An investigation of the eukaryotic picoplankton community in the German North Sea by different methods

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Selbst der Kleinste vermag den Lauf des Schicksals zu verändern

(aus „Der Herr der Ringe“ von John Ronald Reuel Tolkien, 1954/55)

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LIST OF ABBREVIATIONS

18S rRNA = small subunit of the eukaryotic ribosomal RNA

BSH = Bundesamt für Seeschifffahrt und Hydrographie

CARD = catalysed reporter deposition

CHEMTAX = matrix factorisation program for chemical taxonomy analysis by pigments

DGGE = denaturing gradient gel electrophoresis

DNA = deoxyribonucleic acid

DOM = dissolved organic matter

EM = electron microscopy

FCM = flow cytometry

FISH = fluorescence *in situ* hybridisation

FISH-TSA = fluorescence *in situ* hybridisation coupled with tyramide signal amplification

GKSS = Gesellschaft für Kernenergieverwertung in Schiffbau und Schiffahrt mbH

GR = global radiation

HA = heteroduplex analysis

HPLC = high-performance liquid chromatography

HRP = horseraddish peroxidase

MAST = marine stramenopiles

MYA = million years ago

OTU = operational taxonomic unit

PAR = photosynthetic active radiation

PCR = polymerase chain reaction

PICODIV = Project to monitor the diversity of photosynthetic picoplankton in marine waters (EU contract EVK3-CT-1999-00021)

RDA = redundancy analysis

RFLP = restriction fragment length polymorphism

rDNA = ribosomal DNA

rRNA = ribosomal RNA

TGGE = temperature gradient gel electrophoresis

TSA = tyramide signal amplification

VIF = variance inflation factor

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SUMMARY

Picoplankton have evolved diverse physiological benefits and efficient adaptation capabilities to various environmental conditions. They have therefore been described as “probably the oldest, certainly the most widespread and abundant, and arguably the best adapted form of phytoplankton...” (Fogg, 1991). Picoplanktonic organisms are ubiquitous around the world and are able to dominate biomass, production, and metabolic activity in diverse regions of the oceans. It is suggested that they perform important and basic functional roles in the marine ecosystem, although many of their ecological functions and characteristics remain unknown. For example, the reaction of picoplankton to anthropogenic influences, such as eutrophication, pollution and climatic change, or the spatial and temporal differences among clades and ecotypes have not been determined yet.

This thesis examined the identity and ecological abundance of picoeukaryotes in the coastal waters of the German Bight on a weekly frequency over a 17-months period by using diverse currently existing methods. Special emphasis was placed on the comparison of these methods and one species, *Micromonas pusilla* (Mamiellales, Prasinophyceae), which is the best-studied and possibly most abundant phototrophic picoeukaryote. Although the initial intention was to focus on the genus *Micromonas* and on cultivation tests with identical species from different habitats, a detailed approach over the entire and less well-known picoplankton community of the German Bight was decided to be much more beneficial. Thus, the aims of the present study were broadened to encompass a thorough and ecologically relevant analysis of the community of picoeukaryotes by application of diverse analysis methods. The abundance and dynamics of these organisms within temperate coastal waters were analysed and novel insights regarding their identity and niche partitioning were provided.

The picoeukaryotic community exhibited an overall striking taxonomic diversity, with its composition varying over short time scales and between seasons. It consisted of organisms that were consistently present and others that appeared to be more opportunistic. Though the picoautotrophs represented only a minor fraction of the total autotrophic biomass in the eutrophic German Bight, they confirmed their basic role in the marine ecosystem; identical phylotypes of Mamiellales (Prasinophyceae, Chlorophyta) were the most frequently detected. The numerically dominating part of identified phylotypes was attributed to the picoheterotrophs. They were dominated by diverse alveolates and marine stramenopiles, but the sequences analyzed were not restricted to the picoplanktonic size class. Overall, the exact ratio of picoheterotrophs to picoautotrophs, with respect to biomass and cell quantities, remained uncertain.

The integral approach of this doctorate revealed that diverse environmental parameters significantly influenced the composition of the picoplankton community. Furthermore, the variety of methods used to determine the composition of the picoplankton community yielded inconsistent trends regarding the relative strengths of these environmental controls. For example, pigment analyses by high-performance liquid chromatography (HPLC) revealed that radiation had the most prominent effect, whereas water temperature was more important for cell counts by FISH. Nonetheless, this thesis revealed a seasonal alternation among closely related groups (e.g., the Mamiellales *Bathycoccus* and *Micromonas*) and differing responses of *Micromonas*' clades to the investigated abiotic parameters. Fine-scale changes in environmental conditions seemed to influence the abundance of individual picoeukaryotes and groups of higher taxonomic levels. The abundance of the overall community on the other hand, changed simultaneously and exposed the uncertainty of crucial abiotic influences. Thus, the community is suggested to be in direct response to biotic influences imparted by larger-sized organisms such as microalgae and grazers.

The use of different community analysis methods allowed novel comparisons and provided an extensive description of the picoplankton community. Nonetheless, some basic ecological questions remained uncertain. For example, if the occurring clades and ecotypes differed genetically from those at other sites or represented the possibility to adapt physiological and/or phenotypic to various environmental conditions. The present study's approach provided a detailed evaluation of distinct techniques to investigate the community of eukaryotic picoplankton. For example, the use of fluorescence *in situ* hybridisation (FISH) proved to be beneficial for quantitative and qualitative comparisons of the community on a spatial and/or temporal scale. Denaturing gradient gel electrophoresis (DGGE) and genetic sequencing allowed for the identification of occurring phylotypes, and electron microscopy provided morphological structure information for single organisms. Pigment analyses determined the contribution of picophytoplankton to the overall biomass of primary producers, but were most prone to biases from other food web members and organic particles. Larger microalgae, their cell fragments, resting spores, excretion products and/or faecal pellets of grazers may exist in the picoplankton size fraction, largely biasing pigment profiles that attempt to identify the pigment contribution by picoplankton. Thus, the comparison of the different methods not only provided unique insights into the picoplanktonic community of the German Bight, but also revealed the need for careful data interpretation. The findings of the present study may function as a guide for other scientists to choose the optimal method to develop specific research aims to study picoplankton. Furthermore, this study exposed some flaws of existing community analysis methods. For example, future studies could benefit from the development of more specific molecular probes and primers or techniques to isolate and cultivate single picoplanktonic organisms.

ZUSAMMENFASSUNG

Das Picoplankton hat im Laufe seiner Evolution bedeutsame physiologische Vorteile und effiziente Fähigkeiten zur Anpassung an verschiedene Umweltbedingungen entwickelt. Daher wurde es als „die vermutlich älteste, die sicherlich meist verbreitete und vorhandene, und die nachweisbar bestangepasste Form des Phytoplanktons..." (übersetzt nach Fogg, 1991) beschrieben. Das eukaryotische Picoplankton bildet oftmals die dominierende Biomasse, Produktion und metabolische Aktivität in ökologisch unterschiedlichen Regionen der Weltmeere. Wissenschaftliche Untersuchungen der letzten Jahrzehnte haben gezeigt, dass das Picoplankton eine grundlegende Bedeutung für das marine Ökosystem hat, obwohl viele seiner ökologischen Funktionen und Eigenschaften noch nicht beschrieben werden konnten. Ein Beispiel hierfür ist seine Reaktion auf anthropogene Einflüsse, wie Eutrophierung, Verschmutzung und Klimawandel.

Diese Doktorarbeit beschreibt die Gemeinschaft der Picoeukaryoten im Küstenbereich der Deutschen Bucht anhand einer wöchentlichen Probenahme über einen Zeitraum von bis zu 17 Monaten. Im Mittelpunkt der Arbeit lag der Vergleich verschiedener derzeit existierender Analyse-Techniken, welche in einem Parallelansatz auf die identischen Proben des Oberflächenwassers an der Helgoländer Reede angewandt wurden. Diese Kombination unterschiedlicher methodischer Ansätze ermöglichte eine detaillierte Beschreibung der ökologischen Abundanz und taxonomischen Zusammensetzung der Picoeukaryoten-Gemeinschaft. Besondere Berücksichtigung fand hierbei die Untersuchung einer Art, *Micromonas pusilla* (Mamiellales, Prasinophyceae), welche die am besten untersuchte und vermutlich am weitesten verbreitete Art darstellt. Die ursprüngliche Absicht die Doktorarbeit allein auf die Untersuchung von *Micromonas* zu konzentrieren und Kultivierungsversuche mit identischen Arten verschiedener Habitate durchzuführen, wurde zugunsten einer wissenschaftlich bedeutungsvolleren Beschreibung der gesamten und bisher nur wenig untersuchten Picoplankton-Gemeinschaft der Deutschen Nordsee erweitert. Die resultierende detaillierte Analyse der vorkommenden Picoeukaryoten mit Hilfe eines umfassenden methodischen Vergleichs, sowie die Auswertung abiotischer Einflüsse auf die jeweiligen methodischen Ergebnisse, erlaubten neue Einblicke in die Identität und ökologische Abundanz des Picoplanktons in den Küstengewässern gemäßigter Breiten.

Die vorliegende Studie verdeutlicht die auffallend hohe taxonomische Diversität innerhalb der Gemeinschaft der Picoeukaryoten. Obwohl diese Diversität eine saisonale Abhängigkeit zeigte, wurden auch hohe wöchentliche Unterschiede deutlich. Neben kontinuierlich vorkommenden

Arten und Organismengruppen, wie beispielsweise einem Phylotyp von *Micromonas pusilla*, wurden auch in ihren Nischeneigenschaften eher opportun erscheinende Gruppen gefunden, wie beispielsweise Vertreter der Stramenopilen. Insgesamt hatte das autotrophe Picoplankton einen geringen Anteil an der Gesamtbiomasse der vorhandenen Primärproduzenten. Damit bestätigte es seine beschriebene elementare Bedeutung als konstante „Hintergrund-Population" im marinen Ökosystem. Das heterotrophe Picoplankton hingegen zeigte eine hohe und sich verändernde Diversität, und wurde von Organismen der Alveolata und marinen Stramenopilen dominiert. Im Rahmen der vorliegenden Studie blieb das genaue Verhältnis zwischen autotrophen und heterotrophen Picoeukaryoten in Bezug auf ihre Biomassen und Zellzahlen ungeklärt.

Der durchgeführte ganzheitliche Ansatz dieser Doktorarbeit zeigte, dass verschiedene Umweltparameter einen signifikanten Einfluss auf die Zusammensetzung der Picoplankton-Gemeinschaft haben. Die Effektstärke der Parameter variierte jedoch zwischen den verschiedenen Analyse-Techniken. So zeigten beispielsweise die durchgeführten Pigmentuntersuchungen mittels Hochleistungsflüssigkeitschromatographie (HPLC), dass die Einstrahlung den stärksten Einfluss hatte, während Zellzählungen mittels Fluoreszenz *in situ* Hybridisierung (FISH) die Wassertemperatur als den größten Einflussfaktor identifizierten. Die saisonale Sukzession zwischen nahe verwandten Gruppen, wie beispielsweise zweier Gattungen der Mamiellales, *Bathycoccus* und *Micromonas*, sowie die unterschiedlichen Reaktionen einzelner taxonomischer Stämme auf die untersuchten abiotischen Parameter, ließen eine Abhängigkeit von eher fein-skaligen Umweltveränderungen vermuten. Im Gegensatz zu den Veränderungen innerhalb der Picoplankton-Gemeinschaft, ließen die Änderungen in der Abundanz der gesamten Gemeinschaft keine Identifizierung eines zugrundeliegenden Umweltparameters zu. Aufgrund des in dieser Studie nachgewiesenen großen Einflusses anderer Organismen auf die verschiedenen Analyse-Ergebnisse, scheint die Gesamt-Sukzession des Picoplanktons eher in Bezug zu biotischen als abiotischen Einflüssen zu stehen.

Die in der vorliegenden Doktorarbeit dargestellten Ergebnisse unterschiedlicher Analyse-Techniken erlaubten einen bisher nicht durchgeführten methodischen Vergleich und eine detaillierte Beschreibung der Gemeinschaft des Picoplanktons. Dennoch blieben einige grundlegende ökologische Fragen ungeklärt. So zum Beispiel, ob sich die in verschiedenen Meeresregionen vorkommenden taxonomischen Stämme und Ökotypen einzelner Arten genetisch unterscheiden oder eher eine jeweilige Adaptation ihrer Physiologie und/oder ihres Phänotyps repräsentieren. In Bezug auf den durchgeführten methodischen Vergleich zeigten die Ergebnisse der FISH-Analyse deutliche quantitative und qualitative Vorteile in der Untersuchung von Picoeukaryoten. Die Denaturierende Gradienten Gel-Elektrophorese (DGGE) hingegen erlaubte

die taxonomische und phylogenetische Identifizierung vorkommender Organismen, während die Elektronenmikroskopie Aufschluss über morphologische Strukturen gab. Die Pigmentanalysen zeigten sich höchst empfindlich gegenüber äußeren Einflüssen, wie beispielsweise Zellen und Fragmenten von größeren Phytoplankton-Arten oder zooplanktonischen Fäkal-Pellets. Dennoch erlaubten die angewandten Pigment-Methoden einen direkten Vergleich zwischen der Biomasse des Picophytoplanktons und der des Gesamt-Phytoplanktons. Insgesamt ermöglichte die durchgeführte parallele Anwendung verschiedener derzeit existierender Analyse-Techniken auf identische Picoplankton-Proben einen einzigartigen Einblick in die Picoplankton Gemeinschaft der Deutschen Bucht. Zudem konnte die Notwendigkeit einer vorsichtigen Dateninterpretation und die erforderliche Weiterentwicklung von bestehenden Analyse-Techniken verdeutlicht und entsprechende Ansatzpunkte geliefert werden.

~ PART I ~

GENERAL INTRODUCTION - STATE OF KNOWLEDGE

1.) Ecology of picoplankton

Picoplanktonic organisms are highly diverse in the marine environment and have specific physiological characteristics as well as complex abiotic and biotic relationships. Their community consists of single-celled auto-, mixo- and heterotrophic pro- and eukaryotes that can perform different functional roles in the environment that are not yet entirely understood (Fogg, 1991; Guillou et al., 2001; Zubkov et al., 2003; Biegala et al., 2005; Worden and Not, 2008). They are responsible for a wide range of biogeochemical transformations, such as nutrient remineralisation, and they play key roles in tropho-dynamic processes (Eikrem and Throndsen, 1990; Legendre and Le Fèvre, 1995; Massana et al., 2004a; Romari and Vaulot, 2004; Piganeau et al., 2008). Together with larger phytoplankton, they account for less than 1% of the world's photosynthetic biomass but more than 45% of the photosynthetic net primary production (Simon et al., 2009). Thus, this community has an enormous potential as a carbon sink and is an important energy source for diverse grazing organisms.

1.1) Ecological advantages of picoplankton over larger phytoplankton

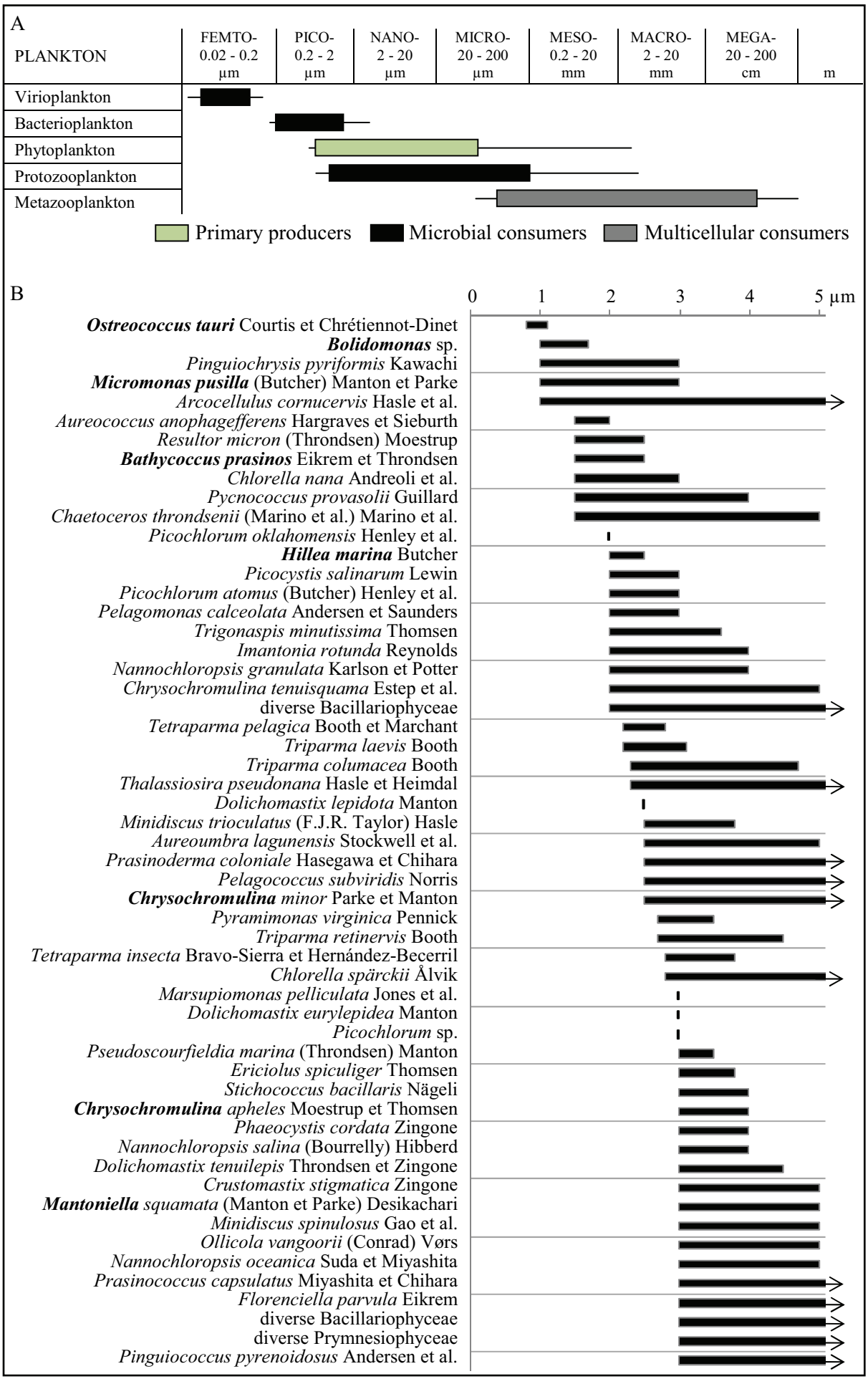
In the marine environment, there is a strong dependency between biological growth and the physical-chemical surroundings. Physical parameters, such as currents, wind, radiation, temperature and salinity (Fogg, 1991; Carr and Kearns, 2003), as well as chemical elements, such as metabolites, nutrients, stimulatory and inhibitory compounds (Boney, 1975), define different ecological conditions within the oceans. These abiotic factors vary spatially and temporally, and plankton communities are forced to adapt to them (Smayda, 1998; Jacquet et al., 2001). Compared to larger phytoplankton, picoplanktonic organisms are better equipped to withstand changing environmental settings. This is because of some basic advantages that even allow their existence under constantly extreme conditions, such as nutrient-deficiency in oligotrophic waters.

Picoplanktonic organisms have evolved over several billion years (Woese and Fox, 1977; Worden and Not, 2008) and have adapted over many generations to be able to respond efficiently to various environmental conditions. Thus, they developed minimal niche requirements (Bryant, 2003) and, because of the movement of ocean waters (Šlapeta et al., 2006), they slowly spread all over the

world's oceans. Today, seemingly identical species can be found in distinct oceanic regions and at different depths; one example are the clades of the eukaryotic picophytoplankton species *Micromonas pusilla* (Simon et al., 2009) (see section "Mamiellales", page 11).

A second advantage of picoplanktonic organisms is their small cell size (Figure 1). Although the operational definition of the upper size limit of picoplankton varies between 2 and 3 µm, often depending on sampling protocols such as the filters used for size fractionation, picoplankton are regarded as being "close to the minimum possible size" (Raven, 1998). Phylogenetic analysis revealed that the ancestral eukaryotic cell was likely to have been larger than the current picoeukaryotes (Raven et al., 2005). Jiang and collaborators (2005) mentioned that the evolution of phytoplankton organisms should generally evolve towards a small cell size because the enhanced surface-to-volume ratio of smaller cells has physiological benefits. Due to these advantages, picoplanktonic organisms have been described as "the ultimate in planktonic existence" (Fogg, 1991). Together with the development of efficient nutrient absorption pathways (Raven, 1998; Simon et al., 2009), picoplankton have, for example, advantageous nutrient acquisition strategies for surviving under oligotrophic conditions (Zubkov and Tarran, 2008). Furthermore, a small cell size results in a lower sinking rate, though it also bestows disadvantages, such as a higher leakage of dissolved substances (Fogg, 1991; Kjørboe, 1993; Raven, 1998; Raven et al., 2005). However, size adaptation to the given environmental parameters can even be seen by the distribution of picoplanktonic organisms within the marine ecosystem: in oligotrophic waters, the cells are smaller than in meso- and eutrophic ones, and in a vertical profile, the smaller cells generally occur at the surface (Masquelier and Vaulot, 2008).

Figure 1: (A) Distribution of different taxonomic-trophic compartments of plankton in a spectrum of size fractions (after Sherr and Sherr, 2008) and (B) sizes of described marine eukaryotes occurring in the picoplankton (after Vaulot et al., 2008). Organisms in bold will be further described in section "The picoplankton community", page 5. Arrows indicate a size range that exceeds 5 µm.


A
PLANKTON
FEMTO- 0.02 - 0.2 µm
PICO- 0.2 - 2 µm
NANO- 2 - 20 µm
MICRO- 20 - 200 µm
MESO- 0.2 - 20 mm
MACRO- 2 - 20 mm
MEGA- 20 - 200 cm
m
Virioplankton
Bacterioplankton
Phytoplankton
Protozooplankton
Metazooplankton
Primary producers
Microbial consumers
Multicellular consumers
B
0
1
2
3
4
5 µm
Ostreococcus tauri Courtis et Chrétiennot-Dinet
Bolidomonas sp.
Pinguiochrysis pyriformis Kawachi
Micromonas pusilla (Butcher) Manton et Parke
Arcocellulus cornucervis Hasle et al.
Aureococcus anophagefferens Hargraves et Sieburth
Resultor micron (Throndsen) Moestrup
Bathycoccus prasinos Eikrem et Throndsen
Chlorella nana Andreoli et al.
Pycnococcus provasolii Guillard
Chaetoceros throndsenii (Marino et al.) Marino et al.
Picochlorum oklahomensis Henley et al.
Hillea marina Butcher
Picocystis salinarum Lewin
Picochlorum atomus (Butcher) Henley et al.
Pelagomonas calceolata Andersen et Saunders
Trigonaspis minutissima Thomsen
Imantonia rotunda Reynolds
Nannochloropsis granulata Karlson et Potter
Chrysochromulina tenuisquama Estep et al.
diverse Bacillariophyceae
Tetraparma pelagica Booth et Marchant
Triparma laevis Booth
Triparma columacea Booth
Thalassiosira pseudonana Hasle et Heimdal
Dolichomastix lepidota Manton
Minidiscus trioculatus (F.J.R. Taylor) Hasle
Aureoumbra lagunensis Stockwell et al.
Prasinoderma coloniale Hasegawa et Chihara
Pelagococcus subviridis Norris
Chrysochromulina minor Parke et Manton
Pyramimonas virginica Pennick
Triparma retinervis Booth
Tetraparma insecta Bravo-Sierra et Hernández-Becerril
Chlorella spärckii Ålvik
Marsupiomonas pelliculata Jones et al.
Dolichomastix eurylepidea Manton
Picochlorum sp.
Pseudoscourfieldia marina (Throndsen) Manton
Ericiolus spiculiger Thomsen
Stichococcus bacillaris Nägeli
Chrysochromulina apheles Moestrup et Thomsen
Phaeocystis cordata Zingone
Nannochloropsis salina (Bourrelly) Hibberd
Dolichomastix tenuilepis Throndsen et Zingone
Crustomastix stigmatica Zingone
Mantoniella squamata (Manton et Parke) Desikachari
Minidiscus spinulosus Gao et al.
Ollicola vangoorii (Conrad) Vørs
Nannochloropsis oceanica Suda et Miyashita
Prasinococcus capsulatus Miyashita et Chihara
Florenciella parvula Eikrem
diverse Bacillariophyceae
diverse Prymnesiophyceae
Pinguiococcus pyrenoidosus Andersen et al.

1.2) Occurrence and function in food webs

Picoplanktonic growth and reproduction are not controlled solely by abiotic parameters, but by overall conditions of the surrounding ecosystem (Hutchinson, 1961). For example, competition for resources, infection by viruses and grazing pressure are additional factors that influence the existence and dynamics of picoplanktonic species (Guillou et al., 2001; Vaulot, 2001a; Mackey et al., 2002; Evans et al., 2003). The high efficiency of grazing on phytoplankton becomes particularly obvious in comparison to terrestrial ecosystems, because a much larger fraction of the generated carbon is directly consumed by grazers (Steele, 1974; Shurin et al., 2006). Although the transfer of carbon from picoplankton to higher trophic levels is still not well understood, data suggest that there is a variety of paths for carbon to be assimilated into higher trophic levels (Legendre and Le Fèvre, 1995; Sherr and Sherr, 2008; Worden and Not, 2008). In addition to the availability, size, nutritional quality and physical condition of prey species, the detection by and physiological state of the predator, as well as its need to compete for the resources define the grazing rate and the trophic efficiency of the occurring food web (Flynn et al., 1996; Caron et al., 1999; Davidson and John, 2001).

Oligotrophic open ocean waters and eutrophic coastal regions exhibit the most contrasting surface food web structures. In the nutrient-depleted regions, such as the central Pacific gyre or the Eastern Mediterranean Sea, picoplankton can account for up to 92% of the chlorophyll *a* concentration, but exist as a background population or dominate only occasionally in coastal regions (Vaulot et al., 2003b; Not et al., 2008). Furthermore, a balanced and homogeneous microbial food web with a strong microbial coupling develops in oligotrophic waters (Legendre and Le Fèvre, 1995). In these regions, heterotrophs take up diverse organic material and remineralise nutrients while the phototrophs use the reactivated nutrients for growth and reproduction. The harmonised interconnection of ecological roles allows the organisms to adapt efficiently to nutritional conditions. Furthermore, the life cycle of the grazing organisms can be shorter than that of their prey; therefore, they can efficiently adapt to the abundant food resources. This effective control of, but at the same time dependency towards the picoplankton abundance ("top-down" control) guarantees a continuous existence of the microbial food web (Guillou et al., 2001; Vaulot, 2001a; Mackey et al., 2002; Evans et al., 2003). Nonetheless, the microbial food web in oligotrophic waters is energetically inefficient and cannot sustain larger organisms because of the very small size and therefore low nutritional value of the abundant prey species (Guillou et al., 2001).

Shelf seas and coastal areas mainly differ from the open ocean because they are shallow (max. 200 m) and more closely connected to land and people. They are mainly affected by high riverine and atmospheric inputs of organic and inorganic material, as well as by rapid changes in environmental conditions and physico-chemical circulation processes (Jacquet et al., 2002a; Qian et al.,

2003; Wynn and Knefelkamp, 2004). Nonetheless, eutrophic coastal waters form optimal abiotic conditions to support blooms of large phytoplanktonic organisms (Jacquet et al., 2002a). These conditions act to increase the trophic efficiency of the eutrophic food web and consequently, coastal waters belong to the most productive natural systems in the world and have high ecological and economic value (Forster et al., 2006). However, larger microalgae and their grazers generally have long generation times (in comparison to picoplankton) that do inhibit a rapid adaptation to environmental changes (Fogg, 1991; Kjørboe et al., 1993; Donald et al., 2001). For example, nutrient exhaustion and self-shading effects stop the excessive growth during a mono-species bloom. The decreasing prey abundance then limits the abundance of grazers. This simplification of the coastal food web illustrates the changing and unstable conditions for individual species in comparison to the previously described oligotrophic waters. However, the picoplankton community forms a steady background population in coastal environments (Vaulot et al., 2000; Jacquet et al., 2002a and b; Bryant, 2003; Massana et al., 2004a; Zhu et al., 2005; Medlin et al., 2006).They can take advantage of changes in abiotic settings associated, for example, with the end of a bloom of larger microalgae. High growth rates and minimal niche requirements, as well as the abiotic restrictions that limit the growth of larger phytoplankton, allow picoplankton to proliferate in eutrophic environments. They may even dominate the community of primary producers for some time (Raven, 1998; Jacquet et al., 2002a and b; Bryant, 2003; Agawin et al., 2004; Jiang et al., 2005; Zhu et al., 2005; Medlin et al., 2006; Zubkov and Tarran, 2008).

1.3) The picoplankton community

Though there are differences in the spatial and temporal abundance of picoplanktonic organisms (Martin et al., 2005), the patterns of genetic and physiological distributions and ecological significances are still under investigation. Furthermore, most picoplankton studies distinguish between the abundance of the two closely related genera of cyanobacteria *Prochlorococcus* and *Synechococcus*, and the overall fraction of picoeukaryotes. Thus, very little information exists regarding the abundance of distinct members of the eukaryotic community, primarily because of a lack of adequate investigation methods (see section “Methods to investigate picoplankton”, page 17). However, cyanobacteria and picoeukaryotes differ in the highest taxonomic category (domain) and cannot be compared phylogenetically (Woese and Fox, 1977). This study aims to provide further detail of the picoeukaryotes and will therefore discuss the cyanobacteria more broadly.

The cyanobacteria

Synechococcus, probably the most ubiquitous phytoplankton genus on earth, was first described in 1979 by Waterbury and colleagues (Waterbury et al., 1979). Its cells have a diameter of approximately 1.0 µm and the genus comprises ten lineages (Scanlan, 2007). Its ecotypes (clades restricted to a distinct niche) can be found from the tropics to the poles, in eutrophic, oligotrophic, estuarine and coastal waters, with decreasing abundance at high latitudes (Partensky et al., 1999a; Vaulot, 2001a; Scanlan and West, 2002; Not et al., 2005; Scanlan, 2007). They mainly occur in surface waters and because some strains are mobile, they exhibit a high taxonomic diversity in their horizontal abundance (Partensky et al., 1999a).

The evolutionary younger *Prochlorococcus* is seen as the most abundant phytoplankton genus on earth and was discovered by Chisholm and collaborators in 1988 (Chisholm et al., 1988; Partensky et al., 1999a; Bryant, 2003; Scanlan et al., 2009). Its cells are slightly smaller than those of *Synechococcus* (0.5 - 0.7 µm), and its ecotypes mainly dominate within warmer and rather unfavourable waters, such as central oligotrophic and stratified waters between 40°N to 40°S. Their taxonomic diversity is rather uniform on a horizontal scale but differs vertically in the water column (Garczarek et al., 2007). This ecotype distribution is mainly a result of adaptations of pigment profiles in response to the availability of light (Partensky et al., 1999a and b; Hess et al., 2001; Garczarek et al., 2007; Scanlan et al., 2009).

Both, *Synechococcus* and *Prochlorococcus*, occupy the marine environment complementarily through overlapping niches and perform a significant contribution to marine and global primary production (Partensky et al., 1999a; Mackey et al., 2002; Scanlan and West, 2002; Vaulot et al., 2004; Scanlan et al., 2009). While *Prochlorococcus* are generally more abundant and extend to greater depths, there is a shift in dominance at higher latitudes and/or when sufficient nutrients become available (Partensky et al., 1999a; Vaulot et al., 2003b; Worden et al., 2004; Garczarek et al., 2007). Thus, *Prochlorococcus* populations dominate oligotrophic warm waters and *Synechococcus* occupies warmer regions from oligotrophic to eutrophic waters, serving as an apparent ecological intermediate between *Prochlorococcus* and the picoeukaryotes. Picoeukaryote abundance, especially of those that are phototrophs, increases from the open ocean towards more estuarine and coastal waters, including polar regions (Not et al., 2005). In these rather eutrophic waters (see previous section), they form a background population with an immense and mostly unknown diversity (Biegala et al., 2003; Massana and Pedrós-Alió, 2008; Masquelier and Vaulot, 2008).

The picoeukaryotes

In 1951, Knight-Jones suggested an overall high abundance of picoeukaryotes in the marine environment, and one year later Butcher published the first description of a picophototrophic species, *Micromonas pusilla* (Knight-Jones, 1951; Butcher, 1952). However, the late 1970s are generally defined as the time of the discovery of picoplankton-sized eukaryotes. Johnson and Sieburth (1979 and 1982) described the importance of picoeukaryotes in oceanic waters, and Azam and colleagues (1983) introduced the concept of a microbial loop (Figure 2). With the subsequent rapid development of adequate detection methods, the picoeukaryotes were finally recognised worldwide. Henceforth, they were no longer ignored and scientists agreed that they provide missing answers concerning the microbial food web and the overall marine ecosystem (Stockner, 1988; Vaulot et al., 2008). Knowledge of the picoeukaryote community has steadily increased, particularly since the 1990s when molecular approaches emerged. Today we know that they are a very important component of the phytoplankton biomass and primary production, and that they play an important role in the recycling processes of organic materials (Vaulot et al., 2002; Massana et al., 2004a and b; Not et al., 2008; Piganeau et al., 2008; Viprey et al., 2008). Compared to the more abundant cyanobacteria (Worden and Not, 2008), picoeukaryotes are taxonomically more diverse and have a slightly larger cell size (Bec et al., 2005; Fuller et al., 2006a). They can be photo-, mixo- and heterotrophic, as well as parasitic and symbiotic (Worden and Not, 2008). Their heterotrophic fraction mainly consists of phagotrophic free-living flagellates, such as marine stramenopiles (MAST)

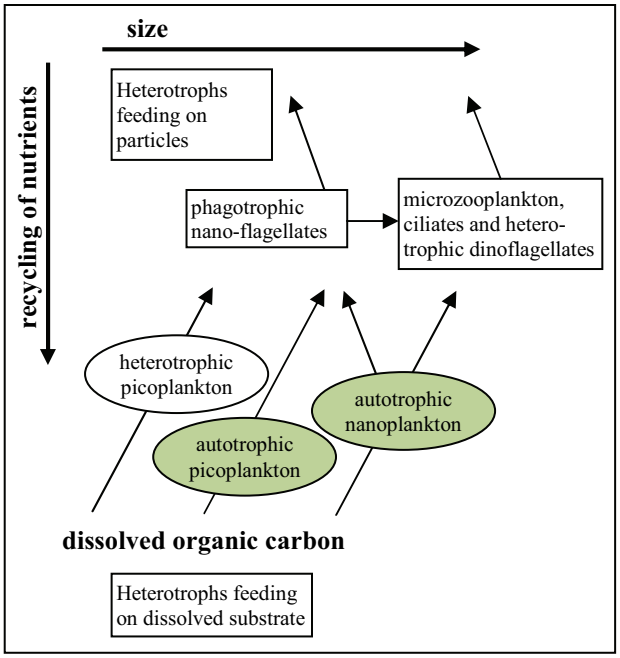


Figure 2: Conceptual diagram of the microbial food web based on the microbial loop idea of Azam et al. (1983) (after Sherr and Sherr, 2008).

(Massana et al., 2004b). These organisms primarily feed on organic molecules, prokaryotes, and other marine microorganisms. Therefore, they are able to adapt to various nutritional resources and exhibit high taxonomic diversity (Vaulot et al., 2002; Massana et al., 2004a and b; Worden and Not, 2008). The phototrophic picoeukaryotes tend to be numerically more abundant than heterotrophs (Worden and Not, 2008). They dominate eutrophic and cold waters, such as the Barents Sea where they make up 75% of the picoeukaryotic community (Not et al., 2002). They dominate the resident phytoplankton biomass and the primary carbon production in coastal regions, particularly in summer (Vaulot, 2001b; Not et al., 2002 and 2005; Massana et al., 2004a and b; Piganeau et al., 2008; Viprey et al., 2008). However, there are a large number of picoeukaryotes that have not yet been described, thus preventing a reliable estimation of species diversity, abundance and niche requirements (Not et al., 2004; Medlin et al., 2006; Massana and Pedrós-Alió, 2008). Furthermore, the underlying genetic distributions have not been described, but several studies have recommended the analysis of biogeographic adaptations of picoeukaryotes through distinct ecotypes, such as those described for cyanobacteria (page 6) (Massana et al., 2004b; Forster et al., 2008; Vaulot et al., 2008).

Nearly every algal division has picoplanktonic representatives (Vaulot et al., 2000 and 2008; Féral, 2002). The presently described species (Figure 1) belong to the stramenopiles (37 species), chlorophytes (23 species), haptophytes (10 species) and cryptophytes (1 species) (Vaulot et al., 2008). This study will focus on the diversity and characteristics of these four phyla and not all picoeukaryotes because this would be beyond the scope of this introduction. An example of a picoplanktonic representative from each phylum is presented below, and emphasis is placed on the Prasinophyceae, especially their order Mamiellales and the genus *Micromonas*.

Stramenopiles (Heterokontophyta) This phylum is very complex because it contains photo-, mixo- and heterotrophic organisms. These may be multi- or unicellular algae, fungal-like cells or parasites (van den Hoek et al., 1995; Massana et al., 2004b; Worden and Not, 2008). The principal ultrastructural features of the stramenopiles (Figure 3A) are a long forward-directed pleuronematic (flimmer or tinsel) flagellum and another short, smooth flagellum directed toward the anterior (van den Hoek et al., 1995; Simon et al., 2009). Their major accessory pigments are fucoxanthin (e.g., in diatoms, Chrysophyceae and Bolidophyceae) or vaucheriaxanthin (e.g., in Eustigmatophyceae); chlorophyll *b* is never present (van den Hoek et al., 1995).

