR

PCR Step	Temperature	Duration	Number of Cycles
Lid	105 °C	/ / /	
Initial Denaturation	94 °C	1 min	
Denaturation	96 °C	10 s	
Annealing	50 °C	5 min	25
Elongation	60 °C	4 min	

The amplicons of the Dye-PCRs were purified using the Agencurt CleanSEQ – Dye Terminator Removal Kit (reference number 000219, Beckman Coulter GmbH, 47807 Krefeld, Germany).

4.3.4 Gel Electrophoresis

To check the amplification success of the standard PCRs, gel electrophoresis was carried out, using agarose gels (3.0 % agarose). Depending on the size of the used electrophoresis chambers and slides, 50 mL, 100 mL or 200 mL of agarose gel were used. To enable the detection of separated DNA bands under UV light, GelRed (Nucleic Acid Gel Stain – 10,000x in water, reference number: 41003, lot number: 13G0306; Biotium, Hayward, CA 94545, USA) was added to the agarose gel (5 µL/100 mL) before casting. The amplicons, obtained by PCR were prepared for electrophoretic separation by adding 2 µl of loading dye to 5 µL of DNA solution. Subsequently, 5 µL of the DNA-loading dye mixtures were applied. The used DNA length marker was prepared in a similar way: 2 µL of Loading Dye were added to 3 µL of DNA length marker "Ultra Low DNA Range Ladder I" (10-300 bp, 50 µg; reference number: 25-3010, lot number: 162417; Peqlab Biotechnology GmbH, 91052 Erlangen, Germany). Subsequently, 4 µL of the marker-loading dye mixtures were applied.The separation process was carried out applying 70 – 85 V for 30 min to 1 h and 20 min (depending on the gel size). To determine the size (in bp) of the DNA bands, the DNA length marker was utilized in each electrophoresis.

4.3.5 Sanger-Sequencing

Sanger-Sequencing was carried out for the DNA samples of *P. pouchetii* 2977, *P. pouchetii* 3039 and *Microcystis aeruginosa* to identify these organisms clearly. The two PCR solutions of each sample were sequenced using ABI 3130xI Genetic Analyzer (Applied Biosystems). The obtained data were collected by the Foundation Data Collection Version 3.0 software (Applied Biosystems). Afterwards, the results of these sequencings were than processed using the software Lasergene® SeqMan Pro™ (DNAStar Inc.). The consensus sequences were compared to the nucleotide database of the online platform NCBI using the BLAST tool.

4.3.6 Quantitative PCR

For quantitative PCR, diluted DNA isolates of 3.3.1 and 3.3.2 (this study) were used. The general pipetting scheme for a qPCR analysis (in triplicates) of both, laboratory culture DNA and environmental sample DNA, is shown in Table 20.

Table 20: Pipetting Scheme of a Triplicate qPCR Analysis of DNA Sample, using a Single Probe Set

Component	Volume	Final Concentration
2 x SYBR® Select Master Mix	37.50 µL	1 x
Forward Primer (10 pmol/µL)	0.75 µL	1 µmol/L
Reverse Primer (10 pmol/µL)	0.75 µL	1 µmol/L
qPCR H2O	28.50 µL	/ / /
DNA Template	7.50 µL	n. a.
Σ	75.00 µL	

n. a. = not available (because not measured)

Of the qPCR mix, triplicates of 20 µL each were transferred into a 96 well microtiter plate (MicroAmp®, Applied Biosystems). For every probe set a negative control as well as a positive control was applied (in triplicates). Instead of a DNA template, qPCR-H_2O was added to the negative control. The DNA templates of the positive controls are listed in Table 21 for each probe set.

Table 21: DNA Templates serving as Positive Controls of the Probe Sets, tested by qPCR

Probe Set	DNA template
Pglo	*P. globosa* CCMP 1524 *
Ppou	*P. pouchetii* 2314 (REPLIg)
Ehux	*E. huxleyi* RCC 1225 *
Mpus	*M. pusilla* CCMP 2306
Csoc	*C. socialis* CCMP 1579
Dino	*A. minutum* CCAP 1119/48
Euk	*P. globosa* CCMP 1524 *

* For qPCRs with laboratory cultures the original isolates were used. For qPCRs with environmental samples REPLIg treated isolates were used

To be used as qPCR positive controls, the DNA templates of the original and REPLIg® treated isolates were diluted 1 : 10 with qPCR-H_2O. Before the loaded 96 well microtiter plate was applied in qPCR, the content was spun down by centrifugation using the “short spin” function of the centrigue. The used qPCR program is shown in Table 22 and is adjusted to the optimal annealing/elongation temperature for each probe set (60 °C: Csoc, Dino; 66 °C: remaining probe sets).

Table 22: qPCR Program

PCR Step		Temperature	Duration	Number of Cycles
Holding Stage	Tempering	50 °C	20 s	
	Initial Denaturation	95 °C	10 min	
Cycling Stage	Denaturation	95 °C	15 s	40
	Annealing and Elongation	60 / 66 °C	1 min	
Melt Curve Stage	Denaturation	95 °C	15 s	
	Annealing and Elongation	60 / 66 °C	1 min	
	Final Denaturation *	95 °C	30 s	
	Tempering	60 / 66 °C	15 s	

The temperature ramp is chosen with 1.6 °C/s for the holding stage, cycling stage and the beginning of the melt curve stage. The final denaturation step (*) of the melt curve stage had a temperature ramp of 0.016 °C/s. For the determination of the C_T-values, a fix threshold of 0.3 ΔRn was applied for all qPCR assays. This enables the comparison of, qPCR runs, performed with different probe sets/ different samples. By doing this, the amount of species specific DNA can be related to the eukaryotic DNA pool directly (comparison of the qPCR signals obtained from the species/phyla specific probe sets and the probe set Euk2). Thereby, an indirect relation between the DNA amounts of certain phytoplanktonic species and by accession their abundance is possible.

5. RESULTS

5.1 Bioinformatical Work

To gain taxon specific molecular probes for the phytoplankton species *Phaeocystis globosa, Phaeocystis pouchetii,* and *Micromonas pusilla*, the software ARB, MEGA and/or Primer Express® were used. Additionally, the online nucleotide database of NCBI and the online tool SILVA were utilized. The same/similar assumptions were made for the design of probes specific for *Emiliania huxleyi, Chaetoceros socialis,* Dinophyta and Eukaryota. The so designed probes were combined with each other or with modified eukaryotic specific probes to gain 18S rDNA fragments of 50 – 150 bps. The probe combinations are shown in Table 23. For further information about the T_m, GC% of the probes see Table 13.

Table 23: Specifications of the Bioinformatically Designed Probe Sets

Probe Set Notation	Specificity	Forward Primer	Reverse Primer	Amplicon Size (bp)	
Euk1	Eukaryotes	1055Fn	EukR18R	62	*1
Euk2		EukR18F	1200Rn	144	
Csoc	*Chaetoceros socialis*	Chae. soc. F	Chae. soc. R	87	*2
Dino	Dinoflagellates	Dino 18S-F	Dino E12 Reverse	133	*3
Ehux	*Emiliania huxleyi*	EHux F-745n	EHux R-803n	64	*4
Mpus1	*Micromonas pusilla*	528F	Primer A	82	*5
Mpus2		528F	Primer B	81	
Mpus3		528F	Primer C	126 / 82	
Mpus4		528F	Primer D	81	
Pglo1	*Phaeocystis globosa*	82F	P1n	137	*6
Pglo2		528F	P4A	112	
Ppou1	*Phaeocystis pouchetii*	528F	P4G	112	*7
Ppou2		82F	P1np	156	

For amplicon sizes, the 18S rDNA fragments flanked by a probe sets were determined using NCBI nucleotide database sequences and the software MEGA. The accession numbers of these sequences are shown below:

*1 EF100712.1 - *Phaeocystis globosa*
*2 JQ217339.1 - *Chaetoceros socialis*
*3 U27499.1 - *Alexandrium minutum*
*4 KC404141.1 - *Emiliania huxleyi*
*5 AY702110 - *Micromonas pusilla*
*6 EF100712.1 - *Phaeocystis globosa*
*7 AJ278036.1 - *Phaeocystis pouchetii*

The results of the probe search within NCBI and environmental sequences (cotnigs) using MEGA are shown in the appendix (Fig. 8-12). Therein, the probe sequences are marked in yellow (or framed by brackets); mismatches of alignments or gaps are marked in black. Matching parts are marked with stars above and are shaded in white. For the probe sets Mpus1-Mpus4, textured bars are used for assignment. The specificity test results of the probe sets, using the SILVA online tool “TestProbe”, are shown in the appendix (Tables 24-28).

5.2 PCR – Specificity Assay

Probe Sets for *Phaeocystis* spp.

First, a temperature gradient PCR with the probe sets Pglo1, Pglo2 and Ppou1 was conducted to determine the optimal annealing temperature (T_a) for the used probe sets. In these PCRs, the DNA of *P. globosa* 1524 and *E. huxleyi* 1225 was amplified. The results of these PCRs are shown in the appendix (Fig. 13 and 14). Subsequently, a Mg^{2+} gradient PCR with the mentioned probe sets and DNA isolates was performed, based on the temperature gradient PCR (T_a = 66 °C). The results of these PCRs are shown in the appendix (Fig. 15 A and B).

The probe set Pglo1 was used for further PCR tests with DNA isolates of several representatives of the main Protista groups as well as the four *P. pouchetii* samples (2314, 2621, 2977, 3039), *P. globosa* 1524 and of *E. huxleyi* 1225. The PCR conditions (66 °C T_a, 1.5 mmol/L Mg^{2+}, 5 % DMSO) were based on the results of the former PCR tests. The result of the PCR is shown in the appendix (Fig. 16 A and B). It was necessary to apply 5 % DMSO to the PCR because amplification of *P. pouchetii* (2314, 2977) DNA with probe set Pglo1 was observed in a former PCR without DMSO (data not shown).

Probe Sets for *Micromonas pusilla*

Based on the PCR conditions of the probe set Pglo1, a PCR assay of the probe sets Mpus1, Mpus2, Mpus3 and Mpus4 was performed (66 °C T_a, 1.5 mmol/L Mg^{2+}, 0 % DMSO). The results of this PCR are shown in the appendix (Fig. 17).

The probe set Mpus2 was chosen for further investigations in a PCR assay (66 °C T_a, 1.5 mmol/L Mg^{2+}, 5 % DMSO), consisting of template DNA obtained by representatives of several picoplankton phyla. The results of this PCR are shown in the appendix (Fig. 18).

Probe Sets for Dinophyta and *Chaetoceros socialis*

The specificity PCR assays of the probe sets Dino and Csoc were performed by Dr. Christian Wolf (data not published). At a T_a of 60 °C, template DNAs of Protista representatives were amplified. For both probe sets, no unspecific amplifications were observed (data not shown). A slightly visible DNA band occurred only for the template DNA of *Bathycoccus prasinos*, tested with the Dino probe set.

Probe Sets for Eukaryota

Here, a PCR assay was performed using the probe sets Euk1 and Euk2. The conditions of the PCR were based on the final PCR conditions of the probe sets Pglo1 and Mpus2 (66 °C T_a, 1.5 mmol/L Mg^{2+}, 5 % DMSO). The results are shown in the appendix (Fig. 19).

5.3 qPCR – Laboratory Cultures

5.3.1 Specificity Tests

To ensure the specificity of the designed probe sets Pglo1, Ppou2, Mpus2, Ehux, Dino, Csoc and Euk2 under qPCR conditions, qPCR assays with template DNA of several phytoplankton phyla were performed for each probe set. Therefore, the DNA isolates were diluted 1 : 2 resp. 1 : 5 with qPCR-H_2O before use. For the qPCR master mix, the SYBR® Select Master Mix (Applied Biosystems®) was used, without adding DMSO or Mg^{2+} additionally. The results (C_T-values and amplicon T_ms) of the performed qPCR assays are shown in Table 29.

5.3.2 Efficiency Tests

Several qPCR assays were performed with the probe sets Pglo1, Ppou2, Mpus2, Ehux Dino, Csoc and Euk2. As template DNA for every assay, a mixture of probe specific DNA (DNA used as positive control) and *E. huxleyi* 1225 DNA was compounded (dilution of each DNA before use = 1 : 5 with qPCR-H_2O). These mixtures were amplified using the probe set specific for the positive control DNA, the probe set Ehux and the probe set Euk2. The results (C_T-values and amplicon T_ms) of the performed qPCR efficiency assays are shown in Table 30.

5.4 qPCR – Environmental Samples

The environmental samples (Table 10) were treated with REPLI-g® and were diluted 1 : 5 with qPCR-H_2O before applied in qPCR. For every environmental sample, a qPCR assay with the probe sets Pglo1, Ppou2, Mpus2, Ehux, Dino and Csoc was performed. As positive controls, diluted DNA (1 : 10) of the organisms *P. globosa* 1524 (probe set Pglo1), *P. pouchetii* 2314 (probe set Ppou2), *M. pusilla* 2306 (probe set Mpus2), *E. huxleyi* 1225 (probe set Ehux) and *A. minutum* (probe set Dino) were used. The results (C_T-values and amplicon T_ms) of the performed qPCR assays are shown in Tables 31-34.

6. EVALUATION

6.1 Bioinformatical Work

6.1.1 Molecular Probe Design

Design of *Phaeocystis* spp. Probes

The binding sites of the Eukaryota specific probes 82F, 528F were searched within aligned *Phaeocystis* spp. 18S rDNA sequences, obtained from NCBI GenBank and environmental sequences (contigs) from Dr. Estelle Kilias and Dr. Katja Metfies (partly published). By doing this, it was demonstrated that probe 82F binds to the 18S rDNA sequences of *P. globosa* (AC: EF100712.1) and *P. pouchetii* (AC: AJ278036.1) without any mismatches (Fig. 9). The *Phaeocystis* spp. sequences of the contigs do not cover the binding region of 82F. Therefore, based on the available data sets, no statement can be given concerning the binding of 82F. Due to this, only the NCBI sequences of *Phaeocystis* spp. are shown in Fig. 9 A. The sequence of probe 528F sequence was found in all contig sequences of *Phaeocystis* spp. and also binds within the NCBI sequence of *P. globosa* (Fig. 9 B). The NCBI sequence of *P. pouchetii* contains a variation of the 528F sequence (one mismatch) to the probe sequence. With a length of 21 nucleotides (nts) and 19 nts, a GC% of 48 % and 53 % and a T_m of 58.5 °C and 57.4 °C, the probes 82F and 528F fulfill the requires for further application in qPCR. The slightly lower T_m of 528F was seen as tolerable. Therefore, the probes 82F and 528F were not modified using Primer Express®.

The *Phaeocystis* spp. probes P4A and P4G differ from each other in only one nucleotide (P4A has an A on position eight, while P4G has a G on this position). This difference shows a diverse binding structure by searching the two probe sequences within the NCBI and contig sequences of *Phaeocystis* spp. using MEGA. P4A binds within the NCBI sequence of *P. globosa* and in 21 of 28 contig sequences (Fig. 10 A). No binding is observed for the NCBI sequence of *P. pouchetii.* In contrast, the probe P4G only binds within the NCBI sequence of *P. pouchetii* and does not bind in any other of the used sequences (Fig. 10 B). With a length of 21 nts, a GC-content of 57 % and 62 %, the probes P4A and P4G fulfill partially the requirements for further qPCR usage. The T_ms of the probes (64.8 °C and 67.1 °C) are higher than the recommended melting temperatures for qPCR usage. Despite this, the sequences have not been modified since modifications (shortening or shifting) would have let to a loss of specificity. Additionally, the used qPCR master mix contains Taq-Polymerase that is not influenced negatively in its DNA synthesis function up to 70 °C.

The *Phaeocystis* spp. probes P1n and P1np differ from each other in two nucleotides (P1n: T at position five and 14; P1np: A at position five, C at position 14). The probe binding sites of P1n and P1np were found within *Phaeocysits* spp. sequences of NCBI but not within the environmental sequences using MEGA (Fig. 8 A and B). The *Phaeocystis* spp. sequences of the contigs do not cover the binding region of P1n and P1np, so no statement can be given if the probes bind there. With a length of 20 nts, a GC-content of 65 % and 70 %, the probes P1n and P1np fulfill the requirements for further qPCR usage. The T_ms of the probes (66.2 °C and 69.1 °C) are higher than the recommended melting temperatures for qPCR usage. For reasons explained above, the probes P1n and P1np were not further modified.

The probes 82F and P1n, 82F and P1np as well as the probes 528F and P4A, 528F and P4G were combined and used as probe sets in further PCR and qPCR applications. The resulting amplicons have sizes between 112 nts and 156 nts (estimated using NCBI-sequences, Table 23). With an amplicon size of 156 nts, the probe set Ppou2 (82F:P1np) is slightly larger than recommended. However, this deviation was tolerated because no other probe set was available.

Design of *Micromonas pusilla* Probes

The binding site of the Eukaryota specific probe 528F was searched within aligned *M. pusilla* 18S rDNA sequences of the NCBI database and contigs (Fig. 11). Here, the 528F sequences were found in all used sequences. The probe 528F is applicable in qPCR without further modification (see above).

The four *M. pusilla* specific probes Primer A – Primer D, obtained by MEGA cover different NCBI and contig sequences (Fig. 12). Primer A binds within four of the 23 used environmental sequences and within six of 11 NCBI sequences. Primer B binds within 16 environmental and three NCBI sequences. Of these three NCBI sequences covered by Primer B, two sequences (AC: DQ02575 and AC: JF794057.1) represent the Arctic isolates *M. pusilla* CCMP 2099 (Lovejoy *et al.* 2007) and *M. pusilla* CCMP 2306. The third NCBI sequence (AC: AY954995) covered by Primer B is related to the Mediterranean isolate *M. pusilla* CCMP 1646 (isolated by Zingone 1993). Primer C binds within two environmental sequences and no NCBI sequence. The probe Primer D binds within one environmental sequence and two NCBI sequences. Looking at the probe size, GC% and T_m, the probes Primer A – Primer C fulfill the requirements for qPCR application. Only Primer D has a slightly higher T_m (60.8 °C). However, this derivation was tolerated due to the temperature range of the Taq-polymerase used in qPCR.

The probes Primer A – Primer D were combined with the Eukaryota specific probe 528F. By doing this, amplicons of approximately 81 bp and 126 bp in size were generated (fulfilling the requirement for qPCR usage) (Table 23). In the case of the probe set Mpus3 (528F:Primer C), two amplicons are obtained in MEGA since Primer C has two binding sites within the used sequences (data not shown). The ΔT_ms (<4 °C) of the combined probes are tolerable for further PCR/qPCR application.

Design of *Emiliania huxleyi* Probes

The probes EhuxF-745 and EhuxR-803 (Nejstgaard *et al.* 2003) were tested for binding sites within *E. huxleyi* sequences using MEGA (data not shown) and modified in their length to fulfill the requirements for qPCR (resulting in EhuxF-745n and EhuxR-803n). Possible specificity decreases resulting out of the modifications were not observed in ARB (data not shown). The two probes EhuxF-745n and EhuxR-803n built the probe set Ehux obtaining an amplicon of around 64 bp in size (Table 23). The ΔT_m (< 1 °C) of the combined probes is tolerable for further PCR/qPCR application.

Design of Dinophyta Probes

The probes Dino18SF1 and Dino E-12 Reverse (Serjie *et al.* 2006 and Medlin *et al.* 2006) were tested for binding sites within Dinophyta spp. sequences using MEGA (data not shown) and modified to fulfill the requirements for qPCR (Dino18SF1N and Dino E-12 ReverseN). Possible specificity decreases resulting out of the modifications were not observed in ARB (data not shown). The two probes Dino18SF1N and Dino E-12 ReverseN built the probe set Dino obtaining an amplicon of around 133 bp in size (Table 23). The ΔT_m (< 3 °C) of the combined probes is tolerable for further PCR/qPCR.

Design of *Chaetoceros socialis* Probes

The probes Chae soc F and Chae soc R (modified after Wollschläger *et al.* 2006 resp. designed by Dr. Christian Wolf (not published)) were tested for binding sites within *Chaetoceros* spp. sequences using MEGA (data not shown) and modified to fulfill the requirements for qPCR. Possible specificity decreases resulting out of the modifications were not observed in ARB (data not shown). The two probes Chae soc F and Chae soc R built the probe set Dino obtaining an amplicon of around 87 bp in size (Table 23). The ΔT_m (< 1 °C) of the combined probes is tolerable for further application.