The picoplanktonic stramenopiles can be separated into the monophyletic phototrophs and at least eight lineages of "unpigmented flagellates" (Massana et al., 2004b) at the basal branches of the stramenopile radiation (Massana et al., 2002). These are grouped into numerous clusters with a wide

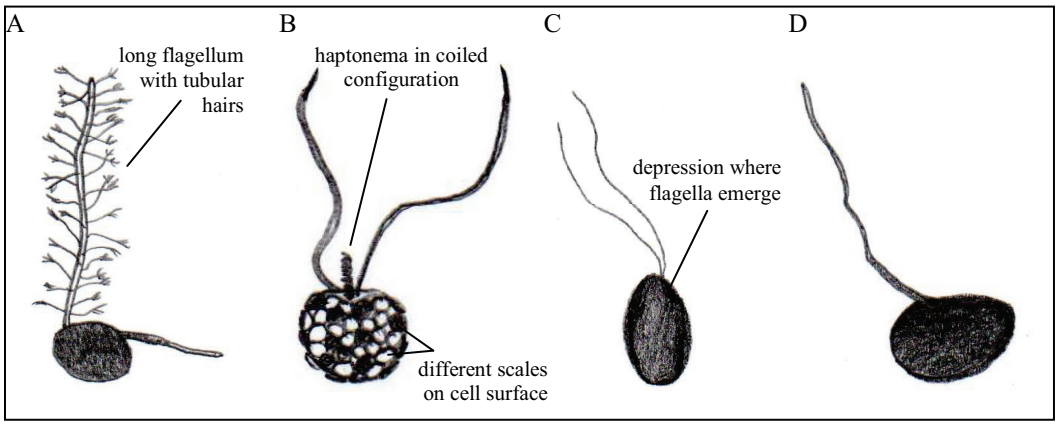


Figure 3: Schematic drawings (not to scale) of (A) *Bolidomonas* (after Guillou et al. (1999b) and own observations), (B) *Chrysochromulina* (after Eikrem and Throndsen (1998) and own observations), (C) *Hillea* (after Butcher, 1952), and (D) *Micromonas* (after Foulon (2005) and own observations).

taxonomic diversity (Massana et al., 2002 and 2004b; Epstein and López-García, 2008). The flagellated and phototrophic Bolidophyceae form a direct sister group to the diatoms (Vaulot et al., 2008), but they do not possess cell walls or any siliceous material, such as scales (Jeffrey and Wright, 2004). Next to fucoxanthin, they contain the chlorophylls *a*, c_1, c_2, c_3, diadinoxanthin and diatoxanthin. Bolidophyceae can be found in offshore and coastal waters, and generally form a minor contribution to the overall picoplankton community (Guillou et al., 1999b; Jeffrey and Wright, 2004; Vaulot et al., 2004). Guillou and collaborators (1999a and b) mentioned that the Bolidophyceae consist of at least three different clades, and determined that the two species *Bolidomonas pacifica* Guillou and Chrétiennot-Dinet, and *B. mediterranea* Guillou and Chrétiennot-Dinet are approximately 1-1.7 µm in diameter (Figure 1). Their cells are spherical or ovoid and differ in the angle of their flagella insertion and pattern of swimming (Figure 3A) (Guillou et al., 1999a). Their long flagellum (4-7 µm) bears tubular hairs, whereas the small one (0.9-2.2 µm) is smooth (Guillou et al., 1999a).

Haptophyta (Prymnesiophyta) The Haptophyta mainly contain unicellular flagellates with two generally smooth flagella of equal or unequal length (van den Hoek et al., 1995). The haptonema is a thin, filamentous appendage that may be used for cell anchoring or prey capture (Simon et al., 2009) and is located between the flagella in either an extended or coiled configuration (Figure 3B) (van den Hoek et al., 1995; Eikrem and Throndsen, 1998). Although the haptonema is considered as a defining feature of this phylum, it is absent in some species and life stages (van den Hoek et al., 1995; Eikrem and Throndsen, 1998). The most important accessory pigments of the haptophytes are the fucoxanthin derivatives 19'-butanoyloxyfucoxanthin and 19'-hexanoyloxyfucoxanthin (van den Hoek et al., 1995).

The Coccolithophyceae (Prymnesiophyceae) form one of the most important classes of this phylum and comprise more than 80 genera and almost 300 described species (Eikrem, 1999; Silva et al., 2007). These organisms dominate the phytoplankton community in coastal, oceanic and freshwater environments, and are generally enumerated as picoplankton as their organic scales and/or coccolithophores (calcium carbonate encrusted structures) are found in this size fraction (Eikrem and Moestrup, 1998; Eikrem, 1999; Eikrem and Edvardsen, 1999). For example, *Chrysochromulina* is a genus of high ecological significance. It is common in diverse marine waters and contains more than 60 species that exhibit micromorphological differences such as the number of scale types on the cell (Figure 3B) (Eikrem and Moestrup, 1998; Eikrem, 1999; Bergesch et al., 2008). Members of *Chrysochromulina* are either photo- or phagotrophic, may bloom in spring and/or summer and can be toxic (Fogg, 1991; Eikrem and Edvardsen, 1999; Bergesch et al., 2008; Vaulot et al., 2003b and 2008). *Chrysochromulina leadbeateri* has been found in different waters, such as the North Atlantic Ocean, the Mediterranean Sea, and from around Australia, and some of its subdivisions differ in morphology of the flagella, haptonema and/or scales (Estep et al., 1984; Eikrem and Throndsen, 1998). The cells described from northern Norway are spherical and 3-8 μm in size (Eikrem and Throndsen, 1998). They bear two flagella of slightly unequal length, a coiling haptonema, and two types of different scales (Figure 3B) (Eikrem and Throndsen, 1998).

Cryptophyta The Cryptophyta contain at least seven distinct clades of mainly nano-sized unicellular flagellates that are common in marine temperate and polar coastal waters (van den Hoek et al., 1995; Vaulot et al., 2003b and 2008). The phylum comprises possibly more than 16 genera, with the members of the Cryptophyceae containing a characteristic set of pigments (e.g., phycoerythrin) (van den Hoek et al., 1995; Beutler, 1998; Clay et al., 1999; Vaulot et al., 2003b and 2008). The flagella are covered with organic scales. The long flagellum bears two rows of lateral hairs, whereas the short one only bears a single row (van den Hoek et al., 1995). The only described picoplanktonic species, *Hillea marina* Butcher (2 - 2.5 μm; Figure 3C), is quite abundant in coastal waters (Butcher, 1952; Romari and Vaulot, 2004; Vaulot et al., 2008). Additional reports have suggested the existence of other picoplanktonic representatives for this group (Medlin et al., 2006; Wojciechowska, 2006).

Chlorophyta This phylum comprises fundamental ecological functions in the open ocean and coastal waters. It consists of Chlorophyceae, Pedinophyceae, Prasinophyceae, Trebuxiophyceae, Ulvophyceae and other unclassified species. Its picoplankton representatives largely dominate the picoeukaryotic community, especially in coastal regions. For example, they make up 85% of the picoeukaryotes in the English Channel (Not et al., 2004). Given the high abundance of these organisms in the marine environment, chlorophytes are the best described picoeukaryotes to date.

The bulk of Chlorophyta is composed of Prasinophyceae, which occur worldwide with high abundances throughout the photic zone in temperate and cold waters and in coastal areas (Vaulot et al., 2008; Viprey et al., 2008; Worden and Not, 2008). The Prasinophyceae are the most primitive class within the Chlorophyta and have no characteristic feature that unifies all of its taxa (Fawley et al., 2000; Guillou et al., 2004; Jeffrey and Wright, 2004; Not et al., 2004; Derelle et al., 2006; Fuller et al., 2006a). This reflects their basic role in the evolution of plants (see section "Ecological advantages of picoplankton over larger phytoplankton", page 1). Picoplanktonic members of the Chlorophyta were initially recognised as scaly green flagellates; today their morphology is known to be primitive and highly diverse, differing in the shape of cells, numbers and structures of flagella, morphologies of scales and cell division features (Simon et al., 2009). This class thus comprises many morphologies, including organisms without scales (e.g., *Micromonas pusilla*; Figure 3D) or without flagella (e.g., *Bathycoccus prasinos*; Figure 4A), and some without scales and flagella (e.g., *Ostreococcus tauri*; Figure 4B) (Skarstad Egeland et al., 1997; Fawley et al., 2000; Backe-Hansen and Throndsen, 2002; Guillou et al., 2004; Jeffrey and Wright, 2004; Worden and Not, 2008). Chlorophyta classes contain a heterogeneous assortment of pigments; although they all contain large amounts of chlorophyll *b*, the general marker pigment, prasinoxanthin, is only present in Mamiellales, Pseudoscourfieldiales (Nephroselmidiaceae) and Prasinococcales (Guillou et al., 2004; Latasa et al., 2004; Vaulot et al., 2008). Among the nine distinct clades of prasinophytes (Pyramimonadales, Marmelliales, Nephroselmidiaceae, Chlorodendrales, Pycnococcaceae, Prasinococcales, and three taxonomically unrecognised clades (VII, VIII and IX)) about 20 genera and 180 species have been recognised (Gescher et al., 2008; Vaulot et al., 2008; Viprey et al., 2008).

Mamiellales After the investigation of marine picoeukaryotes became more intense, it was soon apparent that the order Mamiellales was highly abundant, especially in marine coastal waters. Mamiellales is a well-defined monophyletic clade and contains relatively small-sized species that form the majority of the Prasinophyceae (Guillou et al., 2004; Vaulot et al., 2008). The Mamiellales contain "more primitive characters than any other group of Chlorophyta" (Six et al., 2005). This order contains the genera *Crustomastix*, *Dolichomastix*, *Bathycoccus*, *Ostreococcus*, *Mamiella*, *Mantoniella*, *Micromonas* and a new genera represented by isolate RCC 391 (Guillou et al., 2004; Viprey et al., 2008; Worden and Not, 2008). *Bathycoccus*, *Ostreococcus* and *Micromonas* represent more than 90% of the genes that have been sequenced (Vaulot et al., 2008) and are found most often and continuously in picoeukaryotic studies, especially in temperate coastal waters. For example, *Bathycoccus prasinos* and *Micromonas pusilla* were reported to account for 87% of Mamiellales cells and 60% of the Chlorophyta at Arctic and polar front stations (Not et al., 2005). Detection of the high abundance of the three genera of Mamiellales was supported by improved enumeration methods, and primarily by the molecular fluorescence *in situ* hybridisation (FISH) assay. As a

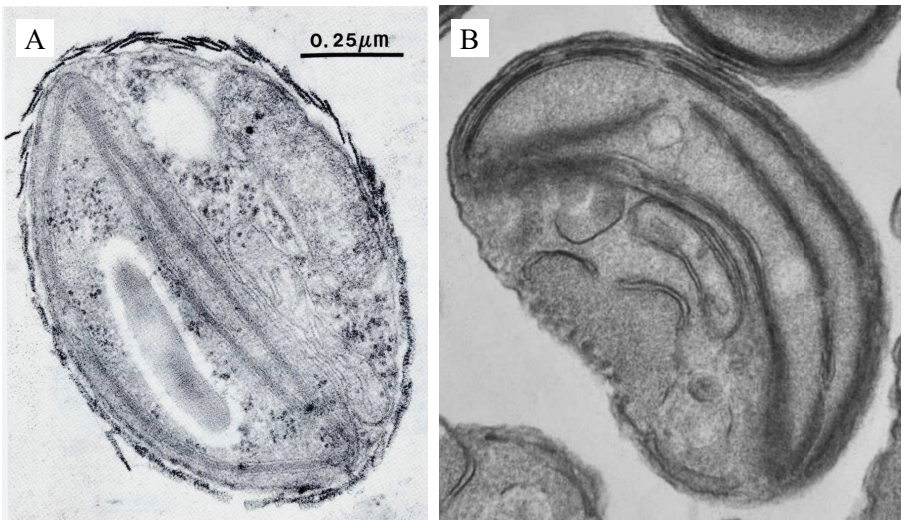


Figure 4: Whole-mount preparation and thin longitudinal section of (A) *Bathycoccus* and (B) *Ostreococcus*. Although a scale bar is lacking for (B), the cell is less than 1.1 µm long (see text). Pictures provided and allowed to show in this thesis by courtesy of Wenche Eikrem and Jahn Throndsen.

consequence, abundances of *Bathycoccus*, *Ostreococcus* and *Micromonas* were analysed in diverse regions of the world's oceans and the existence of diverse ecotypes has been revealed; these ecological adaptations are still poorly characterised however (Worden and Not, 2008).

The genus *Bathycoccus* is taxonomically very homogeneous (Guillou et al., 2004). The non-motile *Bathycoccus prasinos* Eikrem et Throndsen can be found in polar and Mediterranean waters and appear to be mesotrophic and well adapted to low-light conditions (Vaulot et al., 2003b and 2008; Guillou et al., 2004; Marie et al., 2006). These organisms usually occur very sporadic, but are able to form a major part of the picoplankton community (Eikrem and Throndsen, 1990; Not et al., 2004 and 2005; Vaulot et al., 2008). The 1.5 - 2.5 µm long and 1 - 2 µm wide coccoid cells (Figure 4A) are bright-green to olive-green in colour (Eikrem and Throndsen, 1990). The organic scales (0.5 - 1.0 µm in diameter) are arranged in a web-like pattern, which is typical of the order Mamiellales (Eikrem and Throndsen, 1990; Vaulot et al., 2008).

The genus *Ostreococcus* contains the smallest free-living eukaryotic species: *Ostreococcus tauri* Courties and Chrétiennot-Dinet (Courties et al., 1994; Vaulot et al., 2008). Its coccoid cell is only 0.8 - 1.1 µm long and 0.5 - 0.7 µm wide (Figure 4B). Furthermore, it is naked, non-motile, and can hardly be distinguished from prokaryotes (Vaulot et al., 2008). *Ostreococcus* has a cosmopolitan distribution in the marine environment, horizontally as well as vertically. Its four different clades are common among the picoplankton and have adapted to exist as ecotypes with distinct ecological niche requirements, although they only occasionally account for a significant portion of

total picoplankton (Guillou et al., 2004; Rodríguez et al., 2005; Derelle et al., 2006; Six et al., 2008; Vaulot et al., 2008).

The most abundant and best-studied species of the picoeukaryotes is *Micromonas pusilla* (Butcher) Manton and Parke. Butcher (1952) introduced this species as *Chromulina pusilla*, describing the very first phototrophic picoeukaryote and leading to "the real breakthrough" (Vaulot et al., 2008) in picoplankton studies. In 1960, it was renamed as *Micromonas pusilla* (Manton and Parke, 1960). The genus *Micromonas* comprises the oldest group of cryptic species known to date with the first diversification taking place ~65 MYA (Šlapeta et al., 2006). Its five heterogeneous clades occur worldwide (Šlapeta et al., 2006, Viprey et al., 2008; Simon et al., 2009; Worden et al., 2009). Although *Micromonas* is less abundant in oligotrophic open ocean waters, it is the major picoeukaryote in nutrient-rich or coastal regions (Ansotegui et al., 2003; Not et al., 2004 and 2005; Forster et al., 2008, Vaulot et al., 2008). It occurs throughout the year even in tropical and polar regions and can account for ≥ 25% of the picoeukaryotic community (DuRand et al., 2002; Ansotegui et al., 2003; Not et al., 2004, 2005 and 2008; Forster et al., 2008; Vaulot et al., 2008). Furthermore, this genus seems to occur over vast vertical profiles down to 600 m depth (Throndsen and Kristiansen, 1991). Because of its high abundance and broad distribution, Worden and colleagues (2009) suggested *Micromonas* to serve as a sentinel organism for biogeochemical fluxes of modern oceans during climate change. However, the distribution patterns of clades of *Micromonas* have not been reported and correlations between specific ecotypes or well-defined phenotypes with environmental variables are unknown (Forster et al., 2008; Vaulot et al., 2008; Viprey et al., 2008; Simon et al., 2009; Worden et al., 2009). The cell structure of *Micromonas pusilla* is very simple (Figure 3D); cells are generally less than 2 μm in size, pear-shaped and bear one flagellum (Šlapeta et al., 2006; Forster et al., 2008; Vaulot et al., 2008). Recent studies have revealed the possibility of sexual differentiation and the occurrence of a resistant life stage with a thick adhesive zygote wall (Worden et al., 2009).

2.) Picoplankton in the German North Sea

The German Bight is characterised by an average water depth of less than 40 m and a high influence of freshwater inflow from the Eider, Elbe and Weser rivers. It is one of the most extended eutrophic areas in the North Sea (Hickel, 1998). The island of Helgoland lies approximately 60 km off the mainland in the middle of the sea (Figure 5). The first biological station for marine research was founded at this location in 1892.

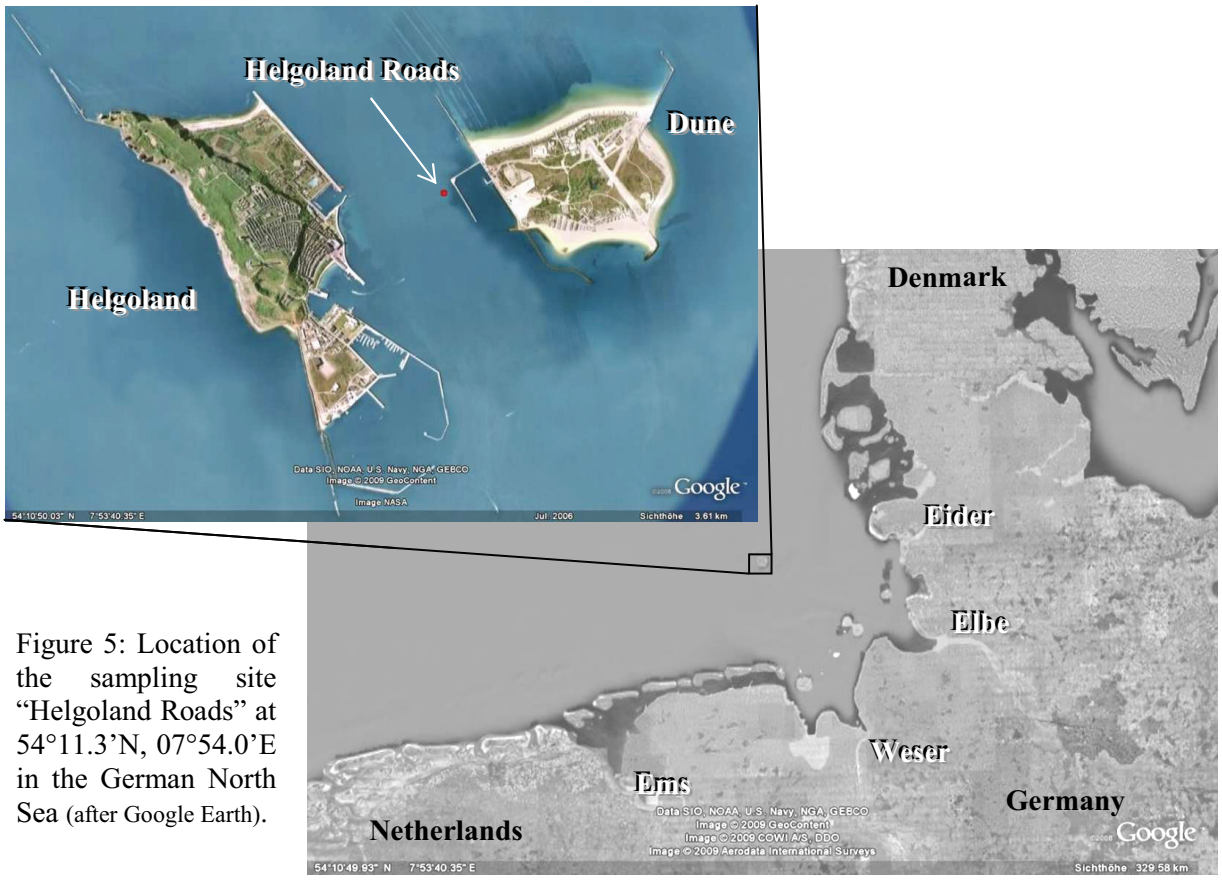


Figure 5: Location of the sampling site "Helgoland Roads" at 54°11.3'N, 07°54.0'E in the German North Sea (after Google Earth).

With the main objective to study ecological dynamics and trophic interactions in the marine ecosystem and to explain these data by laboratory experiments, a long-term time series began in 1962 (Hickel, 1998; Franke et al., 2004). The sampling site, "Helgoland Roads" (54°11.3'N, 07°54.0'E), lies between the island of Helgoland and its dune (Figure 5). Here, the water column has a depth of 5 to 8 metres and is well mixed (Medlin et al., 2006). Although surface samples are representatives of the entire water column, they are quite often loaded with sand and mud, resulting in a clogging of filters during sample preparation (Hickel, 1998; Medlin et al., 2006). Continuous investigations of the abundance and composition of large phytoplankton and abiotic

factors (temperature, salinity, secchi depth, nutrients) provide a unique insight into ecological processes and functions of the resident plankton community (Wiltshire et al., 2008). However, this record does not include investigations of picoplankton. Although bacteria (colony-forming units) and virioplankton have been studied previously (Gerdts et al., 2004), picoeukaryotes were not investigated until the PICODIV project (from 2000 to 2003) (Vaulot et al., 2003b). This project and the study by Medlin et al. (2006) allowed for the first insights into the picoplankton community and corroborated previous reports of these organisms in coastal regions. Picoplankton in the German Bight were composed of a taxonomically diverse background population, exhibited rapid shifts in abundance and occasionally dominated the overall primary production (Medlin et al., 2006). The highest chlorophyll *a* concentrations in the < 3 µm fraction were measured in summer 2000 (July and August) (Medlin et al., 2006). Abundances of individual taxa were highly variable, whereas broader groups remained abundant throughout different seasons (Vaulot et al., 2003b). Medlin et al. (2006) confirmed previous suggestions (Vaulot, 2001b) of the annual succession of the community of coastal picoeukaryotes for the waters around Helgoland. For example, heterotrophic organisms followed the development of photosynthetic organisms. The rare finding of identical sequences in distinct seasons represents the high succession of picoplankton species (Medlin et al., 2006). Thus, a seasonal shift in the community was indicated. Furthermore, an annual reassembling of the picoeukaryotic community seemed to take place (Medlin et al., 2006). As shown by Medlin et al. (2006), the most diverse community occurred in April (with an even community structure) and August 2000 (with a rather uneven community structure). In winter however, one alveolate sequence largely dominated (Medlin et al., 2006). Gescher et al. (2008) found a higher diversity in the winters of 2005/2006 and 2006/2007 and in late summer of 2006 using a DNA microarray.

All major taxonomic assemblages of picoeukaryotes appeared to be present in the waters around Helgoland. They were dominated by non-photosynthetic organisms, such as Alveolata (group II Alveolata, such as *Amoebophyra* and other parasitic protists, and Ciliophora) and stramenopiles (Medlin et al., 2006). This was consistent with the general structure of the picoeukaryotic community, dominated by Alveolata (Ciliophora, Dinophyceae, Perkinsea and Apicomplexa) and stramenopiles (Groisillier et al., 2006; Guillou et al., 2008; Vaulot et al., 2008). In the German Bight, Ciliophora were recorded to dominate in spring 2000, whereas group II Alveolata increased in summer and autumn, possibly in combination with their host cells. The Bolidophyceae were a further highly abundant group, and Cryptophyta, small diatoms and other stramenopiles, such as Dictyochophyceae and Chrysophyceae (primarily freshwater), were also present (Vaulot et al., 2003b and 2008; Medlin et al., 2006; Gescher et al., 2008). The photosynthetic picoeukaryotes were dominated by Prasinophyceae (Mamiellales) with 40% and were most abundant in spring 2000 (Medlin et al., 2006). Medlin et al. (2006) suggested Pycnococcaceae, *Mamiella* and *Mantoniella* as the dominant

Mamiellales as microarrays revealed no signal for *Bathycoccus* or *Ostreococcus*, and the signal for *Micromonas* seemed to be a false positive. In a more detailed study, Gescher et al. (2008) found that *Bathycoccus* formed the largest fraction of Mamiellales off Helgoland, followed by *Micromonas*. During the PICODIV project, novel alveolates (small dinoflagellates), haptophytes, cryptophytes, and novel classes (Rosko II, Picobiliphyta) were found in the waters around Helgoland (Medlin et al., 2006). Gene sequences of obtained cultures were most similar to *Mantoniella squamata* (Prasinophyceae), *Micromonas pusilla* (Prasinophyceae), *Nanochlorum eucayotum* (Trebouxiophyceae), *Methanophrys sinensis* (Alveolata), *Massisteria marina* (Rhizaria), and *Caecitellus parvulus* (stramenopiles) (Vaulot et al., 2003b).

These previous findings on the picoeukaryotic community in the German North Sea provided first information on the presence and succession of its organisms. The present thesis examined the organisms' identities, dynamics and ecological characteristics on a higher sampling frequency, over a longer period, and by the use of a set of currently existing complementing methods. Thus, previous conclusions were confirmed, in part revised and knowledge about the overall community of picoeukaryotes enhanced.

3.) Methods to investigate picoplankton

Methods to investigate microbiological life forms are much more difficult to find than are those for large and visible organisms (Amann et al. 1995). However, those techniques developed to investigate prokaryotes (bacteria and archaea) could also be applied to study eukaryotic organisms, provided their adjustment. Overall, the diverse community of unknown and ecologically similar influenced picoeukaryotes complicated the identification and investigation of individual roles of its components. This section provides an overview of the currently existing methods used to analyse picoplankton communities. General approaches and faults of the methods are discussed in detail. Emphasis is placed on molecular methods, with a focus on techniques used in the present study.

3.1) Sample collection and preparation

The driving question in research studies determines the means and frequency of sample collection. For example, investigation of the physico-chemical influences, seasonal succession and grazing pressures on picoeukaryotes requires a long-term study under recognition of environmental parameters (Falkowski and LaRoche, 1991; Mackey et al., 1998; Guillou et al., 1999b; Partensky et al., 1999a; Vaulot, 2001a; Jacquet et al., 2002b). In general, size fractionation by filtration is the accepted method for separating picoplankton from larger plankton, but they cannot guarantee the isolation of the desired size fraction. For example, larger but slimmer phytoplankton cells may slip through the pre-filters, or larger and fragile cells may break into fragments that are subsequently retained by a filter targeting a smaller size fraction (Not et al., 2005 and 2008). The abundance of resting spores and/or faecal pellets may also bias the results of picoplankton analyses, or larger cells may clog filter pores and thus retain picoplankton cells that would otherwise pass (Díez et al., 2001; Not et al., 2005 and 2008; Vaulot et al., 2008). Furthermore, the picoplankton community covers a broad size range, especially the picoeukaryotes (Figure 1). Therefore, complete isolation of the entire picoplankton size class is difficult (Díez et al., 2001; Vaulot et al., 2008).

The addition of fixatives during sample preparation can cause cell loss, as well as changes in volume, structure and/or chemical properties (Bratbak et al., 1990; Throndsen, 1995; Marie et al., 2000). Glutaraldehyde, for example, is a gentle fixing agent but causes the cells to shrink (Choi and Stoecker, 1989; Throndsen, 1995; Worden et al., 2004). Formaldehyde generates less shrinkage but may bleach pigments (Throndsen, 1995). A mixture of glutaraldehyde and tannic acid (modified van der Veer fixative) seems to cause the smallest change in cell volume and iodine (acid Lugol's solution) will obscure inner cell structures (Choi and Stoecker, 1989; Throndsen, 1995). These examples

represent the importance of cautiously deciding on the best suitable sample preparation in the planned picoplankton project and the need to carefully interpret obtained data in respect to the collection and preparation of samples.

3.2) Isolation and cultivation

Picoeukaryotes are fragile and are easily destroyed when attempting to isolate them from the environment. They are thus difficult to analyse in culture experiments, and therefore, studies of picoeukaryotic physiology and biochemistry have progressed slowly (Scanlan and West, 2002). While very abundant species can be difficult to isolate, scarce species may be isolated without difficulty (Vaulot, 2001b; Massana et al., 2004a). This was the case in the PICODIV project, when *Micromonas* formed the quantitative majority of Mamiellales in the English Channel and *Ostreococcus* was marginal. Nonetheless, *Ostreococcus* was isolated most often and *Micromonas* was only recovered from a single sample (Vaulot et al., 2003b). Furthermore, Massana et al. (2004a) observed that some picoplankton groups were represented in clone libraries and in cultures (Prasinophyceae), whereas others were found either in cultures (Bicosoecida (stramenopiles)) or in clone libraries (novel Alveolata and stramenopiles). Overall, Little et al. (2008) pointed out that cultured community members often constitute less than 1% of the actual community in the field. The more abundant organisms seem to be favoured by isolation attempts using the serial dilution technique (Throndsen, 1978; Vaulot et al., 2008). The difficulty and methodological biases of isolating and cultivating picoeukaryotes make estimates of species abundance using cultivation success impractical. Nonetheless, isolation and culture studies allow for the analysis of individual characteristics of organisms in respect to their genetic and pigment information, and their requirements for optimal growth. These information may allow to track the natural abundances of these organisms (Vaulot et al., 2008) and to adjust analysis techniques, such as molecular probes or pigment calculations, though cultured species may change individual characteristics, such as pigment profiles (Wright and Jeffrey, 2005) or genetic sequences (Massana et al., 2004a).

3.3) Microscopy

Traditional magnification by light microscopy methods is not suitable for detailed investigations of tiny picoplankton organisms. Microscopy can be enhanced when it is used in combination with fluorescence however. Epifluorescence microscopy measures the emission of light by fluorescing cellular compounds, such as pigments or molecular markers (Vaulot et al., 2008). Thus, it allows

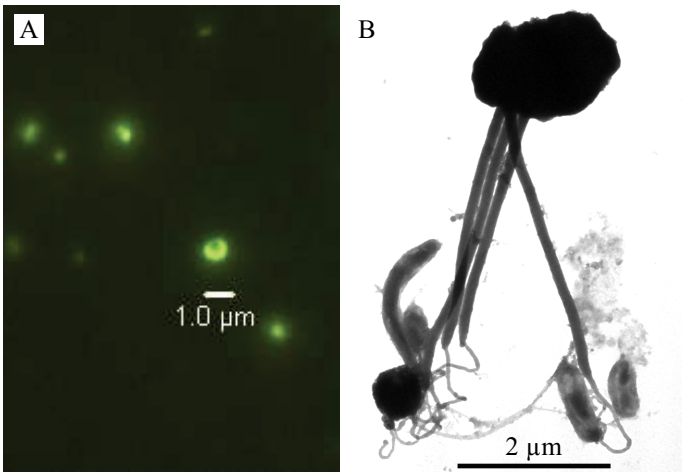


Figure 6: Examples of picoplankton visualisation by (A) epifluorescence microscopy when using FISH-TSA and (B) whole-mount preparation and transmission electron microscopy.

differentiation between auto- and heterotrophic pro- and eukaryotes in natural or cultured samples without the identification of cell morphologies. Major problems associated with epifluorescence microscopy are cell losses in preparation processes, cell clumps and low signal-to-background ratios (Biegala et al., 2003). These complicate the identification of individual cells. However, in combination with molecular staining (Figure 6A; see section "Molecular methods", page 22), epifluorescence microscopy can provide detailed taxonomic information regarding the qualitative and quantitative abundance of organisms that compose the picoplankton community.

Microscopy methods were also enhanced by the development of electron microscopy. Manton and Parke pioneered its use and the analysis of shadow-casted or uranyl-acetate-contrasted whole-cell mounts in the 1950s (Manton and Parke, 1959; Eikrem and Throndsen, 1999). The new techniques were able to reveal morphological details, such as flagella hairs and species-specific scales (Figure 6B) (Eikrem and Throndsen, 1990; Vaulot, 2001a; Backe-Hansen and Throndsen, 2002; Vaulot et al., 2008). An even better resolution of surface features became possible by the development of scanning electron microscopy. In fact, some picoplankton species have been described only by this method (Vaulot et al., 2008). However, electron microscopy cannot be used to quantify cells because preparation procedures cause cell shrinkage and a high cell loss (Throndsen and Kristiansen, 1991; Vaulot et al., 2008). Furthermore, it does not allow correct classification of all picoplankton taxa because similar morphologies occur in genetically and/or ecologically different species and varying morphologies of single species exist at different life stages (Eikrem and Throndsen, 1998; Epstein and López-García, 2008; Simon et al., 2009; Worden et al., 2009). Even characteristic scales, which may be a useful tool for identification, can be similar between different species or differ among organisms of a single species (Eikrem and Throndsen, 1998; Backe-Hansen and Throndsen, 2002). Nonetheless, in combination

with culturing, electron microscopy can provide a detailed description of the morphology of a single picoeukaryote (although scales may be absent in cultures) and may thus improve understanding of its physiological characteristics and ecological functions (Eikrem and Throndsen, 1998; Backe-Hansen and Throndsen, 2002).

3.4) Flow cytometry

Flow cytometry (FCM) allows for easy, fast and accurate estimates of picoplankton abundance. It analyses single particles within a water sample regarding their light-scattering properties, as a function of cell size and refractive index, in combination with the fluorescence of pigments. FCM can thus distinguish and quantify major photosynthetic groups, such as *Prochlorococcus*, *Synechococcus*, as well as small and large autotrophic eukaryotes (Li, 1994; Marie et al., 2000; Medlin et al., 2002). Although FCM allows for the identification of eukaryotic cryptophytes because they possess the distinctive phycoerythrin pigment (see section "Cryptophyta", page 10), further taxonomic determination of the eukaryotic picoplankton is only possible using molecular staining (compare section "Molecular methods", page 22) (Vaulot et al., 2008). For example, in combination with oligonucleotide probes, FCM can provide very detailed information about the taxonomic community composition, including the heterotrophs (Vaulot et al., 2008; Worden and Not, 2008). Furthermore, FCM allows the possibility to sort cells of interest (Li, 1994; Vaulot et al., 2008; Worden and Not, 2008). Thus, it provides a means to cultivate picoplankton-sized organisms or further investigate picoplankton via microscopy (Li, 1994; Vaulot et al., 2008). The general drawbacks of FCM are comparable to those of epifluorescence microscopy; possible loss of cells during sample preparation, the formation of cell clumps or low signal-to-background ratios, both of which lead to bias in cell quantification (Biegala et al., 2003).

3.5) Pigment based methods

Phototrophic picoplankton organisms differ in their content and composition of pigments. Therefore, they exhibit distinguishable absorption spectra and fluorescence chromatograms that can be detected by several methods. For example, the bbe multialgal fluorometer, used in the present study, can detect the entire chlorophyll *a* concentration *in vivo*, including all of its breakdown products within a natural sample. The fluorometer is able to distinguish between spectral groups, such as green-pigmented algae (chloro-, eugleno-, prasinophytes), cyanobacteria, brown-pigmented algae (diatoms and dinophytes), and cryptophytes (Beutler, 1998 and 2003; Beutler et

al., 2000), because excitation of each group at five different wavelengths results in a characteristic emission spectra, or norm spectra, of each group. These norm spectra allow the calculation of the contribution of each spectral group to the measured overall emission intensity of chlorophyll *a* of a water sample by curve-fitting procedures (Beutler et al., 2002). Thus, the fluorometer calculates the relative contribution of the spectral groups to the overall chlorophyll *a* concentration and promotes quantitative conclusions about the relative abundance of each group.

High-performance liquid chromatography (HPLC) is a technique that can separate and quantify pigments that are extracted from cells. It detects absorption spectra *in vitro*, allowing the identification of single pigments, and their quantification by calibration with pigment standards. Thus, in comparison to fluorometry, HPLC methods can calculate the exact concentration of the pure chlorophyll *a*, generally used as a biomass indicator for phytoplanktonic organisms. Specific marker pigments (see section "The picoeukaryotes", page 7) allow a rough distinction of picoplankton classes, for example, chlorophyll *b* for chlorophytes and fucoxanthin for heterokonts and haptophytes. If the general taxonomic composition and pigment profiles of picophytoplankton are known in space and time, the composition of the picoplankton community can be quantified. This is, for example, possible by using the matrix factorisation program CHEMTAX (Mackey et al., 1996; Irigoien et al., 2004). However, because the taxonomic composition and pigment profiles of naturally occurring picophytoplankton are generally unknown, pigment data only provide a first impression of possible community structures.

Accurate analysis of the quantity and quality of picoplankton by pigment concentrations in a water sample, may it be by fluorometry or HPLC, has additional drawbacks. First, low abundances of picoautotrophs may result in pigment concentrations that are below detection limits. Influences of pigmented contaminants, such as zooplankton faecal pellets, ingested phototrophs, endosymbiotic plastids, symbionts, cell fragments, resting spores and detritus are thus a concern (Jeffrey et al., 1999; Jeffrey and Wright, 2004, Zapata et al., 2004, Not et al. 2005 and 2008, Wright and Jeffrey, 2005; Vaulot et al., 2008). Furthermore, pigment ratios can change over the course of days, weeks and seasons, and are influenced by growth, reproduction, senescence, irradiance, nutrients and turbulence (Mackey et al., 1998; Jeffrey et al., 1999; Garrido et al., 2003; Lewitus et al., 2005). Thus, the calculation of the community composition is difficult because individual pigment profiles are not steady. Unequivocal taxonomic delineation is also difficult because there is overlap in the pigment content and composition between distinct taxonomic classes; there may also be different pigment profiles of species of the same class (Jeffrey et al., 1999; Mackey and Higgins, 2001; Vaulot et al., 2003b and 2008; Latasa et al., 2004; Lewitus et al., 2005; Fuller et al., 2006b; Gescher et al., 2008). For example, marker pigments may be absent, as is the case with prasinoxanthin in some prasinophytes (see section

"Chlorophyta", page 10). Furthermore, marker pigments may be detected even in the absence of the associated picoplankton (Jeffrey et al., 1999; Ansotegui et al., 2003; Latasa et al., 2004; Zapata et al., 2004; Lewitus et al., 2005). The influence of larger-sized organisms on picoplankton pigment concentrations within pre-filtered samples cannot be excluded (see section "Sample collection and preparation", page 17). In combination with similarities in pigment profiles of picoeukaryotes and larger microalgae, or fractions and/or life stages of the latter (Mackey et al., 1998; Mackey and Higgins, 2001; Not et al. 2005), this may lead to bias in the interpretation of pigment data and the estimation of picoplankton abundance.