Design of Eukaryota Probes

The probes 1055F and 1200R (Elwood *et al.* 1985 and Giovannoni *et al.* 1988) were tested for binding sites within a *P. globosa* NCBI sequence, using MEGA (data not shown) and modified to fulfill the requirements for qPCR application (resulting in 1055Fn and 1200Fn). Possible specificity decreases, resulting out of the modifications, were not observed in ARB (data not shown).

The probe 1055Fn was combined with the probe EukR18Rn to build the probe set Euk1. EukR18Rn was modified after the probe 18S_internalR (Hardy *et al.* 2011) to fulfill the qPCR requirements. The probe set Euk1 produces an amplicon of around 62 bp within the NCBI sequence of *P. globosa* (Table 23).

The probe 1200Rn was combined with the probe EukR18Fn to build the probe set Euk2. EukR18Fn was modified after the probe 18S_internalR (Hardy *et al.* 2011) to fulfill the qPCR requirements. The probe set Euk2 produces an amplicon of around 144 bp within the NCBI sequence of *P. globosa* (Table 23). The ΔT_ms (<2 °C) of the combined probes are tolerable for further PCR/qPCR application

6.1.2 Probe Specificity Tests

For the probe specificity test using SILVA's online tool "TestProbe", a mismatch range from zero maximal mismatches to two maximal mismatches was applied. This allows little variations in probe sequences and 18S rDNA binding sequences and enables the screening of nearly all organisms, within the SILVA database. A higher mismatch score was not allowed because no probe binding was assumed to occur at mismatch rates higher than two. The probes were evaluated after the hits within the specific taxonomic group and the out-groups. The evaluations are related to the Tables 24 - 28.

Specificity of *Phaeocystis* spp. Probes

For these probes the specific group of Protista is Haptophyta. No unspecific probe match of P1n, P4A, P4G and P1np within the out-groups was observed in SILVA (Table 24).

Specificity of *Micromonas pusilla* Probes

For these probes, the specific group of Protista is Archaeplastida, where *M. pusilla* belongs to. The Primer A probe had unspecific hits within the out-groups of Alveolata, Rhizaria and Opisthokonta. Within the group of Archaeplastida, the most hits of this probe were observed. No hits were obtained within the other groups (Table 25). The Primer B probe had unspecific hits within the groups of Alveolata. Within the specific group Archaeplastida, this probe had hits as well. The Primer D probe had unspecific hits within the groups of Alveolata and Opistokonta. Within the group of Archaeplastida the most hits of this probe were observed.

Specificity of *Emiliania huxleyi* Probes

For these probes, the specific group of Protista is Haptophyta, were *E. huxleyi* belongs to. The probes Ehux F-745n and Ehux R-803n had several unspecific hits within the eukaryotic groups, tested in SILVA (Table 27). Ehux F-745n had hits within the group of Stramenopila, Alveolata, Rhizaria, Archaeplastida and Opisthokonta. Ehux R-803n had unspecific hits within Stramenopila, Amoebozoa, Cryptophyceae and *Inertae Sedis*. Within the group of Haptophyta, Ehux F-745n and Ehux R-803n had hits as well. No hits were observed within prokaryotic taxa.

Specificity of Dinophyta Probes

For these probes, the specific group of Protista is Alveolata. The Dino 18SF1N probe had unspecific out-group hits within all eukaryotic groups tested (except Haptophyta). The range of hits in these out-groups extends from one to 35. Compared to the hits of Dino 18SF1N within the specific group of Alveolata, the unspecific hits were vanishingly low. For the Dinophyta probe Dino E-12 ReverseN, the same pattern can be observed (Table 26). Here, the unspecific out-group hits range from one to 14 except of Haptophyta and *Incertae Sedis,* where no hit is observed. The hits within the specific group of Alveolata were significantly higher.

Specificity of *Chaetoceros socialis* Probes

The probes Cheo soc F and Chae soc R have no unspecific hits within prokaryotic or eukaryotic out-groups (Table 26). Within the specific group of Stramenopila, Chae soc F hits and Chae soc R hits were observable.

Specificity of Eukaryota Probes

Here, all tested eukaryotic groups were assumed as specific groups. This is confirmed by the observed hits of the probes 82F, 528F, 1055Fn, 1200Rn, EukR18Fn and EukR18Rn in all groups (Table 28). The lowest number of hits, assessing two mismatches, were found within the group of Excavata, tested with 82F. The highest number of hits with two allowed mismatches was found within the group of Opistokonta, tested with probe 1055Fn. The out-groups Archaea and Bacteria records no hit for the probes 82F, 528F and 1200Rn. The probe 1055Fn showed a high number of hits within these two out-groups. The probes EukR18Fn as well as EukR18Rn showed six unspecific hits within the group of Bacteria (two maximum mismatches allowed). These unspecific hits consisted of two sequences of the phyla Firmicutes (genus *Asteroleplasma*), two sequences of the phyla Planctomycetes (uncultured organism) and two sequences of the phyla Protebacteria (genus *Sphingomonas*). According to Tully *et al.* (1993), bacteria of the genus *Asteroleplasma* inhabit primarily the pounch of rumiants such as bovines and ovines. As possible habitats of the other bacterial phyla /genera, sea- and fresh-water are reported (Sakai *et al.* 2007, Schlesner 1994).

6.2 Molecular Biological Work

6.2.1 Standard PCR

Probe Sets for *Phaeocystis* spp.

To determine the optimal T_a for the designed probe sets Pglo1, Pglo2, Ppou1 and Ppou2, a temperature gradient PCR was performed using template DNAs of *P. globosa* 1524 and *E. huxleyi* 1225. Hereby, it was tested which annealing temperature (T_a) is applicable in PCR to avoid unspecific amplification of *E. huxleyi* 18S rDNA by the used probe sets. A strain of *E. huxleyi* was chosen for this test, because this species is closely related to *P. globosa* in its phylogeny. The temperature gradient ranged from 53.2 °C to 65.8 °C. Over the whole range, amplification of *P. globosa* 1524 and *E. huxleyi* 1225 DNA was observed for all probes (Fig. 13 and 14). Although the obtained DNA bands, separated by gel electrophoresis, are slightly weaker in their intensity for *E. huxleyi* than for *P. globosa*, no statement about the probe set affinity can be made since the concentration of the applied template DNAs were not considered (this fact takes effect to all other PCR assay performed). In the course of the temperature gradient, the intensity of the DNA bands within *P. globosa* decreases for the probe set Pglo1 with increasing T_a. The other probe sets are less affected in their efficiency, amplifiying *P. globosa* DNA at higher T_as.

In the course of the temperature gradient, the intensity of the DNA bands of *E. huxleyi* decreases for all probe sets with rising T_a. Due to this, a T_a of 66 °C was chosen for *Phaeocystis* spp., related to the results of the T_a gradient PCR at 65.8 °C. Subsequently, a Mg^{2+} gradient PCR with *P. globosa* and *E. huxleyi* DNA was performed (T_a = 66 °C), using the probe sets Pglo1, Pglo2 and Ppou1 (Fig. 15 A and B). This was necessary, because the performed temperature gradient didn't result in a T_a, able to eliminate unspecific *E. huxleyi* amplification. To find the Mg^{2+} concentration, where the annealing specificity of the probe sets is enhanced but the DNA-Polymerase is not influenced negatively in its function, concentrations of Mg^{2+} 1.5 mmol/L, 2.5 mmol/L and 3.8 mmol/L were tested. Firgure 15 A shows, that all probe sets amplified the target 18S rDNA fragment of *P. globosa* at all three Mg^{2+} concentrations. Only the DNA bands amplified by the probe set Ppou1 showed a decrease in their intensity at low (< 3.8 mmol/L) Mg^{2+} concentrations. In Fig. 15 B, the *E. huxleyi* DNA bands amplified by probe set Pglo1 were not visible at Mg^{2+} concentrations of 1.5 mmol/L and 2.5 mmol/L. A slightly visible DNA band were observed at 3.8 mmol/L Mg^{2+} in lane ten. The other probe sets showed more or less intensive *E. huxleyi* DNA bands at all Mg^{2+} concentrations. This observation results in the application of 1.5 mmol/L Mg^{2+} to the following PCRs, using the probe set Pglo1.

To ensure the specificity of the *Phaeocystis* spp. probe set Pglo1, a PCR assay was performed, wherein DNAs of representatives of several phytoplankton phyla (DNA out-group) were tested (T_a = 66 °C, Mg^{2+} = 1.5 mmol/L). In the first assay (data not shown), no DMSO was added. The test revealed unspecific bindings of Pglo1 to two strains of *P. pouchetii* (2314, 2977). To enhance the specificity of the used probe set for *P. globosa,* the PCR assay was repeated with 5 % DMSO (without performing a previous DMSO gradient PCR). The result of this approach is shown in Fig. 16 (A and B). Here, no out-group assay showed a visible DNA band at around 140 bp. In lane 18 an amplification product of *M. pusilla* Clone 170 gained by Pglo1 was visible. Here, three DNA bands of fragments bigger than 300 bp were observed. This unspecific amplification were tolerated for Pglo1`s further use in qPCR, because the amplification of an unspecific fragment two times bigger than the aimed one can be distinguished at the melt curve stage of the qPCR. The data, resulting of such an unspecific qPCR run would be not representative and can be condemned. Lane 8 shows a slightly fluorescent DNA band, attached to the gel pocket. Here, genomic DNA of *E. huxleyi* was stained by GelRed®.

To compare the amplicon sizes gained by PCR to the bioinformatically determined sizes, only Fig. 16 can be used. All other electrophoresis gels show markers without strictly separated band profiles. Just the amplicon size of the probe set Pglo1 was evaluated in Fig. 16 A with around 140 bps. This suits the bioinformatic estimation of 137 bp for a PCR product amplified by Pglo1.

For the probe set Ppou2 consisting of the probes 82F and P1np, no PCR assay was performed. This was justified in the high sequence homology (90 %) of the probes P1n and P1np, differ in two nucleotides. Therefore, similar results were suggested for the probe P1np in combination with 82F within the PCR assay as for the probe P1n.

Probe Sets for *Micromonas pusilla*

For the PCR assays of the probe sets Mpus1 – Mpus4, an annealing temperature of 66 °C, based on the results of the Pglo1 PCR, was applied. The first PCR assay of the four *M. pusilla* probe sets included 1.5 mmol/L Mg^{2+} and 0 % DMSO. The results of this PCR (Fig. 17 A, B, C and D) were not representative, because the negative controls showed clearly visible DNA bands at the same height as the expected specific amplicon. This observation did not change doing several repetitions with solutions freshly prepared.

The second PCR assay included 1.5 mmol/L Mg^{2+} and 5 % DMSO. The T_a was 66 °C. Here, several DNA isolates of representatives of the major phytoplankton phyla were used to determine the specificity of the probe set Mpus2. The result of this PCR assay is shown in Fig. 18. In contrast to the previous PCR, no PCR products were visible within the negative controls. Unspecific DNA bands (similar in size to the specific bands of amplicons of around 81 bps in size) were observed for the template DNAs of *Chaetoceros muelleri, Prorocentrum micans* and *Bathycoccus prasinos*. Within the lanes of *M. pusilla* Clone 179, several unspecific DNA band larger than 150 bps were observed. In lane ten (*M. pusilla* 2306), intense DNA bands were attached next to the gel pocket, probably representing genomic DNA. Beside this, only one DNA band with an approximately size of around 80 bps were seen in lane ten (*M. pusilla* 2306 template DNA). In lane five (*E. huxleyi* 1225 template DNA), a weak unspecific DNA band (larger than 300 bp) was observed. The negative control of this PCR assay showed no DNA bands at all. All efforts (subsequently PCR assays with 0 % DMSO, 0.4 mg/L BSA and 5 % DMSO, 0.4 mg/L BSA) to reduce the unspecific amplifications using Mpus2 were not successful (data not shown). Although the probe set Mpus2, which was bioinformatical preferably, showed no absolute specificity.

Probe Sets for Dinophyta and *Chaetoceros socialis*

The PCR specificity assay (performed with representative of several phytoplankton phyla) of the probe sets Dino and Csoc were performed by Dr. Christian Wolf (data not shown). The PCR assay for the probe set Dino resulted in a single DNA band with an amplicon size around 140-150 bps, amplified from *Alexandrium minutum* and *Prorocentrum micans* template DNA. A weak unspecific DNA band was observed for template DNA of *Bathycoccus prasinos* with amplicons having the same size range. All other template DNA amplifications, performed with the Dino probe set, showed no DNA bands. The PCR assay for probe set Csoc resulted in a single DNA band with amplicon sizes around 80 bps. All other template DNA amplifications, performed with the Csoc probe set, showed no DNA bands.

Probe Sets for Eukaryota

For the PCR assays of the probe sets Euk1 and Euk2, the same PCR conditions as for the probe sets of Phaeocystis spp. and M. pusilla (T_a = 66 °C, 1.5 mmol/L Mg^{2+}, 5 % DMSO) were applied.

The PCR assay of Euk1 (data not shown), performed with representatives of several phytoplankton phyla and one prokaryotic representative *(Microcystis aeruginosa)*, resulted in larger bands (> 300 bps) than expected (62 bps). The same result was observed for all used template DNAs except of *M. aeruginosa*, where no band occurred. Altogether, no DNA band with amplicon sizes around 62 bps were observed in this PCR assay. The negative controls showed no DNA bands.

The result of the PCR assay for the probe set Euk2 amplification (Fig. 19) were not representative, because of the DNA bands observed in the negative control lanes. A rerun of this PCR using freshly prepared/opened solutions did not lead to any other results.

Probe Set for *Emiliania huxleyi*

For the probe set Ehux, no PCR specificity assay was performed in this study. The probes EHux F-745n and EHux R-803n, building this probe set were, tested tested by Nejstgaard *et al.* (2003) for their specificity.

6.2.2 qPCR – Laboratory Cultures

As results of the performed qPCR assays, C_T-values (triplicate values, C_T-means, and C_T standard deviation) as well as the amplicon specific T_ms (triplicate values, T_m-means, T_m standard deviation) were obtained. The means of the C_T-values as well as the T_ms were seen as representative for standard deviations (SDs) smaller 1.0 for C_T- and smaller 0.6 °C for T_m-values. If a C_T-mean or T_m-mean did not meet these SD requirements, a single value of the triplicate was omitted. This was necessary to produce enough comparable and reliable values for further evaluations. A SD of 1.0 for a C_T-mean signifies a twofold higher or lower DNA concentration. This was seen as maximum tolerable deviation from the mean concentration. The maximum SD (± 0.6 °C) of T_m-values was set very high compared to the normally observed SD-values (± 0.2 °C) of qPCR melt curve determinations. This high SD followed from the assumption that 99.7 % of normally distributed T_ms lie within a range of triply standard deviation. By applying a maximum SD of 0.6 °C, enough comparable T_m-values were obtained, without losing reliability.

Specificity Test

To ensure the specificity of the selected probe sets within qPCR, several qPCR assays were performed (results see Table 29). Therefore, defined template DNA (gained by laboratory cultures of several phytoplankton representatives) was used.

Within the potential binding samples (DNA solution of phytoplankton representative to which the tested probe sets should be specific) of all probe set assays, C_T-means were obtained with a SD smaller than 1.0, so they were seen as reliable. In the first run of the probe sets Dino and Euk2, no C_T-values were obtained for the template DNAs of *A. minutum, P. picans, P. globosa* 1524 and *P. pouchetii* 2314 (all diluted 1 : 5). Due to this, a second qPCR run of these probe sets in combination with the mentioned template DNAs (now diluted 1 : 2) was performed. Here, reliable C_T-means were obtained. Within the assumed non-binding samples, tested with each probe set, several unspecific amplifications, resulting in reliable C_Ts, were observed. The probe set Pglo1 cross-hybridized with DNA of *O. aurita* and *P. pouchetii* 2977. After the "unspecific" amplification of *P. pouchetii* 2977 by Pglo1 was observed, the DNA isolate was sequenced (Sanger-Sequencing). Here, *Paraphysomonas imperforata* (EF432519.1, Query cover 90 %, Ident 80 %) was identified within the isolate. A microscopic examination of the referring culture (Dr. Steffi Gäbler-Schwarz, data not shown) declared it as a mixed culture, containing several cells of a *Phaeocystis* sp.

The probe set Ppou2 cross-hybridized with DNA of *P. globosa* 1524, probe set Dino cross-hybridized with DNA of *Bathycoccus prasinos* and *Chrysochromulina ericina* and probe set Ehux cross-hybridized with DNA of *Prorocentrum micans*. The probe set Ehux cross-hybridized with DNA of *P. pouchetii 3039* which was identified as DNA of a *Isochrysis* sp. (NCBI AC: KC888117.1, Query cover 99 %, Ident 99%) after a Sanger-Sequencing approach. This finding explained the lack of qPCR signals of Ppou2 within this isolate. The probe set Euk2 cross-hybridized with DNA of *Microcystis aeruginosa*. The finding was examined by sequencing (Sanger-Sequencing) the DNA isolate. Here, the Amoeba *Hartmannella vermiformis* (AC: AY502959.1, Query cover 99 %, Ident 99 %) was identified.

Due to the fact, that the used template DNA concentrations of the different phytoplankton representatives were not adjusted to meet a specific concentration value, no comparison between the C_T-means of binding and non-binding samples were made. Thereby, no statement regarding to the efficiency differences of the used probe sets, amplifying several phytoplankton representatives (specific and unspecific), were possilbe. The probe sets Mpus2 and Csoc did not show any cross-hybridization within the non-binding sample DNAs so their sustained specificity in qPCR application was shown. The DNA binding sample of *M. pusilla* Clone 179, used in the qPCR assay with the Mpus2 probe set, produced no C_T-mean but did cross the applied threshold at a C_T smaller ten (SD < 1.0). It is assumed that the template DNA concentration of *M. pusilla* Clone 179 was too high to be detected by qPCR appropriately.

Looking at the obtained T_ms for each qPCR assay (probe set amplifying different phytoplankton representatives), there was one specific T_m for each amplicon gained by a probe set. The SDs of the T_ms were smaller 0.6 °C, so these values can be seen as reliable. The results of the melt curve determination (Fig. not shown) are evaluated in follows. Every probe set in combination with its specific binding sample DNA yielded a representative T_m-mean (Table 35).

Table 35: T_m-Means of Template DNA (Positive Controls) amplified by Probe Sets via qPCR

Probe Set	Template DNA	Tm-means
Pglo1	*P. globosa* 1524	79.1
Ppou2	*P. pouchetii* 2314	79.5
Mpus2	*M. pusilla* 2306	76.5
Csoc	*C. socialis*	82.1
Dino	*A. minutum* resp. *P. micans*	77.5 resp. 80.8

Comparing the experimentally determined T_ms with the bioinformatically calculated [*] T_ms (data not shown) latter ones were on average 1.9 °C lower. This deviations were justified by the unknown qPCR master mix composition, which may had stabilizing effects due to its salt and detergent composition. Only the calculated T_m of the amplicon of *P. pouchetii* 2314, obtained from the probe set Ppou2 was higher (1.5 °C) than the measured T_m. The inverse relation of the two T_ms of *P. pouchetii* 2314 may have been caused by a difference within the 18S rDNA sequence of the isolated *P. pouchetii* 2314 and the deposited NCBI sequence.

Efficiency Test – Laboratory Cultures

This efficiency test was performed due to an observation within the specificity test (previous paragraph). Despite the same concentration of one template DNA was used, the amplification using Euk2 resulted in a weaker signal than a qPCR with the species specific probe set did. These partly significant deviations are shown in Table 36. Different amplification efficiencies, depending on the kind/composition of template DNA may result from this observation for the probe set Euk2.

Table 36: Observed Efficiency Deviation (C_T-Deviation) of Euk2 and Species specific Probe Sets, targeting the same Template DNA

Template DNA (1 :5)	C_T-values		
	Specific Probe Set	Euk2 Probe Set	Deviation (C_TEuk2 - $C_T$specific)
P. globosa 1524	32.4	33.4*	1.0
P. pouchetii 2314	23.8	31.3*	7.5
M. pusilla 2306	16.8	26.6	9.8
A. minutum	17.3*	35.9	18.6
C. socialis	20.2	33.7	13.5

* = Calculated C_T-value for dilution 1 : 5 (original dilution 1 : 2);

used formula: $D2/D1 = 2\Delta CT <=> \Delta CT = (\log(D2/D1)/\log(2))$ D2 = Searched Dilution

D1 = Applied Dilution

Such differences in the amplifying efficiencyiy of Euk2 may influence the planned relation method for the determination of phytoplankton species occurrence. Herein, the qPCR signal obtained by the probe set Euk2 shall function as a reference value, which enables the quantitative comparison of the probe set signals specific for single phytoplankton species among themselves. For this purpose, a similar efficiency of Euk2 and the other probe sets was necessary, especially in mixed culture samples (conforming environmental samples).