3.6) Molecular methods

Given the low number of picoeukaryotes that have been described since their discovery (Figure 1) (Vaulot et al., 2008), the difficulty of thorough investigations on them becomes apparent. Molecular methods have made it possible to gain further information regarding picoeukaryote diversity, spatio-temporal abundance, as well as their ecology within natural systems (Amann et al., 1995; Burton, 1996; Medlin et al., 2002; Not et al., 2002, 2004 and 2009; Biegala et al., 2003; Fuller et al., 2006b; Thornhill et al., 2007). This was primarily a result of the ability to identify whole populations in their natural environments and to quantify organisms without identification of morphological features or cultivation of these organisms. Molecular approaches can successfully identify microorganisms with high taxonomic resolution and answer process- and evolution-related questions (Woese and Fox, 1977; Giovannoni et al., 1988; Amann et al., 1995; Lange et al., 1996). These methods even allow the quantification of taxonomically distinct community members in the field. They were the only techniques that suggested the taxonomic diversity of picoplankton to be ecologically significant, and that the dominating part of the picoeukaryotic community may be composed of uncultured groups (Massana and Pedrós-Alió, 2008). Different approaches of molecular assessment can provide either qualitative (cloning) or quantitative (molecular probes) information of plankton communities and individual species.

The small subunit of the ribosomal RNA (18S rRNA) is present in all eukaryotes and is therefore used for taxonomic identification. The genes that encode rRNA are evolutionarily very old, slowly evolving and highly abundant (Woese and Fox, 1977; Medlin et al., 2002; Amann and Fuchs, 2008). They show no lateral gene transfer and contain well-conserved, moderately conserved and extremely variable regions (Woese and Fox, 1977; Medlin et al., 2002; Amann and Fuchs, 2008; Vaulot et al., 2008). All these features encourage the use of rRNA genes to identify the different taxa of picoplankton communities. Furthermore, rRNA probing allows for the discovery of new species or even divisions, as well as the assessment of evolutionary relationships and genetic variations

(Medlin et al., 2002; Metfies et al., 2007; Amann and Fuchs, 2008; Vaulot et al., 2008). General drawbacks of using rRNA are decreased sensitivity for quantifying organisms with robust cell membranes (Not et al., 2008). In addition, the RNA as well as the DNA molecule are instable and differences may exist between the copies of single genomes (Burton, 1996; Medlin et al., 2002; Not et al., 2008 and 2009; Vaulot et al., 2008). The amount of 18S rRNA analysed may be biased by the presence of different numbers of gene copies between organisms; for example, small eukaryotes are known to express fewer copies than larger eukaryotes (Zhu et al., 2005). Massana et al. (2004a) even suggested that the possible dominance of novel Alveolata could simply derive from a higher copy number of the rDNA operon. However, as is the case with pigment profiles, the rRNA content also varies because of environmental conditions, life stages and life strategies (Amann et al., 1995; Burton, 1996; Medlin et al., 2002; Biegala et al., 2003; Zhu et al., 2005; Vaulot et al., 2008; Worden and Not, 2008; Not et al., 2009). These complications may thus bias quantitative analyses of spatio-temporal distributions of eukaryotes. Not et al. (2009) suggested an additional high influence of extracellular DNA that may not be excluded by filtration on 0.2 µm filters, thus biasing the results of the phylotypes in the targeted size fraction.

Fingerprinting methods provide a rapid qualitative overview of the isolated DNA sequences (Díez et al., 2001; Féral, 2002; Medlin et al., 2002; Vaulot et al., 2008). For example, denaturing gradient gel electrophoresis (DGGE) is a rapid and sensitive technique that identifies species diversity of entire communities (Medlin et al., 2002). This is possible by extracting all nucleic acids from a sample, denaturing the strands, identifying target fragments (e.g., on the rRNA genes) by low-level primers (e.g., for all eukaryotes) and cloning (amplifying) the selected gene fragments by polymerase chain reaction (PCR) (Amann et al., 1995; Féral, 2002). The amplified sections are then separated on a polyacrylamide gel that contains a gradient of urea and formamide concentrations (Medlin et al., 2002). This separation is caused by differences in the content of guanine and cytosine within the nucleotide sequences, which define the extent of denaturation of the amplified fragments among the gradient. As a consequence, the different sequences form a characteristic band profile for each environmental sample (Medlin et al., 2002; Marie et al., 2006), that can be visualised by staining of the gel. Then the profile can be compared to other gel patterns and single bands of it can be excised for re-amplification and sequencing (Féral, 2002; Medlin et al., 2002; Vaulot et al., 2008). These sequences can be compared to existing data banks, thus providing an indication of the ecological characteristics or phylogenetic affiliations of organisms in a sample (Medlin et al., 2002). Alignment of sequences allows for phylogenetic reconstruction and design of oligonucleotide probes (Medlin et al., 2002).

However, sample preparation can create bias in the qualitative analysis of the picoeukaryotes using DGGE (Viprey et al., 2008; Not et al., 2008 and 2009). Furthermore, no method using extracted DNA or RNA allows a clear distinction if the obtained sequences originate from free-living organisms, symbionts, parasites or extracellular DNA (Viprey et al., 2008; Not et al., 2009). Amplification by PCR may to lead to an under representation of specific groups, such as autotrophs, and it is possible that the primers used are less specific for certain species or groups (Burton, 1996; Medlin et al., 2002 and 2006; Vaulot et al., 2008; Viprey et al., 2008; Not et al., 2009). Thus, the choice of PCR primers, preparation conditions and machines need special consideration (Medlin et al., 2002; Zhu et al., 2005; Fuller et al., 2006a; Guillou et al., 2008). Additionally, comparison of gel patterns can be imprecise because heteroduplex formations and the formation of chimeric sequences may bias the results (Amann et al., 1995; Burton, 1996; Medlin et al., 2002; Epstein and López-García, 2008; Vaulot et al., 2008). The most abundant phylotypes are generally favoured in excitation and sequencing because more intense bands are easy to detect on the gels and consequently more often excised and sequenced. Thus, phylotypes that contribute fewer DNA fragments, either because of their lower abundance or because of a methodological suppression, can easily be missed (Medlin et al., 2002; Nikolausz et al., 2005; Vaulot et al., 2008). Finally, the alignment of identified sequences towards present data banks for a phylogenetic reconstruction may be hindered or biased by the incomplete data sets and/or disagreement of available sequences (Worden and Not, 2008).

Other, quantitative approaches for identifying members of the picoplankton community use oligonucleotide probes. These consist of 16-24 nucleotides and bind to a specific gene sequence within target organisms. One option is to use the probes as part of a fluorescence *in situ* hybridisation (FISH) assay. This method uses fixed, probe-permeable cells and fluorochrome-labelled probes for analysis with epifluorescence microscopy (Figure 6A) or flow cytometry (see sections "Microscopy", page 18, and "Flow cytometry", page 20). FISH is the most powerful technique for quantitative phylogenetic studies in the natural environment and allows for identification of unknown groups, description of single cells (e.g., their identity and growth rate), as well as their ecological function (Medlin et al., 2002; Biegala et al., 2005; Zhu et al., 2005). Furthermore, FISH is not affected by any of the biases caused by PCR amplification (Alonso-Sáez et al., 2007). Some drawbacks include the possible coincidence of target organisms on non-target organisms or particles, cell losses or lysis, as well as cell aggregation and patchiness on the filter (Medlin et al., 2002; Biegala et al., 2003; Vaulot et al., 2003b; Knefelkamp et al., 2007; Amann and Fuchs, 2008). These may hinder the hybridisation of cells and may bias probe quantification. Furthermore, counts by group-specific probes do not provide information about the group's complexity (Giovannoni et al., 1988). Furthermore, discrepancies in hierarchical probe counts, indicating the occurrence of additional

cells not targeted by the more general probes, or unsuccessful labelling may occur (Gescher et al., 2008; Vaulot et al., 2008).

A drawback of using oligonucleotide probes is the complicated research involved in the probe discovery and the lack of cultured species to serve testing probe success (see section "Isolation and cultivation", page 18) (Medlin et al., 2002; Zhu et al., 2005; Worden and Not, 2008). In addition to membrane-penetration difficulties when used *in situ* and varying hybridisation efficiencies and protocols, unspecific binding, cross-hybridisations and marker saturation with substitutions may bias the results (Medlin et al., 2002; Vaulot et al., 2003b; Amann and Fuchs, 2008; Gescher et al., 2008). It is possible to enhance the fluorescence signal of the probes by using catalysed reporter depositions (CARD), such as tyramide signal amplification (TSA) (Schönhuber et al., 1997; Not et al., 2002; Amann and Fuchs, 2008). These use probe labelling with horseradish peroxidase (HRP) and, in the case of FISH-TSA, fluorescein-tyramide as substrate for the enzyme (Not et al., 2002; Amann and Fuchs, 2008). Nonetheless, a low signal-to-background ratio resulting from a low rRNA content, the binding-loci of the probe, interfering autofluorescence of compounds or fluorescing detritus may still limit the detection of distinct hybridised cells (Medlin et al., 2002; Vaulot et al., 2003b).

Additional semi-quantitative molecular methods exist and include the use of DNA microarrays (phylochips). Similar to FISH, oligonucleotide probes are used. These are attached to a glass slide and the extracted and PCR-amplified DNA fragments are hybridised to them. The previously described drawbacks for the use of DNA or RNA, the amplification by PCR, as well as the use of molecular probes, apply for the use of microarrays. They thus require careful interpretation of results and eventually the reconciliation by additional methods.

FOCUS OF THIS STUDY

The general introduction illustrated the current knowledge of the characteristics, abundances and ecological niches of the picoplankton community. It noted the scarce information available about the abundance and ecological function of single picoeukaryotes. Furthermore, the introduction described the analytical approaches and caveats some of the primary methods used to analyse the eukaryotic picoplankton community. A focus of the present doctorate was to compare these methods. Therefore, the replicate samples of the picoplankton community were collected from the surface waters of the German Bight at Helgoland Roads and were analysed by the different techniques. The comparison of the distinct results yielded conclusions for the success and limitations of individual methods and suggested targets for methodological improvements. Results from all biochemical, molecular and microscopic approaches allowed for a more complete interpretation and provided a more robust overview of the resident picoplankton community of the German North Sea. The weekly sampling frequency over a long period and simultaneous detection of a set of environmental parameters should provide a further insight into the ecological characteristics within the picoplankton community. The present study intended to further our understanding of picoplankton abundance and succession in the German Bight by investigating the following ecological and methodological questions:

1. **How large is the chlorophyll *a* contribution of picophytoplankton to the overall primary production community?**
   This question will primarily be investigated in manuscripts 1 and 2.

2. **How do the methods differ in their description of the picoeukaryotic community?**
   This question will primarily be investigated in manuscripts 2 and 3.

3. **Which are the primary environmental parameters that influence the abundance and composition of the picoplankton?**
   This question will primarily be investigated in manuscript 2.

4. **Are there correlations between the results obtained by the different methods, and which method is best suited for picoplankton investigations?**
   This question will primarily be investigated in manuscripts 2 and 3.

To examine picoeukaryote dynamics in order to answer these questions, water samples were taken weekly for 12 and 17 months, respectively, beginning in March 2005 at Helgoland Roads (54°11.3'N, 07°54.0'E). Samples were analysed for the abundance and composition of picoeukaryotes, with special consideration of Prasinophyceae, especially the Mamiellales and *Micromonas pusilla*. These organisms were suggested to represent a dominant portion of the picoplankton. Simultaneously, a vast amount of environmental parameters was included to assess their influence on the picoplankton community. The methods investigated were flow cytometry (FCM), multialgal fluorometry, high-performance liquid chromatography (HPLC), fluorescence *in situ* hybridisation coupled with tyramide signal amplification (FISH-TSA), denaturing gradient gel electrophoresis (DGGE), rRNA gene sequencing and electron microscopy (EM).

RESULTS AND DISCUSSION

4.) Main findings

4.1) Contribution of picophytoplankton to overall primary production in the German Bight

Chlorophyll *a* is generally used as a biomass indicator for phytoplanktonic organisms. Thus, an estimation of the contribution of the picophytoplankton towards total primary production is possible by analysis of the chlorophyll *a* concentration in the picoplanktonic size fraction and the overall water sample. In general, the chlorophyll *a* concentration of the overall phytoplankton community is analysed by the long-term series at Helgoland (Wiltshire and Manly, 2004; Franke et al., 2004; PANGAEA 2004). This research project uses two methods in parallel: *in vivo* fluorometry, which measures the sum of chlorophyll *a* and its degradation products of the unfiltered water sample, and *in vitro* fluorescence by high-performance liquid chromatography (HPLC), which measures the pure chlorophyll *a* concentration on a 0.2 µm filter (Knefelkamp et al., 2007). In the present doctorate, time-series data taken in parallel to the picoplankton samples were used for a chlorophyll *a* comparison between size classes. Therefore, the picophototrophs were analysed by the same laboratory fluorometer as the long-term series data, but with an improved HPLC method (manuscript 1).

On a seasonal comparison, the total chlorophyll *a* concentration more than doubled within the overall phytoplankton fraction between spring and summer, whereas its pure chlorophyll *a* concentration remained nearly the same (Figure7A). In the pico-sized fraction, both concentrations slightly increased between the two seasons. This indicated a high shift from growth to senescence of larger phytoplankton organisms towards summer, resulting in a higher amount of pigment-breakdown products within the larger microalgae and a lower picoplankton contribution regarding the total chlorophyll *a* (Figure 7B). Towards autumn, there was a decrease in all chlorophyll *a* concentrations of both fractions. This overall decrease of phytoplanktonic biomass indicated an influential change in the environmental settings. Nonetheless, the picophytoplankton were able to better deal with these changes because its contribution to the overall primary production slightly increased. Towards winter, the overall phytoplankton abundance further decreased, whereas the pigment concentrations in the pico-sized fraction increased (pure chlorophyll *a*) or only slightly decreased (total chlorophyll *a*). Thus, the contribution of the picoautotrophs increased and reached its maximum with 19.5% (HPLC) and 38.3% (fluorometry), respectively. This indicated a better adaptation of picoplanktonic organisms towards the given environmental settings in comparison to larger microalgae. Overall, an annual average

contribution of 9% to the pure chlorophyll *a* (HPLC) and of 30.3% to the total chlorophyll *a* (as estimated by fluorometry) was detected for the picophytoplankton. The high proportion in fluorescence is suggested to be caused by the high abundance of degradation products within the smallest size fraction (0 to 3 µm), primarily resulting from cell fragments of larger cells, but eventually also as parts of zooplanktonic faecal pellets (Not et al., 2005 and 2008). HPLC analysis of the 0.2 to 3 µm fraction on the other hand, confirmed the overall low abundance of picoautotrophs in the eutrophic German Bight that was previously calculated to account for 13.6% (Vaulot et al., 2003b).

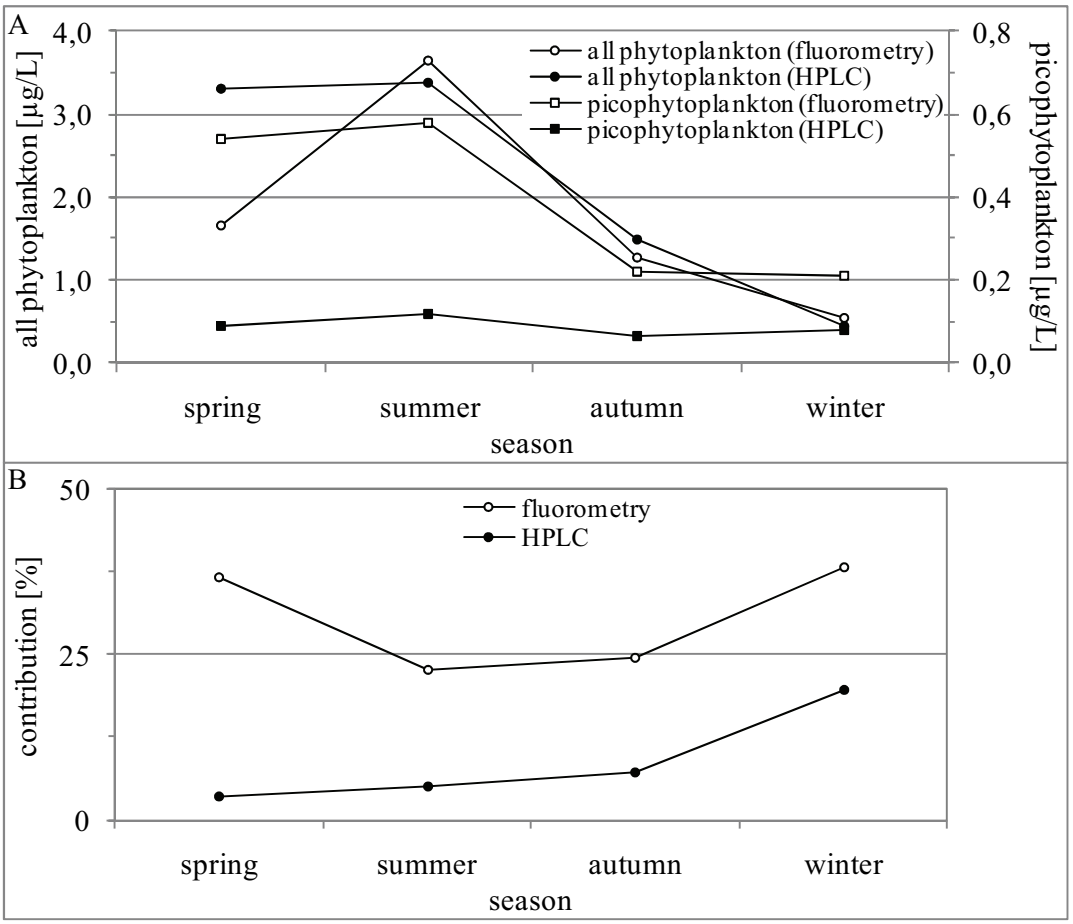


Figure 7: (A) Average seasonal chlorophyll *a* concentrations of the overall phytoplankton community and the picoplanktonic fraction. (B) Average seasonal chlorophyll *a* contribution of the picoplanktonic size class to the overall phytoplankton community. Data estimated by fluorometry (total chlorophyll *a in vivo*) and HPLC (pure chlorophyll *a in vitro*). Data for the overall phytoplankton as analysed for the time series at Helgoland Roads (Wiltshire and Manly, 2004; Franke et al., 2004; PANGAEA 2004).

4.2) Description of the picoeukaryotic community by different methods

The different methods used in the present study approach the picoplankton community in different ways. For example, flow cytometry (FCM) is capable of quantifying phototrophic picoeukaryotes and prokaryotes in natural samples (Medlin et al., 2002; Marie et al., 2005; Vaulot et al., 2008). As described in the general introduction of this thesis, its analysis is based on the size and refractive index of the cell, as well as its pigment content. FCM allows for the distinction of pico- and nanoeukaryotes, cyanobacteria and cryptophytes. Fluorometry and HPLC analyse the pigments within a water sample, but differ in the way of approaching the picophytoplankton community. Fluorometry analyses intact photosystems and estimates the total chlorophyll *a* concentration, including its breakdown products (see previous section). It calculates the concentration of different spectral groups, such as green-pigmented algae (chloro-, eugleno-, prasinophytes), cyanobacteria, brown-pigmented algae (diatoms and dinophytes) and cryptophytes, by measuring their distinct chlorophyll fluorescence intensity when excited by different wavelengths (Beutler, 1998 and 2003; Beutler et al., 2002). HPLC on the other hand, analyses the extracted pigments of a sample and allows for the calculation of their distinct concentrations. Specific marker pigments may be used to quantify the abundance of their respective taxonomic groups. However, because these cannot be definitely defined, which is particularly true for the eukaryotic picoplankton, marker pigments should be used with great care (Jeffrey et al., 1999; Mackey and Higgins, 2001; Ansotegui et al., 2003; Guillou et al., 2004; Latasa et al., 2004; Zapata et al., 2004; Lewitus et al., 2005; Fuller et al., 2006b; Vaulot et al., 2008). Electron microscopy is the only method allowing visualisation of single picoplankton organisms in morphological detail. This method is not quantitative though (Throndsen and Kristiansen, 1991; Vaulot et al., 2008) and it should only be used in combination with another method for an accurate taxonomic classification (Eikrem and Throndsen, 1998; Epstein and López-García, 2008; Simon et al., 2009; Worden et al., 2009). Fluorescence *in situ* hybridisation (FISH) targets specific taxonomic groups, be they auto- or heterotrophic. FISH uses taxon-specific and labelled oligonucleotide probes (Schönhuber et al., 1997; Not et al., 2002), which hybridise to the complementary gene sequences within target organisms. These can then be detected and cells counted under the epifluorescence microscope. Though hybridisation difficulties may occur (Medlin et al., 2002; Vaulot et al., 2003b and 2008; Amann and Fuchs, 2008; Gescher et al., 2008), FISH is the most powerful technique for quantitative phylogenetic studies in the natural environment. It even allows for the further identification of unknown groups, description of single cells, as well as their ecological characterisation (Medlin et al., 2002; Biegala et al., 2005; Zhu et al., 2005). Denaturing gradient electrophoresis (DGGE) gives an overview of the occurrence of distinct phylotypes. It is not quantitative and influenced by methodological drawbacks (Amann et al., 1995; Burton, 1996; Díez et al., 2001; Féral, 2002; Medlin et al., 2002 and 2006; Epstein and López-García, 2008; Vaulot et al., 2008; Viprey et al.,

2008; Not et al., 2009), but it allows sequencing of DNA fragments and the taxonomic identification of occurring organisms.

All these methods target different parts of the picoplanktonic community, and their results may therefore differ and/or partially overlap (Figure 8). Thus, their individual conclusions about the picoplankton community may be difficult to compare, but in conjunction, they seem to give the most reliable picture about its composition and abundance. The distinct results obtained during this doctorate will be described separately on a seasonal basis before an overall conclusion about the picoeukaryotic community of the German Bight will be drawn.

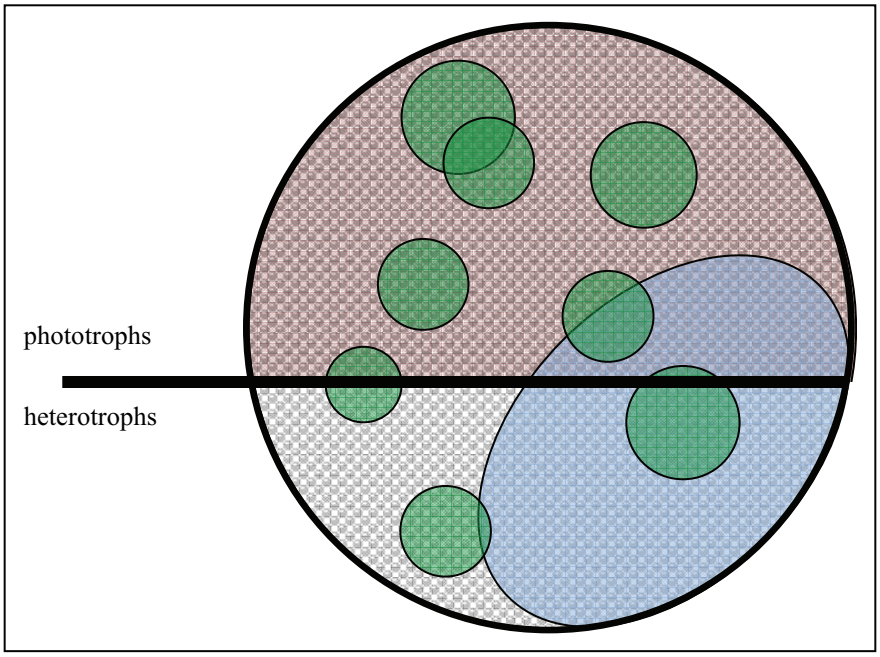


Figure 8: Schematic drawing of the detection limits of the primary methods used in picoplankton investigations. The big circle represents the community of picoeukaryotes; pink area: phototrophic organisms are detected by pigment based methods such as fluorometry and HPLC; blue area: DGGE primarily detects heterotrophs whereas most organisms, especially picoautotrophs, seem to be overlooked; green circles: FISH detects taxonomic groups complementary to the applied molecular probes.

The community by flow cytometry Counts by FCM revealed a clear seasonal trend in the picoplankton abundance with highest numbers in summer, equal ones in spring and autumn, and lowest numbers in winter. Cyanobacteria (*Synechococcus*) dominated the community in summer, autumn and winter, whereas picoeukaryotes dominated in spring (Figure 9). Their numbers were

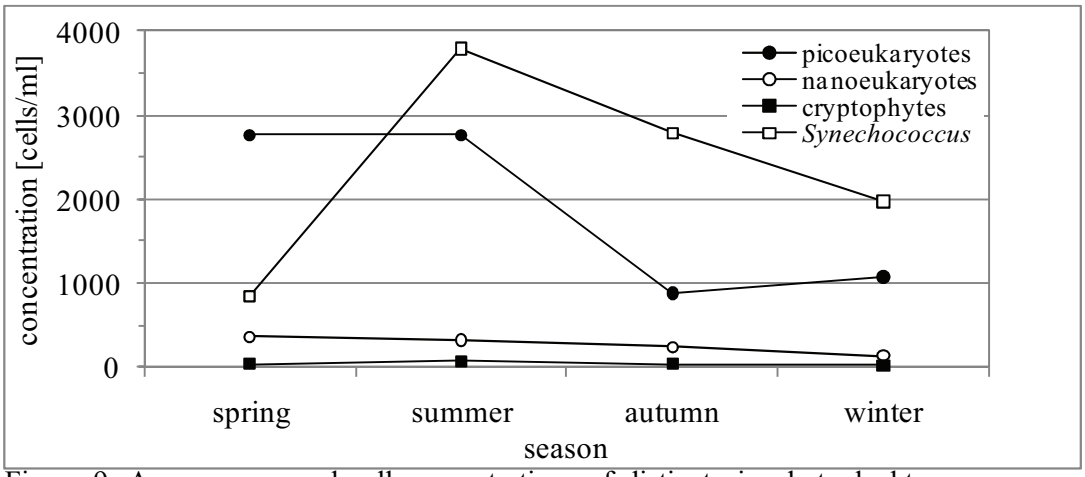


Figure 9: Average seasonal cell concentrations of distinct picophytoplankton groups as estimated by flow cytometry.

equally high in spring and summer, lowest in autumn and slightly higher in winter. Nanoeukaryotes occurred in the prefiltered samples, but in low cell numbers that steadily decreased between spring and winter. Cryptophytes achieved highest concentrations in summer. However, this result is based upon the analysis of only a fraction of their total concentration.

The community by fluorometry The most differing values in the picoplanktonic concentration of total *in vivo* chlorophyll *a* were measured in spring. The highest seasonal average was measured in summer and the lowest in winter (Figure 7A). The spectral groups of the picophytoplankton were clearly dominated by the spectral group of brown-pigmented algae (diatoms and dinoflagellates), making up 79 to 96% from spring to winter. These were abundant in similar concentrations in spring and summer, and autumn and winter, respectively (Figure 10). Green-pigmented algae on the other hand, were most abundant and variable in spring. Their

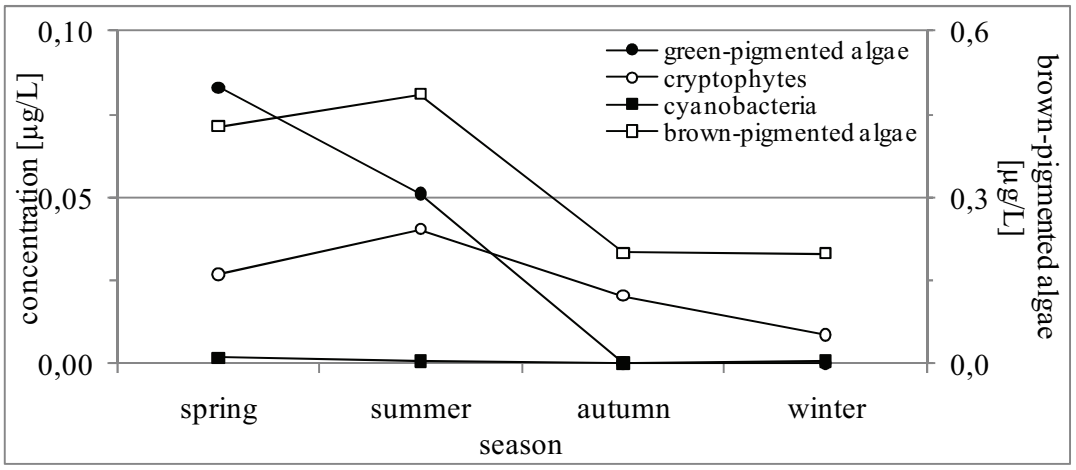


Figure 10: Average seasonal concentrations of distinct spectral groups of the picophytoplankton as estimated by fluorometry.

concentration steadily decreased towards winter. Cryptophytes showed highest values in summer, equal ones in spring and autumn, and lowest in winter. This seasonal succession was congruent to the findings by FCM counts. Cyanobacteria on the other hand, occurred only occasionally and in negligible numbers. This is contradictory to FCM counts that revealed the cyanobacteria to be numerically dominating. However, because picoeukaryotes are larger in their cell size (Bec et al., 2005) and therefore possibly contain higher amounts of pigments, the obtained results by the two methods - one counting cells and the other analysing pigment concentrations - cannot be compared definitely.

The community by HPLC Pigment analysis of the picophytoplankton community was improved before starting the actual investigation regarding the best-suited choice of filters (Knefelkamp et al., 2007) and the HPLC method itself (manuscript 1). Highest average chlorophyll *a* concentrations in the picophytoplankton were found in summer and lower but quite similar values in spring, autumn and winter (Figure 7A). Fucoxanthin (considered as a signature pigment for the diatoms and other stramenopiles) exceeded chlorophyll *a* concentrations most of the time and was highest in summer (Figure 11A). This supports analysis by fluorometry.

Fucoxanthin, peridinin (dinoflagellates), chlorophyll *b* (green algae), neoxanthin (green algae and euglenophytes), alloxanthin (cryptophytes) and βε-Carotene (cryptophytes and green algae) highest in summer. Diadinoxanthin (brown algae) and 19'-butanoyloxyfucoxanthin (pelago-, coccolitho- and chrysophytes) showed highest concentrations in spring, whereas 19'-hexanoyloxyfucoxanthin (haptophytes, some dinoflagellates) was highest in autumn. Prasinoxanthin (prasinophytes) occurred only in winter. Although pigments can only give an impression about the community composition of phytoplanktonic organisms, the results of this study indicate the picoautotrophs to be highly diverse. They indicate a parallel dominance of most groups in summer, whereas only a small amount seemed to dominate within the other seasons. However, because of difficulties in the application of pigments towards taxonomic groups (Jeffrey et al., 1999; Mackey and Higgins, 2001; Vaulot et al., 2003b and 2008; Latasa et al., 2004; Lewitus et al., 2005; Fuller et al., 2006b; Gescher et al., 2008) and because of their high influence by environmental parameters (Mackey et al., 1998; Jeffrey et al., 1999; Garrido et al., 2003; Lewitus et al., 2005), no well-founded conclusions can be made about the picoplanktonic community composition. Furthermore, the already mentioned influence of pigmented compounds, such as zooplanktonic faecal pellets, resting spores, larger cells, and their fragments and detritus, on the investigated picoplankton community cannot be defined quantitatively (Jeffrey et al., 1999; Jeffrey and Wright, 2004; Zapata et al., 2004; Not et al., 2005 and 2008; Wright and Jeffrey, 2005; Vaulot et al., 2008). Thus, it is not possible to definitely attribute all pigment concentrations to picoplanktonic organisms.

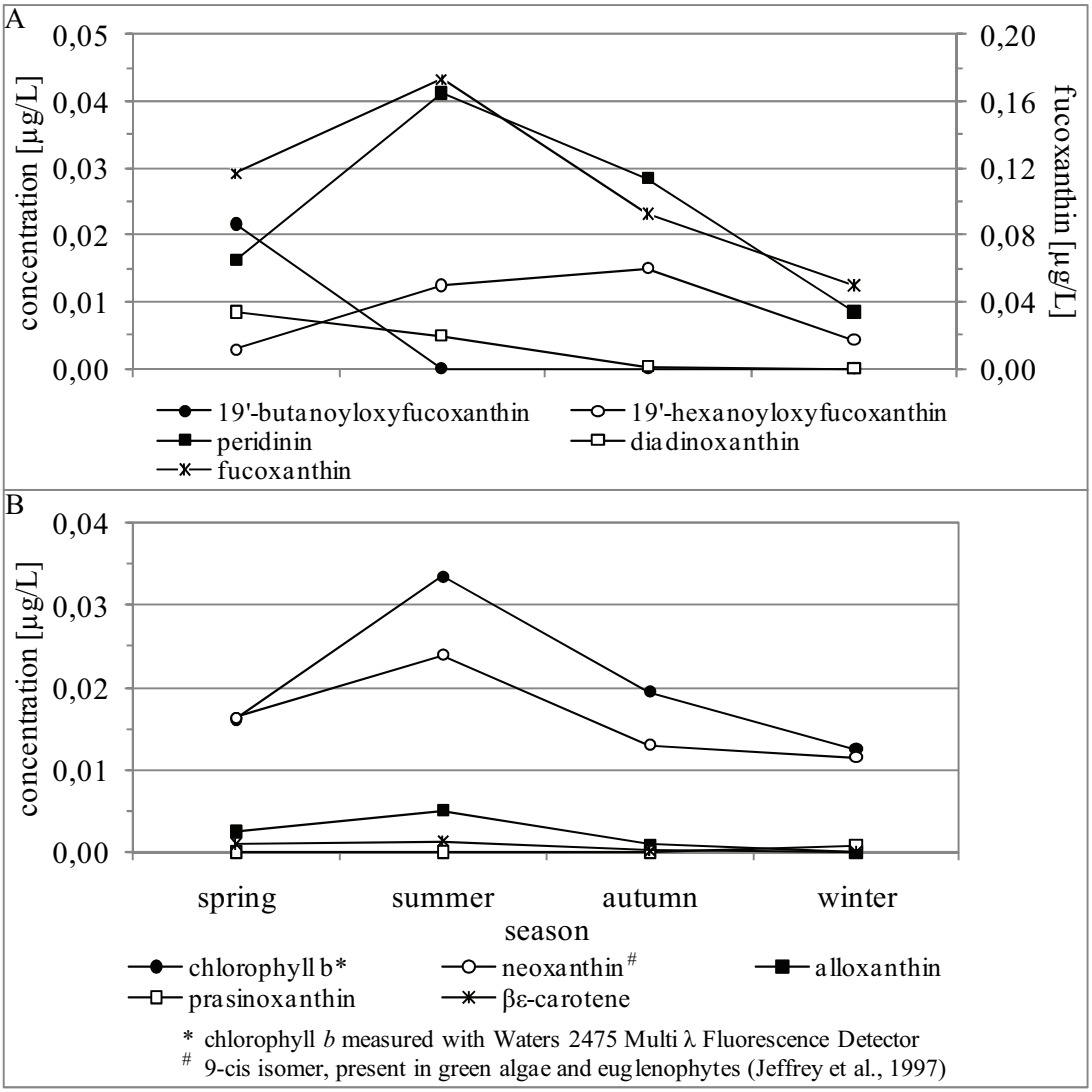


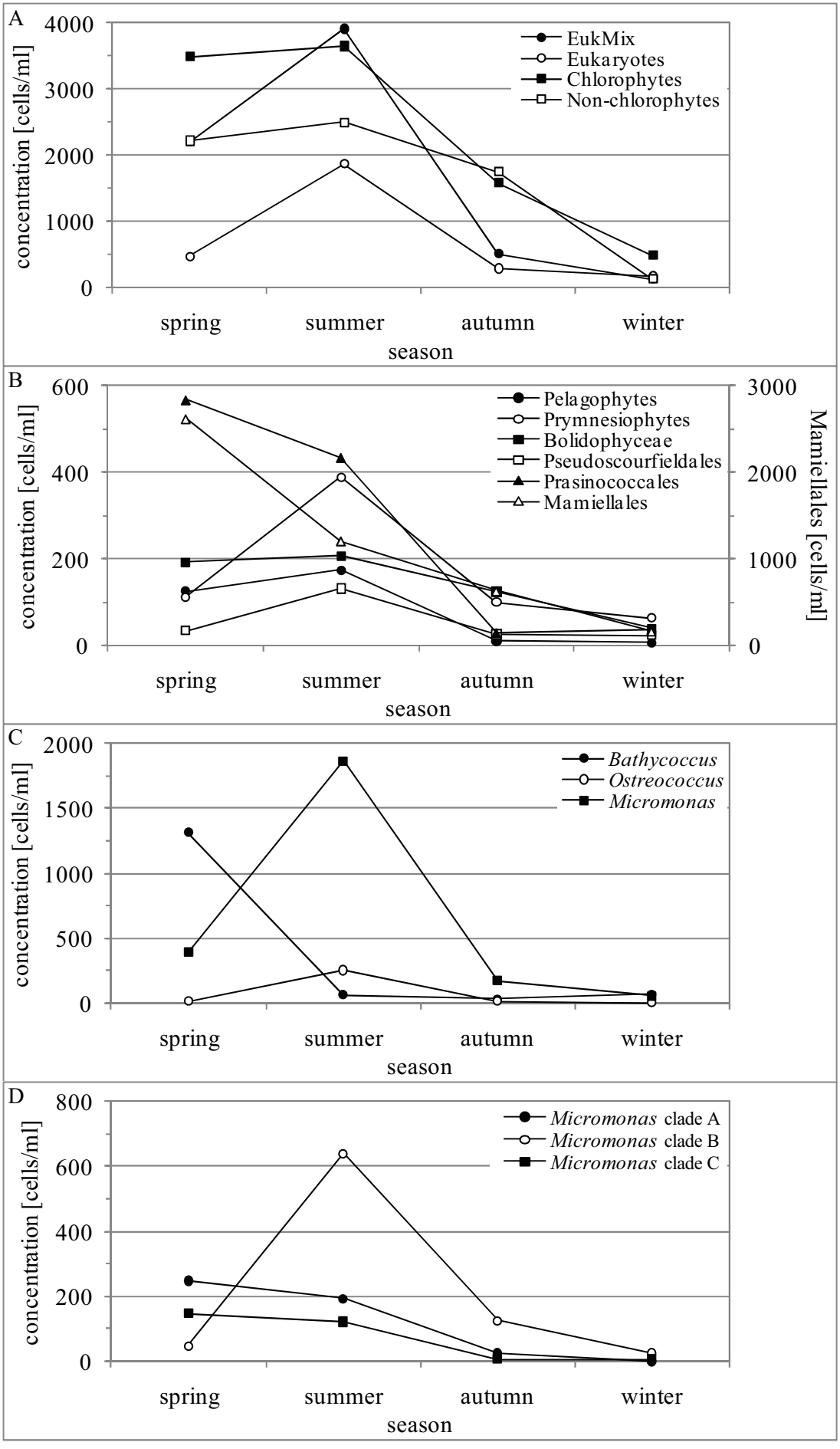
Figure 11: Average seasonal concentrations of distinct pigments of the picophytoplankton as estimated by HPLC. (A) Shows the concentration of those pigments rather belonging to brown-pigmented algae, and (B) those rather belonging to green-pigmented algae.