Therefore, samples were prepared containing DNA of *E. huxleyi* 1225 (dilution 1 : 5) and DNA of one representative of other phytoplankton phyla (dilution 1 : 5). These samples were tested via qPCR, using the probe sets Euk2, Ehux and an alternating probe set in separated reactions. It was assumed that the Euk2 signal should be stronger (smaller C_T-value) than the signal of Ehux and the third probe set. The results of these qPCR efficiency assays are shown in Table 30. Comparing the C_T-values of the species specific probe sets to Euk2 within the template DNA mixture, a deviation was observed (Table 37).

Table 37: C_T-Values of qPCR Assays (Template: DNA Mixture), perfomed with different Probe Sets (Efficiency Test)

Species Specific Probe Sets	Template DNA mixtures* tested with Euk2				
	P. globosa	***P. pouchetii***	***M. pusilla***	***A. minutum***	***C. socialis***
Pglo1	35.4				
Ppou2		25.5			
Mpus2			21.8		
Dino				24.5	
Csoc					22.3
Ehux	22.0	21.7	21.5	21.7	21.5
Euk2	34.5	31.0	27.2	31.9	31.0

* Beside the DNA mentioned above, *E. huxleyi* DNA was added to all reactions

In all cases (except of the probe set Pglo1), the signal obtained by Euk2 within the template DNA mixtures was weaker than the signals gained by the species specific probes. This observation pointed to an efficiency difference of Euk2, amplifying different template DNAs within a DNA mixture. This finding was transferred to planned environmental sample assays, wherein DNA of different origins is present. Here, Euk2 would not be able to amplify these DNA types equally, so a relation of the PCR products and the DNA composition of environmental samples have to be seen in a critical perspective. The probe set Euk2 resulted within all efficiency assays in reliable T_ms. Beside this, T_ms not assignable to any Euk2 amplicon were obtained for the DNA mixtures (basis DNA: *E. huxleyi* 1225) containing *P. globosa*, *P. pouchetii* or *C. socialis* DNA.

Additionally to the Euk2 amplification differences, another observation was made, comparing the C_T-values of species specific probes within the performed specificity and efficiency tests of this study. The data of the specificity and efficiency assays are compared in Table 38.

Table 38: Comparison of C_T-Values of the qPCR Specificity and Efficiency Test using different Probe Sets within DNA Mixtures

Probe Set	C_T-values obtained from the Template DNAs		C_T-Deviation
	Only Probe Set specific DNA (1 : 5) (Specificity Test)	Probe Set specific (1 : 5) + *E. huxleyi* DNA (Efficiency Test)	
Pglo1	32.4	35.4	3.0
Ppou2	23.8	25.5	1.7
Mpus2	16.2	21.8	5.6
Csoc	20.2	22.3	2.1
Dino	17.3*	24.5	7.2
Ehux	20.1 ^	21.7 °	1.6

* = Calculated C_T-value for dilution 1 : 5 (original dilution 1 : 2);
used formula: $D2/D1 = 2^{\Delta CT} <=> \Delta CT = (\log(D2/D1)/\log(2))$ D2 = Searched Dilution
D1 = Applied Dilution

^ Ehux C_T of specificity test (20.1) meets Ehux C_T (template DNA (1 : 5) only from *E. huxleyi*) of efficiency test (20.6)

° Average of Ehux C_Ts within template DNA mixtures (standard deviation = 0.2)

Here, all C_T-values obtained from template DNA mixtures were on average 3.5 C_Ts higher than the C_T-values of mono-DNA samples. This pointed to an interfering effect of DNA mixtures (like they occur in the environment) on the amplification efficiency of the species specific probes. Looking at the T_ms of the amplicons obtained from species/phyla specific probe sets, there was no significant deviation between the amplicon T_ms obtained within the specificity test and the efficiency test (Table 39). Due to this, mono-DNA templates and DNA mixtures resulted in specific amplicons without any byproducts using one probe set.

Table 39: Comparison of T_m-Values of the qPCR Specificity and Efficiency Test using different Probe Sets within DNA Mixtures

Probe Set	T_ms obtained from the Template DNAs		T_m-Deviation
	Only Probe Set specific DNA (1 : 5) (Specificity Test)	Probe Set specific (1 : 5) + *E. huxleyi* DNA (Efficiency Test)	
Pglo1	79.1	78.9	0.2
Ppou2	79.5	79.7	0.2
Mpus2	76.5	76.5	0.0
Csoc	82.1	82.1	0.0
Dino	77.5 resp. 80.8	78	*

* No deviation can be made because the used probe set is phyla specific and therefore, it may result in Tm-ranges and not discrete Tm-values

The probe set Ehux, which was applied to every efficiency assay within this test, showed a similar T_m-relation. All DNA mixture templates, tested with Ehux, only resulted in one specific T_m (76.7 °C ± 0.2 °C), meeting the T_m (76.9 °C) for Ehux, amplifying a mono-DNA template of *E. huxleyi* 1225.

6.2.3 qPCR – Environmental Samples

The environmental samples, tested by the designed probe sets Pglo2, Ehux and Dino, resulted in positive qPCR signals. The other probe sets did not detect their target species. This observation led to the conclusion that these species were not present in the sample at all or they were not that abundant, so the DNA concentrations were too low to be detectable. Due to the fact that a signal was obtained for the probe set Ppou2 (specific for *P. globosa* and *P. pouchetii*) but not for the probe set Pglo1 (specific for *P. globosa*), it was assumed with high degree of certainty, that the *Phaeocystis* spp. detected by qPCR was *P. pouchetii*. For the C_T-values and T_ms resulting of the qPCR of environmental samples, the same criteria for reliability were applied as in 5.2.2 (this study). Thereby, several C_T-values and T_ms were omitted to fulfill these requirements (Table 40 and 41).

Table 40: C_T-Values, omitted to fulfill the Requirements for Reliability; resulting C_T-Means and SDs

Probe Set	Sampling Year	Sample	CT		
			Triplicates	Mean°	SD°
Dino	2010	HG4	34.3 36.1* 34.2	34.3	0.1
		S3	33.7 34.1 30.6*	33.9	0.3
Ehux	2012	HG4	34.4 34.6 37.9*	34.5	0.1
	2013	S3	36.6 33.9* 37.9	37.3	0.9

* omitted value ° Mean and SD calculated without *-marked value

Table 41: T_m-Values, omitted to fulfill the Requirements for Reliability; resulting C_T-Means and SDs

Probe Set	Sampling Year	Sample	T_m (°C)		
			Triplicates	Mean°	SD°
Ehux	2010	S3	79.9* 77.1 77.1	77.1	0.0

* omitted value ° Mean and SD calculated without *-marked value

The probe sets Ppou2, Ehux and Dino, applied in the qPCR test of environmental samples, resulted in respectively one specific amplicon, meeting the T_m of the positive control (Table 42).

Table 42: T_m-Values (°C) of the Environmental qPCR Assay, performed with the Prob Sets Ppou2, Ehux and Dino (compared to the Positive Controls)

T_ms of the Probe Set Ppou2 Tested with Environmental Samples

2010		2011		2012		2013	
Positive Control	Environmental Samples	Positive Control	Environmental Samples	Positive Control	Environmental Samples	Positive Control	Environmental Samples
	79.8		79.4		79.4		79.2
	79.8		79.1		79.5		79.4
79.6	78.5	79.4	79.3	79.3	79.2	79.6	79.7
	79.7		79.1		79.2		79.7
	79.8		n. d.		79.2		n. a.

T_ms of the Probe Set Ehux Tested with Environmental Samples

2010		2011		2012		2013	
Positive Control	Environmental Samples	Positive Control	Environmental Samples	Positive Control	Environmental Samples	Positive Control	Environmental Samples
	77.2		76.6		76.9		76.9
	77.2		76.7		76.9		76.7
77.1	77.1	76.5	77.1	76.5	76.8	76.5	n. d.
	77.1		n. d.		77.0		n. d.
	n. d.		n. d.		n. d.		n. a.

T_ms of the Probe Set Dino Tested with Environmental Samples

2010		2011		2012		2013	
Positive Control	Environmental Samples	Positive Control	Environmental Samples	Positive Control	Environmental Samples	Positive Control	Environmental Samples
	81.0		80.1		79.9		80.3 (73.1)
	81.1		80.0		80.3		81.3
77.9	80.1	78.1	79.8	78.2	81.2	78.1	81.6
	81.1		80.1		80.1		80.9
	n. d.		80.4		80.3		n. a.

For the probe set Dino, a second Tm was found in the year 2013 within one environmental sample. It is listed within brackets.

n. d. = not determined, because no amplification tooks place

n. a. = not available, because no sampling tooks place for HG9 2013

No T_ms were obtained testing the environmental samples with the probe sets Mpus2, Pglo1, since no amplifications took place. For the probe set Csoc, only the environmental sample HG1 of 2013 showed an amplification signal but had two discrete T_ms. For this reason and the fact, that the C_T-mean (39.5) is near the limit of detection (C_T = 40.0), the amplification was seen in a critical perspective and did not represent a reliable quantification of *C. socialis*. For the probe set Dino, a second T_m was observed for the environmental sample HG9, 2013. In this case, the second T_m may have been the result of the amplification of a different representative of Dinophyta within this sample. Since the probe set Dino amplified members of the phylum Dinophyta, the fragments may vary in their sequences and thereby in their T_ms. For this reason, the obtained qPCR signal were seen as reliable for the determination of Dinophyta.

Normalization

For further investigations a normalization of the obtained C_T-values (mean values with SDs ≤ 1.0) was necessary, because the DNA concentration is inversely proportional to the C_T-value: The higher the sample DNA concentration, the lower the corresponding C_T-value. Therefore, the difference of the highest possible C_T-value (40 qPCR cycles) and the measured sample C_T was calculated. The obtained difference was used as exponent to the basis two to consider the DNA concentration factor of two at a ΔCT of 1.0. The used formula is shown below (formula 1):

Formula 1: 2^{40-C_T}

By doing this, the amount of species specific amplicons was quantitatively related to the presence/absence of certain phytoplankton species/phyla. The results of this normalization (only possible for the analyses of the probe sets Ppou2, Ehux and Dino) are shown in Table 43.

Table 43: Normalized C_T-Values of the Environmental qPCR Assay, performed with the Probe Sets Dino, Ppou2 and Ehux

Sampling Year	Dino Probe Set qPCR Signals of the Sampling Sites				
	HG1	HG4	N4	S3	HG9
2010	78.8	34.0	1.0	68.6	194.0
2011	84.4	2521.4	5404.7	90.5	5042.8
2012	7643.4	1024.0	52.0	548.7	194.0
2013	445.7	477.7	5042.8	55.7	n. a.

Continuation of Table 43:

Sampling Year	Ppou2 Probe Set qPCR Signals of the Sampling Sites				
	HG1	HG4	N4	S3	HG9
2010	128.0	104.0	3.2	137.2	39.4
2011	2.8	1.0	7.5	3.2	21.1
2012	16.0	17.1	3.0	7.0	5.7
2013	1663.5	1024.0	3327.0	512.0	n. a.

Sampling Year	Ehux Probe Set qPCR Signals of the Sampling Sites				
	HG1	HG4	N4	S3	HG9
2010	73.5	1.0	7.0	19.7	9.8
2011	1.0	1.0	5.3	21.1	84.4
2012	137.2	45.3	1.0	42.2	11.3
2013	4096.0	1.0	1.0	14.9	n. a.

n. a. = not available, because no sampling tooks place for HG9 2013

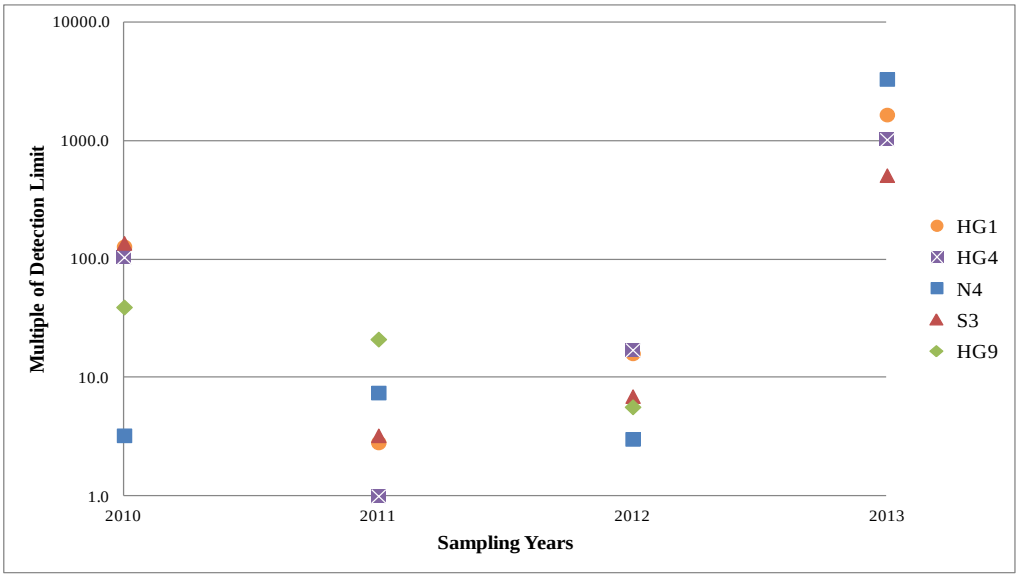
Values of one point zero, shown in Table 43, refer to the detection limit of the qPCR amplification. Here, no signal was obtained, due to the fact that the amount of species specific DNA was under/at the qPCR detection limit, using the designed probe sets. All signals above one point zero represent the multiple value of the detection limit.

Temporal Progress of qPCR Data

The obtained and subsequently normalized qPCR data were used for the determination of possible temporal distribution progresses for the phytoplankton species *Phaeocystis pouchetii, Emiliania huxleyi* and the phylum Dinophyta. These progresses are shown in the Fig. 20 A-C.

Beginning of Figure 10:

A



Continuation of Fig. 10:

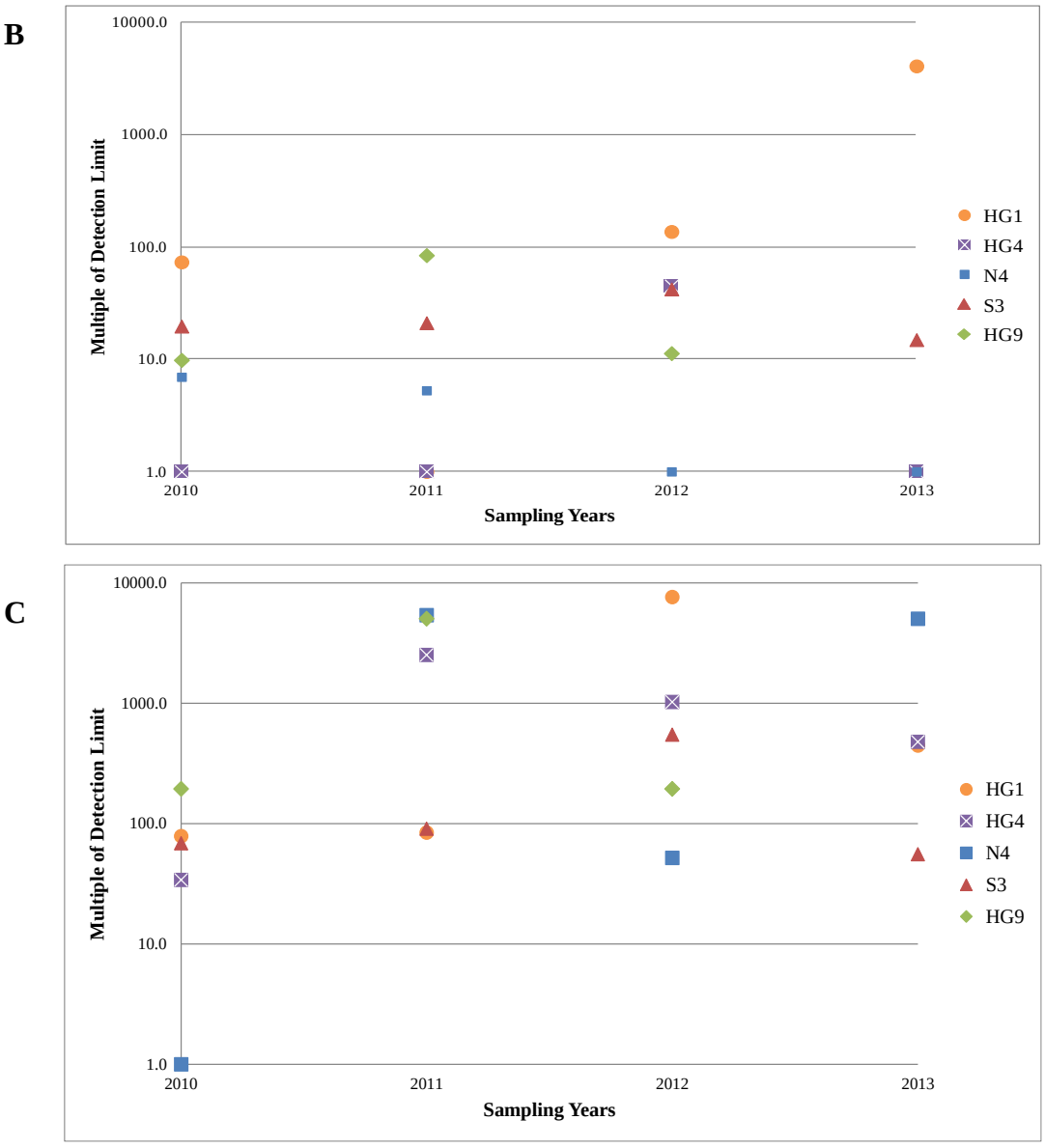


Fig. 20: Temporal Distribution Progress (2010-2013) for Phytoplankton Key Species within the Area of the Deep-Sea Observatory HAUSGARTEN (Stations HG1 – HG9), determined via qPCR

A: Normalized qPCR values, determined for *Phaeocystis pouchetii*

B: Normalized qPCR values, determined for *Emiliania huxleyi*

C: Normalized qPCR values, determined for Dinophyta

5.3.4 Correlation of Environmental qPCR and pyrosequencing Data

Using "R" (software for statistical data analyses), the obtained data of qPCR (normalized C_Ts) and pyrosequencing (processed) were controlled for their correlation behavior. Therefore, the data were plotted against each other and a correlation coefficient (r^2) as well as the probability of correlation (p-value) was calculated. For the year 2010 no pyrosequencing data were available for the HAUSGARTEN station HG1

The qPCR and pyrosequencing data, entered in "R" (software) are shown in Table 44, together with the gained r^2- and p-values for each probe set signal of the sampling sites and years.

Table 44: Correlation Analysis (within "R") of Normalized qPCR Data and Processed Pyrosequencing Data for the Probe Sets Ppou2 and Dino

Probe Set	Sampling Site	Sampling Year	Pyro-Sequencing Data	qPCR Data	p-value	r^2 (adjusted)
Ppou2	HG1	2011	16.8	2.8	0.03	0.29
		2012	7.6	16.0		
	HG4	2010	46.0	104.0		
		2011	4.4	1.0		
		2012	9.6	17.1		
	N4	2010	3.2	3.2		
		2011	1.0	7.5		
		2012	1.0	3.0		
	S3	2010	28.6	137.2		
		2011	38.6	3.2		
		2012	27.4	7.0		
	HG9	2010	23.2	39.4		
		2011	8.4	21.1		
		2012	8.0	5.7		
Probe Set	**Sampling Site**	**Sampling Year**	**Pyro-Sequencing Data**	**qPCR Data**	**p-value**	**r^2 (adjusted)**
Dino	HG1	2011	42.4	84.4	0.15	0.10
		2012	47.2	7643.4		
	HG4	2010	66.0	34.0		
		2011	56.8	2521.4		
		2012	41.0	1024.0		
	N4	2010	1.0	1.0		
		2011	103.2	5404.7		
		2012	21.4	52.0		
	S3	2010	41.0	68.6		
		2011	37.4	90.5		
		2012	79.0	548.7		
	HG9	2010	40.0	194.0		
		2011	46.8	5042.8		
		2012	38.6	194.0		

To check the qPCR and pyrosequencing data sets regarding to possible single outliers, an online calculator was used (http://contchart.com/outliers.aspx, June 20th, 2014), based on the Grubb`s test. Herein, single outliers were defined as values, with a significance probability (p) smaller 0.05. For the qPCR data of Ppou2, the value 137.2 (S3, 2010) fulfills this assumption. Due to this, this vale was removed from the correlation process. All other qPCR data resp. pyrosequencing data showed no p-values smaller than 0.05. As result, the new p-value and r^2-value for the correlation of the Ppou2 data sets was calculated with 0.02 and 0.35. Summing up the correlation results, only the qPCR data obtained from Ppou2 correlates significantly with the processed pyrosequencing data (p-value < 0.05). Despite this, the correlation has to be seen in a critical perspective due to the r^2-value of 0.29 resp. 0.35. The correlation of the two data sets for Dinophyta did not correlate significantly (p-value > 0.05) and the r^2-value of 0.10 did not imply any coherence of the two data sets

7. DISCUSSION

The exploration of biodiversity within the marine microcosm, including multicellular organisms as well as single celled pro- and eukaryotes, is a scientifically relevant field of ecological studies. This is based on the important role, such microorganism play for the marine food webs. Phototrophic organisms are the feeding basis of higher tropic levels. Additionally, they can be used as bio indicators of chaning environmental conditions in the marine realm. These environmental impacts on microorganisms e.g. phytoplankton species, are apperent in Polar Regions. Here, environmental changes appear to be very distinct, due to the fragile prevalent ecosystem structures (Dunbar 1973). In the last decades, this scientific issue got more and more attention, because new molecular methods were able to facilitate biodiversity studies down to species level.

7.1 Methodical and Executional Discussion

One traditional method for biodiversity studies is light microscopy (LM). By this technique, unicellular microorganisms are taxonomically classified using distinguishable morphological features such as cell size and shape. Although LM forms the basis of many ecological studies (e. g., see papers presented in Stroemer and Smol 1999), there are several disadvantages, making the LM insufficient for studies on smaller specimens (2 – 10 µm). Especially within phytoplankton communities, many homomorphic species, lacking special morphologies are abundant, what makes a taxonomic identification very difficult. Even a classification of Diatoma species (Morales *et al.* 2001), showing different morphological specifics like spines, apical pore fields and areolae structures (Silver & Kling 1997), is not always possible due to the limited resolution of LM. Further developments within microscopic applications (e.g. Scanning Electron Microscopy, SEM) improved the sight on the phytoplankton composition, enabling the observation of morphological structures. But even this technique has several disadvantages. A complex preparation of the tested samples, including fixation steps, dehydration and coating with conductive material is required, is necessary and leads to morphological changes or even cell burst. Additionally, the acquisition and maintenance of a SE-microscope is expensive.

A new approach for the study of phytoplankton communities is the use of molecular methods detecting species by their nucleic acid. Here, especially hybridization methods (e. g. fluorescent in situ hybridization, FISH) and PCR based methods (e. g. qPCR) are used.

Several studies deal with the molecular detection of phytoplankton species, e.g. *Micromonas pusilla* (Not *et al.* 2004; Lovejoy *et al.* 2007) and haptophyte species (Not *et al.* 2005). These investigations correlate with HPLC pigment analyses (Not *et al.* 2005). However, FISH application includes one disadvantage in its handling: the required fluorescent microscopic analysis of the FISH samples can be tedious and time consuming due to the fact, that only one probe can be processed at once due to the limited choice of fluorochromes. Therefore, qPCR can be used to avoid these restrictions.

The application of qPCR has its origin in clinical medicin, where it is/was used as a diagnostic tool for the detection of human pathogen viruses, e.g. cytomegalovirus and respiratory syncytical virus (Watzinger *et al.* 2004) or for tumor associated gene monitoring e.g. the NANOG gene, expressed in human germ cell tumors (Hart *et al.* 2005). Due to its sensitivity, specificity and applicability to preserved samples, the use of qPCR has expanded to ecological questions as well. Zhu *et al.* (2005) showed that qPCR has the potentioal to examine the composition of phytoplankton species e.g. the abundance of Mamiellales within Mediterranean seawater samples. Another study found the uncultured Protista MAST-4 within non-polar water samples using qPCR (Rodríguéz-Martínez *et al.* 2009).

Among other things, these established qPCR approaches dealing with (pico-) plankton became the occasion for this thesis. Herein, Arctic/Subarctic plankton species in the area of the deep-sea observatory HAUSGARTEN (Fram Strait) shall be surveillanced via qPCR. Therefore, species specific probe sets were designed bioinformatically and tested in PCR and qPCR assays, using cultured phytoplankton species resp. clone-library representatives. After optimizing the qPCR protocols, environmental samples (sampling years 2010 to 2013) of the HAUSGARTEN stations HG1, HG4, N4, S3 and HG9. The probe sets Pglo1, Ppou2, Mpus2, Ehux, Csoc and Dino were designed to be specific for the phytoplankton key species *Phaeocystis globosa*, *Phaeocystis pouchetii*, *Micromonas pusilla*, *Emiliania huxleyi* resp. *Chaetoceros socialis* as well as for the phylum of Dinophyta. Additionally, a probe set (Euk2) was designed and tested for Eukaryota.

The binding sites of the designed probe sets lie next to a hyper variable region (V4) within the 18S rDNA, encoding for the SSU of ribosomes.This nucleic acid is ubiquitously distributed in eukaryoties and approximately 1800 bps in size.

The V4 region is particularly suitable for phylogenetic classification du to its broad diversitiy even on species level (Zimmermann *et al.* 2011). Other advantages are its size of 350-450 bps, resulting in statistically reliable equations and the presence of conserved DNA sequences flanking the V4 regions. To improve the detectability of a target sequence via qPCR, this sequence should occur in high copy numbers within the genome. The 18S rDNA fulfills this requirement in most cases but is showing a high variation in 18S rDNA copy numbers within differernt phytoplankton representatives. A variation between one and more than 12,000 (Zhu *et al.* 2005) is possible. Such variations can be also seen in different strains of the same species (Galluzzi *et al.* 2010). Therefore, it is advisable to use test laboratory cultures of species within PCR and qPCR optimization assays, inhabiting the sampling site region (Penna & Galluzzi 2013). Due to this, the Arctic culture of *M. pusilla* CCMP 2306 and the isolates of *P. pouchetii* 2314, 2621, 2977, 3039 (isolated by Dr. Steffi Gäbler-Schwartz in Arctic/Subarctic Regions) were used within this study. These isolates might have similar 18S rDNA copy numers than their environmental counterparts inhabiting the sampling site (Fram Strait – HAUSGARTEN) of qPCR examination.

For a successful PCR/qPCR assay, the right choice of primer/probe sets is crucial as well. This was demonstrated by Hong *et al.* (2009), were only 50 % of prokaryotic 16S rDNA of the microbial richness within a sample was recovered, using a single combination of PCR primers. Additionally, Suzuki & Giovannoni (1996) showed that PCR primers may discriminate certain template DNAs in their amplification. This means different amplification efficiencies of a primer set for different templates. In this study, it was attempted to design a Eukaryota specific probe set, amplifying the 18S rDNA of all eukaryotes within an environmental sample. The qPCR signals obtained from the species/phylum specific probe sets should be assigned in ratio to this reference to enable a quantitative comparison between the different species/phyla. The efficiency of the designed eukaryotic probe set Euk2 was tested in a multiple-template DNA qPCR. In all cases, the signals of the Euk2 set were weaker than the ones obtained from the species/phya specific probe sets, detecting the same DNA concentrations. Due to this, signals gained by Euk2 were not seen as representative and the probe set was not used as a reference value for the intention mentioned above. A quantitative comparison between the different phytoplankton key species /phyla in reference to the eukaryotic probe set was not possible.

Beside the primer bias within PCR testings, Hong *et al.* (2009) as well as Peano *et al.* (2004) mentioned the way of DNA extraction as another critical point, influencing further applications. In this step, it is necessary to remomve possible PCR inhibitors to ensure an untroubled amplification via PCR. Many DNA isolation kits are lacking this feature and leave inhibitory substances like humic acid, which can be found in seawater samples (Thurman 1986, Schnitzer *et al.* 1972). Within this study, the DNA isolation kit NucleoSpin® Plant II (Marcherey-Nagel) was used for DNA extraction, having no specific cleaning abilities for PCR inhibitors. Herein, a bias of qPCR results cannot be excluded.

As a further treatment of the isolated genomic DNA of environmental samples a whole genome amplification was performed in this study, using REPLI-g® (Qiagen). This step was necessary, because the original sample volume was limited and the DNA concentration of the different target species was assumed to be quite low within the environmental samples. The impact of REPLI-g® on further quantification uses was examined by Han *et al.* (2012), showing that no significant bias in copy numbers of specific genes occurred, compared to the native unamplified DNA. The only bias was found within GC-rich (> 53 % GC-content) regions of the genome (43 % overall GC-content). According to Escobar *et al.* (2011), the GC-content of 18S rDNA varies between 41.5 % and 52.5 % for the groups Haptophyta (~ 47.5 % – 50.0 %), Stramenopila (~ 41.5 % – 49.5 %), Alveolata (41.5 % – 47.5 %) and Viridiplantae (including green algae; ~ 46.5 % – 52.5 %).

7.2 Discussion of Results

7.2.1 Probe Set Design & Optimization

The design and optimization process of probe sets for phytoplankton key species and Eukaryota resulted in the probe sets Pglo1, Ppou2, Mpus2, Ehux, Csoc, Dino and Euk2. These probe sets showed several advantages compared to other designed probe sets within this thesis, justifying their use in further applications.

Bioinformatical Results

Bioinformatically, the sequences of the designed probes of Pglo1 and Ppou2 can be found in NCBI GenBank sequences (AC: EF100712.1 resp. AC: AJ27836.1). Mpus2 covers NCBI GenBank sequences of two arctic *M. pusilla* strains (*M. pusilla* CCMP 2306, AC: JF794057.1 and *M. pusilla* CCMP 2099, AC: DQ02575) as well as environmental sequences, obtained from arctic isolates (Kilias *et al.*, Metfies *et al.*).

Specificity tests of all probes within SILVA showed high specificity within the target groups of protista (out-group hits occurred as well, maybe influencing their application in environmental samples negatively). Here, the species specific probes of the probe sets Pglo1, Pglo2 and Mpus2 showed the best results. Five probe sets fulfill the requirements of qPCR application (size ~ 20 bps, GC-content 40-70 %, Tm 58-60 °C and amplicon size 50-150 bp) or meet the requirements in a tolerable range. Only the species specific probes of the probe sets Pglo1 and Ppou2 have significantly higher T_ms than recommended. For their use in qPCR, the T_ms of 66.2 °C and 69.1 °C are not obstructive since the used Taq polymerase has a temperature range up to at least 70 °C.

Results of PCR Specificity Assays

The probe sets Pglo1, Pglo2, Mpus2 and Euk2 showed amplification of specific DNA templates within PCR assays at an annealing temperature of 66 °C. This annealing temperature was assumed to meet the optimal annealing condition for the probe set due to its T_ms. The probe sets Csoc and Dino were tested by Dr. Christian Wolf within PCR specificity assays at an annealing temperature of 60 °C, showing specific amplifications. The two annealing temperatures (66 °C and 60 °C) were applied in qPCR settings for the respective probe set.Due to the fact that no probe sets for *Phaeocystis* spp. and *M. pusilla* consist of two species specific probes (one probe is always specific for Eukaryota), unspecific products for non-target DNA samples occurred to be possible. In the case of the probe sets Pglo1 and Ppou2 this occurrence was eliminated using 1.5 mmol/L Mg^{2+} and 5 % DMSO in PCR. These efforts showed no success for the probe set Mpus2 in PCR-based assays. The Mg^{2+} concentration as well as the addition of detergents such as DMSO can be used to create optimal PCR conditions, resulting in high specifity, yield and/ or efficiency. Mg^{2+} stabilizes primer-template bindings with less than 100 % complementarity and enhances the DNA polymerase binding affinity to annealed primers. Due to this, high Mg^{2+} concentrations lead to unspecific primer annealing/ DNA polymerase binding and therefore to unspecific amplicons (Innis *et al.* 1999, p. 8). DMSO is a PCR enhancing additive. It is used for facilitated strand separation complementary base pair interfering (Frackman *et al.* 1998). By doing this, secondary structures, negatively influencing the amplification process, can be reduced by DMSO.

Results of qPCR Specificity Assays

Beside the PCR tests, qPCR specificity assays with DNA of laboratory cultures were performed. This was necessary, because probe sets can hybridize with non-target DNA sequences, resulting in more than one amplicon. In qPCR, this event can be examined by the use of different mono-DNA templates (Yu *et al.* 2005). In this study, the designed probe sets Ppou2, Mpus2 and Csoc showed no amplification of template DNA obtained from non-target organisms (except Ppou2 for *P. globosa*). In contrast, the other probe sets showed amplification for DNA of *O. aurita* (Pglo1), *C. ericina* (Ehux & Dino), *P. micans* (Ehux) or *B. prasinos* (Dino). Hereafter, the non-target signals of the used probe sets within the environmental samples are discussed in regard to their relevance.

The species *P. micans*, detected by Ehux is distributed in all world oceans and can deal with warm and cold water conditions. Lasternas & Agustí (2010) demonstrated low abundance of this species in the northwest of Svalbard (Fram Strait) during the record arctic ice melting in the summer of 2007 by microscopy. Herein, dinoflagellates (including *P. micans*) made up around 4.4 % of the total phytoplankton biomass. Due to the fact that *P. micans* is found in brackish waters (Caroppo 2000) the ice melting of the year 2007 may have enhanced the occurrence of this species in the Fram Strait. Although the abundance of *P. micans* may be low, qPCR signals of Ehux should be critically questioned, consulting the environmental conditions.

The amplification of *O. aurita* DNA using Pglo1 may create difficulties in the qPCR test of environmental samples, collected in the Fram Strait. *O. aurita* is described to life in fresh-, sea-, and brackish waters. From 1992 till 1994, Wiktor *et al.* (1998) examined all three types of waters on the westcoast of Svalbard, to observe the presents of phytoplankton species. Herein, *O. aurita* was only found in brackish water areas. This finding may reduce reservation regarding to the application of Pglo1 in qPCR. As for *P. micans*, *O. aurita* may be influenced negativley in its abundance by the salinity conditions, present within the HAUSGARTEN area. Assuming this, the probe set Pglo1 may be suitable to detect *Phaoecystis* spp. within environmental samples, collected during regular sea-ice situations.

The amplifications of *C. ericina* DNA by the probe sets Ehux and Dino cast doubt on their applicability in examining arctic environmental samples via qPCR. According to Estrep & MacIntyre (1989), *Chrysochromulina* spp. are distributed in marine waters all over the world. Even arctic representatives are observed (Kling & Kristiansen 1983). Despite some coastal blooms of *C. polylepis* Gjøsæter & Johannesen, 1988) and under-ice blooms of *C. birgeri*

(Hällfors & Tomspon 1979), *Chrysochromulina* spp. occur mainly in low cell numbers per mL seawater (Estrep & MacIntyre 1989). Due to this, the qPCR results of the designed probe sets Ehux and Dino for Arctic environmental samples should be questioned critically. The amplification of *B. prasinos* DNA by the probe set Dino creates difficutlies in the detection of Dinophyta in such samples as well. The genus *Bathycoccus* was shown to be present in mixed Atlantic waters of the Fram Strait by using 454-pyrosequencing (Kilias *et al.* 2013). However, the abundance of *Bathycoccus* was observed as quite low (~ 2 % of the sequence reads). A similar observation was made in the study of Not et al. (2005). Herein, *B. parsinos* was recorded (via tyramide signal amplification FISH) in Arctic waters, especially at coastal and Polar front areas, with abundances around 9.3 % of the picoeukaryotic community. In Atlantic waters (southwest of Svalbard) or at coastal areas/Polar front, the *B. prasinos* abundance increased to 11.5 % resp. 12.6 %/18.2 %. Due to the fact that the area of the deep-sea observatory HAUSGARTEN is mainly influenced by Arctic water masses (WSC), *B. prasinos* may interfere the specific detection of Dinophyta using the probe set Dino in qPCR. Thereby, the qPCR signals, obtained by Dino induced amplifications have to be seen in a critical view.

Overall, it has to be claimed that cross-hybridizations of molecular probes (resulting in amplifications of non-target DNA) can never be excluded completely, since only a small selction of environmental relevant organisms can be tested. This is justified in the fact that many organisms are not cultivable/hard to handle in laboratory scale.

7.2.2 Environmental Samples qPCR Testing

Environmental samples of the Polarstern cruises ARK25/2 (2010), ARK26/2 (2011), ARK27/2 (2012) and ARK28/2 (2013), collected in the area of the deep-sea observatory HAUSGARTEN, were tested for the abundance of phytoplankton key species via qPCR, using the designed and optimized molecular probe sets.

Non-Abundant Phytoplankton Key Species

According to the qPCR signals of Pglo1, Mpus2 and Csoc, the phytoplankton key species *Phaeocystis globosa, Micromonas pusilla* and *Chaetoceros socialis* are not present in the tested environmental samples. Additionally, the presence of *Odontella aurita* DNA (amplified by Pglo1) can be excluded as well. These results have to be seen in a critical view. No signal within the qPCR assay may also indicate a present DNA concentration (beneath the detection limit of qPCR), not able to detect.

Abundant Phytoplankton Key Species

As abundant phytoplankton species/phyla in the HAUSGARTEN area, *Phaeocystis pouchetii, Emiliania huxleyi* and representatives of the phylum Dinophyta were identified by qPCR. For the determination of *P. pouchetii,* the results of the probe sets Pglo1 and Ppou2 have to be seen in combination. Due to the fact that Pglo1 (specific for *P. globosa*) served no qPCR signal but Ppou2 (specific for *P. globosa* and *P. pouchetii*) did, the obtained signals of Ppou2 can be assigned explicitly to *P. pouchetii.* The signals pointing to the presence of *E. huxleyi* and Dinophyta have to be seen in a critical view according to the cross-hybridizations mentioned above.

7.2.3 Comparison qPCR Data – Pyrosequencing Data

To verify the validity of the environmental qPCR results, the processed data sets of the different probe set assays were compared to pyrosequencing data. These next generation sequencing data were available for the samples, tested via qPCR and showed the semi-quanitative abundance of phytoplankton sepcies within the abundand biosphere (2010 to 2012). According to the data set of pyrosequening, sequences of *Phaeocystis* spp., the order Mamiellales (including *M. pusilla*), the class of Coscinodiscophyceae (including *C. socialis*) and the phylum of Dinophyta were identified between the years 2010 and 2012. The only phytoplankton order not present within the abundant biosphere is Isochrysidales, including the species *E. huxleyi*. Due to microscopic analyses (Dr. Eva-Maria Nöthig, data not shown) of the samples used for pyrosequencing, the presence of *E. huxleyi* can be approved.

Comparing the pyrosequencing data sets with the qPCR data sets of this thesis, both methods verify the presents of *Phaeocystis* spp. and of representatives of the phylum Dinophyta. In contrast, the two data sets make different statements for the presence of Mamiellales, Isochryidales and Coscinodiscophyceae. While qPCR did not detect any *M. pusilla,* the pyrosequencing data may show its occurrence within the order Mamiellales. The composition of representatives within this order is reported to be mostly dominated by *M. pusilla* within the Fram Strait (Kilias *et al.* 2013). One possible reason for this variation could have been a low DNA concentration of *M. pusilla* within the environmental (REPLI-g® treated) samples, lying beneath the detection limit of qPCR. In the case of *E. huxleyi*, both detection methods might contain errors. As mentioned above, the designed probe set for *E. huxleyi* showed cross-hybridization with DNA of *C. ericina* and *P. micans,* both potentially inhabiting the sampling site area. That would mean a false positive result, while *E. huxleyi* is absent or under-presented.

As a possible reason for the absence of *E. huxleyi* sequences within the pyrosequencing data, the the high GC-content of its genome (Liu *et al.* 2009) can be quoted. Due to this, sequencing approaches may be problematic. Like *M. pusilla*, *C. socialis* was not detected using qPCR approaches but was present in the pyrosequencing data (Coscinodiscophyceae). A similar reason as for *M. pusilla* is assumed.

The two data sets of *P. pouchetii/ Phaeocystis* spp. and Dinophyta were undergone a correlation analysis using the statistical software "R". Plotting the normalized qPCR data of one phytoplankton key species/phylum against the corresponding processed pyrosequencing data, a linear correlation was assumed due to the semi-quantitative character of both methods. For the *P. pouchetii* plot, a significant correlation ($p > 0.05$) was calculated. However, the obtained correlation coefficient r^2 (0.35) is too low to see this correlation as unquestionable. The plot of Dinophyta data resulted in no significant correlation ($p > 0.05$). The obtained r^2-value (0.10) confirms a not significant data context and shows the high deviations of the data sets.