The community by FISH The majority of probes did result in highest cell counts in summer or spring (Figure 12), with the overall picoeukaryotic community being dominated by chlorophytes. Non-chlorophyte counts dominated over chlorophytes only in autumn, though their highest seasonal abundance occurred in summer. More than three quarters of the occurring non-chlorophytes were not detected by the more specific class probes. When comparing the abundance of prymnesiophytes, pelagophytes and bolidophytes, prymnesiophytes dominated in summer and winter, with highest cell numbers in summer. Bolidophytes dominated in spring and autumn, though they showed highest abundances in summer. Pelagophytes were of lesser abundance and never dominated the counts of the three non-chlorophyte classes. Their numbers were highest in summer. FISH revealed prasinophytes making up nearly all of the chlorophytes in spring, whereas

unlabelled chlorophytes dominated in summer, autumn and winter. Within the Prasinophyceae, the Mamiellales were dominating throughout the year with highest numbers in spring. Prasinococcales and Pseudoscourfieldales accounted for lesser amounts and showed highest abundances in spring and summer, respectively. The Mamiellales were dominated by *Bathycoccus prasinos* in spring and winter, whereas *Micromonas pusilla* dominated in summer and autumn. *Ostreococcus* made up only a smaller part and showed its highest abundance in summer. Among *Micromonas pusilla*, clade A dominated in spring and clade B in summer, autumn and winter. Clade C never dominated. It accounted for its largest cell abundance in spring.

These findings indicated a taxonomically highly diverse picoplankton community whose composition changed among seasons. Its abundance was highest in spring and summer and decreased in autumn and winter. These results are comparable to chlorophyll *a* findings by fluorometry and HPLC, as well as to counts by FCM. Chlorophytes and especially the Mamiellales seemed to dominate in the German Bight, whereas most of the non-chlorophytes remained hidden. The inferiority of non-chlorophytes towards chlorophytes revealed that green-pigmented algae, not the brown-pigmented algae, dominated the picoplankton. This was not congruent to findings by HPLC and fluorometry.

Figure 12: Average seasonal cell concentrations of (A) general taxonomic groups, (B) classes and orders, (C) genera, and (D) clades within the picophytoplankton, as estimated by FISH. EukMix represents a mixture of different probes to target all eukaryotes (see manuscript 2).


A
concentration [cells/ml]
4000
3000
2000
1000
0
EukMix
Eukaryotes
Chlorophytes
Non-chlorophytes
spring
summer
autumn
winter
season
B
concentration [cells/ml]
600
400
200
0
3000
2000
1000
0
Mamiellales [cells/ml]
Pelagophytes
Prymnesiophytes
Bolidophyceae
Pseudoscourfieldales
Prasinococcales
Mamiellales
spring
summer
autumn
winter
season
C
concentration [cells/ml]
2000
1500
1000
500
0
Bathycoccus
Ostreococcus
Micromonas
spring
summer
autumn
winter
season
D
concentration [cells/ml]
800
600
400
200
0
Micromonas clade A
Micromonas clade B
Micromonas clade C
spring
summer
autumn
winter
season

The community by DGGE The fingerprinting method DGGE revealed a high taxonomical abundance and diversity within the picoeukaryotic fraction. Most of the phylotypes were abundant throughout the year, but with some phylotypes being of sporadic occurrence, whereas others exhibited a rather continuous presence. The lack of distinct seasonal maxima in their occurrence indicated an independency towards environmental parameters. Overall, the taxonomic diversity of the picoeukaryotes was highest in autumn, lowest in spring, and highly variable in summer, with changes in the seasonal composition of the community. Furthermore, high changes in the weekly phylotype composition were observed, especially in summer. Although the seasonal composition of DGGE profiles differed, an annual reassembling, as previously suggested (Medlin et al., 2006), was supported. The high amount of identified phylotypes that were not sequenced previously highlights the still-incomplete discovery of existing species and clades among the picoeukaryotes (Massana and Pedrós-Alió, 2008). Obtained phylotypes were related to species and uncultured clones previously found in diverse regions of our world's oceans and at various environmental conditions, such as hydrothermal vents or at 3000 m depth of the Antarctic polar front. Identified sequences were dominated by heterotrophs (Figure 13), primarily alveolates and stramenopiles. Their frequency of occurrence seemed to depend on the abundance of larger microalgae, such as host cells of parasitic group II alveolates. The occurrence of phototrophic phylotypes was dominated by picobiliphytes and *Micromonas pusilla*, and remained steady throughout distinct seasons. In addition, *Micromonas pusilla* presented the most abundant band class throughout all seasons with a continuous occurrence over 55 weeks.

Because phylotype occurrences give no information about organism quantities, the described findings by DGGE cannot be compared towards FCM, fluorometry, HPLC or FISH. However, used in conjunction, DGGE supported the occurrence of larger-sized organisms in the picoplankton fraction and the steady occurrence of green-pigmented picoautotrophs. Thus, it

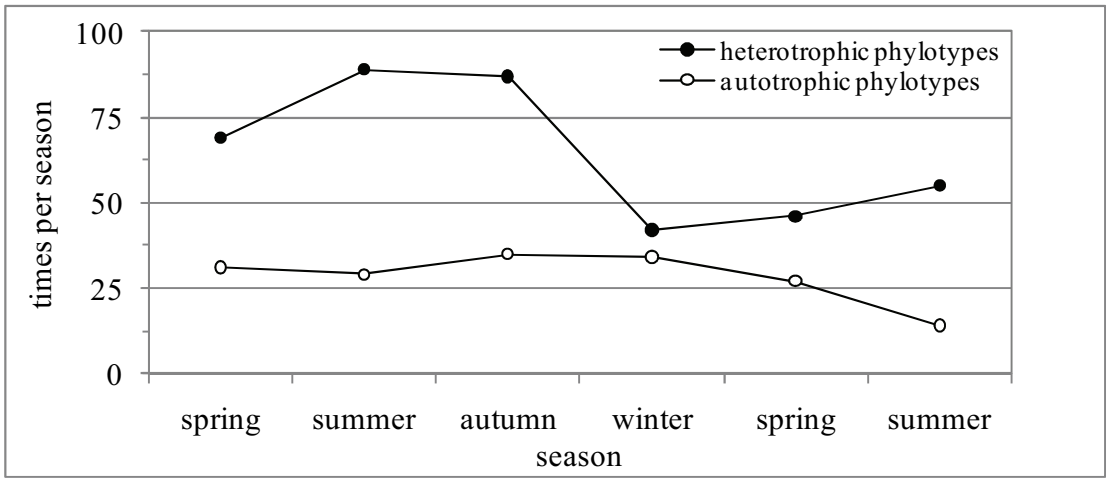


Figure 13: Times of seasonal occurrences of identified autotrophic and heterotrophic phylotypes as estimated by DGGE and sequencing.

supports the assumption of a low dependency of distinct phylotypes on environmental parameters.

The community by electron microscopy Those organisms found under the electron microscope were diverse bacteria (including *Synechococcus*) and flagellates, which included cf. *Micromonas*, cf. *Bathy*- and/or *Ostreococcus*, cf. *Bolidomonas*, cf. *Chrysochromulina*, cf. *Florenciella parvula*, and cf. *Pachysphaera* or cf. *Pterospaera*. Furthermore, scales were detected and seemed to belong to cf. *Mantoniella*, cf. *Pyramimonas disomata*, cf. *Mamiella gilva* or *Pyromonas*, cf. *Cymbomonas tetramitiformis* or cf. *Pyramimonas disomata*, cf. *Pyramimonas grossii* and cf. *Chrysochromulina*. Although these results were not quantitative, most organisms were found in samples taken in summer, which may indicate higher cell abundance. Overall, electron microscopy gave an impression about the morphological appearance of the picoplankton community and revealed the occurrence of larger-sized microalgae within the prefiltered samples, thus supporting FCM counts and DGGE findings.

Overall, the entire picoplankton community of the German Bight was most abundant in spring and summer; it was formed by a high diversity of different taxonomies. The phototrophic fraction formed a steady background population among the primary producers, with highest contribution in winter. It never dominated the community of primary producers, although this is expected for coastal temperate regions, especially in summer (Vaulot, 2001b; Not et al., 2002 and 2005; Massana et al., 2004a and b; Piganeau et al., 2008; Viprey et al., 2008). The results of this study were not able to confirm a higher numerical abundance of phototrophic picoeukaryotes and a parallel higher taxonomic diversity among the heterotrophs. This difference is generally suggested for the community of picoeukaryotes (Vaulot et al., 2002; Massana et al., 2004a and b; Worden and Not, 2008). However, sequencing of eukaryotic rRNA gene fragments primarily identified heterotrophs, giving the impression of a low abundance of phototrophs, but this relation of nutritional groups may be caused by PCR biases (Burton, 1996; Medlin et al., 2002 and 2006; Vaulot et al., 2008; Viprey et al., 2008; Not et al., 2009). Overall, previous findings (Medlin et al., 2006) on the picoeukaryotes of the German Bight were confirmed and complemented, and the understanding of these previous results improved. This includes, for example, the low contribution of picoplankton to primary production, the fast taxonomic and numerical changes within the community and the annual reassembling of the community. Furthermore, the general dominance of chlorophytes was supported (Not et al., 2004; Vaulot et al., 2008; Viprey et al., 2008; Worden and Not, 2008; Simon et al., 2009; Worden et al., 2009). Although pigment studies (fluorometry and HPLC) indicated the dominance of brown-pigmented algae in the picoplanktonic size fraction, fucoxanthin-containing picoplankters, such as bolidophytes and/or prymnesiophytes, or non-chlorophytes in general, were not detected in corresponding cell

numbers by FISH. Furthermore, the stramenopiles sequenced primarily belonged to lineages of unpigmented flagellates and not to phototrophic ones (such as diatoms or bolidophytes). It is therefore suggested that the dominance of brown pigments in the pico-sized fraction originated from zooplanktonic faecal pellets, cell fragments, detritus, resting spores and/or larger but slim cells that slipped through the filters of prefiltration (Jeffrey et al., 1999; Jeffrey and Wright, 2004; Zapata et al., 2004; Not et al., 2005 and 2008; Wright and Jeffrey, 2005; Vaulot et al., 2008). This is supported by the occurrence of larger cells (cf. *Rhizosolenia*) and detritus in the picoplanktonic size fraction, as was observed under the electron microscope. Furthermore, FCM revealed the abundance of nanoeukaryotes and most identified phylotypes belonged to larger organisms, such as ciliophores. Statistical analyses (data not shown) revealed Dinoflagellata (thecate and nonthecate) and Flagellata to be significantly correlating to pigment analyses of the picoplankton, whereas Coccolithophores and Noctiluca were significant for FCM counts, and nonthecate Dinoflagellata for group counts by FISH probes. However, a deeper analysis of the relation between larger microalgae and picoplankton was not possible due to the restricted framework of this study.

4.3) Environmental factors influencing the picoeukaryotic community

To understand how abiotic factors can influence the results of distinct methods, individual findings were regressed against a set of environmental parameters. These parameters included the water temperature, secchi depth, salinity and nutrient concentrations (nitrite, nitrate, ammonium silicate and phosphate), regularly analysed in the framework of the long-term series at Helgoland Roads (Wiltshire and Manly, 2004; Franke et al., 2004; PANGAEA 2004). Further data, kindly provided by the GKSS Institute for Coastal Research (Geesthacht, Germany), were the global and photosynthetic radiation, as well as the wind direction and speed.

Spectral group variances (as estimated by fluorometry) were explainable for 76% when environmental parameters were used, and the distinct groups were primarily influenced by the weekly change in salinity (Figure 14A). A further significant factor was the concentration of silicate. When pigment concentrations (as estimated by HPLC) of the picoplankton community were described by environmental parameters, the explained variance of inter-species distances was 78%. The photosynthetic active radiation (PAR), being in co-linearity with the water temperature, showed the highest significant effects. Further significant factors were the wind direction, secchi depth, the change in concentration of ammonium and global radiation towards the previous sampling (Figure 14B). The only significant factors with negative correlations were the concentration in silicate and the change in salinity. Regarding group counts by FCM, only the

change in water temperature towards the previous sampling was significant (Figure 14C), and the overall explainable variance of inter-species distances was 51.1%. The weekly change in water temperature was also the only significant factor on chlorophyte and non-chlorophyte counts by FISH (Figure 15A). Overall, environmental parameters explained a variance of 76.3% of the inter-species distances. Class and order counts were mostly influenced by the concentration of phosphate, but also by the weekly change in water temperature (Figure 15B). The overall explainable variance of inter-species distances was 75%. Genera counts were most significantly influenced by the weekly change in water temperature, but also by the change in salinity and nitrate concentrations at sampling (Figure 15C). The overall explainable variance of inter-species distances was 90.5%. Counts of *Micromonas* clades were significantly influenced by the global radiation (Figure 15C), with an overall explainable variance of inter-species distances of 52.3%.

These findings represented the change in water temperature and salinity to be the most significant environmental parameters with high effects on the picoplankton community. Nonetheless, there was no single environmental parameter significantly influencing all findings as obtained by the different methods. Thus, it can be concluded that this fact represents a rather unspecific influence of abiotic factors on the picoplankton. This supports the organisms' characteristic minimal way of living and their high capability to adapt to diverse environmental settings (Fogg, 1991; Guillou et al., 2001; Vaulot, 2001a; Mackey et al., 2002; Vaulot et al., 2002 and 2008; Bryant, 2003; Massana et al., 2004a and b; Worden and Not, 2008). However, possible small-scale influences (Simon et al., 2009) could not be defined in detail, though differences between genera (Figure 15C) and clades (Figure 15D) can be suggested. Conclusions on whether the observed correlations and influences on the analyses of the picoplankton were direct or indirect could not be estimated. However, significant negative correlations of the silicate concentration and the change in salinity towards brown-pigmented algae (Figure 14A), fucoxanthin and peridinin concentrations (Figure 14B) was detected. Because of the higher dependency of larger phytoplankton on sufficient nutrient amounts, coming into the German Bight by freshwater inflow, there is a possible relation between salinity values and silicate concentrations (Fogg, 1991; Hickel, 1998; Jiang et al., 2005; Raven et al., 2005; Medlin et al., 2006; Masquelier and Vaulot, 2008; Worden and Not, 2008; Zubkov and Tarran, 2008). Because phytoplanktonic cell counts, as obtained by the long-term series (Wiltshire and Manly, 2004; Franke et al., 2004; PANGAEA 2004), only showed significant correlations towards salinity values, the wind direction and the photosynthetic active radiation (data not shown), an indirect influence on spectral group abundance and pigment concentrations within the picoplankton can be expected. Nonetheless, further investigations are needed to answer the degree of larger microalgae' influences on the picoplanktonic size fraction.

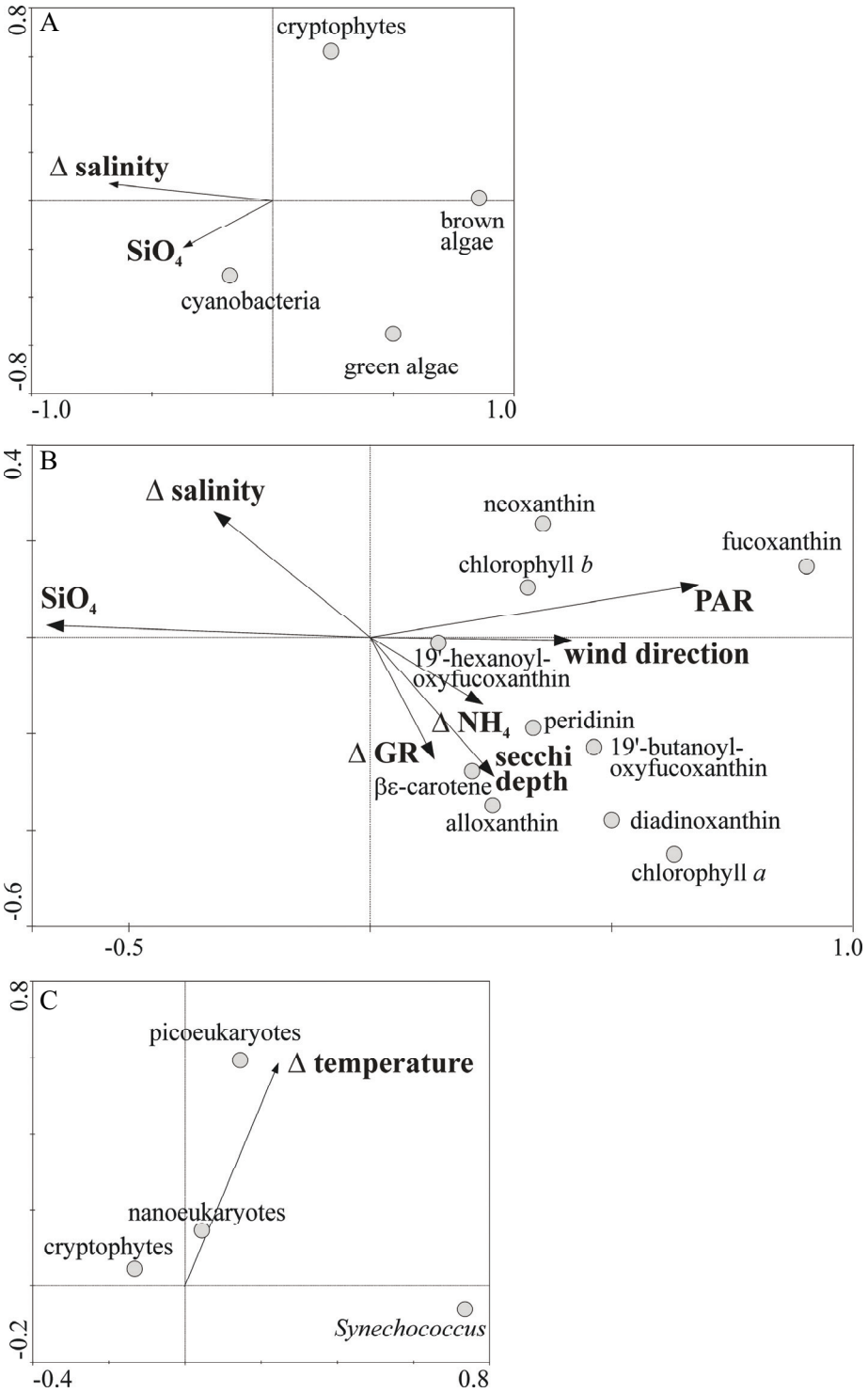


Figure 14: RDA biplots showing inter-species distances of (A) spectral groups by fluorometry, (B) pigments by HPLC and (C) counts by FCM using environmental parameters. Highest variance inflation factors (VIF): (A) insignificant value of PAR, co-linearity with water temperature; (B) significant ($p < 0.05$) value of PAR, co-linearity with water temperature; (C) insignificant value of PAR. Only significant ($p < 0.05$) explanatory variables are included in the biplots. Arrows indicate the direction of their increasing values and the degree of their correlation with response variables. Abbreviations: GR = global radiation; PAR = photosynthetic active radiation; Δ = delta (weekly change).

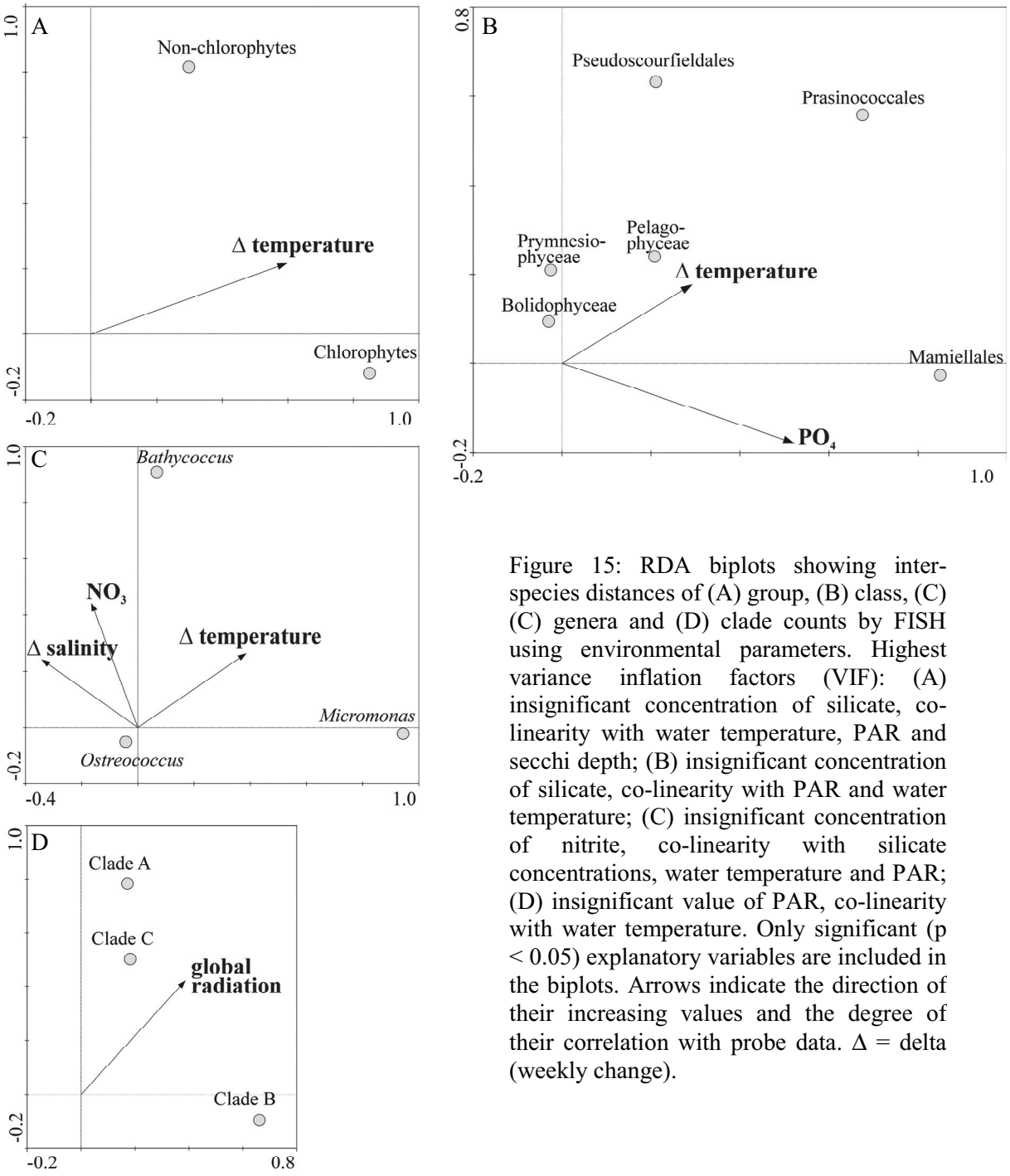


Figure 15: RDA biplots showing inter-species distances of (A) group, (B) class, (C) (C) genera and (D) clade counts by FISH using environmental parameters. Highest variance inflation factors (VIF): (A) insignificant concentration of silicate, co-linearity with water temperature, PAR and secchi depth; (B) insignificant concentration of silicate, co-linearity with PAR and water temperature; (C) insignificant concentration of nitrite, co-linearity with silicate concentrations, water temperature and PAR; (D) insignificant value of PAR, co-linearity with water temperature. Only significant ($p < 0.05$) explanatory variables are included in the biplots. Arrows indicate the direction of their increasing values and the degree of their correlation with probe data. Δ = delta (weekly change).

4.4) Statistical correlations between quantitative methods and an evaluation of their suitability to analyse picoeukaryotes

Different methods can be used to approach the community of eukaryotic picoplankton, as shown in the present study. The decision as to which method to apply on the picoplankton samples depends on the research aim, the differing methodological perspectives on the community (Figure 8, page 31), and the individual advantages and flaws of the methods.

The present doctorate applied diverse currently existing analysis methods on the same samples of picoplankton, evaluated their possible biases, and analysed their different results statistically. Redundancy analyses (RDA), for example, revealed similar explainable variances (47-48%) between FCM counts towards pigments (HPLC) and between pigments (HPLC) towards spectral groups (fluorometry). Because these three methods all use pigments for analysis of organisms, these low redundancies were not expected. In this thesis, FISH was considered the best method to combine quality and quantity analysis of the picoeukaryotic community in the German North Sea. When comparing the other methods' results to findings by FISH, HPLC explained most of the variances occurring among hierarchical probe counts (groups: 66.6%; classes and orders: 72.9%; genera: 79.7%; clades: 44.4%). The explained variances by FCM were lower for most levels (groups: 63.5%; classes and orders: 77.4%; genera: 62.9%; clades: 40.7%) and fluorometry was least able to explain total variances among FISH counts (groups: 47.6%; classes and orders: 51.8%; genera: 46.6%; clades: 13.9%). Regarding the significance of single explanatory variables on FISH, chlorophyll *b* and neoxanthin (HPLC), the cell numbers of picoeukaryotes and *Synechococcus* (FCM), as well as the green spectral group (fluorometry) were most often significant. These correlations support the dominance of chlorophytes, not brown-pigmented organisms, within the picoplankton community. This confirms the previously stated hypothesis of a high abundance of larger, brown-pigmented, algae, their cell fragments, resting spores, and/or faecal pellets of their grazers within the pico-sized fraction (Jeffrey et al., 1999; Jeffrey and Wright, 2004; Zapata et al., 2004; Not et al., 2005 and 2008; Wright and Jeffrey, 2005; Vaulot et al., 2008).

Taking the qualitative methods, electron microscopy and DGGE, into account as well, the molecular methods (FISH and DGGE) are suggested to be the most informative and beneficial for picoplankton studies. In the present doctorate, they were able to allow for answers regarding the individual ecological occurrence and succession. DGGE, for example, allowed for the identification of abundant phylotypes and gave an impression about their succession in the waters of the German Bight. FISH allowed for a specific and quantitative determination of single taxonomic groups and revealed the abundance of non-targeted organisms. Furthermore, both analysis methods allow a taxonomic and quantitative (in the case of FISH) comparison to findings

at other sites, as well as a direct confirmation and/or complementation of previous findings. FCM, HPLC and fluorometry on the other hand, provided only a broad impression, no reliable details, about the composition of the picoplankton community. Furthermore, HPLC and fluorometry results were suggested to be biased by different abiotic and biotic parameters.

5.) Ecological embedding

Picoplankton abundance Coastal waters are characterised by high amounts of organic and inorganic compounds, fast-changing environmental conditions and a general dominance of larger phytoplankton (Fogg, 1991; Raven, 1998; Jacquet et al., 2002a; Qian et al., 2003; Wynn and Knefelkamp, 2004; Raven et al., 2005). In the eutrophic water of the German Bight, the chlorophyll *a* concentration of the picophytoplankton was of minor abundance, thus supporting these general suggestions of dominating larger microalgae. Furthermore, the picophytoplankton appeared to be more balanced in its abundance than the overall phytoplankton (Figure 7A). Although the highest picophytoplanktonic chlorophyll *a* concentrations were measured in summer, it never dominated the primary production community. These findings were congruent with previous measurements at Helgoland (Medlin et al., 2006) but contradictory to general suggestions of biomass dominance in summer (Vaulot, 2001b; Not et al., 2002 and 2005; Massana et al., 2004a and b; Piganeau et al., 2008; Viprey et al., 2008). Nonetheless, these results supported the picophototrophs to fulfil a basic ecological role as a steady background population (Vaulot et al., 2000 and 2003b; Jacquet et al., 2002a and b; Biegala et al., 2003; Bryant, 2003; Massana et al., 2004a; Zhu et al., 2005; Medlin et al., 2006; Masquelier and Vaulot, 2008; Not et al., 2008). A timely succession between larger-sized microalgae and picophytoplankton, with the latter being able to grow as soon as the former starts to decline (Fogg, 1991; Kjørboe et al., 1993; Vaulot et al., 2000; Donald et al., 2001; Jacquet et al., 2002a and b; Bryant, 2003; Massana et al., 2004a; Zhu et al., 2005; Medlin et al., 2006), was not observed in the present study. Therefore, further investigations, including the application of quantitative methods such as FCM, are recommended to complement and enhance the current understanding of these interactions within the marine food web. They may even allow an estimation of differences among size classes in carbon transport towards higher trophic levels (Steele, 1974; Legendre and Le Fèvre, 1995; Shurin et al., 2006; Worden and Not, 2008).

Overall, the picoeukaryotes were most abundant in spring and summer and decreased towards autumn and winter. Their community was able to change significantly within one week in respect to its abundance and taxonomic diversity (data presented in manuscripts), supporting previous findings in the German Bight (Medlin et al., 2006). Furthermore, this is congruent with the general view of a fast change in picoplankton abundance because of a high growth rate and adaptation capability towards changing environmental conditions (Jacquet et al., 2002a and b; Bryant, 2003; Agawin et al., 2004; Massana et al., 2004a; Jiang et al., 2005; Zhu et al., 2005; Medlin et al., 2006; Zubkov and Tarran, 2008). Regarding abiotic influences, the picoplankton generally seems to be minimally opportunistic (Fogg, 1991; Bryant, 2003). In the present study, this view was supported by the overall influence of different significant environmental parameters and the lack of one prevailing parameter influencing all methods' results (Figures 14 and 15), though the change in water temperature seemed to be important for the community of picoeukaryotes. However, the distinct taxonomic

groups of the community increased and decreased in their abundance at the same time. This, for example, is supported by highest cell counts of diverse FISH probes in summer and overall lowest counts in winter. Some indications of differing responses among distinct taxonomic groups were found nonetheless; for example, between the three investigated genera of the Mamiellales (Figure 15C). These findings support the suggestion of influential small-scale changes in environmental settings (Simon et al., 2009). Overall, whether the observed correlations between the abundance of picoeukaryotes and environmental factors were direct or indirect cannot be concluded. This allows for the assumption that other parts of the food web, such as larger microalgae, grazers and/or viruses, generally suggested to influence the picoplankton abundance (Guillou et al., 2001; Vaulot, 2001a; Mackey et al., 2002; Evans et al., 2003), may control the community as well. Their influence possibly biases conclusions about the pico-sized organisms' reactions towards abiotic parameters.

Picoplankton composition Overall, the present study's findings confirmed and enhanced previous suggestions about the picoplankton community in temperate coastal waters. First of all, Cyanobacteria (*Synechococcus*) were numerically dominating most of the year (Figure 9, page 32), while the picoeukaryotes showed a high and varying diversity with lower cell numbers (Vaulot et al., 2000 and 2003b; Jacquet et al., 2002a and b; Biegala et al., 2003; Bryant, 2003; Massana et al., 2004a; Zhu et al., 2005; Medlin et al., 2006; Masquelier and Vaulot, 2008; Not et al., 2008). Especially in comparison to *Prochlorococcus*, this confirms the possible high abundance of *Synechococcus* in temperate regions (Partensky et al., 1999a; Vaulot, 2001a; Scanlan and West, 2002; Worden et al., 2004; Not et al., 2005; Garczarek et al., 2007; Scanlan, 2007). The detected diversity of picoeukaryotes confirms the development of a high variety of taxonomically different species, possibly resulting from their long evolutionary existence, physiological benefits, and minimal niche requirements (Woese and Fox, 1977; Fogg, 1991; Raven, 1998; Bryant, 2003; Raven et al., 2005; Vaulot et al., 2008; Worden and Not, 2008). The organisms are suggested to have spread around the world having adapted to diverse environmental settings. Nonetheless, the underlying genetic circumstances are not entirely known (Massana et al., 2004b; Šlapeta et al., 2006; Forster et al., 2008; Masquelier and Vaulot, 2008; Zubkov and Tarran, 2008; Simon et al., 2009).

Among the picoeukaryotes, previous clone libraries as well as DGGE attempts in the waters around Helgoland revealed the heterotrophs to have a particularly high abundance (Vaulot et al., 2003b; Medlin et al., 2006). In the present study, no method directly targeted the proportion between photo- and heterotrophic organisms. Thus, their quantitative and qualitative differences could not be definitively estimated. The only method that allowed for an identification of single phylotypes and their attribution to one nutritional group was DGGE (data presented in manuscript 3). Nonetheless, DGGE is highly biased in the sense of under representation of phototrophs (Burton,

1996; Medlin et al., 2002 and 2006; Vaulot et al., 2008; Viprey et al., 2008; Not et al., 2009). Thus, although a higher diversity of picoheterotrophs may be possible, especially because they are suggested to adapt to the various nutritional resources in coastal waters, they are generally recorded to form a contribution of around 25% (Andersen et al., 1996; Vaulot et al., 2002; Massana et al., 2004a and b; Not et al., 2005; Worden and Not, 2008).

Overall, the present doctorate was able to confirm the abundance of highly variable as well as very stable fractions within the picoeukaryotic community (Vaulot et al., 2003b) and their annual reassembling (Medlin et al., 2006) (data presented in manuscript 3). The different analysis methods applied in this thesis did not always give a congruent view about the composition of the picoeukaryotes within the waters of the German Bight (see section "Description of the picoeukaryotic community by different methods", page 30). Nonetheless, FISH counts confirmed the suggested vast dominance of Prasinophyceae in the phototrophic fraction (Medlin et al., 2006; Vaulot et al., 2008; Viprey et al., 2008; Worden and Not, 2008) and their principal composition of Mamiellales (Guillou et al., 2004; Vaulot et al., 2008). Their previously described highest abundance in spring (Medlin et al., 2006) was supported, and their recorded morphological diversity (Skarstad Egeland et al., 1997; Fawley et al., 2000; Backe-Hansen and Throndsen, 2002; Guillou et al., 2004; Jeffrey and Wright, 2004; Not et al., 2004; Derelle et al., 2006; Fuller et al., 2006a; Worden and Not, 2008; Simon et al., 2009) confirmed by electron microscopy; especially in respect to flagella numbers. Although the dominance of Pycnococcaceae, *Mamiella* and *Mantoniella* among the Mamiellales (Medlin et al., 2006) could not be checked, previous disagreeing findings in the abundance of the three genera *Bathycoccus*, *Micromonas* and *Ostreococcus* (Medlin et al., 2006; Gescher et al., 2008) were clarified for the waters of the German Bight. The present study investigated the abundance and composition of these three genera in detail and revealed *Micromonas* to be the most abundant genus on annual average. This is congruent with findings at other sites (DuRand et al., 2002; Ansotegui et al., 2003; Not et al., 2004, 2005 and 2008; Forster et al., 2008, Vaulot et al., 2008).

6.) Comparison to other sites and previous findings

Longer-term studies on picoplankton abundance and annual succession were previously only carried out during the PICODIV project at Helgoland, Roscoff (English Channel) and Blanes (Mediterranean Sea). Although a direct comparison with the present study's data was sometimes difficult because of the different methods used, FISH counts were obtained in a comparable form at all three sites and allowed an accurate quantitative comparison of the occurring picoeukaryotic community. That is why focus will be laid on the comparison of FISH findings at the three coastal stations.

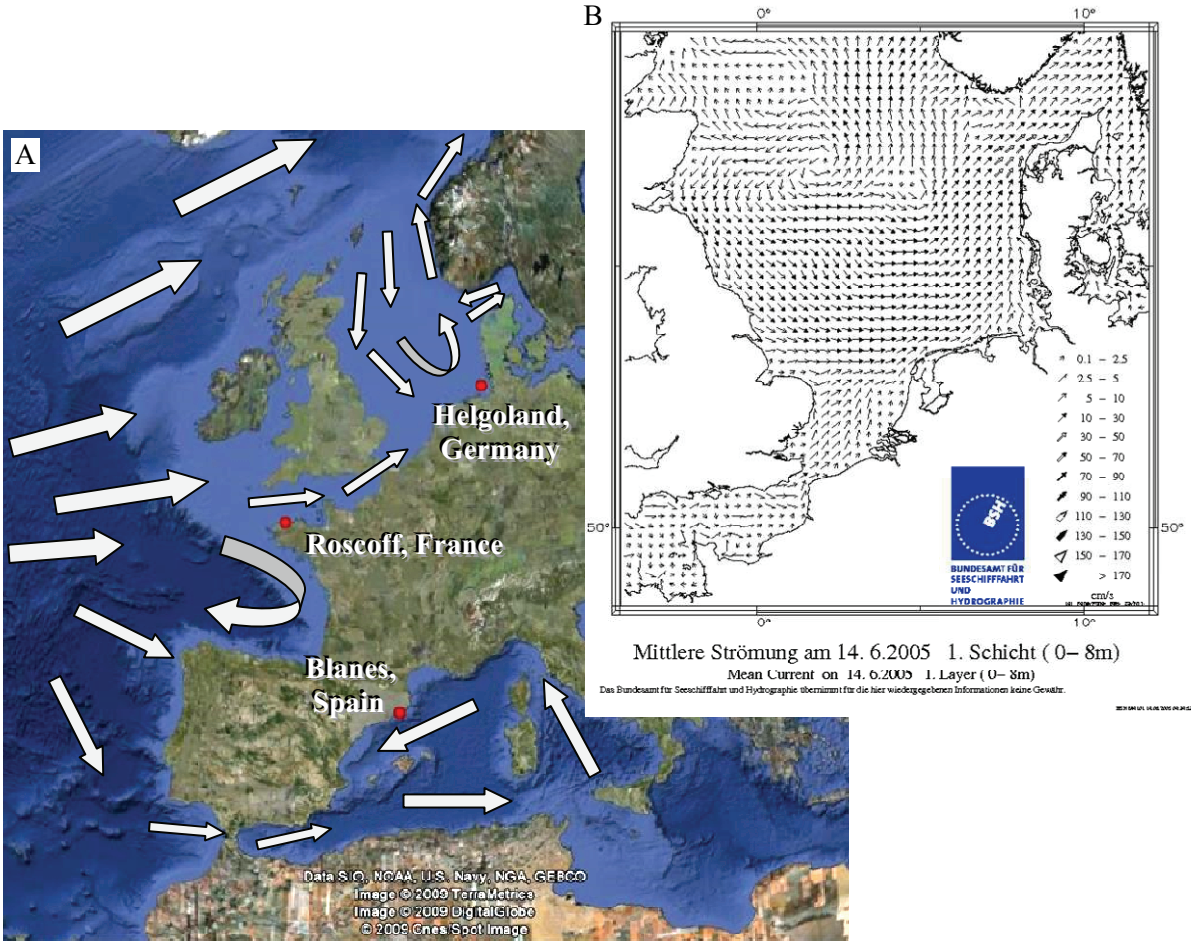


Figure 16: Map (A) shows the location of the three study sites Helgoland, Roscoff and Blanes. The main surface currents under flood conditions represent the influence of water from the open Atlantic on the North Sea, the English Channel and the Mediterranean Sea (after Google Earth and BSH). Map (B) shows the mean surface currents on June 14th 2005 (BSH), when *Micromonas* was highly abundant in the present study's samples.

Picoplankton abundance Whereas the water bodies at Helgoland and Roscoff are similar, the Mediterranean Sea is widely unaffected by mixing processes with fresh water from the open Atlantic (Figure 16) and is exposed to a higher evaporation. That is why its salinity is higher, nutrient concentrations lower, and the resulting picoplankton community differs from that at the northern stations (Vaulot et al., 2003b and 2004; Massana et al., 2004a and b; Romari and Vaulot, 2004; Zhu et al., 2005; Medlin et al., 2006). Within the sampling period of this doctorate, salinity values at Helgoland were below the values previously measured at Roscoff (station Astan, 48°46'N, 3°56'W) and Blanes (Blanes Bay, 41° 40' N, 2°48' E) (Vaulot et al., 2003b). Furthermore, the minimum water temperature was lower at Helgoland, with its maximum values being only slightly higher than those at Roscoff (Vaulot et al., 2003b; Massana et al., 2004a; Not et al., 2004). The nutrient concentrations were higher than in the English Channel and can only be suggested to exceed those in the Mediterranean by far (Vaulot et al., 2003b, Massana et al., 2004a; Not et al., 2004). These environmental conditions explain that the highest overall chlorophyll *a* concentration was found in the German Bight during the PICODIV project (Vaulot et al., 2003b) because the basic conditions favour the growth of larger phytoplankton (Fogg, 1991; Raven, 1998; Jacquet et al., 2002a; Raven et al., 2005). Thus, the average picoplanktonic chlorophyll *a* contribution was 37% at Roscoff and 52.7% at Blanes, whereas at Helgoland it was only 13.6% (Vaulot et al., 2003b), or 9% as calculated by HPLC in the present study. Maximal picoplankton concentrations at Helgoland and Roscoff occurred in summer and at Blanes in winter. Despite these differences in the overall abundance of picoeukaryotes, all sites have a highly diverse and variable picoplankton community (Vaulot et al., 2003 a and b; Massana et al., 2004a; Not et al., 2004; Zhu et al., 2005).

Picoplankton composition The picoplankton community in the Mediterranean is similar to the one reported for warm oligotrophic regions: cyanobacteria, especially *Prochlorococcus*, dominate numerically, while eukaryotes are of lesser abundance (Legendre and Le Fèvre, 1995; Partensky et al., 1999a; Vaulot et al., 2003b; Massana et al., 2004a; Worden et al., 2004; Zhu et al., 2005; Garczarek et al., 2007; Not et al., 2008). *Synechococcus* on the other hand, revealed highest concentrations at the northern stations (Vaulot et al., 2003b). This is congruent with the ecological distribution of the two cyanobacteria (Partensky et al., 1999a; Mackey et al., 2002; Scanlan and West, 2002; Vaulot et al., 2004; Garczarek et al., 2007; Scanlan et al., 2009). The main groups of the picoeukaryotic community were present at all three sites, supporting the organisms' ubiquity by different clades and/or ecotypes (Vaulot et al., 2003b and 2008; Romari and Vaulot, 2004; Zhu et al., 2005; Medlin et al., 2006; Gescher et al., 2008). Genetic libraries revealed alveolates (58%), stramenopiles (10%), prasinophytes (5%), and cryptophytes (4%) to be the better represented phylogenetic groups at Blanes (Massana et al., 2004a), whereas alveolates (34.8%), stramenopiles (23.4%), prasinophytes (17.6%), cryptophytes (8.6%), metazoans (8%), and prymnesiophytes (1.5%) were found at Roscoff (Romari and Vaulot, 2004). At Helgoland, non-

photosynthetic organisms, such as alveolates and stramenopiles, were reported to dominate, while phototrophs were dominated by prasinophytes (40%). Referring to the identified phylotypes of the present thesis, alveolates (40.3%) dominated stramenopiles (26.4%), and prasinophytes only accounted for a minor part (4.2%) of the population. Regarding the detected and identified band classes, phototrophs accounted for 30.5%, with Picobiliphyta 39.4% and *Micromonas pusilla* (36.4%) dominating.

Regarding the community composition revealed by FISH analyses, chlorophytes dominated with 85% over non-chlorophytes at Roscoff (Not et al., 2004), whereas at Helgoland they only dominated with 58.3% and at Blanes with 52% (Zhu et al., 2005). At Roscoff, prymnesiophytes, pelagophytes, bolidophytes and cryptophytes were detected at around 1000 cells/ml in winter and 5000 cells/ml in summer, whereas prymnesiophytes and cryptophytes were counted at Blanes at maximum values of 2000 cells/ml in summer (Vaulot et al., 2003b). At Helgoland, prymnesiophytes, pelagophytes and bolidophytes accounted for maximum values of nearly 3000 cells/ml in summer. Although these data are not directly comparable, they represent overall lower cell abundances in the Mediterranean Sea and the German Bight, and highly variable ones in the English Channel. Overall, the chlorophytes were generally investigated in much more detail than the picoplanktonic non-chlorophytes. This is also represented by the availability of diverse FISH data (Vaulot et al., 2003b; Not et al., 2004; Zhu et al., 2005). However, Mamiellales dominated the Chlorophyta with 78.6% on average in the English Channel (Prasinococcales: 3.4%), with 74.4% in the German Bight (Prasinococcales: 19.9%, Pseudoscourfieldiales: 5.8%) and with 53.9% in the Mediterranean (Vaulot et al., 2003b; Not et al., 2004). *Micromonas* was the best represented and most abundant group of the three investigated genera of the Mamiellales at all three sites (Vaulot et al., 2003b; Not et al., 2004; Zhu et al., 2005) (data represented in manuscript 2). These findings supported its role as a species with simple and basic niche requirements, allowing for its adaptation to dissimilar and changing environmental conditions (Ansotegui et al., 2003; Not et al., 2004 and 2005; Šlapeta et al., 2006; Forster et al., 2008, Vaulot et al., 2008; Viprey et al., 2008; Simon et al., 2009; Worden et al., 2009). Because *Micromonas*' average contribution to the Mamiellales was lower than that of *Bathycoccus* and *Ostreococcus* at Helgoland, *Bathycoccus* and *Ostreococcus* might have a higher ecological importance in the German Bight (Eikrem and Throndsen, 1990; Guillou et al., 2004; Not et al., 2004 and 2005; Rodríguez et al., 2005; Derelle et al., 2006; Six et al., 2008; Vaulot et al., 2008). Nonetheless, the reasons for this are still hidden (Forster et al., 2008; Vaulot et al., 2008; Viprey et al., 2008; Simon et al., 2009; Worden et al., 2009).

Picoplankton ecology When comparing the results of the three sampling sites, some ecological suggestions about the picoeukaryotes can be drawn up. For example, at all sites the

picoeukaryotes showed the highest annual abundance at water temperatures around 15°C (summer in Roscoff and Helgoland, winter/spring at Blanes) (Vaulot et al., 2003b; Not et al., 2004; Zhu et al., 2005). This resembles preference of temperate waters (Vaulot, 2001b; Not et al., 2002 and 2005; Biegala et al., 2003; Massana et al., 2004a and b; Piganeau et al., 2008; Masquelier and Vaulot, 2008; Viprey et al., 2008) and supports the present study's finding of a significant influence of the change in water temperature on the abundance of picoeukaryotes. Furthermore, the picoeukaryotes reached overall highest abundances at moderate nutrient conditions (as at Roscoff) that did not allow a vast dominance of larger phytoplankton (as at Helgoland). The community composition revealed a higher abundance of non-chlorophytes when a higher amount of organic compounds was available (Helgoland) or a close coupling of the microbial loop developed (as at Blanes). The proportions between *Bathycoccus*, *Micromonas* and *Ostreococcus* at the three sites showed that *Bathycoccus* and *Ostreococcus* were better established under the eutrophic conditions in the German Bight. Furthermore, their higher abundance may also be caused by their lower affection by changing salinity values (Figure 15B), whereas *Micromonas* is negatively correlated to it.

The comparison of the picoeukaryotic community at Helgoland, Roscoff and Blanes gave a first impression about its differing composition in different ecosystems. Nonetheless, the overall lack of knowledge and data on the picoeukaryotes (Not et al., 2004; Massana and Pedrós-Alió, 2008; Medlin et al., 2006) inhibit any further conclusions.

7.) Problems in picoplankton monitoring and outlook

Those characteristics that make the picoplankton successful in its abundance and survival, such as their minimal opportunistic character, seem to be the main obstacles in monitoring (Fogg, 1991; Jacquet et al., 2002a and b; Vaulot et al., 2002 and 2008; Bryant, 2003; Massana et al., 2004a and b; Zhu et al., 2005; Medlin et al., 2006; Worden and Not, 2008). For example, their small cell size necessitates the use of biasing prefiltration steps for further analyses and limits the observation of specific morphological characteristics (Díez et al., 2001; Vaulot, 2001a; Backe-Hansen and Throndsen, 2002; Not et al., 2005 and 2008; Vaulot et al., 2008). The long evolutional existence of picoeukaryotic organisms has allowed them to develop a high adaptation capability to various environmental conditions (Fogg, 1991; Guillou et al., 2001; Vaulot, 2001a; Mackey et al., 2002; Vaulot et al., 2002 and 2008; Bryant, 2003; Massana et al., 2004a and b; Worden and Not, 2008). The majority of their community does not exhibit distinct ecological preferences and therefore the recognition of single groups or classes is not possible in respect to environmental changes. In addition, the picoplankton developed a high taxonomical diversity that has not yet been entirely understood (Not et al., 2004; Medlin et al., 2006; Massana and Pedrós-Alió, 2008; Worden and Not, 2008; Worden et al., 2009). Therefore, monitoring of picoplanktonic organisms, and especially of picoeukaryotes, can be described as the attempt to find something invisible and taxonomically unknown that very easily gets lost or remains unseen in existing methods (Biegala et al., 2003; Massana et al., 2004b; Not et al., 2004; Medlin et al., 2006; Forster et al., 2008; Masquelier and Vaulot, 2008; Vaulot et al., 2008; Worden and Not, 2008; Worden et al., 2009).

The present study's findings were not always congruent among each other regarding the composition of the picoplankton community. As already seen, there exist differences in FISH counts and DGGE findings regarding the dominating taxonomic group. Furthermore, the composition of non-chlorophytes, especially the abundance of fucoxanthin-containing organisms, remained uncertain. For example, DGGE revealed a clear dominance of stramenopiles within the picoplankton community, whereas FISH counts revealed a rather low abundance of haptophytes and stramenopiles (bolidophytes and pelagophytes) but a high abundance of unlabelled non-chlorophytes. Pigment analyses did not allow for a definite estimate of the contributions of single groups, such as, for example, haptophytes, primarily because of the lack of knowledge of picoplanktonic pigment profiles and the uncertainties involved in using pigments as a taxonomic distinction parameter (Jeffrey et al., 1999; Mackey and Higgins, 2001; Garrido et al., 2003; Latasa et al., 2004; Lewitus et al., 2005; Fuller et al., 2006b; Gescher et al., 2008; Vaulot et al., 2008). Furthermore, electron microscopic investigations revealed the abundance of cells and scales comparable to *Chrysochromulina* (Prymnesiophyceae) but did not allow for quantification. Thus, the findings of this thesis were only able to expose parts of the non-chlorophyte community in the German Bight, but no definite conclusion about the overall community.

Trustworthy conclusions about interspecies relationships, individual functional roles and characteristic ecological behaviour are only possible when our overall knowledge about picoplankton members is improved. Therefore, we need to monitor the entire picoplankton community by diverse methods in various regions of the marine environment. Fingerprinting methods, such as DGGE and sequencing, should be used in the first step to identify the occurring species, though their methodological biases need to be reduced (see next section). Identified sequences then allow for the improvement of quantitative molecular methods, such as FISH by the possibility to develop specific oligonucleotide probes. These allow the more specific monitoring of defined taxonomic groups and organisms in the field. This will then aid in gathering information about their individual occurrence and their reaction to differing environmental settings. Thus, for the near future, a concurrent approach is recommended. On the one hand, more monitoring projects have to be carried out in general to identify abundant taxa in diverse regions of our world's oceans. On the other hand, the knowledge of identified species has to be deepened. For example, the occurrence, functional roles and ecological preferences of the clades of *Micromonas pusilla* (Forster et al., 2008; Vaulot et al., 2008; Viprey et al., 2008; Simon et al., 2009; Worden et al., 2009) should be discovered. Nonetheless, it is not possible implement such a project successfully without methodological adjustments and improvements.

8.) Problems with methods and outlook

Problems in monitoring start with the sampling technique, proceed with the preparation steps for the specific methods that shall be applied, and end with inestimable biases in the detection of organisms and data analysis. Thus, the results obtained may not resemble the actual picoplankton community abundant in the field. Because of our poor knowledge of the picoplankton (Biegala et al., 2003; Massana et al., 2004b; Not et al., 2004; Medlin et al., 2006; Forster et al., 2008; Masquelier and Vaulot, 2008; Vaulot et al., 2008; Worden and Not, 2008; Worden et al., 2009), an adequate adjustment of the current methods is quite difficult. Nonetheless, some insights on improving those methods used in the present doctorate will now be given.

Sample collection and preparation Because of the broad size spectrum of the picoplankton and because of larger cells, fragments of them and/or reproductive stages that may slip through the filters (Not et al., 2005 and 2008), a reliable separation between different-sized organisms is not possible. This problem, especially regarding contaminating larger organisms, was unequivocally identified by EM and phylotype analyses in this study and was suggested to be the primary cause of biases in pigment analyses. Biases of preparation steps can in part be estimated by comparative tests. For example, the influence of fixation and storage time in FCM analyses can be verified in direct comparison to analysis made on comparative natural samples. However, the influence of the sample preparation in FISH investigations cannot be estimated because preparation is needed to carry out the method itself. Only comparative tests towards other methods may allow for the estimation of the preparation bias. This could be done, for example, by examining the filter carrying the organisms by electron microscopy after the ethanol series at the end of filtration and before fixing the filter on a glass slide at the end of the preparation for FISH.

Isolation and cultivation The possibilities to isolate and cultivate picoplankton need urgent enhancement. Most picoplanktonic groups are still not represented in culture (Little et al., 2008; Piganeau et al., 2008), although only cultivation tests allow for the investigation of structural and physiological characteristics in detail. For example, their changes under different environmental conditions can only be observed and a fine-scale description of their ecological niches and functional roles (Forster et al., 2008; Vaulot et al., 2008; Viprey et al., 2008; Simon et al., 2009; Worden et al., 2009) is only possible, when identified species are analysed separately. Next to the modelling of their reaction to environmental changes, such as climate change, eutrophication and hypoxia, cultivation tests will also allow for the investigation on and conclusions about picoplankton grazers, viral attacks, and the ways in which picoplanktonic carbon reaches higher trophic levels (Figure 2, page 7). Furthermore, the definition of individual pigment profiles allows for the specification and adjustment of quantification capabilities of the community by HPLC analyses.

Electron microscopy Electron microscopic investigations revealed the occurrence of large phytoplankton cells (cf. *Rhizosolenia longiseta*) within the size-fractionated picoplankton samples in this study. Furthermore, they are suggested to be exposed to high cell losses during sample preparation. However, this method allows for the examination of morphological and systematic features (Eikrem and Throndsen, 1990; Vaulot, 2001a; Backe-Hansen and Throndsen, 2002; Vaulot et al., 2008), and, in combination with cultural investigations or ecological monitoring, it may reveal morphological differences in space and time. Thus, its use is highly recommended. Nonetheless, a different approach should be chosen. Instead of the chosen method to concentrate the cells and to dry and wash them on a grid (details in manuscript 2), the direct examination of cells concentrated on 0.2 µm filters is suggested to result in a much lower cell loss.

Flow cytometry Flow cytometry led to a quantitative separation of four different organism groups (Li, 1994; Marie et al., 2000; Medlin et al., 2002) within the waters of the German Bight, but did not differentiate their composition further. This is suggested to lead to a more detailed analysis of the quantity and quality of the picoeukaryotic community when used in combination with molecular markers (Vaulot et al., 2008; Worden and Not, 2008). As already mentioned, its cell losses caused by fixation and storage should be analysed in more detail to allow a more precise estimation of its quantitative biases.

Pigment based methods Chlorophyll *a* can be used as a biomass indicator of picophytoplankton abundance. Because it is difficult to estimate the influence of larger organisms and their fragments on the pico-sized fraction (Not et al., 2005 and 2008), complementary investigations by flow cytometry and/or molecular approaches are advisable. Overall, pigment analyses by HPLC gave a rough and rather qualitative overview of the community composition in the present study, whereas fluorometry allowed for the quantification of spectral groups. Pigments are highly variable and influenced by diverse outer factors (Jeffrey et al., 1999; Mackey and Higgins, 2001; Jeffrey and Wright, 2004; Lewitus et al., 2005; Not et al. 2005 and 2008; Wright and Jeffrey, 2005; Fuller et al., 2006b; Vaulot et al., 2008). In combination with the lack of knowledge of picoplanktonic pigment profiles, pigment analyses, such as HPLC or fluorometry investigations, do not allow a reliable quantification of the distinct parts of the autotrophic picoeukaryotes. To allow for the use of pigments as quantitative class-indicators in picoplankton studies, knowledge about pigment compositions and ratios in space and time needs to be enhanced. This would allow the application of quantitative calculation programmes, such as CHEMTAX (Mackey et al., 1996), on the obtained pigment concentrations of natural samples.

Molecular methods The applied fingerprinting technique, DGGE, allowed a broad overview of the overall taxonomic abundance of the picoeukaryotes, whereas FISH counts provided specific

and quantitative investigations on single taxonomy levels. However, both methods run the risk of not detecting all organisms. DGGE, for example, runs the risk of biases in the extraction of DNA and its amplification by PCR (Burton, 1996; Medlin et al., 2002 and 2006; Zhu et al., 2005; Fuller et al., 2006a; Vaulot et al., 2008; Viprey et al., 2008; Not et al., 2009). Furthermore, the DNA analysed may not belong to the organisms aimed for (Viprey et al., 2008; Not et al., 2009). Thus, the phylotype diversity obtained may not be representative of the picoeukaryotic community analysed. On the other hand, application of FISH has the general drawback of hybridisation difficulties, unspecific labelling and counting errors by patchiness of the cells, for example (Medlin et al., 2002; Biegala et al., 2003; Vaulot et al., 2003b and 2008; Amann and Fuchs, 2008). These problems became obvious in the present study by the discrepancies among hierarchical probe counts (discussed in manuscript 2). Thus, the overall methodological problems in molecular studies can be characterised as occurring in extraction, amplification and hybridisation procedures. This is already known (Burton, 1996; Medlin et al., 2002 and 2006; Vaulot et al., 2008; Viprey et al., 2008; Not et al., 2008 and 2009), but because of our restricted knowledge about picoplankton identities, we cannot estimate the possible methodological biases in total. For example, the reliability of probes cannot be provided for an organism community that is not yet entirely discovered taxonomically. Thus, it is highly recommended to enhance the knowledge about and number of genetic sequences occurring in space and time and to evaluate the representativeness of the obtained sequences (Vaulot et al., 2008). Data banks need permanent updating to enhance the reliability of the phylogenetic trees constructed. Furthermore, the generation of oligonucleotide probes specific for smaller and more specific groups is suggested to lead to an increased understanding of picoplankton abundance, diversity and ecology. These specialised markers, could be combined into "regional probe sets" that can be used for regular monitoring or observations over environmental gradients. Furthermore, microarrays might be a possibility to fasten molecular analyses, although this method still needs improvement in detection reliability and in the quantitative calculation of results. A further promising method is the use of metagenomics with direct cloning, thus avoiding PCR, and shotgun sequencing of DNA in picoeukaryotic studies (Massana and Pedrós-Alió, 2008; Not et al., 2009).

GENERAL OUTLOOK

The biggest obstacles that became obvious during this intensive and comparative study on the coastal picoeukaryotes around Helgoland were methodological uncertainties. These did not allow for definite conclusions about the composition of the community or the diversity of species, nor the definition of individual niche compartmentalisations. The primary reason for this is suggested to be the lack of knowledge about the highly diverse picoplankton community, especially in respect to the organisms' taxonomy. This inhibits the adjustment and specification of currently existing methods.

Future goals include learning more about the picoeukaryotes by identifying them and by gathering information about them in the natural environment. Monitoring approaches including diverse abiotic and biotic conditions in combination with identification techniques should be carried out to obtain reliable estimation about the picoplankton abundance and ecology, even though it is a challenge to take samples at ecologically relevant scales (Amann et al., 1995; Worden and Not, 2008). However, these identification and monitoring attempts are most beneficial with a parallel improvement of analysis. Thus, the general approach on picoplankton regarding its sampling, preparation and cultivation, as well as the permanent adjustment towards novel discoveries, for example, regarding the specificity of molecular markers, need to be improved in a continuous manner. This will provide the possibility to go deeper into specific details, such as the occurrence of different ecotypes, the roles of viral attack, viral symbioses and grazers, as well as the identification and calculation of trophic and ecologic roles of single picoeukaryotes in the marine ecosystem. Furthermore, methods for picoplankton investigations need to become more unaffected towards methodological and environmental biases, such as the suppression of phototrophic sequences in the PCR, the influence of larger algae in pigment studies or the unintended inclusion of extracellular DNA in DGGE analyses. Only then, a trustworthy quantitative calculation and discrete analyses of the picoplankton community, or single fractions of it, will be possible.

As the findings of this thesis showed, only a mutual and therefore time-consuming development between gaining knowledge and improving methods seems to lead to an overall understanding of the eukaryotic picoplankton, its ecology and its role in the marine ecosystem. This information is fundamental for the definite description of its wide-ranging significance and ecological potential for our world's oceans in the future.

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~ PART II ~

MANUSCRIPTS

This part contains the main manuscripts that were produced during this doctorate work. The author's contribution to each of them ranged from the overall organisation, implementation and analyses of samples, to the writing of the first drafts of the manuscripts. The short summary at the beginning of each manuscript documents its contextual positioning within the present project and a description of the author's specific contribution.

Manuscript 1: HPLC determination of picophytoplankton pigments from the North Sea

Manuscript 2: Assessment of picoplankton abundance and dynamics in the German North Sea by FISH, flow cytometry, fluorometry, HPLC and electron microscopy

Manuscript 3: Feasibility to assess the diversity, occurrence and ecology of picoplanktonic eukaryotes by DGGE and sequencing

Manuscript 1:

HPLC determination of picophytoplankton pigments from the North Sea

Authors: Britta Knefelkamp, Kristine Carstens, Karen H. Wiltshire

Published in: Journal of Experimental Marine Biology and Ecology, submitted

This manuscript reports the improvement of a fast and user-friendly HPLC method to analyse phytoplanktonic pigments. The described method is based on the work of Zapata et al. (2000) as well as Garrido et al. (2003), and combines these strategies by using a monomeric c_8 column and a pyridine-containing mobile phase. The method was adjusted for picoplankton investigations in the waters of the German Bight and allows for the detection and analysis of very low pigment concentrations as well as the accurate distinction between main phytoplankton pigments. The newly designed technique offers a high sample throughput in only a quarter of the time needed by most of the previously established methods. This drastically reduces costs for material and work force and may even encourage future studies of pigments in the picoplanktonic size class.

Based on a concept by K. Wiltshire, the contribution of the author included the development, adjustment and enhancement of the method. Furthermore, the author wrote the first draft of the manuscript describing the improved method and the results on picoplankton investigations.

ABSTRACT

Pigments are routinely used as an indicator of biomass, primary productivity and composition of phytoplankton communities in marine environments. The most frequently used method to characterise and quantify pigments is high-performance liquid chromatography (HPLC). HPLC allows the separation of pigments based on their individual characteristics, such as molecule size and polarity. The separation quality and elution order of pigments depend on the chosen column, mobile phases, solvent gradients, flow rates and run lengths. While particulate matter and microalgal pigment analyses are common in the literature, pigment analyses of natural picoplankton populations by HPLC are uncommon in the literature. This study's goals were twofold: (1) presentation of a fast and user-friendly HPLC method allowing picoplankton evaluation by quantifying important marker pigments, and (2) its application to the Picophytoplankton community of the German Bight. The described method is a modification of the methods described by Zapata et al. (2000) and Garrido et al. (2003). It uses a monomeric c_8 column with a pyridine-containing mobile phase and allows a specific separation of major phytoplankton pigments in 9 minutes. Using this method, we found the highest picoplankton abundance and the highest contribution to the overall phytoplankton population in winter. The picophytoplankton seemed to be dominated by brown-pigmented algae, though a high influence of larger cells (nanoflagellates and diatoms) and organic particles was suggested. Green-pigmented algae on the other hand, seemed to form a permanent part of the picophytoplanktonic background population in the coastal waters of the German Bight at Helgoland Roads.

Key words: HPLC, pigments, c_8 column, pyridine, picophytoplankton, coastal ecosystem, North Sea, Helgoland Roads.

INTRODUCTION

Picoplankton is fundamental in aquatic ecosystems and their high diversity implies a high functional diversity that is still not entirely understood (Fogg, 1991; Vaulot et al., 2008; Worden and Not, 2008; Not et al., 2009). While the cyanobacteria (prokaryotes) constitute the numerical majority of this picoplanktonic size-fraction, diversity is due to eukaryotes (Worden et al., 2004; Bec et al., 2005; Worden and Not, 2008). In coastal waters, the picoeukaryotes occur as background populations, but are occasionally able to dominate the biomass of the overall autotrophic phytoplankton (Not et al., 2008). In this study, we investigate picophytoplankton at Helgoland Roads (54°11.3' N, 07° 54' E).

Picophytoplankton is very abundant in aquatic systems and can contribute significantly to overall microalgal biomass and productivity (Vaulot et al., 2008; Worden and Not, 2008; Not et al., 2009). However, they have been seldom studied due to their extremely small cell size and fragility, and consequent difficulties in their detection by traditional methods. Adjusted pigment determination may help here as with appropriate filtering and high-performance liquid chromatography (HPLC) assessments can be carried out relatively easily. They can provide reliable information on the abundance of the community and give an impression about its taxonomic diversity. The main hindrance of calculating the concentrations of single picoautotrophic groups is the lack of knowledge about their characteristic pigment contents and pigment ratios. Thus, the improvement of cultivation attempts (Vaulot, 2001; Massana et al., 2004a and b; Little et al., 2008; Piganeau et al., 2008; Vaulot et al., 2008) is needed to define these individual pigment profiles by cultivation tests. These will allow a specification and adjustment of quantification capabilities of the picoplankton community by HPLC analyses, for example, by the matrix factorisation program CHEMTAX (Mackey et al., 1996; Irigoien et al., 2004). However, many HPLC methods are available in the literature (e.g., Gieskes and Kraay, 1983; Mantoura and Llewellyn, 1983; Daemen, 1986; Deventer, 1985; Wright and Jeffrey, 1987; Wiltshire, 1992; Zapata and Garrido, 1991; Wilhelm et al. 1991; Pinckney et al., 1994; Wiltshire and Schroeder, 1994; Wiltshire et al., 2000; Garrido et al., 2003). The decision for a specific HPLC method depends upon different criteria. These are, for example, the type of substrate to be analysed and the analytical needs, such as pigment resolution, sensitivity and/or speed of analysis (Jeffrey, 1997; Mendes et al., 2007). Because columns differ in their separation capabilities due to their bonding chemistry, pore size, surface area, coverage and carbon load (Zapata et al., 2001; Jeffrey and Wright, 2004; Wiltshire, 2009), the choice of column, as well as solvents, depend on these factors. Reversed-phase chromatography, in which the mobile phase is polar and the non-mobile phase is non-polar, is generally the method of choice for pigment separation. Reversed-phase columns are characterized by alkyl chains (e.g., octyl (c_8) or octadecyl (c_{18}) chains) bonded covalently to the support surface of polar packing material resulting in a non-polar surface. To date we carried out most pigment

separations using c_{18} columns and acetone, methanol gradients (see also Mantoura and Lewellyn, 1983 and Wiltshire et al., 2000). The advantage of these methods is that relatively untoxic solvents are used. The disadvantage is that they are slow, taking up to 40 minutes for separations.

The determination of picoplankton pigments is a challenge because of their very low cell densities and small biomass of these. Thus, we needed a sensitive method. Additionally, as we had to deal with a large number of picoplankton samples, we needed a method that was fast. We decided to test the method of Zapata et al. (2000), which uses a C_8 column and acetonitrile and pyridine as liquid phase (Jeffrey et al., 1999; Zapata et al., 2000). Additionally, we tested the c_8 column in combination with methanol, pyridine and acetone as liquid phase by applying a modified gradient to the one described by Garrido et al. (2003) and a simpler gradient than that of Zapata et al. (2000). Our aim was to test a quick method for determining picoplankton pigments in seasonal samples from Helgoland Roads.

METHODS

Before analyses of samples could be carried out, two HPLC methods were tested. Method 1, the method of Zapata et al. (2000), uses a two-solvent gradient as given in Table 1. Solvent A consists of metanol:acetonitrile:aqueous pyridine in a volume ratio of 50:25:25 and solvent B acetonitrile:acetone (80:20). Method 2 (gradient used is given Table 2) involves 3 solvents; methanol as solvent A, 0.025 M aqueous pyridine solution (pH 5.0 with acetic acid) as solvent B and 100% acetone (HPLC grade) as solvent C. The flow rates were 1.3 ml/min. It was found that Method 2 resulted in better separations of the major pigments (see Table 3) in less than a quarter of time and thus this method was then implemented from then on.

Samples were taken weekly at Helgoland Roads (54°11.3’ N, 07° 54’ E) between March 2005 and March 2006. Sequential filtration was carried out to separate the picoplankton from the larger microalgae using 10 and 3 µm isopore membrane filters (*Millipore*, TCTP and TSTP, diameter: 47 mm). The pressure of the filtration never exceeded 200 mbar in order to prevent cell disruption. The resultant filtrate was gently sucked through a 0.2 µm nylon membrane filter (*Whatman*) using a water-tap-based vacuum pump (see also Knefelkamp et al., 2007). The filtered volume ranged between 1100 and 7000 ml depending on the ease of filtration. Each 0.2 µm filter was placed in 2 ml of 90% acetone (HPLC grade) and stored frozen at -80 °C for at least 48 hours. Thereafter, filters were homogenised mechanically by adding a pinch of quartz sand and disrupting with a Teflon pestle (Wiltshire et al., 2000). After this, the samples were sonicated for 90

minutes in an ultrasound bath (*Bandelin Sonorex Super Rk 103/H*) filled with ice water. Each sample was then syringed through a 0.2 µm filter (*Spartan 30/0.2 RC*) into a 1.5 ml brown-glass HPLC vial. The whole preparation process was carried out in dim light.

The filtrate was injected into a Waters 2695 Separation Module. The autosampler was cooled to 4°C and a 200 µl sample loop was used. To get sharper peaks, distilled water (millipore) was injected before and after 100 µl of each sample (Wiltshire et al., 2000). The column used in both methods was a reversed-phase monomeric Waters Symmetry C_8 (150 x 4.6 mm, 3.5 µm spherical particle size, 100 Å pore size, end-capped) and was thermostated in the column oven at 25 °C (compare Zapata et al., 2000). Separated pigments were detected using both, a Waters 2475 Multi λ Fluorescence Detector (excitation 430 nm, emission 650 nm) and a Waters 996 Photodiode Array (PDA) Detector (absorption wavelength range from 400 to 700 nm). As baseline variations within the chromatogram may result from the solvents, a calibration run with the injection of the pure extraction solvent (90% acetone) was carried out each run and used for blank subtraction. Samples were analysed in a random order to avoid linear measurement mistakes (e.g., ageing of column) and every 4 weeks a threefold measurement with three samples was carried out. The HPLC-system was calibrated with external pigment standards (*DHI, Water and Environment*) to allow the quantification of marker pigments. Every chromatogram was manually checked for incorrect peak assignment (for integration problems see Wright and Mantoura, 1997b). The peak purity was also checked (Rodríguez et al., 2006; Zapata et al., 2000). The chromatographic detection was at 430 nm and the identification of substances was by their PDA absorption spectra between 400 and 700 nm, band ratios and their retention times within the separate runs (see Table 3 and Figure 1). Standard concentrations were calculated using the Lambert-Beer law with the known extinction coefficients from the calibration pigments (see Jeffrey et al., 1997 and Wiltshire et al., 2000).

As a backup to the HPLC methods, a laboratory multialgal fluorometer (*bbe Moldaenke)* was used to determine different algal pigment classes. Each sample was measured five times and the following spectral algal groups were identified: green-pigmented (chloro-, eugleno-, prasinophytes), blue-green-pigmented (cyanobacteria) and brown-pigmented algae (diatoms and dinophytes), as well as cryptophytes. For further methodological details, see Beutler (1998 and 2003) and Beutler et al. (2002).

Chlorophyll *a* concentrations, generally used as a biomass indicator for phytoplankton, were compared towards measurements of the overall phytoplankton as analysed by the long-term series

at Helgoland Roads. However, only concentrations of the PDA detector and the bbe multialgal fluorometer were used for comparison.

RESULTS AND DISCUSSION

Method choice Phytoplankton pigments include chlorophylls, carotenoids and phycobilins, and represent a complex mixture of at times nearly identical molecular structures and polarities (Garrido and Zapata, 1996; Porra et al., 1997; Zapata et al., 2000; Garrido et al., 2003). Based on the HPLC methods of Zapata et al. (2000) and Garrido et al. (2003) we set up a simple 3-solvent gradient that separates and detects most pigments described in marine phytoplankton (Table 3). These cover a wide range of chromatographic polarities. Clear separation of chlorophylls *a* and *b*, monovinyl chlorophyll *a*, chlorophyllides *a* and *b*, as well as diverse xanthophylls and carotenes. However, monovinyl chlorophyll *b*, phaeophorbides, phaeophytins and chlorophylls *c* at times co-eluted and were not always identified clearly (Table 3).

The method was successfully applied to picophytoplankton samples from the German Bight. Figure 2A shows the picoplanktonic chlorophyll *a* concentrations as detected by the two HPLC detectors. The threefold filtration had an average variation of -14.1/+12.5% with the fluorescence detector and -16.6/+14.8% with the PDA detector. This could have been a result of the greater detection sensitivity of the fluorescence detector at lower chlorophyll concentrations. However, it was found that the percentage deviation was independent of the chlorophyll *a* concentration (tested for linear and rank correlation $p < 0.05$). Results of both detectors were linear with 90.5%.

In the case of the bbe multialgal fluorometer (Figure 2B), variations of -8.1/+8.8% were detected and they significantly correlated ($p < 0.05$) to the mean chlorophyll *a* concentrations (Spearman's rank $\rho = -0.35$; Table 4). Thus, showing a decrease in variation when concentrations increased. The results from the fluorometric measurements were only with 57.1% linear to the HPLC results (PDA detector), and 45.3% (fluorescence detector), respectively. The Spearman ranks (Table 4) showed a significant correlation ($p < 0.05$) to the HPLC absorption ($\rho = 0.489$) and, to a lesser extent, to the HPLC fluorescence ($\rho = 0.371$). This is not surprising as the measuring principle of the bbe multialgal fluorometer deals with pigment signals from intact algal cells and not from pigments extracted from these. Such differences have been shown (Wiltshire et al. 2000).

In comparison with the larger phytoplankton, picophytoplankton is generally found in much lower abundance in terms of both cell numbers and biomass, especially in eutrophic coastal regions

(Jacquet et al., 2002; Biegala et al., 2003; Massana and Pedrós-Alió, 2008; Masquelier and Vaulot, 2008). This makes the detection of pigments in the < 3 µm fraction difficult for reasons of concentration alone. In the low-concentration range, then such aspects as, zooplanktonic faecal pellet extracts, cell fragments from larger plankton and detritus will influence the pigment determination substantially (Jeffrey et al., 1999; Jeffrey and Wright, 2004; Zapata et al., 2004; Not et al., 2005 and 2008; Wright and Jeffrey, 2005; Vaulot et al., 2008). Furthermore, picoplankton studies constitute a relatively "young" area of scientific investigation and therefore, the highly variable community with the associated pigment diversity and relative pigment concentrations are still unknown (Ansotegui et al., 2003; Jeffrey and Wright, 2004; Not et al., 2005; Rodríguez et al., 2006). This hinders the conclusive definition of picoplankton classes from the pigments present. The method described in this study resulted in sufficient pigment concentrations, meaningful chromatograms (Figure 1), and allowed the identification of distinct pigments (Figure 2) within the picoplanktonic size class. Thus, we achieved a reliable impression of the picoplankton community composition and succession (Figures 2, 3 and 4).