Overall, the results of the performed environmental qPCR assays and pyrosequencing data in general (Sogin *et al.* 2006) enable only a semi-quantitative assessment of the abundance of the examined phytoplankton key species. Due to this, a correlation of both data sets is hard to proof. Several approaches can be attempted to improve the precision of quatitification: calibration curves (Einspanier *et al.* 1999) of DNA isolates from laboratory cultures (cell number for isolation known); nested (q)PCR approaches (Gosiewski *et al.* 2014), see 7.2 of this thesis.

7.3 Ecological Discussion – Temporal Progress

Due to the observed correlation of the pyrosequencing and qPCR data of *P. pouchetii*, verifying the results of this thesis, a semi-quantitative statement about the abundance of *P. pouchetii* is made in the following. Herein, the abundances (shown by normalized qPCR signals) of *P. pouchetii* at the sampling sites of the deep-sea observatory HAUSGARTEN are compared over the years 2010 to 2013 (Fig. 20 A). The environmental samples were collected between mid June and mid July, so a comparison of qPCR signals (normalized) between the sampling years is possible. According to Bauernfeind *et al.* (1994), blooms of *Phaeocystis pouchetii* occur in the Fram Strait near the ice edge or in open water zones. In the early summer of 2007, Saiz *et al.* (2013) reported a bloom of *P. pouchetii* in the Fram Strait area, while examining the distribution and feeding rates of zooplankton.

Within the year 2010, the sampling sites HG1, HG4, and S3 show nearly identical abundances of *P. pouchetii* (Ø deviation by the factor 1.2 (± 0.1)). Only the stations HG9 (westernmost station) and N4 (northernmost) showed lower abundances. HG9 varies by the factor 3.1 from the mean value of the stations HG1, HG4 and S3 while N4 varies by the factor 38.5. To examine the possible reasons (e.g. environmental conditions such as salinity, water temperature, ice situation) for the qPCR deviation of HG9, further investigations are needed

In the year 2011, the sampling sites HG1 and S3 showed nearly identical abundances of *P. pouchetii* (deviation by the factor 1.1). The stations N4 and HG9 vary by the factors 2.5 and 7.0 from the mean value of the stations HG1 and S3. Only for station HG9, no qPCR signal for *P. pouchetii* was obtained.

Within the year 2012, the sampling sites HG1 and HG4 as well as the sampling sites S3 and HG9 showed nearly identical abundances of *P. pouchetii* (deviation factor between HG1 and HG4 = 1.1; between S3 and HG9 = 1.2). The means of the sampling sites HG1, HG4 and S3, HG9 differ by the factor 2.6. Here, station N4 showed again a lower normalized qPCR signal than the other stations. The factor, varying from the mean of HG1 and HG4 is calculated with 5.5. The variation between the mean of S3, HG9 and N4 was observed with 2.1.

In the year 2013, the stations HG1, HG4, N4 and S3 differed by the average factor of 3.3 (± 1.7). The station HG9 was not sampled in this year.

Overall, the sampling sites within a single year have similar abundances for the phytoplankton key species *P. pouchetii*, according to the obtained qPCR data. Due to this, an abundance profile for the HAUSGARTEN area (represented by the sampling sites) over the years 2010 to 2013 can be made. Therefore, the mean was built by the normalized qPCR signals of all stations for each sampling year. By doing this, a mean of 82.4 for 2010, 7.1 for 2011, 9.8 for 2012 and 1631.6 for 2013 was calculated. This approach showed that the relative abundance of *P. pouchetii* over the years 2010 till 2013 reached its maximum in 2013, while the years 2011 and 2012 showed a comparable minimum. The relative *P. pouchetii* abundance of the year 2010 lay between the observed qPCR signal minimum (2011 and 2012) and maximum (2013). No publications, confirming/disproving these observations were found to date.

8. OUTLOOK

8.1 Applicability of Results

The probe sets Pglo1 and Pglo2 can be applied in a "tandem qPCR assay" to identify *Phaeocystis pouchetii* or *Phaeocystis globosa* in culture collections or in the environment and to make semi-quantitative statements about its abundance.

During former Polarstern cruises to the Arctic and Subarctic Regions, Dr. Steffi Gäbler-Schwarz *et al.* isolated various potential cells of *Phaeocystis* spp. out of seawater samples using light microscopy. These isolations are now part of a *Phaeocystis* spp. collection, cultivated in laboratories of the Alfred-Wegener-Institute, Bremerhaven, Germany. Due to the morphological similarity of the different *Phaeocystis* spp., molecular approaches are helpful to identify *Phaeocystis pouchetii* within this collection. Beside sequencing techniques (e.g. Sanger-Sequencing), the use of qPCR in combination with the here designed probe sets Pglo1 and Ppou2 is a promising tool to realize this approach. The advantage of qPCR over sequencing techniques is its sensitivity. Hence, *P. pouchetii* can be detected even in contaminated cultures, where it constitutes a minority. This advantage was observed within this study as well. Whereas Sanger-Sequencing identified the *P. pouchetii* 2977 isolate as DNA of *Paraphysomonas imperforata,* the qPCR signal using the designed probe sets posited the presents of *P. pouchetii.* For quantifying DNA, even next generation sequencing (NGS) techniques (e.g. pyrosequening, illumina) are laborious, costly and partially unriable compared to qPCR. A reason for this is the need of downstream methods e.g. electrophoresis or spectrophotometry, having *"[…] low sensitivity, consuming nanograms of precious samples and are not suitable for high-troughput workflows"* (information sheet of KAPABiosystems).

The probe sets Pglo1 and Ppou2 can be used in qPCR of environmental samples, collected from the Arctic and Subarctic Regions, to identify the algae *P. pouchetii.* Since the presence or absence of *P. pouchetii* gives something in evidence about the current environmental conditions were the sample was taken, this qPCR approach can be a very promising tool to examine environmental changes. Due to the semi-quantitative character of the developed qPCR assay, even a statement about the increase or decrease in *P. pouchetii* abundance can be made. Due to the optimal growth of *P. globosa* at 15-20 °C (Schoemann *et al.* 2005), this species is not inhabiting the Polar Regions to date. Using the developed qPCR assay, a possible invasion of *P. globosa* in this regions caused by rising water temperatures can be examined.

8.2 Improvements & Further Inestigations

To enhance the sensitivity of the developed qPCR assays, especially for the detection of *M. pusilla* and *C. socialis* within environmental samples of the Fram Strait, the application of a nested PCR seems promising. In the first step, the entire 18S rDNA within a sample will be amplified using Eukaryota specific primers, known for their high amplification efficiency (Lopez-Garcia *et al.* 2001, Elwood *et al.* 1985). By doing this, an amplicon of defined size is generated, which can be used as template within qPCR approaches, using the here designed probe sets. The result of this nested PCR is a 100- to 1000-times higher sensitivity in detecting low concentrated DNA samples [*]. Additionally, the efficiencies of the probe sets within the qPCR may be positively influenced due to the elimination of iterfering sequences by the prior PCR. This may result in the applicability of the designed Eukaryota probe set Euk2, enabling a quantitative comparison between the abundances of different phytoplankton key species via qPCR.

Additionally, the semi-quantitative character of the qPCR can be improved by the production of amplicons having defined sizes. Hereby, the amount of DNA (amplicon molecules) of a laboratory culture isolate can be calculated due to the molecular weight of the produced amplicon (PCR) and related to the obtained fluorescence signal (qPCR). This potential improvement needs further investigation.

[*] http://www.labor-gaertner.de/Nested-PCR.nested_pcr.0.html (June 12th, 2014)

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9.2 Picture Source (June 30th, 2014)

[1] **A: Modified after Agnieszka Beszczynska-Möller; year not known, not published:** http://www.awi.de/fileadmin/user_upload/News/Press_Releases/2008/3._Quartal/Graphik_p.jpg

B: Soltwedel et al.; 2005: *HAUSGARTEN: multidisciplinary investigations at deep-sea, long-term observatory in the Arctic Ocean.* Oceanography, 18, p. 46-61.

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[3] **Riebesell, U.; Zondervan, I.; Rost, B.; Tortell, P. D.; Zeebe, R. E.; Morel, F. M. M.; 2000:** *Reduced calcification of marine plankton in response to increased atmospheric CO_2.* Nature, 407, p. 364-367.

[4] **Foulon, E.; 2009, not published:** http://www.obsvlfr.fr/gallery2/v/Aquaparadox/album319/agMicromonas_RCC114_EF.jpg.

[5] **A: Vaulot, D.; Birrien, J. L.; Marie, D.; Casotti, R.; Veldhuis, M. J. W.; Kraay, G. W.; Chretiennotdinet, M. J.; 1994:** *Morphology, Ploidy, Pigment Composition, and Genome Size of Cultured Strains of Phaeocystis (Prymnesiophyceae).* Journal of Phycology, 30, p.1022-1035

B: Hamm, C. E.; 2000: *Architecture, ecology and biogeochemistry of Phaeocystis colonies.* Journal of Sea Research, 43, p. 307–315.

[6] **Rodríguez-Lázaro, D.; Hernández, M.:** *Real-Time PCR in Food Science: Current Technology and Applications – Introduction to the Real-time PCR.* Caister Academics Press/ Horizon Scientific Press, Poole (UK), 2013.

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10. APPENDIX

10.1 Appended Figures

MATERIAL – Microorganisms

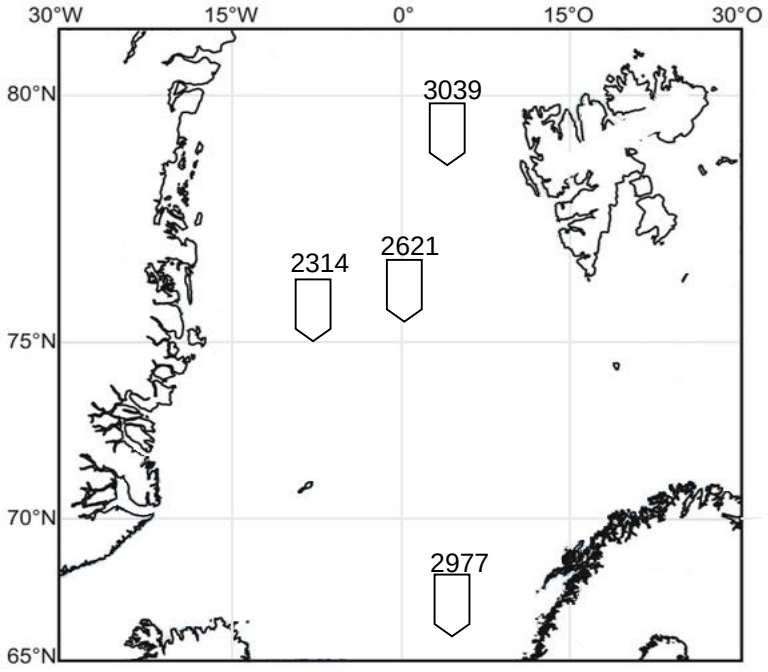

Fig. 7: Isolation Sites of *Phaeocystis* spp. Cells during former FS Polarstern Cruises

RESULTS – Bioinformatical Work (MEGA)

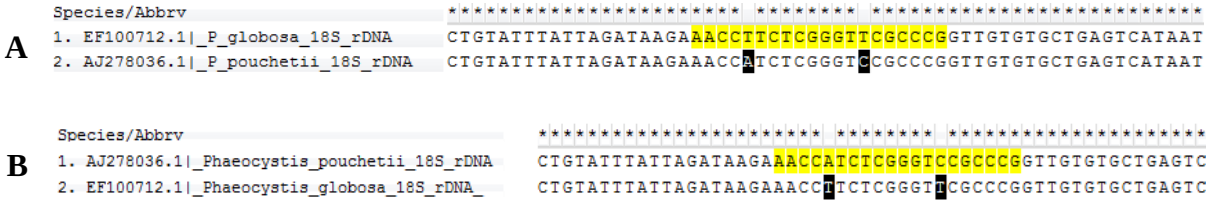

Fig. 8: MEGA Search Results for Probe Sequences (P1n, P1np) within NCBI and Contig 18S rDNA Sequences of *Phaeocystis* spp. (Screenshot)

A: Probe P1n (reverse complementary)

B: Probe P1np (reverse complementary)

Probe binding sites are shaded in yellow, mismatches are shaded in black. Matches are shaded in white and are star marked.

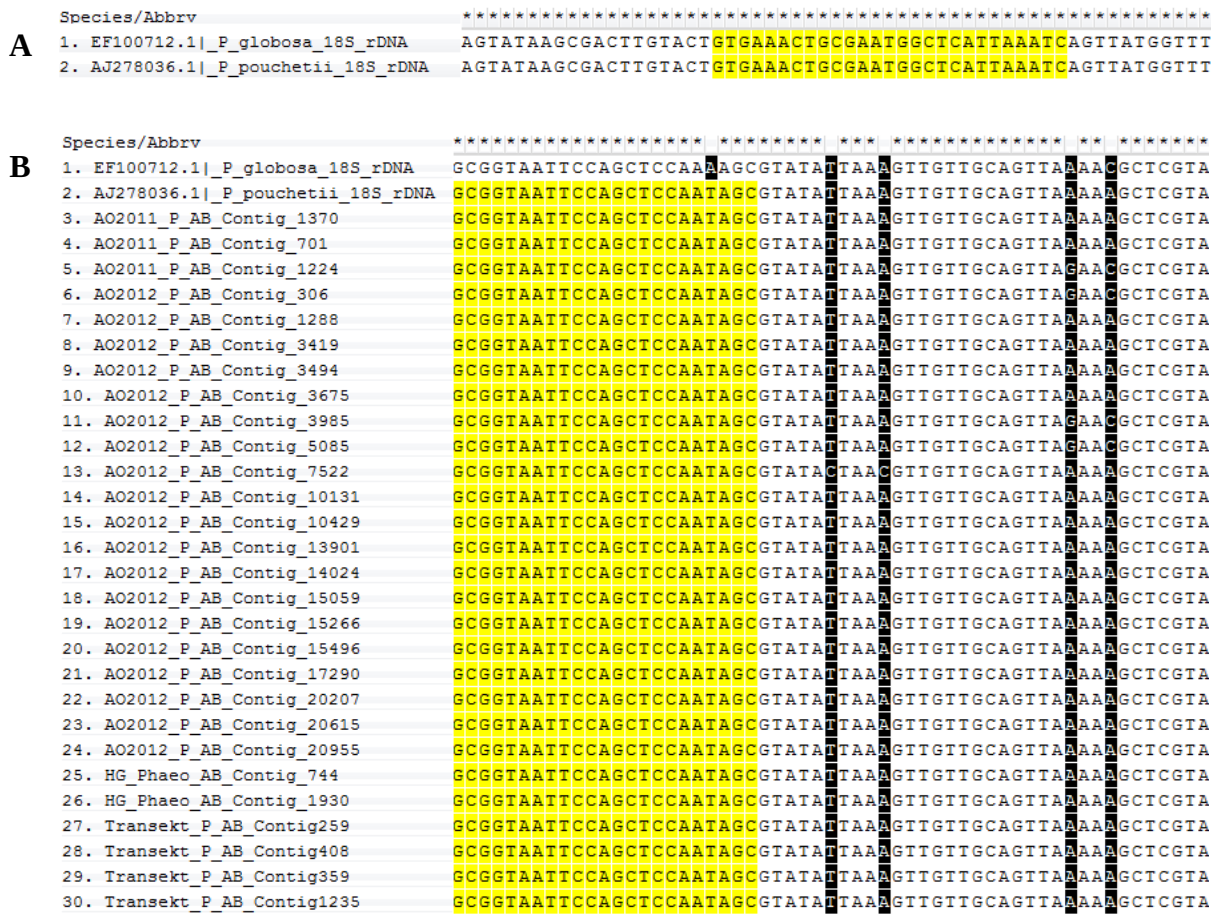

Fig. 9: MEGA Search Results for Probe Sequences (82F, 528F) within NCBI and Contig 18S rDNA Sequences of *Phaeocystis* spp. (Screenshot)

A: Probe 82F

B: Probe 528F

Probe binding sites are shaded in yellow, mismatches are shaded in black. Matches are shaded in white and are star marked.

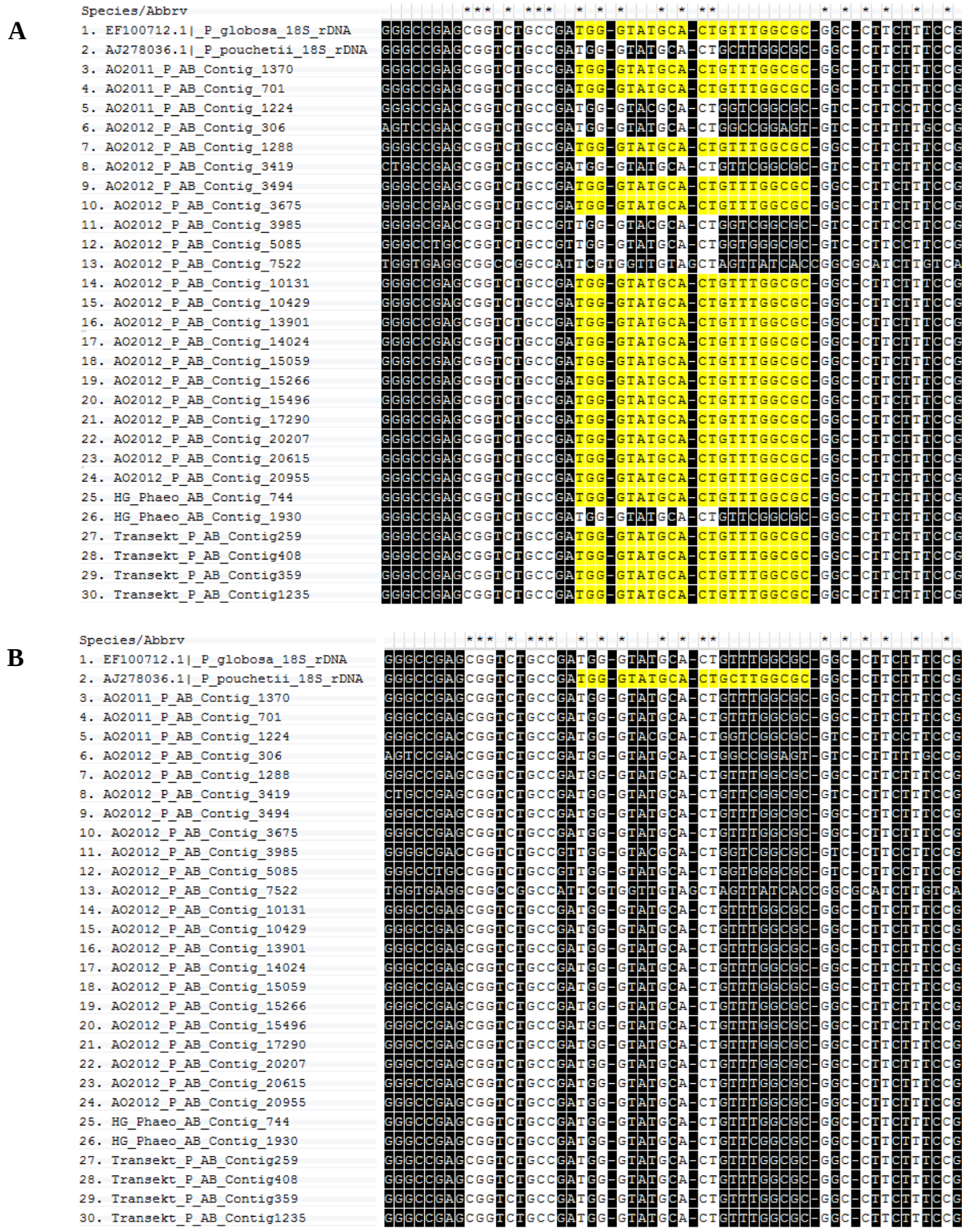

Fig. 10: MEGA Search Results for Probe Sequences (P4A, P4G) within NCBI and Contig 18S rDNA Sequences of *Phaeocystis* spp. (Screenshot)

A: Probe P4A (reverse complementary)

B: Probe P4G (reverse complementary)

Probe binding sites are shaded in yellow, mismatches are shaded in black. Matches are shaded in white and are star marked.