Picoplankton succession Measurements with the bbe multialgal fluorometer revealed an average concentration of total chlorophyll *a* in the pico-sized fraction (< 3 µm) of 0.54 µg/L in spring (Figure 3). This made up a contribution of 36.7% towards the whole phytoplankton fraction. Chlorophyll *a* as estimated by HPLC (0.2 to 3 µm; PDA detector) made up only 0.09 µg/L and 3.6%. Towards summer, total chlorophyll *a* concentration reached its highest concentration (0.578 µg/L) in the picophytoplankton, but its lowest contribution (22.6%) to the overall primary production community. Chlorophyll *a* as estimated by HPLC revealed the highest seasonal average as well (0.12 µg/L) with a resulting higher contribution of the picoplankton in comparison to spring (5%). This difference between the two seasons and two methods was possibly caused by an increase of picoplankton towards summer (compare Knefelkamp et al., submitted 1) in combination with a decrease of cell debris. Towards autumn, the fluorometrical chlorophyll *a* concentration decreased by 62% to 0.22 µg/L, though the contribution to the overall total chlorophyll *a* remained nearly the same (24.3%). Chlorophyll *a* by HPLC decreased by 46% to 0.06 µg/L, resulting in a contribution of picoplanktonic chlorophyll *a* of 7.2%. This seasonal difference in concentration, but at the same time steady contribution to the overall phytoplankton, was possibly caused by an overall decrease in the abundance of phytoplankton. Towards winter, the concentrations of total (0.21 µg/L; fluorometer) and pure (0.08 µg/L; HPLC) chlorophyll *a* remained the same in the picoplankton fraction, while its contribution to the overall chlorophyll *a* values increased to maximum values (fluorometry: 38.3%, HPLC: 19.5%). These findings represent the further decline of chlorophyll *a* within the larger microalgae (> 3 µm), whereas the picoplankton remained steady in its abundance, or even slightly increased (HPLC). As

picoplankton is known to deal with unfavourable environmental conditions, particularly with regard to light, this result was to be expected.

Picoplankton composition Regarding the pigments in spring (Figure 4), diadinoxanthin (a marker for brown-pigmented algae) was at 0.008 µg/L at its highest seasonal average concentration. 19'-butanoyloxyfucoxanthin (a marker pigment of Pelago-, Prymnesio- and Chrysophyceae) occurred only twice (in May) and thus had its highest mean concentration (0.022 µg/L) in spring as well. A closer look at the spring data using both the HPLC pigments and fluorometric spectral groups (latter data not shown) revealed that green-pigmented algae dominated in March, while brown-pigmented algae (Dinophyceae) and Cryptophyceae were dominant in April. In May, Bacillario-, Pelago- and Prymnesiophyceae were found in higher concentrations, while the Dinophyceae decreased and green-pigmented algae increased.

In summer, brown-pigmented algae had an average concentration of 0.486 µg/L and made up 88.8% of the chlorophyll *a* estimated by the fluorometer. The pigments of brown-pigmented algae were also highest in summer. Peridinin, a marker pigment for Dinophyceae, had a seasonal average of 0.041 µg/L; fucoxanthin, a marker pigment of Bacillariophyceae and other stramenopiles, had an average of 0.173 µg/L.

Molecular analyses of the same samples (Knefelkamp et al., submitted 1) revealed that the unexpectedly high abundances of brown-pigmented algae and fucoxanthin possibly did not result from the picoplanktonic Bolidophyceae, Prymnesiophyceae or Pelagophyceae. Instead, an influence from larger-sized microalgae, such as nanoflagellates and/or diatoms, fragments of them, resting spores and/or faecal pellets of their grazers is suggested (Jeffrey et al., 1999; Jeffrey and Wright, 2004; Zapata et al., 2004; Not et al., 2005 and 2008; Wright and Jeffrey, 2005; Medlin et al., 2006; Vaulot et al., 2008).

Green marker pigments were also particularly abundant in summer. Alloxanthin, a marker pigment for Cryptophyceae had an average concentration of 0.005 µg/L, chlorophyll *b*, a marker for Eugleno-, Prasino- and Chlorophyceae, an average of 0.033 µg/L, and neoxanthin (green-pigmented algae and Euglenophyceae) an average of 0.024 µg/L. A closer look at the monthly values showed a high abundance of Bacillariophyceae and green-pigmented algae in June (Prymnesiophyceae were present to a small degree). In July and August, the picoplankton community was mainly composed of Bacillariophyceae, green-pigmented algae, Dinophyceae and Prymnesiophyceae. This remained the case throughout autumn and winter but with overall lower pigment concentrations.

In the only study ever carried out previously at Helgoland Roads regarding picophytoplankton, Vaulot et al. (unpublished) also found a clear seasonality of Prasino- and Cryptophyceae with a maximal abundance in summer. Although it was not possible to determine the green-pigmented picoplankton organisms of 2005/2006 in further detail by their pigments, they mainly comprise Prasinophyceae (Knefelkamp et al., submitted 1). This supports their suggested predominant position and consequently important ecological role in the microbial food web of temperate coastal waters (Vaulot et al., 2008; Viprey et al., 2008; Worden and Not, 2008; Simon et al., 2009). In the present study, Prasinoxanthin was not reliably detected, a situation that has been described in other studies by Not et al. (2007). They determined a significant presence of Prasinophyceae even when the prasinoxanoxanthin:chlorophyll *b* ratio was low. Some class members do not contain prasinoxanthin (Jeffrey et al., 1999; Ansotegui et al., 2003; Guillou et al., 2004; Latasa et al., 2004; Zapata et al., 2004; Lewitus et al., 2005; Vaulot et al., 2008).

19'-hexanoyloxyfucoxanthin, the marker pigment for Prymnesiophyceae, and certain Dinoflagellates showed the highest seasonal average concentration in autumn (0.015 µg/L), resulting from the high value on November 1st (0.08 µg/L). Though for the Prymnesiophyceae only a minor number of picoplanktonic representatives have been recorded until now (Vaulot et al., 2008), they were recorded at being present throughout the year at Helgoland Roads in significant abundance (Medlin et al., 2006). This is supported by recent findings of Liu et al. (2009), who discovered haptophytes to be more abundant and ecologically important in the marine phytoplankton than previously thought. Carotene implies the presence of Rhodophyceae or Cryptophyceae in our samples but occurred only occasionally in minimal concentrations. No seasonal maximum could be defined. The same is true for diatoxanthin (marker for brown-pigmented algae). Zeaxanthin (Cyanobacteria, Prochlorophyceae) and divinyl chlorophyll *a* (Prochlorophyceae) were detected, but not in reliable amounts or only in single instances. As was to be expected, only low concentrations of Prochlorophyceae were found, as they are known to be more abundant in oligotrophic and stratified waters between 40°N to 40°S (Partensky et al., 1999a and b; Hess et al., 2001; Garczarek et al., 2007; Scanlan et al., 2009).

CONCLUSIONS

In this study, the clear separation of different marker pigments allowed quantification and a concrete picture of the annual succession of picoplanktonic pigments (Figure 4). Furthermore, the findings are within the expectations on picoplankton pigments in coastal waters: the highest numbers were found in summer and the highest chlorophyll *a* contribution to the overall

phytoplankton community in winter. Pigments revealed a fast change in the composition of the picoplankton within one week (Figures 1, 2 and 4) and thus supported previous suggestions (Medlin et al., 2006) and findings by other methods (Knefelkamp et al., submitted 1 and 2). No distinct blooms of the picoplankton community were recorded throughout the year, supporting their role as a background population in coastal waters (Not et al., 2002, 2005 and 2008; Medlin et al., 2006, Rodríguez et al., 2005; Derelle et al., 2006; Vaulot et al., 2008). The annual picoplankton succession described here for Helgoland Roads provides a first impression about the dynamics of this important community in the waters of the German Bight and findings will serve as the basis for further studies on the aquatic food web. A HPLC method of high resolution and short runtime (less than 9 minutes) was used to analyse the pigments of the picoplankton size fraction from samples at Helgoland Roads. The methodological and ecological findings of this study represent an important step forward towards investigating and understanding of picophytoplankton ecology.

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LIST OF TABLES AND FIGURES

Table 1

Time	A	B
0	100%	0%
18	60%	40%
22	0%	100%
38	0%	100%
40	100%	0%

Table 2

Time	A	B	C
0	80%	12%	8%
4	80%	0%	20%
8.5	80%	0%	20%
8.51	80%	20%	0%
9	80%	20%	0%

Table 3

Peak	Pigment	Method 1 Retention	Maxima (nm)					Method 2 Retention	Maxima(nm)				
1	Chlorophyll c_3	**5,157**		457,6	590,2	668,4		**2,551**		469	593,8	699	
2	Chlorophyllide *a*	**6,804**	429,8	489,1	619,5	664,7		**2,864**		431		619,5	666
3	Peridinin	**7,806**		474,5				**2,59**		473,3			
4	Chlorophll c_2	**8,262**	452,7	582,8	634,1			**2,9**	455,2	586,6	635,4	700,3	
5	Fucoxanthin	**10,947**		450,3				**3,063**		450,3			
6	Pheophorbide *a*	**11,397**	409,3	504,9	535,3	607,3	664,7	**3,57**	408,1	506,1	536,5	608,5	664,7
7	Neoxanthin	**13,445**	414,1	438,2	466,1			**3,354**	412,9	435,8	464,9		
8	Diadinoxanthin	**14,905**		447,9	477			**3,76**		445,5	475,8		
9	Diatoxanthin	**18,146**		454	485,5			**4,2**		451,5	479,4		
10	Lutein	**21,604**		446,7	475,8			**4,408**		445,5	473,3		
11	Zeaxanthin	**20,861**		454	480,6			**4,368**		451,5	478,2		
12	Chlorophyll *b*	**24,583**		455,4	496,3	645,2		**5,79**		468,5	601,1	651,3	652,5
13	Chlorophyll *a*	**25,488**	429,8	532,9	579,2	662,3		**6,359**		431		618,3	664,7
14	Pheophytin *a*	**27,101**	409,3	506,1	535,3	607	664,7	**6,919**	408,1	506,1	535,3	608,5	664,7
15	ß-Carotene	**27,436**		454	480,6			**7,628**		451,5	478,2		

Table 4

	HPLC											Fluorometer			
	Chlorophyll *a* (PDA)	Chlorophyll *a* (abs.)	19'-Butanoyloxyfuco-xanthin	19'-Hexanoyloxyfuco-xanthin	Alloxanthin	Chlorophyll *b*#	Diadinoxanthin	Fucoxanthin	Neoxanthin*	Peridinin	βε-Carotene	Chlorophyll *a*	green-pigmented algae	cyanobacteria	brown-pigmented algae
HPLC															
Chlorophyll*a* (abs.)	0,949														
19'-Butanoyloxyfucoxanthin	n.s.	0,344													
19'-Hexanoyloxyfucoxanthin	n.s.	n.s.	n.s.												
Alloxanthin	0,349	0,546	n.s.	n.s.											
Chlorophyll *b*#	0,475	0,507	n.s.	0,426	0,293										
Diadinoxanthin	0,436	0,596	0,358	n.s.	0,563	n.s.									
Fucoxanthin	0,479	0,619	n.s.	0,417	0,334	n.s.	0,643								
Neoxanthin*	0,333	0,360	n.s.	0,404	n.s.	0,712	n.s.	0,356							
Peridinin	n.s.	n.s.	n.s.	n.s.	0,403	n.s.	0,363	0,394	n.s.						
βε-Carotene	0,406	0,535	n.s.	n.s.	0,766	n.s.	0,533	n.s.	n.s.	0,322					
Fluorometer															
Chlorophyll *a*	0,371	0,489	0,342	n.s.	0,344	0,377	0,405	0,368	0,487	n.s.	n.s.				
green-pigmented algae	n.s.	n.s.	0,319	n.s.	n.s.	n.s.	n.s.	n.s.	0,383	-0,305	n.s.	0,527			
cyanobacteria	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		
brown-pigmented algae	0,408	0,528	0,354	n.s.	0,400	0,332	0,458	0,427	0,440	n.s.	n.s.	0,922	0,439	-0,298	
cryptophytes	n.s.	n.s.	n.s.	-0,289	n.s.	n.s.	n.s.	n.s.	n.s.	0,348	n.s.	0,343	n.s.	n.s.	n.s.

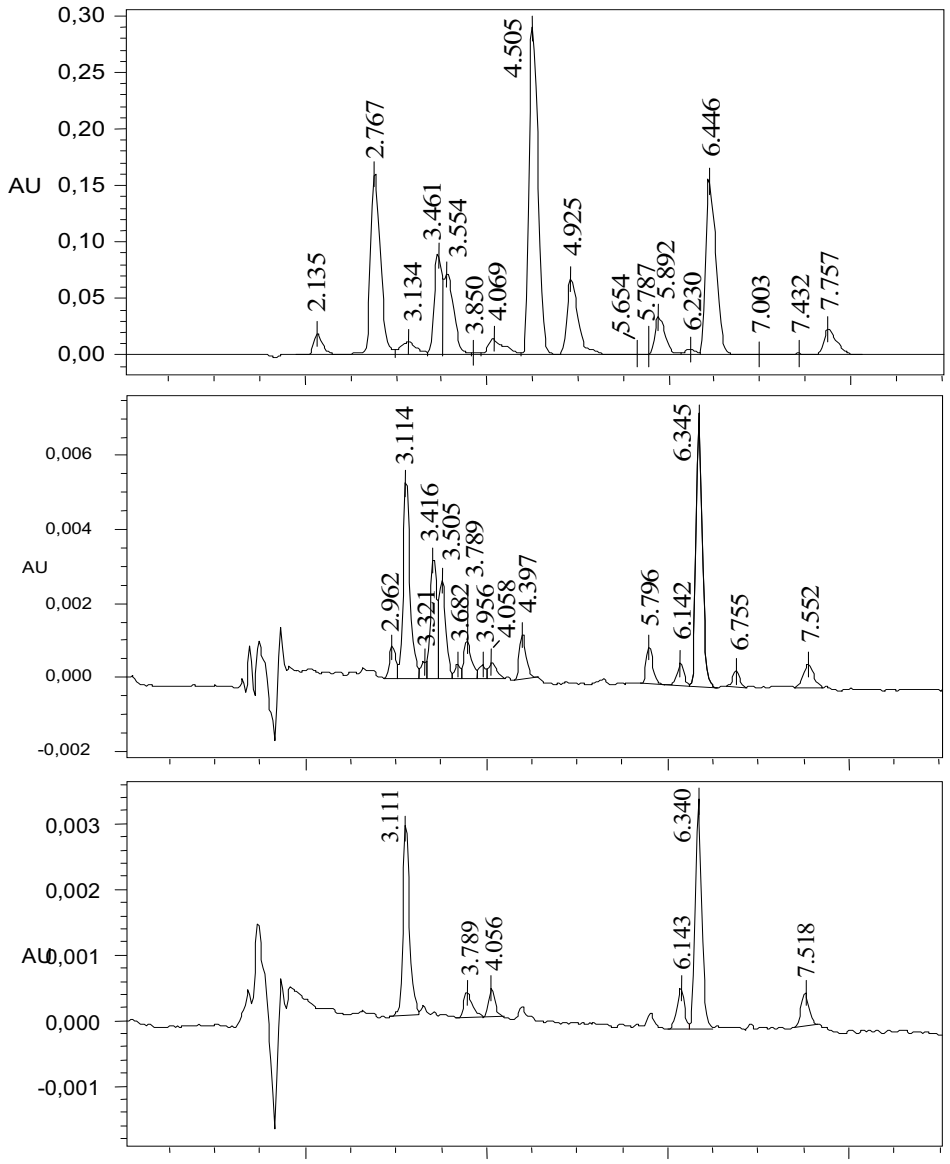
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Figure 1

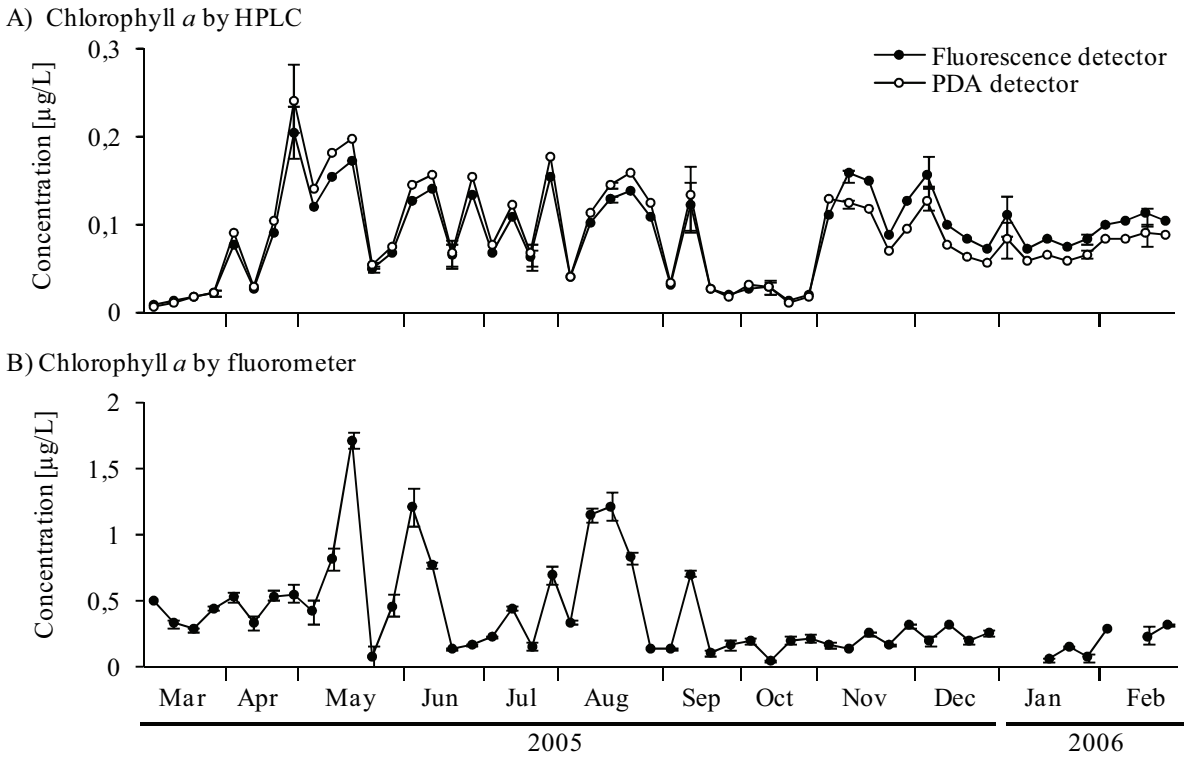


Figure 2

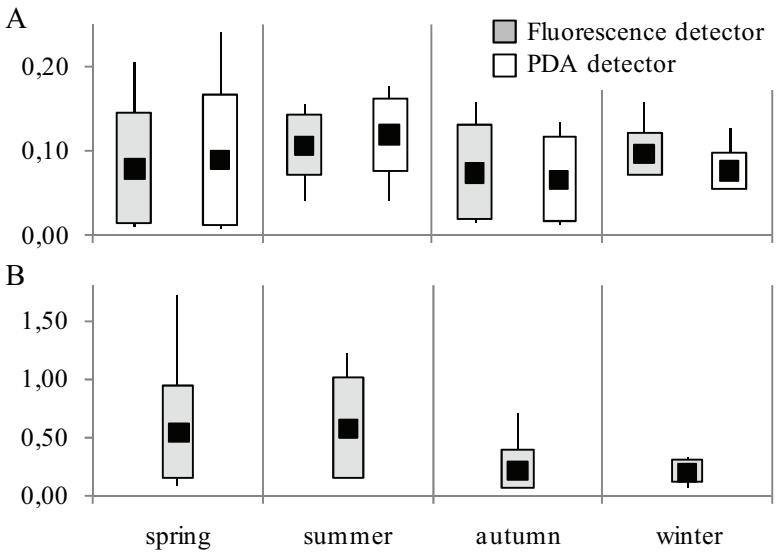
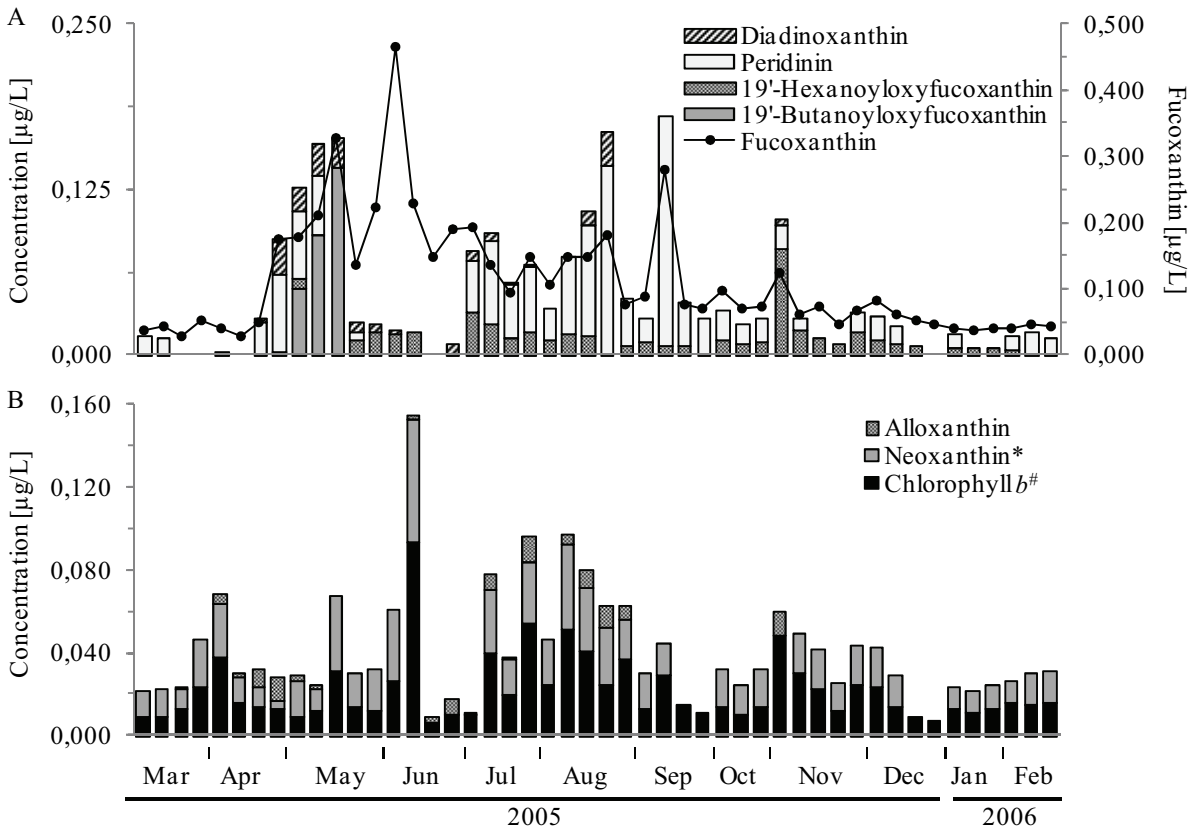


Figure 3



Chlorophyll *b* measured with Waters 2475 Multi λ Fluorescence Detector

* 9-cis isomer, present in green algae and Euglenophytes (Jeffrey et al., 1997).

Figure 4

Manuscript 2:

Assessment of picoplankton abundance and dynamics in the German North Sea by FISH, flow cytometry, fluorometry, HPLC and electron microscopy

Authors: Britta Knefelkamp, Daniel Vaulot, Nathalie Simon, Wenche Eikrem, Elodie Foulon, Sylvie Masquelier, Karen H. Wiltshire, Jahn Throndsen, Rudolf I. Amann, Gunnar Gerdts

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This manuscript combines the main part of the present doctorate work. It displays the findings of different, primarily quantitative, methods used to characterise the eukaryotic picoplankton community and provides statistical comparisons. The different approaches of each method allows for an estimation of specific advantages and flaws, as well as a very detailed description of the picoeukaryotes in the German Bight, including seasonal succession. Because FISH counts provided a qualitatively and quantitatively reliable picture about the picoplankton community, they were the focus of this study. They were compared to FCM, fluorometry and HPLC analyses, and assessed in relation to diverse environmental parameters. In addition, observations by EM were consulted to support distinct findings and to answer remaining questions. Thus, it was possible to confirm and enhance previous results regarding the abundance, diversity and succession of the picoplankton in temperate coastal ecosystems. Findings were compared to previous observations at Roscoff (English Channel) and Blanes (Mediterranean Sea).

Based on the concept developed together with the thesis committee and under the care of the corresponding supervisors of each method, the author organised and implemented the practical work and analyses of the results, and wrote the first draft of the manuscript.

ABSTRACT

Picoplankton samples were collected weekly in the German Bight at Helgoland Roads (54°11.3' N, 07° 54' E) over one year. The eukaryotic picoplankton community composition and dynamics were analysed by fluorescence *in situ* hybridisation (FISH), flow cytometry (FCM), pigment analysis (fluorometry and high-performance liquid chromatography (HPLC)) and electron microscopy (EM). Multivariate statistical analyses (detrended correspondence (DCA) and redundancy analyses (RDA)) were applied for inter-comparison of methods and estimation of the effects of environmental factors on the community. Using pigment analyses, we found that the pico-sized phytoplankton represented only a minor part of the total autotrophic biomass in the German Bight. Examinations by FISH revealed the eukaryotic picoplankton community to be taxonomically highly diverse but more "balanced" in its composition when compared to other coastal sites. Nonetheless, the picoeukaryotes were very dynamic in their composition and abundance in the German Bight throughout all seasons. Overall, the picoeukaryotes were dominated by chlorophytes, primarily Mamiellales (Prasinophyceae). Regarding the influence of environmental parameters, the change in water temperature correlated most to our group counts by FISH.

Key words: picoplankton, FISH, microbial diversity, microbial dynamics, microbial ecology, RDA

INTRODUCTION

The picoplankton consists of single-celled auto-, mixo- and heterotrophic prokaryotes and eukaryotes. These perform different functional roles in the marine ecosystem that are still not entirely understood (Vaulot et al., 2008; Worden and Not, 2008; Not et al., 2009). Although generally outnumbered by cyanobacteria, the picoeukaryotes are taxonomically more diverse and have a larger cell size (Worden et al., 2004; Bec et al., 2005; Worden and Not, 2008). Ecologically, they are very successful because of their simple niche requirements and high adaptability to diverse environmental conditions. For example, their heterotrophic fraction is able to efficiently use various energy resources (Vaulot et al., 2002), and the autotrophs are an important component of the primary production community (Not et al., 2008). This is particularly the case in estuarine and coastal waters, where they can occasionally even dominate the biomass of the overall autotrophic phytoplankton (Not et al., 2008). Though picoeukaryotes seem to be an essential part of the microbial food web, investigations on them are difficult, especially when using traditional methods. This is because of their small cell size, simple and uniform morphology, as well as their fragility. In contrast, molecular tools make it possible to reveal their high taxonomic diversity and complex ecological linkages in the marine environment (Viprey et al., 2008; Worden and Not, 2008).

In the present study, we used different complementary methods to obtain a reliable picture of the eukaryotic picoplankton community of the German Bight (North Sea). For example, flow cytometry (FCM) is a widely used method in biological oceanography and capable to quantify phototrophic picoeukaryotes and prokaryotes in natural samples (Li, 1994; Zubkov et al., 2000; Medlin et al., 2002; Marie et al., 2005; Vaulot et al., 2008). On the other hand, *in vivo* fluorescence and *in vitro* absorption techniques allow an estimation of the picophototrophic biomass contribution by analyses of size-fractionated chlorophyll *a* concentrations. Furthermore, fluorescence profiles and the use of so-called marker pigments give an impression about the composition of the autotrophic community, though an exact classification is not possible. This is because pigment concentrations and compositions are very heterogeneous and the individual pigment content and ratio of picoplanktonic algae have not been discovered yet (Guillou et al., 2004; Latasa et al., 2004; Vaulot et al., 2008). Whole-cell fluorescence *in situ* hybridisation (FISH) is a molecular method that allows detailed studies on the taxonomical composition (domain, division, order, genus and species) of the entire picoplankton community (Amann et al., 1995). It uses taxon-specific and labelled oligonucleotide probes, which hybridise to the complementary gene sequences within target organisms (Schönhuber et al., 1997; Not et al., 2002; Amann and Fuchs, 2008). They allow an easy counting of labelled cells under the epifluorescence microscope. The visualisation of single picoplankton cells in morphological detail is possible by using electron microscopy (EM), though it is not

suitable as a stand-alone identification method. This is because of similar morphologies of taxonomically different species and different morphologies within one taxonomic group or even one species due to different life stages.

The objective of the present study was to give a detailed analysis of the picoplankton community composition and its seasonal dynamics at Helgoland Roads by different methodological approaches. Statistical analyses were used to analyse correlations between different methods' results with a primary focus on FISH counts. Moreover, the influences of environmental factors on the picoplanktonic community composition were investigated to gain an impression about its ecological behaviour.

MATERIAL AND METHODS

Surface samples were taken weekly at Helgoland Roads (54°11.3' N, 07° 54' E) between March 2005 and March 2006. Water temperature (T) and secchi depth (secchi) were measured directly at sampling. Determination of salinity was performed using an inductive salinometer (GDTAutosal8400B Salinometer, Guildline, Ontario, Canada) followed by conversion to a salinity value using UNESCO tables (Cox, 1966; Grasshoff et al., 1999). The concentrations of nutrients (ammonium, nitrite, nitrate, silicate and phosphate) were measured photometrically (Grasshoff & Johannsen, 1974; Grasshoff et al., 1999). Global (GR) and photosynthetic active radiation (PAR), as well as wind data (speed (WS) and direction (WD)) are measured continuously by a land station on Helgoland. Data were kindly provided by the GKSS Institute for Coastal Research (Geesthacht, Germany). Wind-direction data were converted clockwise into numerical data (0 = North, 15 = NNW). For statistical analyses, environmental parameters measured at sampling time and their differences towards the previous sampling (Δ) were applied.

We prefiltered our seawater samples by two separate prefiltration steps over 10 and 3 µm isopore membrane filters (*Millipore*, TCTP and TSTP, diameter: 47 mm) with 200 mbar at the most to prevent cell disruption. The < 3 µm filtrate was used for the following methodological approaches.

Flow cytometry Sample aliquots of 1.5 ml were fixed with 6 µl of commercial 25% glutaraldehyde (final concentration: 0.1%; Marie et al., 2005). After remaining at room temperature for at least 15 minutes, samples were frozen at -80°C until analysis. A FACScalibur flow cytometer (BD Bioscience) was used with the CellQuest Pro software (BD Bioscience).

Light scattering was measured in a forward (FSC: 488/10 nm; related to cell size) and right angle (SSC: 488/10 nm; related to cell size and refractive index) to the light source. Fluorescence of the particles passing the laser beam was measured at FL 1 530/30 nm (bandpass, green fluorescence), FL 2 585/42 nm (bandpass, orange fluorescence from phycoerythrin), and FL 3 670 nm (longpass, red fluorescence from chlorophyll). Samples were analysed randomly and were briefly stirred before measurement. FACSFlow (BD Bioscience) was used as sheath fluid and the sample line was cleaned with distilled water after each sample. Results were quantified by weighting each sample before and after analysis. On average, a volume of 290 µl was measured within 3 minutes by a medium flow rate. According to their differing fluorescence profiles, cells were identified as picoeukaryotes, nanoeukaryotes, *Synechococcus* or cryptophytes, and quantitatively analysed in all samples. Since the samples were prefiltered onto 3 µm, the populations of cells identified as nanoeukaryotes and cryptophytes probably represent only the smallest size fraction of the whole nanoeukaryote and cryptophyte assemblages. These categories will be referred to as small nanoeukaryotes and small cryptophytes in the following text.

Fluorometry In the present study, subsamples of 25 ml each were measured five times by a laboratory fluorometer (bbe Moldaenke GmbH, Germany). This was used to measure the entire chlorophyll *a* concentration of the pre-filtered samples *in vivo* and to quantify the contribution of four spectral groups (Knefelkamp et al., submitted 1).

According to the similar content of accessory pigments in the light-harvesting complexes in phylogenetically related spectral groups of algae and cyanobacteria, these spectral groups exhibit characteristic excitation spectra of chlorophyll-fluorescence. The applied fluorometer defined 5 different wavelengths suitable for distinguishing the excitation spectra specific for spectral groups (Beutler et al., 2002). Thus, the excitation of each group at these 5 wavelengths results in a characteristic emission spectra, or norm spectra. These norm spectra allow the calculation of the contribution of each spectral group to the measured overall emission intensity (of chlorophyll *a* at 685 nm) of a water sample by curve-fitting procedures (Beutler et al., 2002). Spectral groups are defined as green-pigmented algae that contain chlorophyll *a*, *b* and xanthophyll (chloro-, eugleno-, prasinophytes), cyanobacteria that contain phycocyanin, brown-pigmented algae that contain chlorophyll *a*, *c* and xanthophyll, such as fucoxanthin or peridinin (diatoms and dinophytes), and reddish cryptophytes that contain chlorophyll *a*, *b* and phycoerythrin (Beutler et al., 2002; Beutler, pers. comm.).

HPLC For pigment analysis by high-performance liquid chromatography (HPLC), up to 7 l of each prefiltered sample, depending on the ease of filtration, were passed through 0.2 µm nylon membrane (*Whatman*) filters (Knefelkamp et al., 2007) with a water-tap-based vacuum pump. To each

membrane filter, 2 ml of 90% acetone (HPLC grade) was added and the filter was stored frozen at -80 °C. For pigment extraction, samples were sonicated for 90 min. on ice (*Bandelin Sonorex Super Rk 103/H*) and passed through a 0.2 µm filter (*Spartan 30/0.2 RC*). For pigment analysis, a Waters 2695 Separation Module and two different detectors were used: a Waters 2475 Multi λ Fluorescence Detector (excitation filter: 430 nm, emission filter: 650 nm) for a better analyses of chlorophyll *b*, and a Waters 996 Photodiode Array (PDA) Detector. The PDA detector measured the entire absorption within the wavelength range of 400 to 700 nm (PAR). The system ran with a Waters Empower software programme. The reversed-phase monomeric Waters Symmetry c_8 column (150 x 4.6 mm, 3.5 µm spherical particle size, 100 Å pore size, end-capped) was thermostated in the column oven at 25 °C (Zapata et al., 2000). The autosampler was cooled to 4°C and a 200 µl sample loop was used. To get sharper peaks, 50 µl distilled water (millipore) was injected before and after 100 µl of each sample (Wiltshire et al., 2000). For separation of the different polar pigments, the following mobile phases were used (Garrido et al., 2003): methanol (super gradient grade) as solvent A, 0.025 M aqueous pyridine solution (pH 5.0 with acetic acid) as solvent B and 100% acetone (HPLC grade) as solvent C. The gradient used is given in Table S1 in the supplementary material. Each run was performed with a flow rate of 1.3 ml min^{-1} and was completed after 9 minutes. 10% methanol was used to clean the HPLC system. Samples were analysed randomly, a blank subtraction with 90% acetone was carried out, and every 4 weeks a threefold measurement with three separate sample filtrations was done to enhance the reliability of the results (Knefelkamp et al., submitted 1). Every chromatogram was checked manually and if necessary, the baseline was adjusted to exclude wrong peak integrations caused by the software (for integration problems see Wright and Mantoura, 1997). The peak purity was checked (Rodríguez et al. 2006, Zapata et al., 2000) and pigments were identified by their PDA absorption spectra at 430 nm, their individual band ratios and their retention times within the separate runs (Jeffrey et al., 1997). The HPLC system was calibrated with external pigment standards (*DHI, Water & Environment*) to allow the quantification of marker pigments. To generate response factors (calibration curve), the wavelengths for photometric measurements and the extinction coefficients were taken from the according product information or Jeffrey et al. (1997), respectively. Standard concentrations were calculated with the Lambert-Beer law ($E_\lambda = \varepsilon_\lambda * c * d$).

FISH Samples (90 ml) were fixed with paraformaldehyde (1% final concentration) for one hour in the dark and filtered onto 0.2 µm GTTP filters. For removing cellular pigments, filters were subjected to an ethanol series (50, 80, 100%, 3 minutes each) and finally stored at -80 °C until further analysis. To encompass a wide range of eukaryotic picoplankton organisms, the HRP-labelled oligonucleotide probes listed in Table 1 were used. *In situ* hybridisation, signal amplification and target cell detection were carried out according to Not et al. (2002) and Biegala

et al. (2002), respectively. Filters were prepared and analysed randomly in respect to sample taking, but with all probes on the distinct filter pieces of one sample in parallel. Each probe was analysed under an epifluorescence microscope (Olympus Optical BX51) by counting of at least 10 squares (100 x 100 µm each) chosen arbitrarily on the filter sections. Hierarchical counts were checked for validity by comparing cell counts of the higher taxonomic level towards cell counts of the according lower taxonomic levels.

Of our non-zero counts of distinct probes, 270 were analysed by counting 30 squares (counting area of 0.3 mm^2) and 30 by counting 50 squares (counting area of 0.5 mm^2). Counting the threefold area resulted in an average decline of 5% and counting the fivefold area in an average decline of 30% towards the first count (Figure S1). Next to cell patchiness on the filters (Knefelkamp et al., 2007), we suggest that cells at the periphery of the analysed filter sections were lost more easily during sample preparation, whereas those in the centre possibly accumulated. While the centres of the filters were counted first because of the adjustment of the microscope, successive counts were carried out further towards the periphery. This can explain the decline in the average cell concentration. Nonetheless, the influence of preparation steps on the distribution of cells has to be further analysed to draw final conclusions.