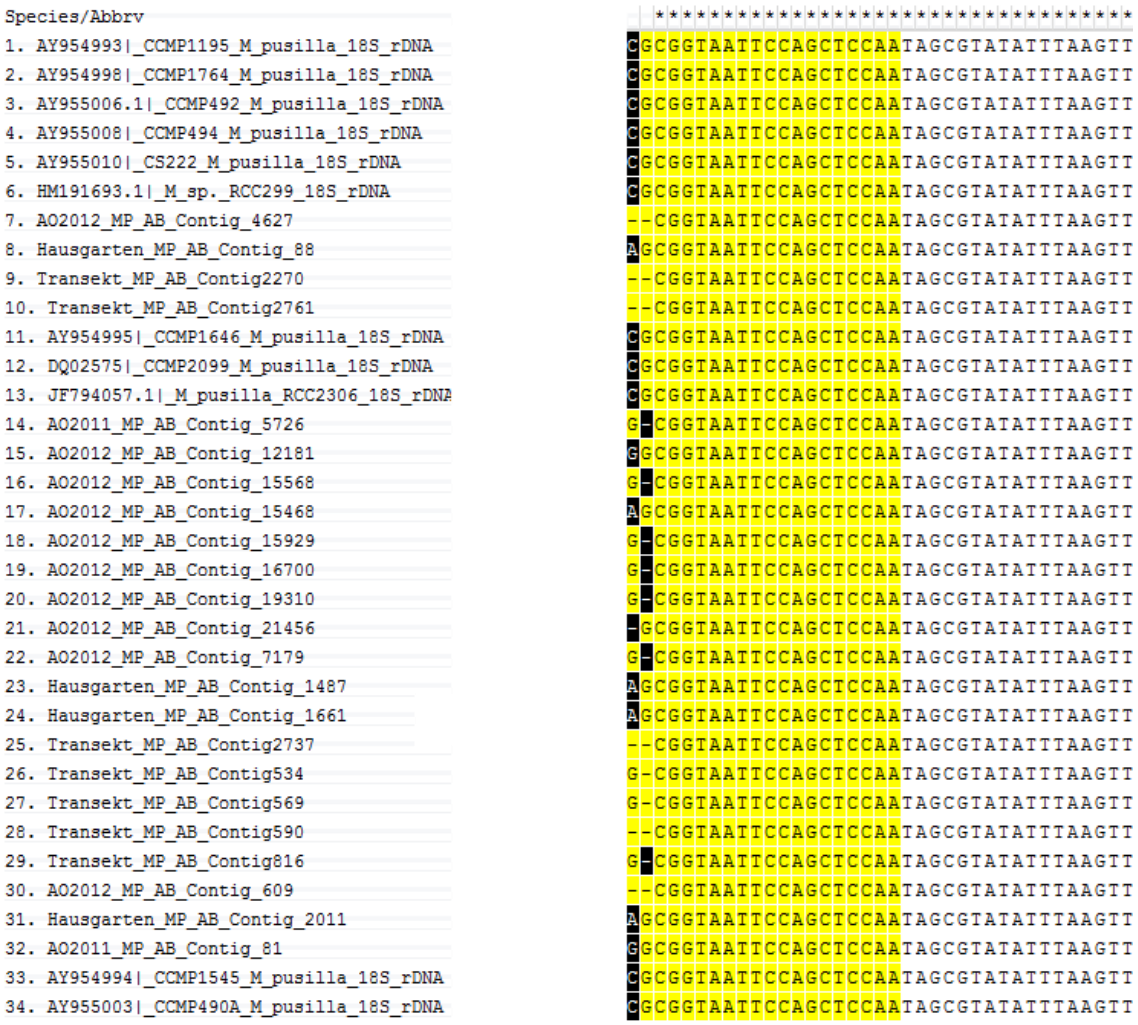

Fig. 11: MEGA Search Results for 528F Probe Sequences within NCBI and Contig 18S rDNA Sequences of *Micromonas* spp. (Screenshot)

Probe binding sites are shaded in yellow, mismatches and gaps are shaded in black.

Matches are shaded in white and are star marked.

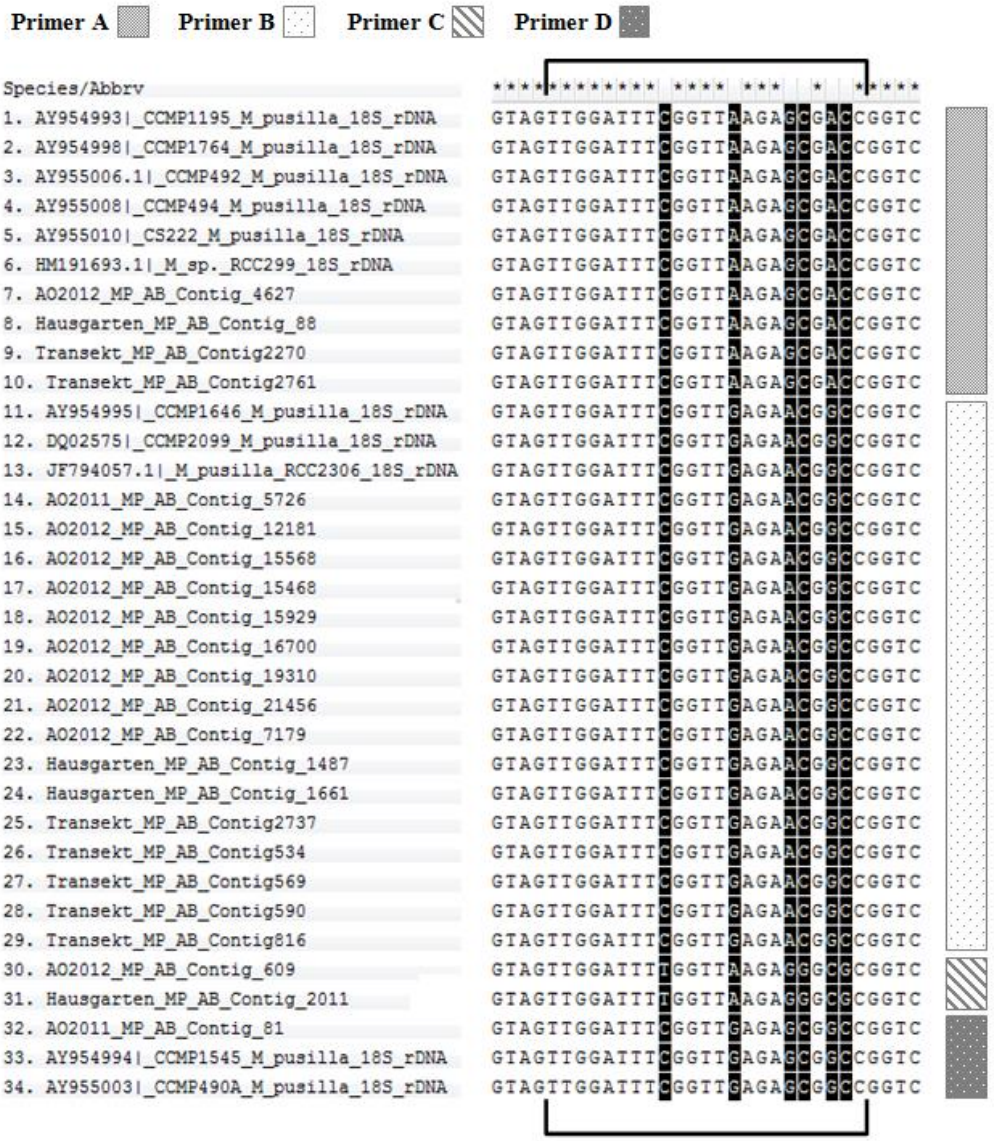

Fig. 12: MEGA Search Results for Primer A-D Probe Sequences within NCBI and Contig 18S rDNA Sequences of *Micromonas* spp. (Screenshot)

Probe binding sites are framed by brackets. Primers A-D are assigned to the NCBI and contig sequences by bars with different structures.

Mismatches and gaps are shaded in black. Matches are shaded in white and are star marked.

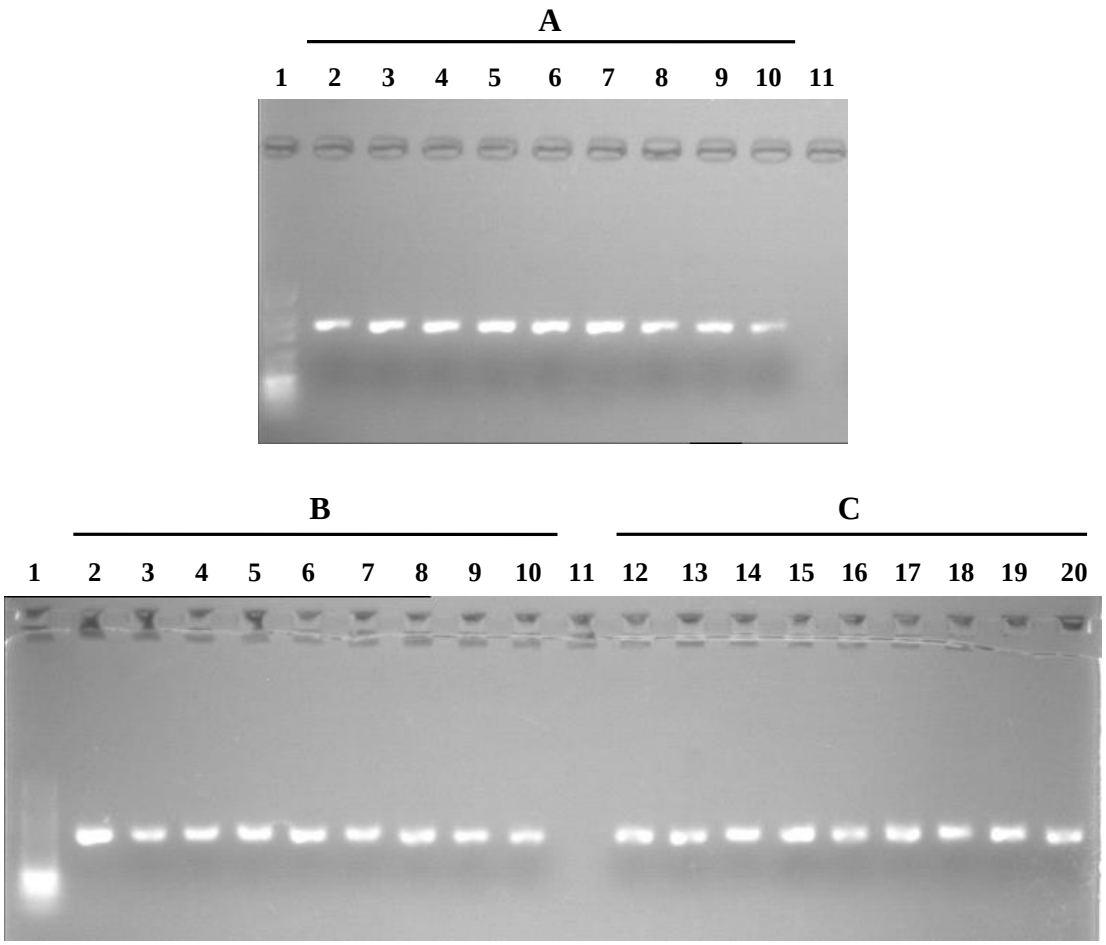

Fig. 13: Agarose Gel (3 %) of Gradient (53.2 – 65.8 °C) PCR for the *P. globosa* Probe Sets Pglo1, Pglo2 and Ppou1. 5.0 mmol/L Mg^{2+}, 0 % DMSO. Used DNA Length Marker: Ultra Low Range DNA Ladder I; Power Supply: (A) 70 V, 30 min; (B, C) 70 V, 50 min. Stained with GelRed®

A: *P. globosa* 1524 DNA amplified with Pglo1

B: *P. globosa* 1524 DNA amplified with Pglo2

C: *P. globosa* 1524 DNA amplified with Ppou1

Lane 1:	DNA length marker	**Lane 6 (16):**	58.7 °C T_a	**Lane 11:**	Empty
Lane 2 (12):	53.2 °C T_a	**Lane 7 (17):**	60.6 °C T_a		
Lane 3 (13):	54.0 °C T_a	**Lane 8 (18):**	62.5 °C T_a		
Lane 4 (14):	55.2 °C T_a	**Lane 9 (19):**	64.3 °C T_a		
Lane 5 (15):	56.8 °C T_a	**Lane 10 (20)**:	65.8 °C T_a		

The DNA isolates were not diluted before use. T_a = Annealing Temperature.

The negative controls are not seen on Fig. 11. They showed no DNA bands.

No statement can be given about the fragment sizes of the DNA bands, due to the bad performance of the DNA length marker.

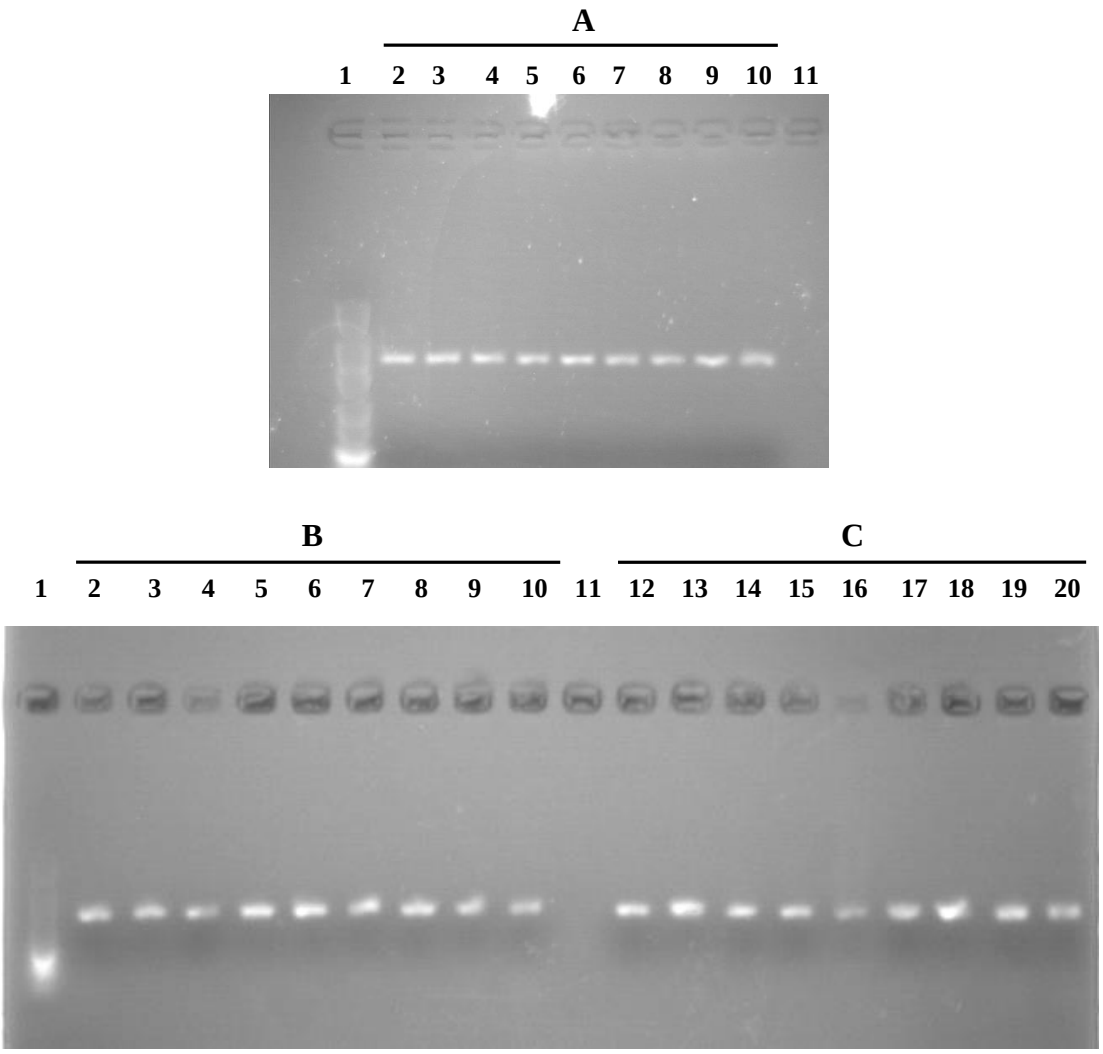

Fig. 14: Agarose Gel (3 %) of Gradient (53.2 – 65.8 °C) PCR for the *P. globosa* Probe Sets Pglo1, Pglo2 and Ppou1. 5.0 mmol/L Mg^{2+}, 0 % DMSO. Used DNA Length Marker: Ultra Low Range DNA Ladder I; Power Supply: (A) 70 V, 30 min; (B, C) 70 V, 50 min. Stained with GelRed®

A: *E. huxleyi* 1225 DNA amplified with Pglo1

B: *E. huxleyi* 1225 DNA amplified with Pglo2

C: *E. huxleyi* 1225 DNA amplified with Ppou1

Lane 1:	DNA length marker	**Lane 6 (16):**	58.7 °C T_a	**Lane 11:** Empty
Lane 2 (12):	53.2 °C T_a	**Lane 7 (17):**	60.6 °C T_a	
Lane 3 (13):	54.0 °C T_a	**Lane 8 (18):**	62.5 °C T_a	
Lane 4 (14):	55.2 °C T_a	**Lane 9 (19):**	64.3 °C T_a	
Lane 5 (15):	56.8 °C T_a	**Lane 10 (20)**:	65.8 °C T_a	

The DNA isolates were not diluted before use. T_a = Annealing Temperature.

The negative controls are not seen on Fig. 11. They showed no DNA bands.

No statement can be given about the fragment sizes of the DNA bands, due to the bad performance of the DNA length marker.

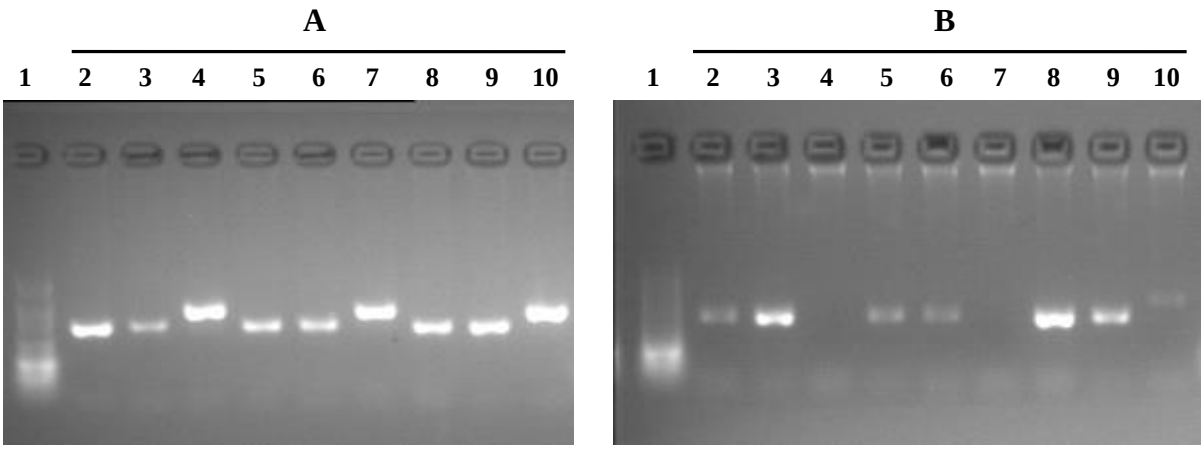

Fig. 15: Agarose Gel (3 %) of Gradient (1.5 mmol/L, 2.5 mmol/L, 3.8 mmol/L Mg^{2+}) PCR for the *P. globosa* Probe Sets Pglo1, Pglo2 and Ppou1. 0 % DMSO. Annealing Temperature: 66 °C. Used DNA Length Marker: Ultra Low Range DNA Ladder I; Power Supply: 70 V, 30 min. Stained with GelRed®

A: Template DNA = *P. globosa* 1524 DNA

B: Template DNA = *E. huxleyi* 1225 DNA

Lane 1:	DNA length marker	**Lane 6:**	Probe set Ppou1, 2.5 mmol/L Mg^{2+}
Lane 2:	Probe set Pglo2, 1.5 mmol/L Mg^{2+}	**Lane 7:**	Probe set Pglo1, 2.5 mmol/L Mg^{2+}
Lane 3:	Probe set Ppou1, 1.5 mmol/L Mg^{2+}	**Lane 8:**	Probe set Pglo2, 3.8 mmol/L Mg^{2+}
Lane 4:	Probe set Pglo1, 1.5 mmol/L Mg^{2+}	**Lane 9:**	Probe set Ppou1, 3.8 mmol/L Mg^{2+}
Lane 5:	Probe set Pglo2, 2.5 mmol/L Mg^{2+}	**Lane 10:**	Probe set Pglo1, 3.8 mmol/L Mg^{2+}

The DNA isolates were not diluted before use.

The negative controls are not seen on Fig. 15. They showed no DNA bands.

No statement can be given about the fragment sizes of the DNA bands, due to the bad performance of the DNA length marker.

NOTE: The visible shift of the DNA bands in the lanes four, seven and ten (amplified using probe set Pglo1) compared to the remaining bands (amplified using probe sets Pglo2 resp. Ppou1) shows the suspected deviation in fragment size (Pglo1 amplicons: 137 bps; Pglo2/Ppou1 amplicons: 112 bps).

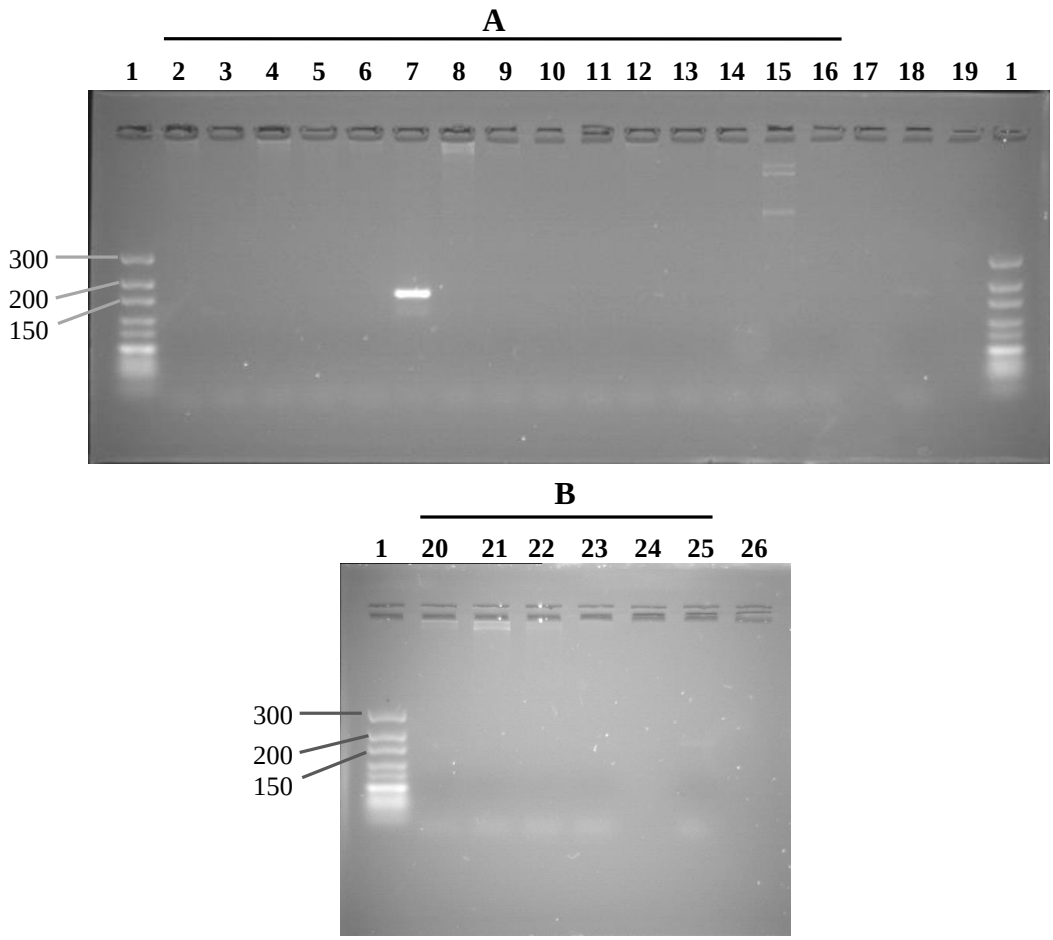

Fig. 16: Agarose Gel (3 %) of PCR using Pglo1 Probe Set. 1.5 mmol/L Mg^{2+}, 5 % DMSO. Annealing Temperature: 66 °C. Used DNA Length Marker: Ultra Low Range DNA Ladder I; Power Supply: (A) 75 V, 50 min; (B) 75 V, 30 min. Stained with GelRed®

A: Amplification of DNA isolates of several representatives of the main phytoplanktonic Protista groups including *P. globosa 1524* and *E. huxleyi* 1225

B: Amplification of DNA isolates of *P. pouchetii* (2314, 2621, 2977, 3039)

Lane 1: DNA length marker
Lane 2: *Odontella aurita*
Lane 3: *Thalassiosira weissflogii*
Lane 4: *Chaetoceros socialis*
Lane 5: *Chaetoceros muelleri*
Lane 6: *Chrysochromulina ericina*
Lane 7: *Phaeocystis globosa* 1524
Lane 8: *Emiliania huxleyi* 1225
Lane 9: *Ceratium longipes*
Lane 10: *Alexandrium minutum*
Lane 11: *Prorocentrum micans*
Lane 12: *Pyramimonas parkeae*
Lane 13: *Prasinococcus capsulatus*
Lane 14: *Bathycoccus prasinos*
Lane 15: *Micromonas pusilla* Clone 170
Lane 16: *Microcystis aeruginosa*
Lane 17 (19, 24, 26): Empty
Lane 18 (25): Negative Control
Lane 20: *Phaeocystis pouchetii* 2314
Lane 21: *Phaeocystis pouchetii* 2621
Lane 22: *Phaeocystis pouchetii* 2977
Lane 23: *Phaeocystis pouchetii* 3039

The DNA isolates were not diluted before use.

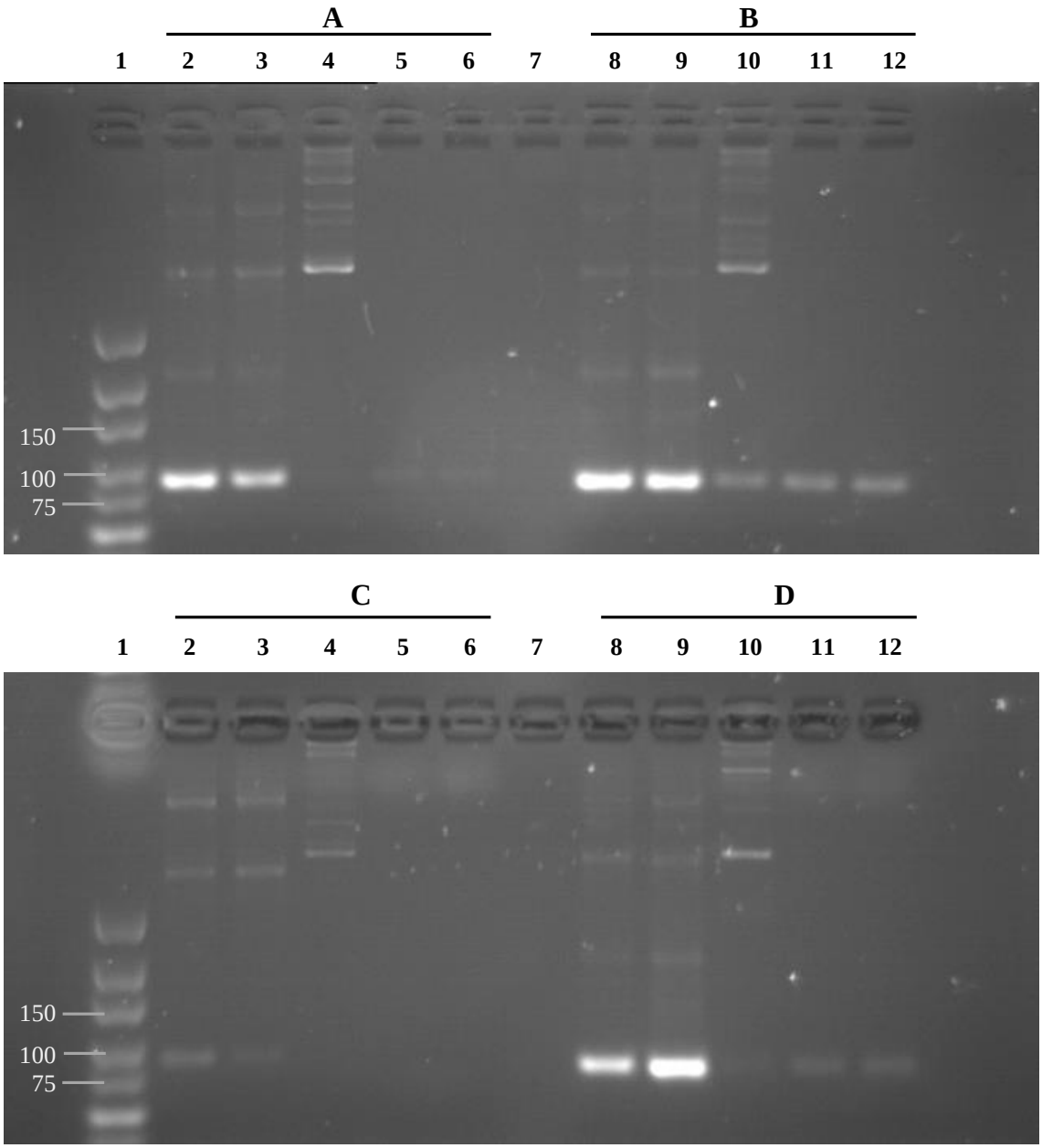

Fig. 17: Agarose Gel (3 %) of PCR using Probe Sets Mpus1 – Mpus4. 1.5 mmol/L Mg^{2+}, 0 % DMSO. Annealing Temperature: 66 °C. Used DNA Length Marker: Ultra Low Range DNA Ladder I; Power Supply: 80 V, 60 min. Stained with GelRed®

A: Probe set Mpus1 **C:** Probe set Mpus3

B: Probe set Mpus2 **D:** Probe set Mpus4

Lane 1: DNA length marker

Lane 2 (8): *Micromonas pusilla* Clone 170

Lane 3 (9): *Micromonas pusilla* Clone 179

Lane 4 (10): *Emiliania huxleyi* 1225

Lane 5 (11): Negative Control

Lane 6 (12): Negative Control

Lane 7: Empty

The DNA isolates were not diluted before use.

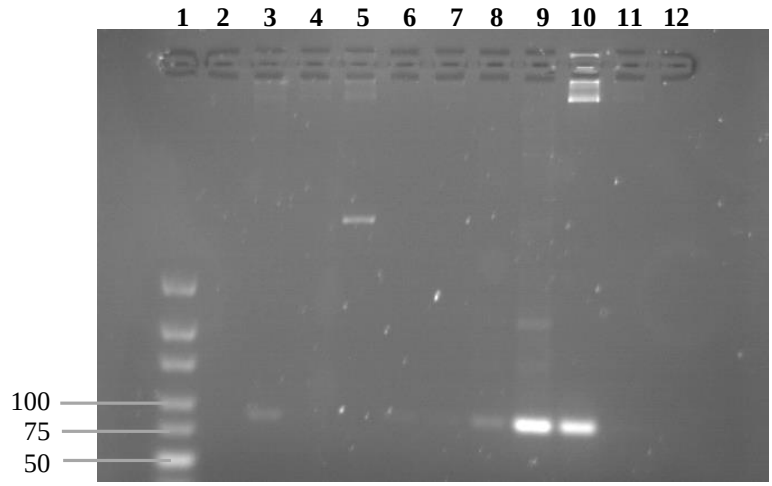

Fig. 18: Agarose Gel (3 %) of PCR using Mpus2 Probe Set. 1.5 mmol/L Mg^{2+}, 5 % DMSO. Annealing Temperature: 66 °C. Used DNA Length Marker: Ultra Low Range DNA Ladder I; Power Supply: 70 V, 50 min. Stained with GelRed®

Lane 1:	DNA length marker	**Lane 7:**	*Bathycoccus prasinos*
Lane 2:	*Thalassiosira weissflogii*	**Lane 8:**	*Micromonas pusilla Clone* 170
Lane 3:	*Chaetoceros muelleri*	**Lane 9:**	*Micromonas pusilla Clone* 179
Lane 4:	*Phaeocystis globosa* 1524	**Lane 10:**	*Micromonas pusilla* 2306
Lane 5:	*Emiliania huxleyi* 1225	**Lane 11:**	*Microcystis aeruginosa*
Lane 6:	*Prorocentrum micans*	**Lane 12:**	Negative Control

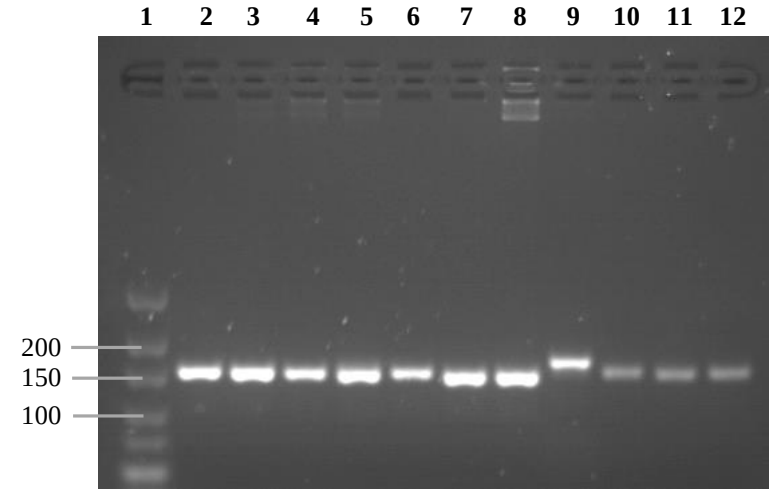

Fig. 19: Agarose Gel (3 %) of PCR using Euk2 Probe Set. 1.5 mmol/L Mg^{2+}, 5 % DMSO. Annealing Temperature: 66 °C. Used DNA Length Marker: Ultra Low Range DNA Ladder I; Power Supply: 70 V, 40 min. Stained with GelRed®

Lane 1:	DNA length marker	**Lane 6:**	*Prorocentrum micans*
Lane 2:	*Thalassiosira weissflogii*	**Lane 7:**	*Bathycoccus prasinos*
Lane 3:	*Chaetoceros muelleri*	**Lane 8:**	*Micromonas pusilla* 2306
Lane 4:	*Phaeocystis globosa* 1524	**Lane 9:**	*Microcystis aeruginosa*
Lane 5:	*Emiliania huxleyi* 1225	**Lane 10-12:**	Negative Control

10.2 Appended Tables

RESULTS – Bioinformatical Work (SILVA)

Table 24: Specificity Test Results of Probes for *Phaeocystis* spp. using SILVA (TestProbe)

Probe	P4A			P4G			P1n			P1np		
Allowed Mismatches	0	1	2	0	1	2	0	1	2	0	1	2
Stramenopila												
Alveolata												
Rhizaria												
Haptophyta	3	9	21	5	5	21	3	7	8	1	5	8
Archaeplastida												
Amoebozoa												
Opisthokonta												
Cryptophyceae												
Excavata												
Incertae Sedis												

There was no hit observed within the SILVA sequences of archaea or bacteria

Table 25: Specificity Test Results of Probes for *Micromonas pusilla* using SILVA (TestProbe)

Probe	Primer A			Primer B			Primer C			Primer D		
Allowed Mismatches	0	1	2	0	1	2	0	1	2	0	1	2
Stramenopila												
Alveolata	10	10	10	2	2	31				1	2	19
Rhizaria	1	1	1									
Haptophyta												
Archaeplastida	22	23	31	13	21	23	11	12	12	8	21	46
Amoebozoa												
Opisthokonta	1	1	1							1	1	1
Cryptophyceae												
Excavata												
Incertae Sedis												

There was no hit observed within the SILVA sequences of archaea or bacteria

Table 26: Specificity Test Results of Probes for Dinophyta and *C. socialis* using SIVA (TestProbe)

Probe	Dino 18SF1			Dino E-12 Reverse			Chae soc F			Chae soc R		
Allowed Mismatches	0	1	2	0	1	2	0	1	2	0	1	2
Stramenopila	1	4	5	2	2	5	3	3	3	2	3	12
Alveolata	612	936	1212	1041	1788	2267						
Rhizaria	1	1	3	1	2	14						
Haptophyta												
Archaeplastida	1	1	2	1	2	2						
Amoebozoa	1	1	1	1	1	1						
Opisthokonta	1	13	35	5	6	8						
Cryptophyceae	0	1	1	1	1	1						
Excavata	1	1	4	1	3	5						
Incertae Sedis			8									

There was no hit observed within the SILVA sequences of archaea or bacteria

Table 27: Specificity Test Results of Probes for *Emiliania huxleyi* using SIVA (TestProbe)

Probe	Ehux F-745n			Ehux R-803n		
Allowed Mismatches	0	1	2	0	1	2
Stramenopila	26	26	26	5	5	5
Alveolata	1	1	64			
Rhizaria	10	10	10			
Haptophyta	11	12	14	40	153	171
Archaeplastida	1	1	10			
Amoebozoa				2	2	2
Opisthokonta	3	3	75			
Cryptophyceae				1	1	1
Excavata						
Incertae Sedis				3	3	3

There was no hit observed within the SILVA sequences of archaea or bacteria

Table 28: Specificity Test Results of Eukaryota Specific Probes using SIVA (TesProbe)

Probe	82F			528F			1055Fn			1200Rn			EukR18Fn / EukR18Rn		
Allowed Mismatches	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2
Stramenopila	13	895	1322	2378	2522	2587	2695	2752	2764	2557	2659	2686	105	2541	2698
Alveolata	25	1877	2414	3939	4821	4947	4948	5264	5318	4040	4565	5096	666	4621	4928
Rhizaria	18	55	215	651	1083	1194	1409	1491	1503	1077	1405	1441	45	1104	1188
Haptophyta	157	165	167	184	194	195	202	206	206	195	201	202	5	198	205
Archaeplastida	4065	4658	4750	5126	5355	5402	5741	6006	6041	5144	5910	5983	196	3592	4298
Amoebozoa	66	110	147	287	464	485	335	483	492	271	317	415	102	285	398
Opisthokonta	6799	11561	15639	18249	21418	22809	23134	24323	24460	20017	23053	23519	13738	17063	22960
Cryptophyceae	41	125	182	55	221	227	230	234	234	222	225	226	6	219	230
Excavata	10	12	19	49	456	621	274	869	895	56	91	105	21	50	123
Incertae Sedis	33	92	102	117	131	135	138	143	143	125	138	139	53	93	133
Archaea							2521	7395	11465						
Bacteria							300	45666	359211						6

EukR18Rn is the reversed and complementary sequence of EukR18Fn

RESULTS – qPCR Specificity Assays

Table 29: qPCR Specificity Test of Designed Probe Sets using Template DNA of Laboratory Cultures

Probe Set	DNA Isolates of Laboratory Cultures		C_T Triplicates	C_T Mean	C_T SD	T_m (°C) Triplicates	T_m (°C) T_m Mean	T_m (°C) SD
Pglo1	Binding Sample	*P. globosa* 1524	33.6			78.9		
			32.1	**32.4**	1.0	79.1	**79.1**	0.2
			31.5			79.3		
	Non-Binding Sample	*B. prasinos*						
		C. ericina						
		O. aurita	35.3			76.5		
			36.1	**36.1**	0.5	76.5	**76.5**	0.0
			36.8			76.5		
		P. micans						
		P. pouchetii 2314						
		P. pouchetii 2621						
		P. pouchetii 2977	30.6			79.1		
			29.9	**30.0**	0.6	79.3	**79.3**	0.2
			29.5			79.4		
		P. pouchetii 3039						

Continuation 1 of Table 29:

Probe Set	DNA Isolates of Laboratory Cultures		C_T Triplicates	C_T Mean	C_T SD	T_m (°C) Triplicates	T_m (°C) T_m Mean	T_m (°C) SD
Ppou2	Binding Sample	*P. pouchetii* 2314	24.4			79.3		
			23.9	**23.8**	0.7	79.5	**79.5**	0.2
			23.1			79.6		
		P. pouchetii 2621	23.9			79.8		
			23.9	**23.8**	0.2	79.8	**79.8**	0
			23.5			79.8		
		P. pouchetii 2977	22.5			79.3		
			22.0	**22.1**	0.4	79.5	**79.5**	0.2
			21.7			79.6		
		P. pouchetii 3039						
	Non-Binding Sample	*B. prasinos*						
		C. ericina						
		O. aurita						
		P. micans						
		P. globosa 1524	38.3			79.3		
			37.2	**37.5**	0.7	79.5	**79.5**	0.2
			36.9			79.6		
Mpus2	Binding Sample	*M. pusilla* 2306	17.5			76.4		
			16.8	**16.8**	0.7	76.5	**76.5**	0.2
			16.2			76.7		
		M. pusilla Clone 179 °	Strong Signal (< 10)	/ / /	/ / /	76.5		
						76.5	**76.5**	0.0
						76.5		
	Non-Binding Sample	*B. prasinos*						
		C. ericina						
		O. aurita						
		P. micans						
Ehux	Binding Sample	*E. huxleyi* 1225	20.1			77.0		
			20.0	**20.1**	0.1	77.0	**77.0**	0.0
			20.2			77.0		
	Non-Binding Sample	*B. prasinos*						
		C. ericina	19.8			76.8		
			20.0	**20.0**	0.2	77.0	**76.9**	0.1
			20.2			77.0		
		O. aurita						
		P. micans	36.2			76.8		
			34.9	**35.6**	0.9	76.6	**76.7**	0.1
		P. globosa 1524						
		P. pouchetii 2314						
		P. pouchetii 2621						
		P. pouchetii 2977						
		P. pouchetii 3039	19.2			77.2		
			19.1	**19.1**	0.1	77.0	**77.1**	0.1
			19.0			77.0		
Csoc	Binding Sample	*C. socialis*	20.6			82.2		
			19.7	**20.2**	0.5	82.2	**82.1**	0.1
			20.3			82.0		
	Non-Binding Sample	*B. prasinos*						
		C. ericina						
		O. aurita						
		P. micans						