Electron microscopy Subsamples of 100 ml each were transferred into a blown glass bottle, fixed with 1 ml lugol and 1 ml 25% glutaraldehyde, carefully mixed and stored in the dark at room temperature for at least one month. After settling of all picoplankton cells, the upper volume of 90 to 95 ml was pipetted off and the remaining sample was transferred into a 10 ml glass vial for further settlement. The upper sample was again pipetted off and the remaining 1-2 ml at the bottom were transferred into a 2 ml brown glass vial. After a settling period of a few days, a drop from the bottom of each vial was placed on a grid used for transmission electron microscopy. The grids were left to dry, and subsequently washed in distilled water to remove salt. Then they were dried again and contrasted on a drop (cell-side down) of 2% uranylacetate for 20 minutes. After washing and drying, samples were analysed with a CM 100 (*Philips*) transmission electron microscope. Whole grids were investigated and pictures of all organisms and scales were taken.

Statistics Multivariate analyses using CANOCO (version 4.53, Biometris 1997-2004) were performed with a detrended correspondence analysis (DCA). Because of linear gradients, we carried out direct redundancy analyses (RDA) for further analysis of relationships between response and explanatory variables. FISH counts, checked for validity and sorted by their hierarchical levels, served as response variables. Group counts by flow cytometry, spectral group concentrations, distinct pigment concentrations and environmental parameters were defined as

explanatory variables. Statistical settings of the CANOCO programme were carried out as described by Sapp et al. (2007).

RESULTS

Environmental conditions Within the sampling period, salinity at Helgoland Roads varied between 30.4 and 34.1 PSU, whereas the water temperature varied between 3.2 °C in February/March, and 17.4 °C between July and September (Figure S2). Secchi depth was lowest in December (1 m) and highest in June (12 m). Silicate and phosphate rapidly decreased from their highest values in March (SiO_4: 8.5 µmol l^{-1}, PO_4: 1.1 µmol l^{-1}) to their lowest in May (both ≤ 0.01 µmol l^{-1}). Nitrite and nitrate were lowest (both ≤ 1 µmol l^{-1}) in July/September and September, respectively. Highest values were measured in winter (NO_2: 3.9µmol l^{-1}, NO_3: 36 µmol l^{-1}). Ammonium was lowest in January, February and July (≤ 0.01 µmol l^{-1}) and highest in September (8.2 µmol l^{-1}). At sampling time (between 6 and 9 a.m.), the global radiation (GR) ranged between 0.4 W m^{-2} and 618.2 W m^{-2}, and the photosynthetic active radiation (PAR) between 21.9 µmol s^{-1} m^{-2} and 1564.0 µmol s^{-1} m^{-2}. Wind speed and direction were variable but most often the wind came from WNW (Figure S2). This indicates that the sampling site primarily lay at the lee coast of the island, with the possible consequence of it being less affected by wind perturbations at the surface.

Flow cytometry The sample counts by FCM (Figure 1) were used to calculate seasonal averages (13 samples per season). These showed a clear seasonal trend within the abundance of picoplankton. The highest average cell numbers were found in summer (June - August), when the four groups (picoeukaryotes, small nanoeukaryotes, *Synechococcus* and small cryptophytes) made up ~7,000 cells ml^{-1}. In spring (March - May) and autumn (September - November), they accounted for ~4,000 cells ml^{-1} and in winter (December - February) for ~3,000 cells ml^{-1}. *Synechococcus* cells showed a clear increase between spring (~840 cells ml^{-1}) and summer (~3,790 cells ml^{-1}) and slowly decreased again in autumn (~2,790 cells ml^{-1}) and winter (~1,970 cells ml^{-1}). Their highest concentration (> 16,000 cells ml^{-1}) occurred in July (Table S2 and Figure 1). Picoeukaryotes showed similar cell counts in spring and summer (~2,760 cells ml^{-1}), sharply decreased in autumn (~880 cells ml^{-1}) and slightly increased again in winter (~1,070 cells ml^{-1}). Their highest concentration (> 11,000 cells ml^{-1}) was found in June (Table S2 and Figure 1). Small nanoeukaryotes did not vary much in their seasonal average, but steadily decreased from ~360 cells ml^{-1} in spring towards ~130 cells ml^{-1} in winter. Their maximum value (~1,700 cells ml^{-1}) was measured in May (Table S2 and Figure 1). The region defined for counting small

cryptophytes represented only a fraction of the total concentration of pico-sized cells. Therefore, their equal numbers in spring and autumn, highest in summer (especially in August) and lowest in winter, can only be used as a proxy for the sum of all small cryptophytes in the picoplanktonic size fraction.

Fluorometry The *in vivo* fluorescence of the < 3 µm fraction revealed an annual average contribution of 30.3% to the total chlorophyll *a* in the waters around Helgoland (Figure S3). Please note that the measurement of fluorescence does not result in the concentration of pure chlorophyll *a* but in the sum with its degradation products, occurring, for example, in small cell fragments. Thus, the seasonal contributions of 36.7% in spring, 22.6% in summer, 24.3% in autumn and 38.3% in winter are not related to the mere chlorophyll *a* concentration of picoplanktonic organisms. However, brown-pigmented algae (cells and/or fragments of cells with brown accessory pigments) clearly made up the major part of the total chlorophyll concentration (82.9% on annual average). Their concentration peaked in spring (1.403 µg l^{-1}) and again in summer (1.137 µg l^{-1}) (Table S2, Figure 2A). Their respective average chlorophyll concentration was highest in summer (0.486 µg l^{-1}), slightly lower in spring (0.428 µg l^{-1}), and equally low in autumn and winter (0.200 and 0.202 µg l^{-1}, respectively). Green-pigmented algae (cells and/or fragments of cells with green accessory pigments) were most abundant in spring (0.083 µg l^{-1}) and summer (0.051 µg l^{-1}), and were not detected in autumn and winter. Small cryptophytes showed highest respective chlorophyll concentrations in summer (0.040 µg l^{-1}), lower ones in spring (0.027 µg l^{-1}) and autumn (0.020 µg l^{-1}), and were nearly absent in winter (0.006 µg l^{-1}). Cyanobacteria occurred only occasionally in very low concentrations. Results of replicate samples are discussed in detail in Knefelkamp et al. (submitted 1).

HPLC *In vitro* pigment analysis of the 0.2 - 3 µm fraction revealed highest average concentrations of the pure pigment chlorophyll *a* in summer (0.12 µg l^{-1}), whereas spring (0.09 µg l^{-1}), autumn (0.06 µg l^{-1}) and winter (0.08 µg l^{-1}) values were quite similar (Table S2, Figure 2B). Compared to the chlorophyll *a* concentration of the whole phytoplankton (all above 0.2 µm size; data not shown), the picoplankton contribution was 9% on annual average with the highest seasonal contribution in winter (19.5%). In spring (3.6%), summer (5%) and autumn (7.2%), the contribution was much lower. Results of replicate samples and the different detectors are discussed in detail in Knefelkamp et al. (submitted 1).

Fucoxanthin (considered as a signature pigment for diatoms and other stramenopiles) showed higher concentrations in spring (0.12 µg l^{-1}) and summer (0.17 µg l^{-1}), and slightly lower ones in autumn (0.09 µg l^{-1}) and winter (0.05 µg l^{-1}). Other pigments showing highest seasonal averages in summer were peridinin (0.04 µg l^{-1}; dinoflagellates), chlorophyll *b* (0.03 µg l^{-1}; green algae),

neoxanthin (0.02 µg l^{-1}; green algae and euglenophytes), alloxanthin (0.01 µg l^{-1}; cryptophytes) and βε-carotene (0.007 µg l^{-1}; Cryptophyta and green algae) (Table S2, Figure 2B). Diadinoxanthin (0.01 µg l^{-1}; brown algae) and 19'-butanoyloxyfucoxanthin (0.02 µg l^{-1}; pelago-, prymnesio- and chrysophytes) showed highest concentrations in spring, whereas 19'-hexanoyloxyfucoxanthin showed highest in autumn (0.01µg l^{-1}; prymnesiophytes, some dinoflagellates). The concentrations of diatoxanthin, prasinoxanthin and zeaxanthin were excluded from further analyses because these pigments were measured fewer than three times within the whole annual cycle and in negligible concentrations. Results of distinct pigment concentrations and successions are discussed in detail in Knefelkamp et al. (submitted 1).

FISH All probes revealed highest counts in summer or spring (Table S2, Figure 3). The mixture of three probes (EukMix = EUK1209R, CHLO01 and NCHLO01), meant to detect all eukaryotes within each sample, showed highest counts in summer (~3,910 cells ml^{-1}), second highest in spring (~2,200 cells ml^{-1}), and lowest in autumn (~500 cells ml^{-1}) and winter (~120 cells ml^{-1}). Probe EUK1209R alone, revealed a sharp increase in average cell counts between spring (~460 cells ml^{-1}) and summer (~1,860 cells ml^{-1}), and lower counts in autumn (~280 cells ml^{-1}) and winter (~160 cells ml^{-1}). However, counts by the EukMix and EUK1209R were found to be invalid. In comparison to counts by the according proportionate (in the case of the EukMix) or higher-level probes, respectively, they accounted for only a fraction of cell numbers. For example, the EukMix accounted for 54.5% of the counts by probe CHLO01 on annual average, and EUK1209R for 34.9% of the combined counts of probes NCHL01 and CHLO02. This indicated the missing of target cells by the EukMix and probe EUK1209R. Therefore, they were excluded from further statistical analyses. CHLO02 counts were similar in spring (~3,490 cells ml^{-1}) and summer (~3,650 cells ml^{-1}), and then declined in autumn (~1,580 cells ml^{-1}) and winter (~480 cells ml^{-1}). NCHLO01 counts were also similar in spring (~2,220 cells ml^{-1}) and summer (~2,490 cells ml^{-1}) and then decreased in autumn (~1,750 cells ml^{-1}) and winter (~120 cells ml^{-1}).

The sum of cell counts by PRAS01 (Pseudoscourfieldales), PRAS03 (Prasinococcales) and Pras04 (Mamiellales) corresponded to the CHLO02 counts with 92, 48, 43 and 48% (from spring to winter). This indicates their dominance in spring, but the dominance of other chlorophytes not detected by the applied class probes (Table 1) between summer and winter. Overall, the counts of all PRAS probes were highest in spring (~3,220 cells ml^{-1}), second highest in summer (~1,770 cells ml^{-1}), and lower in autumn (~690 cells ml^{-1}) and winter (~230 cells ml^{-1}). Between spring and winter they accounted for 88, 70, 75 and 68% of the sum of all class and order counts (Bolidophyceae, Pelagophyceae, Prasinophyceae and Prymnesiophyta), representing their clear dominance. Pelagophyceae accounted for 1 to 7% of the class and order counts throughout the

year with similar counts in spring (~130 cells ml^{-1}) and summer (~170 cells ml^{-1}) and negligible ones in autumn and winter (≤ 10 cells ml^{-1}). Prymnesiophyta have had a contribution of 3% in spring (~110 cells ml^{-1}), 15% in summer (~390 cells ml^{-1}), 11% in autumn (~100 cells ml^{-1}), and a peak of 19% in winter (~60 cells ml^{-1}). Bolidophyceae showed the lowest contributions to the class and order counts but highest counts in spring (~5%; 190 cells ml^{-1}) and summer (~8%; ~210 cells ml^{-1}), and higher contributions with lower counts in autumn (~13%; 120 cells ml^{-1}) and winter (12%; ~40 cells ml^{-1}). Towards counts by NCHL01, the sum of BOLI02 (Bolidophyceae), PELA01 (Pelagophyceae) and PRYM02 (Prymnesiophyceae) accounted for 19, 31, 13 and 92% between spring and winter.

Among all Prasinophyceae, Mamiellales (PRAS04) accounted for 47 and 50% in summer (~1,210 cells ml^{-1}) and winter (~170 cells ml^{-1}), and for 69 and 72% in spring (~2,620 cells ml^{-1}) and autumn (~630 cells ml^{-1}). Prasinococcales (PRAS03) on the other hand accounted for 16% (~570 cells ml^{-1}) in spring, 17% (~430 cells ml^{-1}) in summer, 3% (~30 cells ml^{-1}) in autumn and 11% (~40 cells ml^{-1}) in winter. Pseudoscourfieldales (PRAS01) showed the overall lowest contribution to the Prasinophyceae counts: 1% (~34 cells ml^{-1}) in spring, 5% in summer (~130 cells ml^{-1}), 3% in autumn (~30 cells ml^{-1}) and 7% (~20 cells ml^{-1}) in winter.

Looking at the sum of the separate genus probes of the dominating order Mamiellales (BATHY01 (*Bathycoccus prasinos*), OSTREO01 (*Ostreococcus*) and MICRO01 (*Micromonas pusilla*)), we found their highest seasonal abundance in summer (~2,180 cells ml^{-1}), the second highest in spring (~1,730 cells ml^{-1}) and lower ones in autumn (~230 cells ml^{-1}) and winter (~130 cells ml^{-1}). In summer, these sums exceeded counts by probe PRAS04, which should label all Mamiellales. Looking at the three probes targeting genera of the Mamiellales, *Bathycoccus prasinos* showed its highest contribution in spring (76% of genera counts, ~1,320 cells ml^{-1}), the lowest in summer (3%, ~60 cells ml^{-1}), and higher ones again in autumn (15%, ~30 cells ml^{-1}) and winter (53%, ~70 cells ml^{-1}). *Ostreococcus* accounted for only 3% of the Mamiellales genera in spring (~20 cells ml^{-1}) and winter (< 10 cells ml^{-1}), for 11% in summer (260 cells ml^{-1}) and 6% in autumn (~20 cells ml^{-1}). *Micromonas pusilla* reached its highest genera percentage of 85% in summer (~1,865 cells ml^{-1}) and made up 77% in autumn (~170 cells ml^{-1}), whereas its spring (~390 cells ml^{-1}) and winter (~60 cells ml^{-1}) contributions were 23 and 45%, respectively.

Micromonas clades A, B and C, represented by the probes MICROA01, MICROB01 and MICROC01 showed a clear shift among each other. Whereas clade A had its seasonal maximum in spring (~250 cells ml^{-1}) and accounted for 56% of *Micromonas* clade counts, it decreased during summer (~190 cells ml^{-1}, 20%) and autumn (~20 cells ml^{-1}, 15%) before it diminished in winter. Clade B showed low cell abundances in spring (~50 cells ml^{-1}, 11%), increased ones in

summer (~640 cells ml^{-1}, 67%) and decreased ones again in autumn (~125 cells ml^{-1}) and winter (~20 cells ml^{-1}) accounting for 80% of *Micromonas* counts in both seasons. Clade C showed similar counts in spring (~150 cells ml^{-1}, 33%) and summer (~120 cells ml^{-1}, 13%), and remained under 10 cells ml^{-1} in autumn (4%) and winter (20%). The sum of the three clade probes exceeded counts by MICRO01 in spring (112%), but were lower in summer (51%), autumn (90%) and winter (51%).

Electron microscopy Under the transmission electron microscope, we found bacteria, diverse flagellates, cells larger than 10 µm and various scales occurring within the samples. Detected organisms with their possible identity are listed in Table S3 and photo examples of organisms and scales are shown in Figure 4. EM results cannot be used for quantitative estimations because of a low settling rate of picoplankton and a possible high cell loss during preparation. Therefore, EM observations had to be excluded from further statistical analyses. Nonetheless, we found the highest diversity of picoplankton organisms in the sample taken on August 23rd 2005, whereas winter samples primarily revealed bacteria (Table S3).

Statistics On the whole, 52 samples (13 per season) were taken. Two had to be excluded, because FISH counts were zero for each probe and thus implied a methodological failure. For spectral group analyses, four additional samples had to be excluded because of lacking data. Furthermore, only probe counts valid towards the according lower-level counts were included. This resulted in the exclusion of 11 NCHL01 counts, 8 CHLO02 counts, 18 PRAS04 counts and 21 MICRO01 counts. Multivariate analyses (RDA) between methods (Table S4) showed the highest explainable total variance between FISH counts of genera (BATHY01, OSTREO01, MICRO01) and HPLC pigment concentrations (79.7%). Chlorophyll *b* had the highest marginal and conditional effects on the FISH counts. Compared to fluorometry, the highest explainable variance (51.8%) was found towards class and order counts (BOLI02, PELA01, PRYM02, PRAS01, 03, 04). Green-pigmented algae showed the highest effects. The methodological comparison between FISH and FCM counts showed the highest total explainable variance of 77.4% towards class and order counts and with picoeukaryotes being the only significant group. With pigment concentrations (HPLC), less than 50% of the total variance of FCM counts was explainable. The same percentage was true when explaining pigment concentrations by fluorometry.

Regarding the influence of environmental parameters of the distinct hierarchical FISH counts (Table 2), the change in water temperature towards the previous sampling was the only significant factor on group counts (NCHL01 and CHLO02). The highest explainable total variance with 90.5% was possible for the genera counts (BATHY01, OSTREO01, and MICRO01). The change

in water temperature showed highest marginal and conditional effects, but the change in salinity and nitrate concentrations were significant as well.

DISCUSSION

Methodological considerations Within our 10 and 3 µm prefiltered water samples we found diatoms larger than 10 µm (Table S3) and nano-sized eukaryotes (Figure 1). In addition, recent phylotype investigations revealed a dominance of nano- and microplanktonic organisms (alveolates (40.3%) and marine stramenopiles (26.4%)) among the identified phylotypes in the same filtrates (Knefelkamp et al., submitted 2). These findings indicate the abundance of larger-sized organisms in our picoplankton samples and allow the assumption that these organisms may have an influence on the analyses of the picoplankton. The high abundance of brown-pigmented algae (Figure 2A) and high concentrations of fucoxanthin (Figure 2B) did not correspond to our molecular cell counts. FISH counts by the probes that target some of the brown-pigmented algae, such as BOLI02, PELA01 and PRYM02, did not result in comparable findings. Furthermore, FISH counts revealed a dominance of chlorophytes (including green-pigmented algae) over non-chlorophytes (including brown-pigmented algae) (Figure 3). Because this proportion is not equivalent to our findings by HPLC and fluorometry, we suggest an influence of brown-pigmented algae from outside the picoplankton community. Furthermore, counts of non-chlorophytes rather correlated towards small nanoeukaryotes, fucoxanthin, 19'-hexanoyloxyfucoxanthin and brown-pigmented algae (data not shown). In combination with the occurrence of larger-sized organisms in the pico-size fraction, we conclude a biasing influence from larger-sized microalgae, such as nanoflagellates and/or diatoms (Medlin et al., 2006), fragments of them, resting spores and/or faecal pellets of their grazers on our analyses by HPLC and fluorometry. The occurrence of possible influences by the mentioned parameters on the smallest size fraction has been suggested before (Jeffrey et al., 1999, Jeffrey and Wright, 2004, Zapata et al., 2004, Not et al. 2005 and 2008, Wright and Jeffrey, 2005; Vaulot et al., 2008).

In our study, we found discrepancies between hierarchical probe counts in separate samples. These occurred between a) EukMix (EUK1209R + NCHLO01 + CHLO01) and the sum of separate EUK1209R, CHLO01 and NCHLO01 counts, b) NCHLO01 and the sum of BOLI01, PELA01 and PRYM02 counts, c) PRAS04 and the sum of BATHY01, MICRO01 and OSTREO01 counts, and d) between MICRO01 and counts of the three clade probes MICROA01, B01 and C01 (Figure 3). Such biases are known and generally attributed to difficulties in the labelling with and recognition of probes (Not et al., 2002 and 2004; Amann and Fuchs, 2008). For

example, an overlapping in the hybridisation by the more specific probes would lead to higher cumulative cell concentrations than counts by a more general probe. An irregular distribution of corresponding cells on the distinct filter sections is able to lead to discrepancies in counting as well. The reason for this can be the patchiness on the filter caused by filtration (Knefelkamp et al., 2007) or changes in the abundance of cells on the filter pieces during preparation (compare above). Nonetheless, biases in hierarchical probe counts may also result from the lack of knowledge about the actual species diversity in natural samples. This taxonomic diversity is suggested to be not yet discovered (Not et al., 2004; Medlin et al., 2006; Massana and Pedrós-Alió, 2008; Worden and Not, 2008; Worden et al., 2009) and therefore, it is possibly not yet represented by the amount of available sequences used to develop molecular probes.

The picoplankton community at Helgoland Three quarters of the detected non-chlorophytes remained unknown in our study (Figure 3). The rest was composed of prymnesiophytes, pelagophytes and bolidophytes that made up rather equal concentrations on annual average. Pelagophyceae did not reach previously described cell concentrations from the English Channel, but Bolidophyceae accounted for double the reported concentrations (Vaulot et al., 2008). Prymnesiophytes accounted for 10.1% of the non-chlorophytes in the present study and possibly comprised species of the genus *Chrysochromulina* (Table S3). Because only four *Chrysochromulina* species are reported to be smaller than 3 µm (Vaulot et al., 2008) and since we only found scales under the EM (Figure 4, Table S3), we cannot estimate their actual contribution to the pico-sized prymnesiophytes. Nonetheless, haptophytes were recently discovered to be more abundant and ecologically important in the marine phytoplankton than previously thought (Liu et al., 2009). This supports the previous suggestion of a possibly larger species number within the picoplanktonic size fraction as well (Medlin et al., 2006).

Our probe counts (Figure 3) and microscopic observations (Table S3) revealed chlorophytes and especially prasinophytes to be the most abundant picoeukaryotes in the German Bight. Their characteristic pigments (chlorophyll *b* and neoxanthin) and spectral group (green) additionally showed significant influences on our overall FISH counts (Table S4). Furthermore, DGGE and sequencing investigations on the same samples identified the most often occurring phylotype to be *Micromonas pusilla*, a prasinophyte (Knefelkamp et al., submitted 2). All these congruent findings by diverse methods support the suggested predominant position and consequently important ecological role of chlorophytes, particularly prasinophytes, in the microbial food web of temperate coastal waters (Vaulot et al., 2008; Viprey et al., 2008; Worden and Not, 2008; Simon et al., 2009). Nonetheless, our findings do not allow any further conclusions about their relationships towards other organisms, such as larger microalgae or grazers, and dependencies on environmental parameters.

Among the chlorophytes, we found Mamiellales dominating with 57.5% on annual average (Figure 3). In comparison, Pseudoscourfieldiales clade V was of minor quantitative importance for the picoeukaryotic community, though it was abundant throughout all seasons (Figure 3, Table S2). Because it was reported with even lower numbers from the Skagerrak and the English Channel (Vaulot et al., 2008), we suggest it to have a minor role in microbial food webs, though we cannot draw sustainable ecological conclusions yet. Prasinococcales clade VI made up 11.6% of our chlorophyte counts, but it was not a permanent part of the picoplanktonic community (Figure 3). It primarily occurred in spring and summer, with its overall abundance correlating to the positive change in water temperature (data not shown). At times, it even dominated the cumulative counts of the three prasinophyte classes and moreover, we found higher cell concentrations (Figure 3, Table S2) than previously reported for its abundance in coastal waters (Vaulot et al., 2008). We therefore suggest its higher abundance in more eutrophic waters.

Ostreococcus, one of the three investigated Mamiellales genera, was present throughout the year but only once accounted for higher numbers (Figure 3). In comparison to *Bathycoccus* and *Micromonas*, it showed the lowest correlation towards environmental parameters (data not shown) and therefore we suggest it to be an opportunistic picoeukaryote with minimal niche requirements. These findings are congruent with the current knowledge and support its ecological role as a background organism (Rodríguez et al., 2005; Derelle et al., 2006; Vaulot et al., 2008). *Bathycoccus* and *Micromonas* showed an alternation in their seasonal abundance and dominance among genera counts. Whereas *Bathycoccus* accounted for higher abundances in spring and winter, *Micromonas* dominated in summer and autumn. These findings confirm and expand previous suggestions about their differing ecology (Worden and Not, 2008; Simon et al., 2009). For example, *Bathycoccus* is known to occur from polar to mediterranean waters and to efficiently adapt to low-light conditions (Vaulot et al., 2008; Guillou et al., 2004; Marie et al., 2006). In our study, it was able to better deal with low temperatures and radiation values in comparison to *Micromonas* (Figures 3 and S2). *Micromonas* on the other hand, has always been considered to be the major and dominating picoeukaryote in nutrient-rich and/or coastal regions, especially in cold waters (Not et al., 2004 and 2005; Foulon et al., 2008, Vaulot et al., 2008; Worden et al., 2009). Although it dominated the Mamiellales in the German Bight with 47.1% on annual average, it was in an inferior position towards *Bathycoccus* in winter and spring, the nutrient-rich and cold seasons (Figures 3 and S2). We therefore suggest its occurring ecotypes or clades to be better adapted to summer conditions, or a higher influence of other controlling factors, such as grazers and/or viruses, on *Bathycoccus* during summer. Overall, our probe counts revealed the additional occurrence of organisms labelled by the more universal probes but not the more specific ones (Figure 3). Regarding the Mamiellales, our EM observations

(Table S3) indicated the abundance of cf. *Mantoniella* and cf. *Mamiella*, but we were not able to quantify or analyse their occurrence any further.

Our results of the *Micromonas* clades show their numbers to be similar in spring, with clade B dominating for the rest of the year (Figure 3). This is congruent with the difference between clades A and C towards clade B that we found in our statistical analyses: clades A and C were similarly influenced by the investigated environmental parameters, whereas clade B seemed to prefer different niche conditions (data not shown). Nonetheless, our findings do not support the suggestion of clade B being adapted to warm, well-illuminated coastal waters (Foulon et al., 2008), because it also dominated our winter counts. Thus, it either can adapt to a broad temperature range or different subclades occur in parallel (Foulon et al., 2008) in the German Bight. Clade A did not sustain its role as the most abundant clade in coastal waters (Foulon et al., 2008) and rather showed a steady decrease in its abundance from spring to winter around Helgoland. On the other hand, clade C was of much higher abundance in our samples than expected from previous observations (Foulon et al., 2008). It made up 33% of the clade counts in spring and 20% in winter, supporting its suggested low-light adaptation (Foulon et al., 2008).

Ecological considerations RDA of inter-species distances of taxa-specific FISH counts using environmental factors (Table 2) revealed that the change in water temperature, the concentration of phosphate and nitrate, as well as the change in salinity and the global radiation at sampling did have significant influences on the picoplankton community. Co-linearity existed towards the water temperature in all analyses, and occasionally also towards the photosynthetic active radiation (PAR), the secchi depth and the silicate concentration, indicating their additional importance. When considering the environmental correlations to the other methods' results (data not shown), pigment concentrations (HPLC) showed highest significant influences by the PAR at sampling but with fucoxanthin correlating more than chlorophyll *b*. Other significant factors were the wind direction, secchi depth, silicate concentrations, global radiation, changes in the ammonium concentration and salinity values. Towards spectral group abundances, only changes in salinity and silicate concentrations were significant. Regarding FCM counts, the change in temperature was significant and positively correlated to the abundance of picoeukaryotes. This variation of different significant environmental parameters represents the overall low dependency of the picoplankton on abiotic factors. This is in conjunction with their high adaptability and minimal niche requirements (Vaulot et al., 2002; Worden and Not, 2008). Nonetheless, community members exhibit an annual succession that seems to be caused by more individual and specific preferences, including the possible occurrence of grazers and viruses. We found picoplanktonic chlorophytes, especially Mamiellales including *Bathycoccus prasinos*, to be highly abundant in

early spring. This is congruent to their observed higher correlation to high nitrate concentrations (data not shown) and the overall high correlation of picoeukaryotes towards positive changes in temperature (Table 2). Non-chlorophytes, detected by the non-chlorophyte probe but not by the class and order specific probes, were highly abundant in late spring. Early summer on the other hand, showed high concentrations of fucoxanthin (Figure 2B) and small nanoeukaryotes (Figure 1). Because we did not observe correlating changes in our FISH counts (Figure 3), we suggest larger microalgae (diatoms, including *Rhizosolenia* (Table S3)), their cell fragments and/or faecal pellets of their grazers to bias our analyses by HPLC and FCM. However, within the course of summer, the picoeukaryotic community presented very high chlorophyte concentrations, especially of *Micromonas pusilla* (Figure 3). This is congruent with its clades showing a significant correlation towards global radiation values (Table 2). In addition, we were able to find most organisms under the EM in August, but without clear dominances (Table S3). In late summer, peridinin concentrations (Figure 2B), as well as numbers of small nanoeukaryotes and small cryptophytes (Figure 1) increased. We therefore suggest higher abundances of nano-sized dinoflagellates around Helgoland that biased our pigment analyses by their cell fragments or faecal pellets of their grazers. In autumn and winter, the picoeukaryotic community was rather "balanced" in its composition and no definite conclusions about the dominance or succession of distinct groups were possible (Figures 1, 2 and 3). Furthermore, EM observations primarily revealed the exclusive presence of bacteria (Table S3).

Comparisons to previous findings At three European coastal sites, in the surface waters at Helgoland (Helgoland Roads, 54°11.3' N, 07° 54' E) and Roscoff (Astan, 48°46'N, 3°56'W) and in the deeper waters (-20 m) at Blanes (Blanes Bay, 41° 40' N, 2°48' E), the picoplankton community has been shown to be highly diverse and to change within short time scales (Not et al., 2004; Medlin et al., 2006). At Blanes, it is dominated by the two cyanobacteria genera *Prochlorococcus* and *Synechococcus*, with its eukaryotic fraction showing maximal numbers in winter (Zhu et al., 2005). In the German Bight and the English Channel, only *Synechococcus* occurs as a representative of the cyanobacteria and seems to consist of different clades with changing seasonal abundances (unpublished data). The picoeukaryotes on the other hand, show maximal numbers in spring and summer, with a suggested dominance of their heterotrophs (Medlin et al., 2006). This is supported by recent phylotype findings on the same samples like those analysed in the present study (Knefelkamp et al., submitted 2). To find out about the size relation within the primary production community, we analysed the contribution of picoautotrophs to the overall chlorophyll *a* concentration, used as a proxy of photosynthetic biomass (Figure S3) (Knefelkamp et al., submitted 1). The eutrophic German Bight generally enhances the growth of larger microalgae. It therefore shows the highest overall chlorophyll *a* concentration when compared to the English

Channel and the Mediterranean Sea. The picophototrophs account for only a small fraction compared to the other sites. We calculated a contribution of 9% to the pure chlorophyll *a* (HPLC) and of 30.3% to the total chlorophyll *a* (as estimated by fluorometry) on annual average. The high proportion recorded with fluorometry is probably caused by the excessive abundance of degradation products within the smallest size fraction (0 to 3 μm) that contribute to the analysed picoplanktonic fluorescence. Previous investigations found a picoplanktonic chlorophyll *a* contribution of 13.6% in the German Bight, whereas at Roscoff they accounted for 37% and at Blanes for 52.7% (unpublished data). Thus, the present study supports the picophototrophic role as a background population in temperate coastal waters (Not et al., 2002, 2005 and 2008; Medlin et al., 2006, Rodríguez et al., 2005; Derelle et al., 2006; Vaulot et al., 2008).

At all sites, the autotrophic picoeukaryotes were dominated by chlorophytes, Mamiellales (Prasinophyceae) in particular. The Mamiellales accounted for 78.6% of the chlorophytes at Roscoff (Prasinococcales: 3.4%), for 53.9% at Blanes, and for 46% at Helgoland (Prasinococcales: 9%, Pseudoscourfieldiales: 3%). Regarding the composition of its genera *Micromonas*, *Bathycoccus* and *Ostreococcus*, our results differ towards previous findings (Medlin et al., 2006), although these discrepancies are possibly caused by methodological variations and/or biases. However, *Micromonas* is the best-represented group at all three sites, but with different average proportions (Figure 5). It seems to be in a rather suppressed position in the German Bight, whereas the other sites enhance its growth. Regarding its clades, clade A and B seem to vary in their dominance in the English Channel, whereas clade C is rather negligible (Foulon et al., 2008). In the German Bight, clade B clearly dominates, whereas clades A and C account for more similar numbers and react equally towards environmental parameters.

CONCLUSIONS

In the present study, we used different complementary methods to obtain a reliable picture of the eukaryotic picoplankton community of the German Bight (North Sea). The methods applied reflected different perspectives and thus, it was possible to describe the composition and succession of the picoeukaryotes in great detail. Our findings highlight the need for the careful interpretation of pigment profiles in natural samples, and for methodological improvements. For example, FISH probes targeted at narrower taxonomic groups appear to provide more reliable results. Thus, future improvements of this method should involve the higher specification of probes towards smaller groups of target organisms to enhance the probes' reliability.

In addition, the possibility to cultivate picoeukaryotes need urgent enhancement. Most picoplanktonic groups are still not represented in culture (Little et al., 2008; Piganeau et al., 2008), although only cultivation tests allow the investigation of structural and physiological adaptation characteristics and fine-scale descriptions of ecological niches and functional roles of picoplankton organisms (Foulon et al., 2008; Vaulot et al., 2008; Viprey et al., 2008; Simon et al., 2009; Worden et al., 2009). Thus, identification of morphological differences by electron microscopy, the checking of probe specificity, and the definition of individual pigment profiles to specify and adjust quantification capabilities by HPLC, for example, by the matrix factorisation program CHEMTAX (Mackey et al., 1996; Irigoien et al., 2004), are only possible when organisms are present in culture. Furthermore, cultivation tests allow investigation on and conclusions about picoplankton grazers and viral attacks (Guillou et al., 2001; Vaulot, 2001; Evans et al., 2003), and consequently an estimation of the ways in which picoplanktonic carbon reaches higher trophic levels (Shurin et al., 2006; Worden and Not, 2008).

Overall, our results confirmed and enhanced current knowledge about the eukaryotic picoplankton within temperate coastal waters. The picoeukaryotes at Helgoland Roads seemed to form a steady background population, as it was suggested and reported previously for coastal temperate waters (Not et al., 2002, 2005 and 2008; Medlin et al., 2006, Rodríguez et al., 2005; Derelle et al., 2006; Vaulot et al., 2008). Although their community is suggested to exhibit an immense and mostly unknown taxonomic diversity (Biegala et al., 2003; Massana and Pedrós-Alió, 2008; Masquelier and Vaulot, 2008), it was largely dominated by chlorophytes and Mamiellales (Prasinophyceae). The taxonomic composition of this group was more "balanced" than at other coastal sites such as the coastal English Channel or the Blanes bay, where *Micromonas pusilla* dominated the assemblage all year round and with higher proportions. Our findings improved the understanding of the abundances and dynamics of distinct groups, for example, the seasonal alternation between *Bathycoccus* and *Micromonas*, and the dominance of *Micromonas* clade B around Helgoland. Nonetheless, the ecological characterisation of the spatial and temporal occurrence of single groups, clades or even ecotypes, needs further investigation. The cause of growth limitation of the picoeukaryotes still needs to be definitely identified as well. In our study, diverse environmental parameters showed significant influences on the growth and mortality of the pico-size fraction. Because the infection by viruses is suggested to account for only part of the population decline (Zingone et al., 1999; Evans et al., 2003), we suggest grazers to have the most influential impact on the abundance of picoeukaryotes. To prove this hypothesis, further investigations on the trophic interactions in the microbial food web of the marine ecosystem need to be carried out.

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Table S4: Tables showing the marginal and conditional effects of significant variables ($p \leq 0.05$) in methodological RDAs of (A) FISH versus FCM, (B) FISH versus HPLC pigments, (C) FISH versus fluorometrical spectral groups, and (D) FCM versus HPLC and HPLC versus fluorometry. FISH counts were grouped by their taxonomic level and checked for validity by higher level probe counts prior to analyses. RDA's were carried out with inter-species distances and no variance inflation factors above 20 occurred. The total explainable variance corresponds to the sum of the canonical Eigenvalues.

Figure S1: Difference between counting 10 squares of a sample in FISH analyses by epifluorescence microscopy and counting 30 or 50 squares. On average, 30 squares resulted in a decline of 5% of the initial cell concentration calculated by counting 10 squares, and 50 squares in a decline of 30%. Calculations are based on 270 samples for 30 squares and 30 samples for 50 squares.

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Figure S3: Chlorophyll *a* contribution of picoplanktonic organisms to the overall phytoplankton community, measured by HPLC (pure chlorophyll *a*) and fluorometry (total chlorophyll *a*).