Continuation 2 of Table 29:

Probe Set	DNA Isolates of Laboratory Cultures		C_T Triplicates	C_T Mean	C_T SD	T_m (°C) Triplicates	T_m (°C) T_m Mean	T_m (°C) SD
Dino	Binding Sample	*A. minutum* *	15.9			77.4		
			19.7	**16.3**	0.5	77.6	**77.5**	0.1
			16.6			77.6		
		P. micans *	26.2			80.8		
			25.7	**25.9**	0.3	80.8	**80.8**	0.1
			25.9			80.7		
	Non-Binding Sample	*B. prasinos*	33.8			78.4		
			35.2	**34.2**	0.9	78.4	**78.4**	0.1
			33.6			78.3		
		C. ericina	37.2			78.3		
			36.5	**37.0**	0.4	78.4	**78.4**	0.1
			37.3			78.4		
		O. aurita						
Euk2	Binding Sample	*B. prasinos*	28.7			81.2		
			28.5	**28.5**	0.2	81.2	**81.1**	0.1
			28.3			81.0		
		M. pusilla 2306	26.8			79.4		
			26.6	**26.6**	0.3	79.5	**79.5**	0.1
			26.2			79.5		
		C. ericina	30.9			82.8		
			31.2	**31.0**	0.2	82.9	**83**	0.2
			31.0			83.1		
		E. huxleyi 1225	31.8			83.3		
			32.0	**31.9**	0.1	83.2	**83.2**	0.2
			32.0			83.0		
		P. globosa 1524 *	32.5			82.5		
			31.5	**32.1**	0.5	82.6	**82.6**	0.2
			32.3			82.8		
		P. pouchetii 2314 *	30.0			82.1		
			30.2	**30.0**	0.2	82.0	**82**	0.1
			29.9			82.0		
		P. micans *	37.4			80.2		
			38.5	**37.9**	0.6	80.2	**80.1**	0.1
			37.7			80.0		
		A. minutum	35.7			78.6		
			36.2	**35.9**	0.3	78.6	**78.6**	0.1
			35.8			78.7		
		O. aurita	26.3			81.3		
			26.2	**26.4**	0.3	81.2	**81.2**	0.2
			26.8			81.0		
		C. socialis	34.4			77.9		
			33.9	**33.7**	0.8	78.1	**78**	0.1
			32.8			78.1		
	Non-Binding Sample	*M. aeruginosa*	39.2			83.9		
			38.2	**38.4**	0.8	84.3	**84.2**	0.2
			37.7			84.3		

° = undiluted

In general, template DNA isolations were diluted 1 : 5 before use

* = Template DNA, diluted 1 : 2 before use

The DNA isolations of *E. huxleyi 1225*, *P. globosa* 1524 and the four isolates *P. pouchetii* (2314, 2621, 2977, 3039) were treated with REPLI-g® before diluted and subsequently used in qPCR

= undetermined

/ / / = no data

SD = Standard Deviation

RESULTS – qPCR Efficiency Assays

Table 30: Efficiency Test of the Designed Probe Sets (especially Euk2) using Mixed Template DNA Solutions

Template DNA-mix	Probe Set	C_T			T_m1 (°C)			T_m2 (°C)			T_m3 (°C)		
		Triplicates	Mean	SD	Triplicates	Mean	SD	Triplicates	Mean	SD	Triplicates	Mean	SD
P. globosa + *E. huxleyi*	Euk2	34.6			82.7			72.5					
		34.5	34.5	0.1	82.7	82.8	0.1		72.4	0.2			
		34.5			82.9			72.2					
	Ehux	21.9			76.8								
		22.1	22.0	0.1	76.8	76.8	0.1						
		22.1			76.9								
	Pglo1	35.1			79.1								
		35.4	35.4	0.3	78.9	78.9	0.2						
		35.6			78.8								
M. pusilla + *E. huxleyi*	Euk2	27.1			79.7								
		27.1	27.2	0.2	79.6	79.6	0.1						
		27.4			79.6								
	Ehux	21.6			76.6								
		21.5	21.5	0.1	76.7	76.7	0.2						
		21.5			76.9								
	Mpus2	21.5			76.4								
		21.7	21.8	0.4	76.6	76.5	0.1						
		22.3			76.6								
P. pouchetii + *E. huxleyi*	Euk2	31.3			83.0			72.8					
		31.2	31.0	0.5	83.0	83.0	0.0	73.1	73.1	0.3			
		30.4			83.0			73.4					
	Ehux	21.7			77.1								
		21.8	21.7	0.1	76.9	76.9	0.2						
		21.7			76.8								
	Ppou2	26.1			79.6								
		25.2	25.5	0.6	79.7	79.7	0.1						
		25.1			79.7								
A. minutum + *E. huxleyi*	Euk2	32.1			83.5			79.2					
		31.7	31.9	0.2	83.5	83.5	0.1	79.2	72.7	0.1			
		31.9			83.4			79.1					
	Ehux	21.7			77.1								
		21.7	21.7	0.0	76.9	76.9	0.2						
		21.7			76.8								
	Dino	24.5			77.9								
		24.7	24.5	0.2	78.1	78.0	0.1						
		24.4			78.1								
C. socialis + *E. huxleyi*	Euk2	31.5			83.2			78.3			72.8		
		30.8	31.0	0.5	83.4	83.3	0.1	78.3	78.3	0.1	73.1	73.1	0.3
		30.5			83.4			78.4			73.3		
	Ehux	21.6			77.1								
		21.5	21.5	0.5	77.3	77.2	0.1						
		21.5			77.3								
	Csoc	22.2			82.1								
		22.4	22.3	0.1	82.1	82.1	0.0						
		22.2			82.1								
E. huxleyi	Euk2	32.7			83.4								
		31.9	32.2	0.4	83.4	83.3	0.1						
		32.0			83.2								
	Ehux	20.6			76.9								
		20.6	20.6	0.1	76.8	76.8	0.2						
		20.7			76.6								

not determined

RESULTS – qPCR Environmental Samples

Table 31: qPCR Results of Environmental Sample Assays (Sampling Year 2010) using the Probe Sets Pglo1, Ppou2, Mpus2, Ehux, Csoc and Dino

Sample	C_T			T_m1 (°C)			T_m2 (°C)			T_m3 (°C)		
	Triplicates	Mean	SD	Triplicates	Mean	SD	Triplicates	Mean	SD	Triplicates	Mean	SD
***Phaeocystis pouchetii* specific Probe Set Ppou2**												
	20.3			79.4								
Positive	19.8	20.0	0.3	79.6	79.6	0.2						
	19.8			79.7								
	32.5			79.7								
HG1	32.9	33.0	0.5	79.7	79.8	0.1						
	33.5			79.9								
	33.6			79.9								
HG4	33.4	33.3	0.4	79.7	79.8	0.1						
	32.8			79.7								
	38.0			78.6								
N4	37.5	38.0	0.5	78.4	78.5	0.1						
	38.5			78.6								
	32.8			79.6								
S3	32.8	32.9	0.2	79.7	79.7	0.1						
	33.2			79.7								
	34.2			79.8								
HG9	35.0	34.7	0.5	79.8	79.8	0.0						
	35.0			79.8								
***Phaeocystis globosa* specific Probe Set Pglo1**												
	31.1			79.1								
Positive	29.6	30.1	0.9	79.3	79.2	0.1						
	29.5			79.3								
HG1												
HG4												
N4												
S3												
HG9												
***Micromonas pusilla* specific Probe Set Mpus2**												
	21.8			76.4								
Positive	22.8	22.6	0.7	76.4	76.4	0.0						
	23.1			76.4								
HG1												
HG4												
N4												
S3												
HG9												
***Emiliania huxleyi* specific Probe Set Ehux**												
	20.8			76.9								
Positive	20.6	20.7	0.1	77.1	77.1	0.2						
	20.6			77.3								
	33.9			77.2								
HG1	33.3	33.8	0.4	77.2	77.2	0.0						
	34.1			77.2								
HG4												
	36.6			77.3								
N4	37.5	37.2	0.5	77.6	77.2	0.5						
	37.4			76.7								
	36.0			79.9								
S3	35.3	35.7	0.4	77.1	78.0	1.6						
	35.7			77.1								
HG9	36.9	36.7	0.1	77.1	77.1	0.0						
	37.0			77.1								

Continuation of Table 31:

Sample	C_T			T_m1 (°C)			T_m2 (°C)			T_m3 (°C)		
	Triplicates	Mean	SD	Triplicates	Mean	SD	Triplicates	Mean	SD	Triplicates	Mean	SD
***Chaetoceros socialis* specific Probe Set Csoc**												
Positive	21.7 21.7 21.6	21.7	0.1	82.0 82.1 82.3	82.1	0.2						
HG1												
HG4												
N4												
S3												
HG9	39.2 39.9 39.5	39.5	0.4	82.3 82.3 82.1	82.2	0.1	61.9 62.1	62.0	0.1			
Dinophyta specific Probe Set Dino												
Positive	22.6 22.6 23.1	22.8	0.3	77.8 77.8 78.0	77.9	0.1						
HG1	33.4 33.8 34.0	33.7	0.3	81.0 81.0 81.0	81.0	0.0						
HG4	34.3 36.1 34.2	34.9	1.1	81.1 81.1 81.1	81.1	0.0						
N4	39.2			80.3			67.1					
S3	33.7 34.1 30.6	32.8	1.9	80.0 80.1 80.1	80.1	0.1						
HG9	32.4 32.5 32.4	32.4	0.1	81.1 81.1 81.1	81.1	0.0						

not determined

The negative controls showed no qPCR signals (C_T-, T_m-values) in all probe set assays.

Table 32: qPCR Results of Environmental Sample Assays (Sampling Year 2011) using the Probe Sets Pglo1, Ppou2, Mpus2, Ehux, Csoc and Dino

Sample	C_T			T_m1 (°C)			T_m2 (°C)			T_m3 (°C)		
	Triplicates	Mean	SD	Triplicates	Mean	SD	Triplicates	Mean	SD	Triplicates	Mean	SD
***Phaeocystis pouchetii* specific Probe Set Ppou2**												
Positive	27.0 26.0 26.3	26.4	0.5	79.3 79.4 79.4	79.4	0.1						
HG1	38.4 38.4 38.7	38.5	0.2	79.4 79.4 79.4	79.4	0.0						
HG4												
N4	36.8 36.6 37.8	37.1	0.6	79.3 79.1 79.0	79.1	0.2						
S3	39.4 38.2 37.4	38.3	1.0	79.1 79.3 79.3	79.3	0.1						
HG9	35.5 35.7 35.7	35.6	0.1	79.1 79.1 79.1	79.1	0.0						
***Phaeocystis globosa* specific Probe Set Pglo1**												
Positive	37.1 35.7 34.4	35.7	1.3	76.2 76.4 76.6	76.4	0.2						
HG1												
HG4												
N4												
S3												
HG9												
***Micromonas pusilla* specific Probe Set Mpus2**												
Positive	25.2 23.8 23.8	24.3	0.8	76.2 76.4 76.6	76.4	0.2						
HG1												
HG4												
N4												
S3												
HG9												
***Emiliania huxleyi* specific Probe Set Ehux**												
Positive	21.1 21.3 21.3	21.2	0.1	76.4 76.4 76.6	76.5	0.1						
HG1	37.7											
HG4												
N4	38.5 36.8 37.5	37.6	0.9	76.6 76.6 76.4	76.6	0.1						
S3	35.9 35.6 35.4	35.6	0.3	76.6 76.7 76.9	76.7	0.2						
HG9	33.2 33.2 34.5	33.6	0.8	77.1 77.1 77.2	77.1	0.1						
***Chaetoceros socialis* specific Probe Set Csoc**												
Positive	21.7 21.6 21.7	21.7	0.1	82.2 82.2 82.0	82.1	0.1						
HG1												
HG4												
N4												
S3												
HG9												

Continuation of Table 32:

Sample	C_T			T_m1 (°C)			T_m2 (°C)			T_m3 (°C)		
	Triplicates	Mean	SD	Triplicates	Mean	SD	Triplicates	Mean	SD	Triplicates	Mean	SD
Dinophyta specific Probe Set Dino												
Positive	22.4 22.4 22.3	22.4	0.1	77.9 78.1 78.2	78.1	0.2						
HG1	33.7 33.5 33.7	33.6	0.1	80.1 80.1 80.1	80.1	0.0						
HG4	28.7 28.8 28.6	28.7	0.1	79.9 79.9 80.1	80.0	0.1						
N4	27.6 27.7 27.6	27.6	0.1	79.9 79.9 79.7	79.8	0.1						
S3	33.4 33.8 33.3	33.5	0.3	80.2 80.1 80.1	80.1	0.1						
HG9	27.7 27.8 27.7	27.7	0.1	80.2 80.4 80.6	80.4	0.2	62.7					

not determined

The negative controls showed no qPCR signals (C_T-, T_m-values) in all probe set assays.

Table 33: qPCR Results of Environmental Sample Assays (Sampling Year 2012) using the Probe Sets Pglo1, Ppou2, Mpus2, Ehux, Csoc and Dino

Sample	C_T			T_m1 (°C)			T_m2 (°C)			T_m3 (°C)		
	Triplicates	Mean	SD	Triplicates	Mean	SD	Triplicates	Mean	SD	Triplicates	Mean	SD
***Phaeocystis pouchetii* specific Probe Set Ppou2**												
Positive	23.8 23.8 23.8	23.8	0.0	79.2 79.4 79.4	79.3	0.1						
HG1	35.2 35.8 36.9	36.0	0.9	79.4 79.4 79.4	79.4	0.0						
HG4	36.6 36.0 35.1	35.9	0.8	79.6 79.4 79.4	79.5	0.1						
N4	38.3 38.5 38.3	38.4	0.1	79.2 79.2 79.1	79.2	0.1						
S3	37.7 36.9 36.9	37.2	0.5	79.2 79.2 79.2	79.2	0.0						
HG9	37.2 37.7 37.5	37.5	0.3	79.2 79.2 79.2	79.2	0.0						
***Phaeocystis globosas* specific Probe Set Pglo1**												
Positive	35.6 34.6 34.2	34.8	0.7	78.9 79.1 79.2	79.1	0.2						
HG1												
HG4												
N4												
S3												
HG9												

Continuation of Table 33:

Sample	C_T			T_m1 (°C)			T_m2 (°C)			T_m3 (°C)		
	Triplicates	Mean	SD	Triplicates	Mean	SD	Triplicates	Mean	SD	Triplicates	Mean	SD
***Micromonas pusilla* specific Probe Set Mpus2**												
Positive	20.6 19.3 19.6	19.8	0.7	76.3 76.4 76.6	76.4	0.2						
HG1												
HG4												
N4												
S3												
HG9												
***Emiliania huxleyi* specific Probe Set Ehux**												
Positive	21.4 21.6 21.5	21.5	0.1	76.5 76.5 76.6	76.5	0.1						
HG1	32.8 33.0 32.7	32.9	0.2	76.8 77.0 77.0	76.9	0.1						
HG4	34.4 34.6 37.9	36.7	2.0	77.0 77.0 76.8	76.9	0.1						
N4												
S3	35.1 34.8 34.0	34.6	0.6	76.6 76.8 77.0	76.8	0.2						
HG9	35.8 37.2	36.5	1.0	77.0 77.0	77.0	0.0						
***Chaetoceros socialis* specific Probe Set Csoc**												
Positive	21.6 21.6 21.6	21.6	0.0	81.7 81.7 81.9	81.8	0.1						
HG1												
HG4												
N4	38.0			81.9								
S3												
HG9												
Dinophyta specific Probe Set Dino												
Positive	22.4 22.5 22.5	22.5	0.1	78.2 78.2 78.2	78.2	0.0						
HG1	27.1 27.1 27.2	27.1	0.1	79.7 79.9 80.1	79.9	0.2						
HG4	30.1 30.0 30.0	30.0	0.1	80.2 80.2 80.4	80.3	0.1						
N4	34.2 34.5 34.3	34.3	0.2	81.4 80.9 81.4	81.2	0.3	72.3					
S3	31.0 30.8 30.8	30.9	0.1	80.2 80.1 79.9	80.1	0.2						
HG9	32.4 32.6 32.2	32.4	0.2	80.2 80.4 80.4	80.3	0.1						

not determined

The negative controls showed no qPCR signals (C_T-, T_m-values) in all probe set assays.

Table 34: qPCR Results of Environmental Sample Assays (Sampling Year 2013) using the Probe Sets Pglo1, Ppou2, Mpus2, Ehux, Csoc and Dino

Sample	C_T			T_m1 (°C)			T_m2 (°C)			T_m3 (°C)		
	Triplicates	Mean	SD	Triplicates	Mean	SD	Triplicates	Mean	SD	Triplicates	Mean	SD
***Phaeocystis pouchetii* specific Probe Set Ppou2**												
Positive	22.7 22.5 22.2	22.5	0.3	79.7 79.6 79.6	79.6	0.1						
HG1	29.1 29.4 29.4	29.3	0.2	79.4 79.2 79.1	79.2	0.2						
HG4	30.2 29.9 29.8	30.0	0.2	79.2 79.4 79.6	79.4	0.2						
N4	27.8 28.4 28.8	28.3	0.5	79.6 79.7 79.7	79.7	0.1						
S3	31.5 31.0 30.5	31.0	0.5	79.7 79.7 79.7	79.7	0.0						
HG9	n. a. for 2013											
***Phaeocystis globosas* specific Probe Set Pglo1**												
Positive	31.9 31.3 31.0	31.4	0.5	78.9 78.9 79.1	79.0	0.1						
HG1												
HG4												
N4												
S3	39.3			79.6								
HG9	n. a. for 2013											
***Micromonas pusilla* specific Probe Set Mpus2**												
Positive	18.6 17.8 18.3	18.3	0.4	76.2 76.3 76.3	76.3	0.1						
HG1												
HG4												
N4	39.8			75.8								
S3												
HG9	n. a. for 2013											
***Emiliania huxleyi* specific Probe Set Ehux**												
Positive	20.4 20.6 20.5	20.5	0.1	76.5 76.5 76.6	76.5	0.1						
HG1	27.8 28.1 28.1	28.0	0.2	76.8 76.8 77.0	76.9	0.1						
HG4												
N4	36.7			76.5								
S3	36.6 33.9 37.9	36.1	2.1	76.5 76.8 76.8	76.7	0.2						
HG9	n. a. for 2013											
***Chaetoceros socialis* specific Probe Set Csoc**												
Positive	21.8 21.8 21.8	21.8	0.0	81.5 81.5 81.7	81.6	0.1						
HG1												
HG4												
N4												
S3												
HG9	n. a. for 2013											

Continuation of Table 34:

Sample	C_T			T_m1 (°C)			T_m2 (°C)			T_m3 (°C)		
	Triplicates	Mean	SD	Triplicates	Mean	SD	Triplicates	Mean	SD	Triplicates	Mean	SD
Dinophyta specific Probe Set Dino												
Positive	23.5 23.5 23.5	23.5	0.0	78.2 78.0 78.0	78.1	0.1						
HG1	31.2 31.2 31.2	31.2	0.0	80.5 80.3 80.2	80.3	0.2	73.1 73.0	73.1	0.1			
HG4	31.1 31.0 31.2	31.1	0.1	81.2 81.3 81.5	81.3	0.2						
N4	27.6 27.7 27.8	27.7	0.1	81.5 81.7 81.7	81.6	0.1						
S3	34.4 34.1 34.2	34.2	0.2	81.0 80.8 80.8	80.9	0.1						
HG9	n. a. for 2013											

not determined

n. a. = not available

The negative controls showed no qPCR signals (C_T-, T_m-values) in all probe set assays.

10.3 List of Figures

10.4 List of Tables