Table 1

Probe	Sequence (5‘ → 3‘)	Target group	Source
EUK1209R	GGG CAT CAC AGA CCT G	Eukaryotes[#]	Giovannoni et al. (1988)
CHLO01	GCT CCA CGC CTG GTG GTG	Chlorophyta[#]	Simon et al. (1995)
CHLO02	CTT CGA GCC CCC AAC TTT	Chlorophyta[#]	Simon et al. (2000)
NCHLO01	GCT CCA CTC CTG GTG GTG	Non-Chlorophyta[#]	Simon et al. (1995)
BOLI02	TAC CTA GGT ACG CAA ACC	Bolidophyceae (Heterokontophyta)	Guillou et al. (1999)
PELA01	ACG TCC TTG TTC GAC GCT	Pelagophyceae (Heterokontophyta)	Simon et al. (2000)
PRYM02	GGA ATA CGA GTG CCC CTG AC	Prymnesiophyta (Haptophyta)	Simon et al. (2000)
PRAS01	ACG GTC CCG AAG GGT TGG	Pseudoscourfieldiales clade V*	Not et al. (2004)
PRAS03	GCC ACC AGT GCA CAC CGG	Prasinococcales clade VI*	Not et al. (2004)
PRAS04	CGT AAG CCC GCT TTG AAC	Mamiellales clade II except *Dolichomastix**	Not et al. (2004)
BATHY01	ACT CCA TGT CTC AGC GTT	*Bathycoccus prasinos**	Not et al. (2004)
OSTREO01	CCT CCT CAC CAG GAA GCT	*Ostreococcus**	Not et al. (2004)
MICRO01	AAT GGA ACA CCG CCG GCG	*Micromonas pusilla**	Not et al. (2004)
MICROA01	CCG TCA AGA GGC CGC GGT	*Micromonas pusilla clade A**	Foulon et al. (2008)
MICROB01	CAC GAC CAA CAG ACG GTT	*Micromonas pusilla clade B**	Foulon et al. (2008)
MICROC01	ACG GCG GCG AAC CGC AAT	*Micromonas pusilla clade C**	Foulon et al. (2008)

[#] The specificity of probes has been revised in Not et al. (2002 and 2004).
* Chlorophyta

Table 2

	Marginal effects	Conditional effects		
	Lambda1	LambdaA	P	F
A) FISH groups (NCHL01 and CHLO02): total explainable variance: 76.3%; significance: 0.042				
Delta temperature	0.20	0.20	0.004	8.45
B) FISH classes and orders (BOLI02, PELA01, PRYM02, PRAS01, 03 and 04): total explainable variance: 75%; significance: 0.38				
PO_4	0.20	0.20	0.012	7.33
Delta temperature	0.06	0.16	0.022	7.20
C) FISH genera (BATHY01, OSTREO01 and MICRO01): total explainable variance: 90.5%; significance: 0.052				
Delta temperature	0.12	0.12	0.046	3.64
Delta salinity	0.09	0.13	0.046	4.46
NO_3	0.06	0.11	0.028	4.22
D) FISH clades (MICROA01, B01 and C01): total explainable variance: 52.3%; significance: 0.288				
Global radiation	0.08	0.08	0.028	4.15

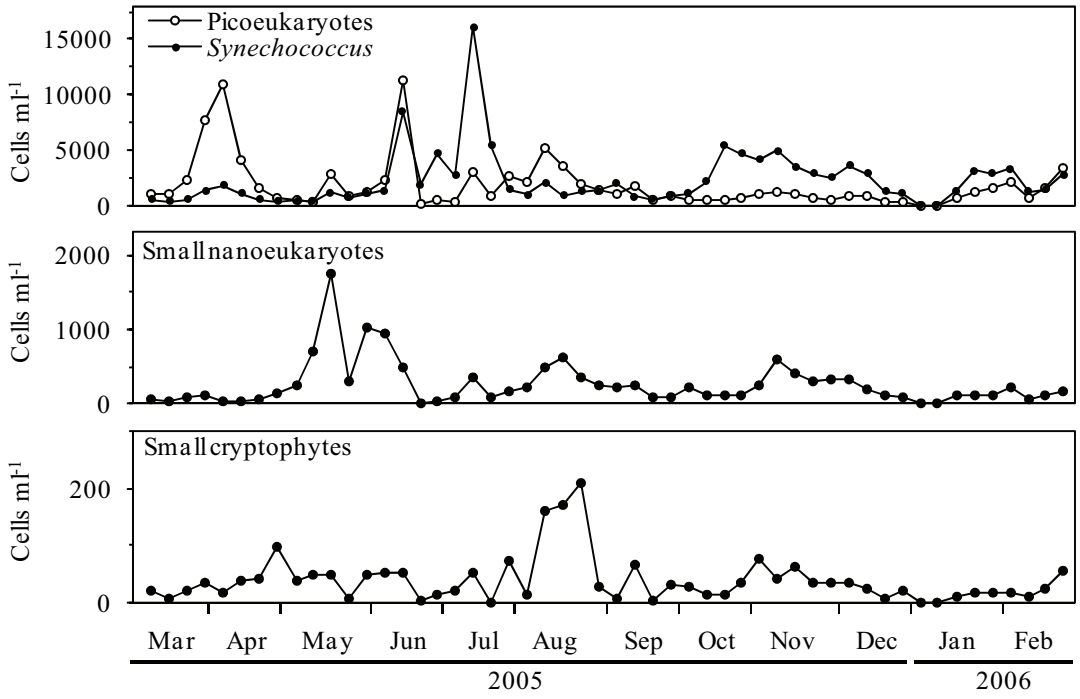
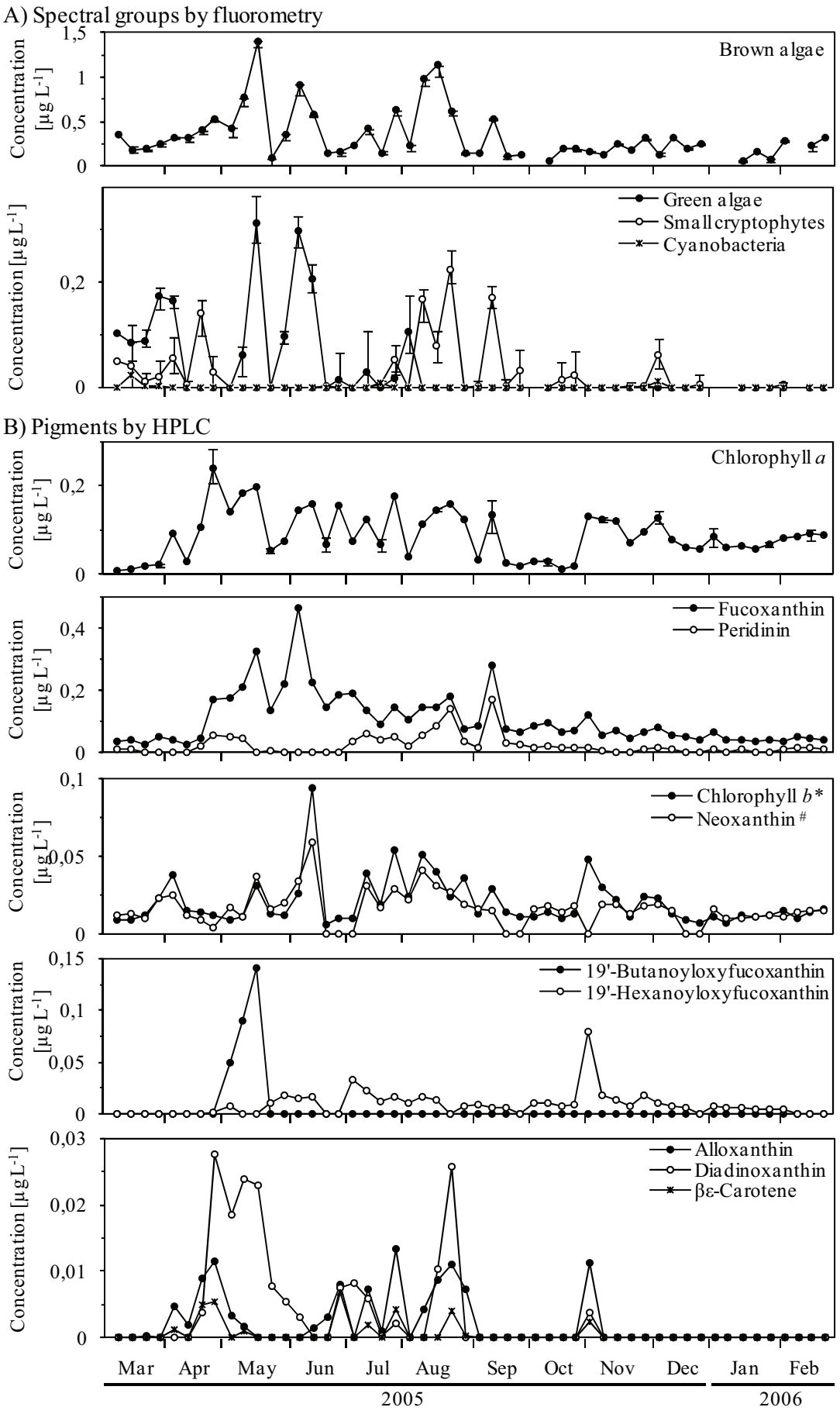

Picoeukaryotes
Synechococcus
Cells ml-1
15000
10000
5000
0
Small nanoeukaryotes
2000
1000
Small cryptophytes
200
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Feb
2005
2006

Figure 1



* Chlorophyll *b* measured with Waters 2475 Multi λ Fluorescence Detector

9-cis isomer, present in green algae and Euglenophytes (Jeffrey et al., 1997).

Figure 2

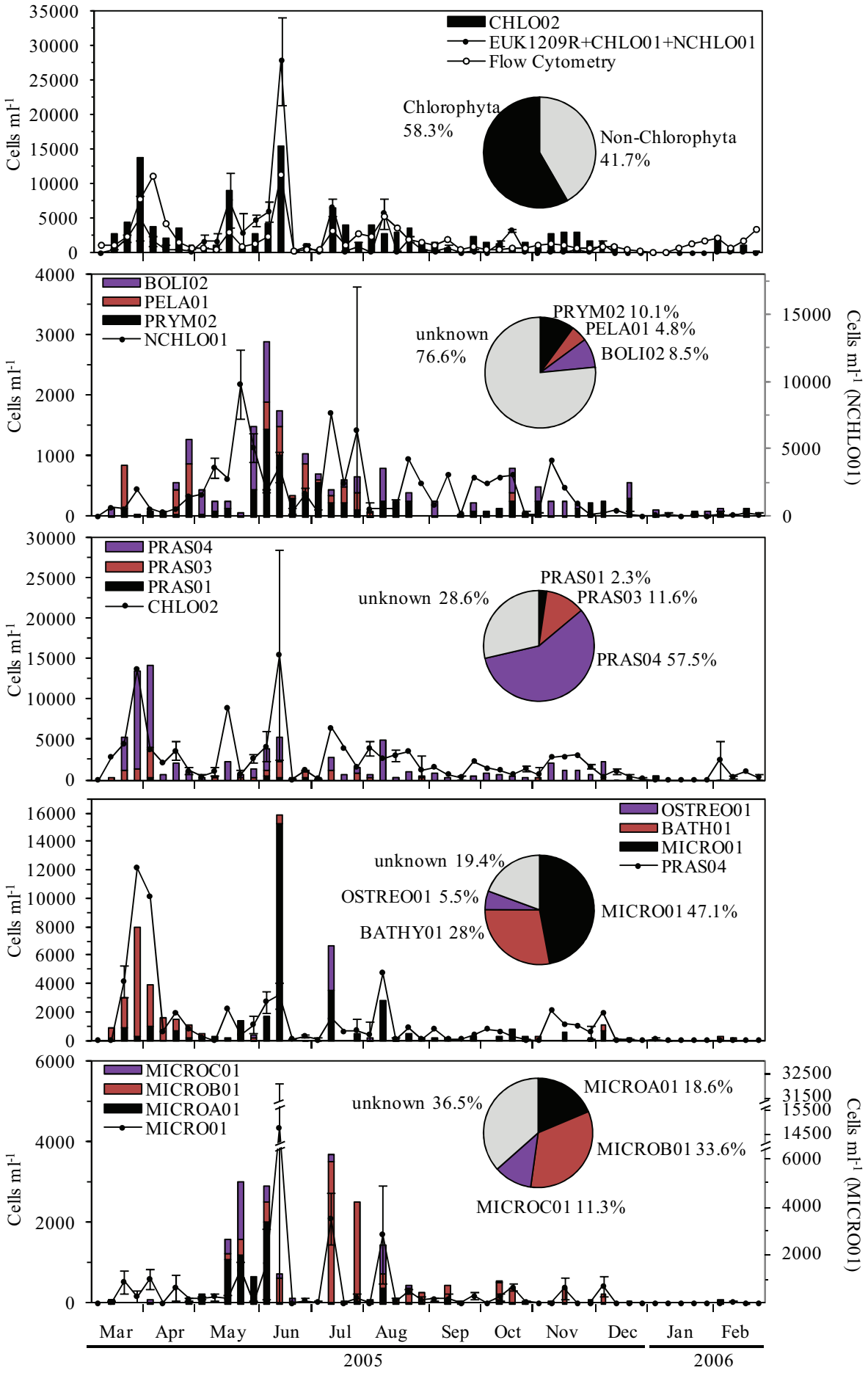

CHLO02
EUK1209R+CHLO01+NCHLO01
Flow Cytometry
Chlorophyta 58.3%
Non-Chlorophyta 41.7%
Cells ml-1
BOLI02
PELA01
PRYM02
NCHLO01
unknown 76.6%
PRYM02 10.1%
PELA01 4.8%
BOLI02 8.5%
Cells ml-1 (NCHLO01)
PRAS04
PRAS03
PRAS01
CHLO02
unknown 28.6%
PRAS01 2.3%
PRAS03 11.6%
PRAS04 57.5%
OSTREO01
BATH01
MICRO01
PRAS04
unknown 19.4%
OSTREO01 5.5%
BATHY01 28%
MICRO01 47.1%
MICROC01
MICROB01
MICROA01
MICRO01
unknown 36.5%
MICROA01 18.6%
MICROB01 33.6%
MICROC01 11.3%
Cells ml-1 (MICRO01)
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Feb
2005
2006

Figure 3

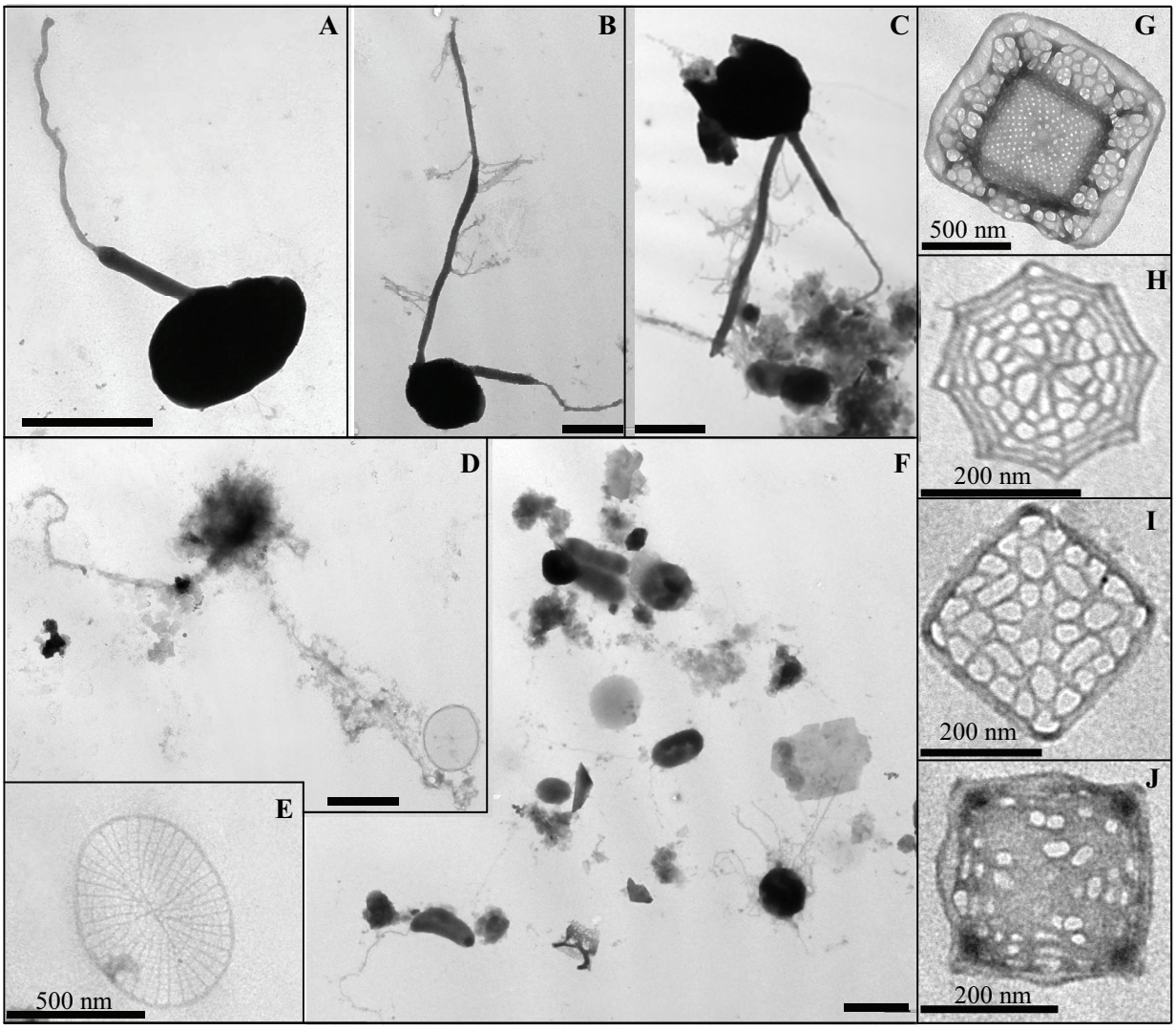


Figure 4

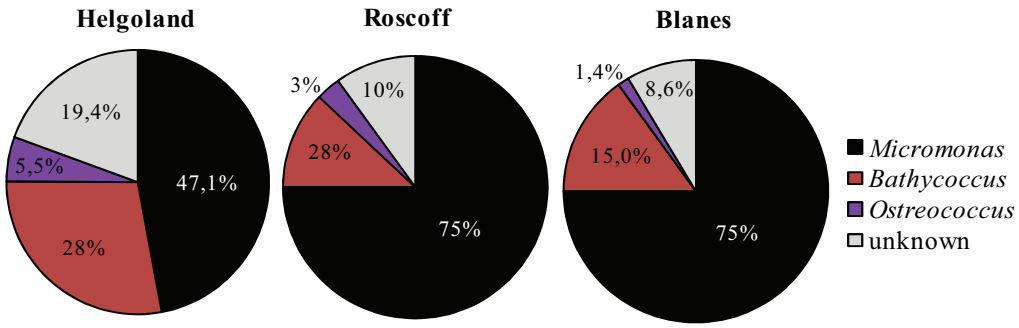


Figure 5

Table S1

Time	**A**	**B**	**C**
0	80	12	8 %
4	80	0 %	20
8.5	80	0 %	20
8.51	80	20	0 %
9	80	20	0 %

Table S2

	Minimum					Maximum		Average			
		number of occurrence in									
	Concentration	spring	summer	autumn	winter	Concentration	Date	spring	summer	autumn	winter
FISH [cells ml^{-1}]											
EukMix	0	1	0	3	9	27731	14.06.2005	2201	3912	499	115
EUK1209R	0	6	3	5	6	19368	14.06.2005	458	1864	276	155
CHLO02	0	1	0	0	5	15508	14.06.2005	3491	3652	1580	477
NCHL01	0	1	0	0	3	9810	24.05.2005	2217	2490	1747	116
BOLI02	0	5	3	6	9	1056	31.05.2005	191	207	124	39
PELA01	0	10	4	12	11	704	22.03.2005	125	174	10	6
PRYM02	0	3	1	4	8	1453	07.06.2005	112	389	100	63
PRAS01	0	10	3	10	10	440	07.06.2005	34	131	25	24
PRAS03	0	5	3	9	8	3830	05.04.2005	566	433	29	37
PRAS04	0	2	0	1	9	12149	29.03.2005	2616	1206	631	166
BATHY01	0	4	9	10	8	7659	29.03.2005	1317	63	34	68
OSTREO01	0	12	9	10	12	3169	12.07.2005	17	255	18	3
MICRO01	0	2	3	6	11	15252	14.06.2005	393	1865	174	58
MICROA01	0	8	9	9	13	2025	07.06.2005	247	193	24	0
MICROB01	0	9	3	8	10	3521	12.07.2005	46	640	125	24
MICROC01	0	9	7	11	11	1453	24.05.2005	147	122	7	6
Flow [cells ml^{-1}]											
Picoeukaryotes	0	0	0	0	2	11262	14.06.2005	2766	2761	878	1070
Small nanoeukaryotes	0	0	0	0	2	1754	17.05.2005	356	323	242	129
Synechococcus	13	0	0	0	0	16111	12.07.2005	842	3792	2786	1970
Small cryptophytes	0	0	1	0	2	209	23.08.2005	37	66	35	19
HPLC [µg L^{-1}]											
Chlorophyll *a*	0,007	1	0	0	0	0,240	25.04.2005	0,009	0,120	0,065	0,077
Chlorophyll *b**	0,006	0	1	0	0	0,094	14.06.2005	0,016	0,033	0,019	0,013
19-Butanoyloxyfucox.	0,000	9	13	13	13	0,141	17.05.2005	0,022	0,000	0,000	0,000
19-Hexanoyloxyf ucox.	0,000	9	3	1	4	0,080	01.11.2005	0,003	0,012	0,015	0,004
Alloxanthin	0,000	6	2	12	12	0,013	26.07.2005	0,002	0,005	0,001	0,000
Diadinoxanthin	0,000	6	5	12	13	0,027	25.04.2005	0,008	0,005	0,000	0,000
Fucoxanthin	0,027	0	0	0	0	0,465	07.06.2005	0,117	0,173	0,093	0,050
Neoxanthin[#]	0,000	0	3	3	2	0,059	14.06.2005	0,016	0,024	0,013	0,011
Peridinin	0,000	6	4	2	5	0,174	13.09.2005	0,016	0,041	0,028	0,008
βε-Carotene	0,000	9	8	12	13	0,007	28.06.2005	0,001	0,001	0,000	0,000
Spectral groups [µg L^{-1}]											
Green	0,000	5	7	11	9	0,311	17.05.05	0,083	0,051	0,000	0,000
Cyanobacteria	0,000	10	12	12	9	0,021	15.03.05	0,002	0,001	0,000	0,001
Brown	0,054	0	0	1	0	1,403	17.05.05	0,428	0,486	0,200	0,202
Small cryptophytes	0,000	5	7	5	8	0,223	23.08.05	0,027	0,040	0,020	0,006

* Chlorophyll *b* measured with Waters 2475 Multi λ Fluorescence Detector

[#] 9-cis isomer, present in green algae and Euglenophytes (Jeffrey et al., 1997).

Table S3

Date	detected organisms and scales
22.03.2005	(cyano-)bacteria
29.03.2005	(cyano-)bacteria, flagellates (cf. *Micromonas*), cf. *Bathycoccus*
05.04.2005	(cyano-)bacteria
12.04.2005	(cyano-)bacteria
19.04.2005	(cyano-)bacteria
26.04.2005	(cyano-)bacteria
04.05.2005	(cyano-)bacteria, flagellates
10.05.2005	(cyano-)bacteria, flagellates
17.05.2005	(cyano-)bacteria, flagellates
24.05.2005	(cyano-)bacteria
31.05.2005	(cyano-)bacteria, cf. *Rhizosolenia longiseta*, scales (cf. *Mantoniella*)
07.06.2005	(cyano-)bacteria, cf. *Micromonas*, cf. *Bathycoccus* or *Ostreococcus*, scales (cf. *Pyramimonas disomata*)
14.06.2005	(cyano-)bacteria
21.06.2005	(cyano-)bacteria
28.06.2005	(cyano-)bacteria, flagellates
05.07.2005	(cyano-)bacteria, flagellates, cf. *Rhizosolenia longiseta*
19.07.2005	(cyano-)bacteria, cf. *Bathycoccus* or *Ostreococcus*, scales (cf. *Pyramimonas disomata*)
02.08.2005	(cyano-)bacteria, flagellates, cf. *Micromonas*, scales (cf. *Mamiella gilva* or *Pyromonas*; dinoflagellates)
09.08.2005	(cyano-)bacteria, flagellates, cf. *Bathycoccus* or *Ostreococcus*, scales (cf. *Mamiellales*)
16.08.2005	(cyano-)bacteria, cf. Anabaena, scales (cf. *Mamiella gilva*)
23.08.2005	(cyano-)bacteria, flagellates, cf. *Micromonas*, cf. *Bathycoccus* or *Ostreococcus*, cf. *Chrysochromulina*, cf. *Florenciella parvula*, cf. *Rhizosolenia longiseta*, cf. *Pachysphaera* or *Pterosperma*, scales (cf. *Cymbomonas tetramitiformis* or *Pyramimonas disomata*, cf. *Pyramimonas grossii*)
30.08.2005	(cyano-)bacteria
06.09.2005	(cyano-)bacteria
13.09.2005	(cyano-)bacteria, flagellates, cf. *Bathycoccus* or *Ostreococcus*, cf. *Rhizosolenia longiseta*, scales (cf. *Cymbomonas tetramitiformis* or *Pyramimonas disomata*, cf. *Chrysochromulina*)
20.09.2005	(cyano-)bacteria, cf. *Bathycoccus* or *Ostreococcus*, cf. Rhizosolenia longiseta
27.09.2005	(cyano-)bacteria, flagellates, cf. *Bathycoccus* or *Ostreococcus*, cf. Rhizosolenia longiseta, scales (cf. Pyramimonas grossii)
04.10.2005	(cyano-)bacteria
18.10.2005	(cyano-)bacteria, cf. *Bathycoccus* or *Ostreococcus*
25.10.2005	(cyano-)bacteria, cf. *Rhizosolenia longiseta*
01.11.2005	(cyano-)bacteria
15.11.2005	(cyano-)bacteria, flagellates
29.11.2005	(cyano-)bacteria
06.12.2005	(cyano-)bacteria
20.12.2005	(cyano-)bacteria
27.12.2005	(cyano-)bacteria
24.01.2006	(cyano-)bacteria
31.01.2006	(cyano-)bacteria
07.02.2006	(cyano-)bacteria, flagellates
14.02.2006	(cyano-)bacteria
21.02.2006	(cyano-)bacteria
28.02.2006	(cyano-)bacteria, cf. *Rhizosolenia longiseta*

Table S4

	Marginal effects	Conditional effects		
	Lambda1	LambdaA	P	F
A) FISH versus FCM				
FISH groups (NCHL01 and CHLO02): total explainable variance: 63.5%; significance: 0.002				
picoeukaryotes	0.40	0.40	0.002	17.69
Synechococcus	0.30	0.17	0.002	10.64
FISH classes and orders (BOLI02, PELA01, PRYM02, PRAS01, 03 and 04): total explainable variance: 77.4%; significance: 0.002				
picoeukaryotes	0.76	0.76	0.002	94.64
FISH genera (BATHY01, OSTREO01 and MICRO01): total explainable variance: 62.9%; significance: 0.002				
picoeukaryotes	0.40	0.40	0.002	17.69
Synechococcus	0.30	0.17	0.002	10.64
FISH clades (MICROA01, B01 and C01): total explainable variance: 40.7%; significance: 0.004				
Synechococcus	0.26	0.26	0.006	17.18
small nanoeukaryotes	0.11	0.12	0.010	8.40
B) FISH versus HPLC				
FISH groups (NCHL01 and CHLO02): total explainable variance: 66.6%; significance: 0.006				
Neoxanthin	0.41	0.41	0.002	23.24
Chlorophyll *b*	0.34	0.08	0.020	4.66
FISH classes and orders (BOLI02, PELA01, PRYM02, PRAS01, 03 and 04): total explainable variance: 72.9%; significance: 0.006				
Neoxanthin	0.15	0.15	0.028	5.26
19'- Butanoyloxyfucoxanthin	0.00	0.19	0.002	15.33
FISH genera (BATHY01, OSTREO01 and MICRO01): total explainable variance: 79.7%; significance: 0.004				
Chlorophyll *b*	0.65	0.65	0.002	50.22
Diadinoxanthin	0.01	0.03	0.040	3.80
FISH clades (MICROA01, B01 and C01): total explainable variance: 44.4%; significance: 0.054				
Fucoxanthin	0.15	0.15	0.018	8.72
Chlorophyll *b*	0.13	0.11	0.018	6.85
C) FISH versus fluorometry				
FISH groups (NCHL01 and CHLO02): total explainable variance: 47.6%; significance: 0.006				
Green algae	0.43	0.43	0.002	22.84
FISH classes and orders (BOLI02, PELA01, PRYM02, PRAS01, 03 and 04): total explainable variance: 51.8%; significance: 0.012				
Green algae	0.26	0.26	0.008	9.59
Brown algae	0.03	0.19	0.006	10.14
FISH genera (BATHY01, OSTREO01 and MICRO01): total explainable variance: 46.6%; significance: 0.006				
Green algae	0.39	0.39	0.002	14.98
FISH clades (MICROA01, B01 and C01): total explainable variance: 13.9%; significance: 0.156				
D)				
FCM versus HPLC: total explainable variance: 47.6%; significance: 0.016				
Chlorophyll *b*	0.29	0.29	0.002	19.39
HPLC versus fluorometry: total explainable variance: 47.1%; significance: 0.002				
Brown algae	0.37	0.37	0.002	25.49
Green algae	0.21	0.07	0.004	5.54

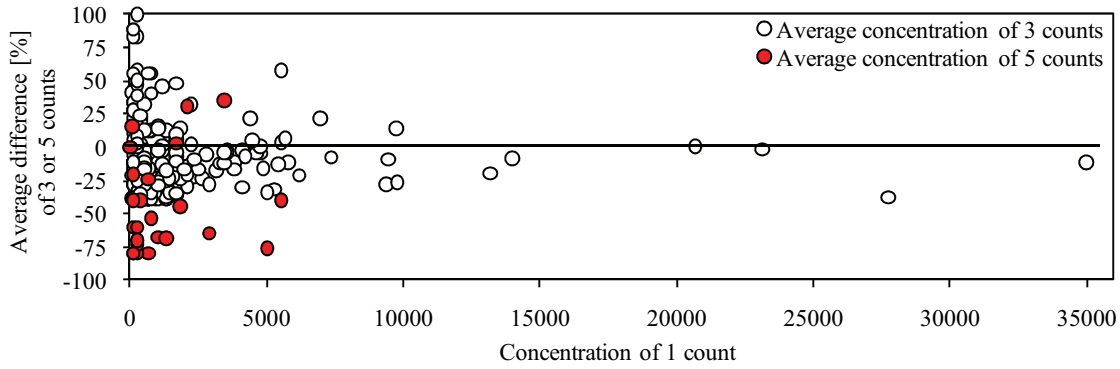

Average concentration of 3 counts
Average concentration of 5 counts
Average difference [%] of 3 or 5 counts
100
75
50
25
0
-25
-50
-75
-100
0
5000
10000
15000
20000
25000
30000
35000
Concentration of 1 count

Figure S1

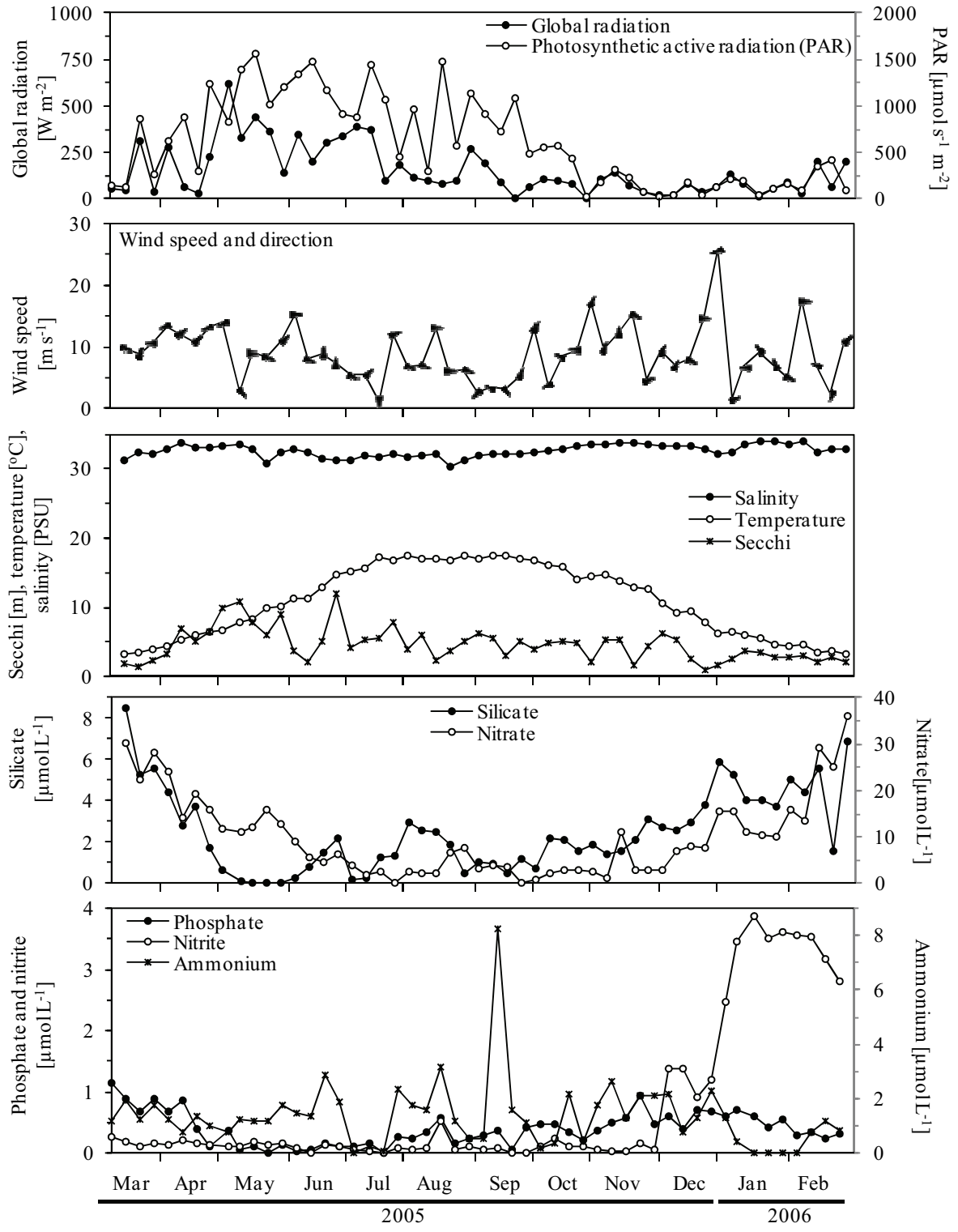

Global radiation
Photosynthetic active radiation (PAR)
Global radiation [W m-2]
PAR [µmol s-1 m-2]
Wind speed and direction
Wind speed [m s-1]
Salinity
Temperature
Secchi
Secchi [m], temperature [°C], salinity [PSU]
Silicate
Nitrate
Silicate [µmol L-1]
Nitrate [µmol L-1]
Phosphate
Nitrite
Ammonium
Phosphate and nitrite [µmol L-1]
Ammonium [µmol L-1]
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Feb
2005
2006

Figure S2

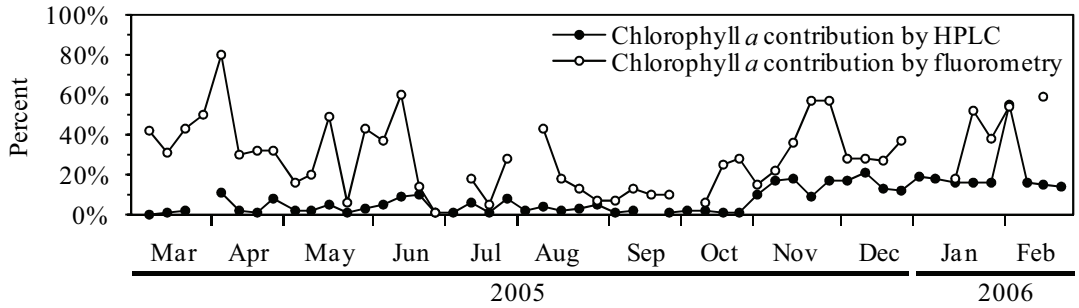

100%
80%
60%
40%
20%
0%
Percent
Chlorophyll a contribution by HPLC
Chlorophyll a contribution by fluorometry
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Feb
2005
2006

Figure S3

Manuscript 3:

Feasibility to assess the diversity, occurrence and ecology of picoplanktonic eukaryotes by DGGE and sequencing

Authors: Britta Knefelkamp, Gunnar Gerdts, Robert Niestroj, Karen H. Wiltshire, Antje Wichels

Published in: Aquatic Microbial Ecology, submitted

This publication presents the qualitative results obtained by DGGE and sequencing analyses. Findings provided a detailed insight into the taxonomic diversity of the picoeukaryotes of the German Bight, but were not quantitatively comparable to those results presented in the previous manuscripts. They were used complementarily and supported distinct findings and suggestions discussed in the previous manuscripts.

The fingerprints of each sample were used for comprehensive community analyses. The number of occurring phylotypes served for the estimation of taxonomic diversities in the samples. Identified phylotypes were tracked in their natural abundance throughout the sampling period. Thus, the seasonal diversity and succession of phylotypes were described and analysed to support and deepen previous findings.

Based on a concept by A. Wichels and G. Gerdts, the author's contribution was the organisation and implementation of DGGE analyses and sample preparation for sequencing, as well as analyses of the results and writing of the first draft of the manuscript.

ABSTRACT

Picoeukaryotic organisms are known to be basic for the marine ecosystem, but their identity and ecological function are still far from being understood. Hence, the present study aims to analyse the identity, taxonomic diversity and succession of the picoeukaryotes in the German Bight. For this approach, samples were taken weekly at Helgoland Roads (54°11.3' N, 07° 54' E) between March 2005 and July 2006 and investigated by Denaturing Gradient Gel Electrophoresis (DGGE). Bands of partial 18S rRNA gene fragments were excised, sequenced and, in conjunction with the obtained fingerprints of each sample, used for comprehensive community analyses. In our study, we observed a high number of different band classes (DGGE bands of identical nucleotide sequence within different samples). Their majority occurred only occasionally but were not restricted to distinct seasons. Overall, the taxonomic diversity of the picoeukaryotic community was highest in autumn, lowest in spring, and highly variable in summer. The seasonal composition of DGGE profiles differed, though the identified phylotypes were largely dominated by heterotrophs, primarily alveolates (especially ciliophores and group II alveolates) and marine stramenopiles (MAST). Nevertheless, the phylotypes most often found were attributed to the prasinophytes and picobiliphytes.

Key words: picoeukaryotes, marine ecosystems, diversity, ecological function, community dynamics, DGGE, sequencing

INTRODUCTION

The taxonomic diversity of the picoplankton (< 3 µm) community is harboured by the auto- and heterotrophic picoeukaryotes (Worden and Not, 2008). In comparison to the prokaryotic fraction, these are more abundant in eutrophic coastal regions (Biegala et al., 2003; Masquelier and Vaulot, 2008) with a high diversity of distinct taxa coexisting (Massana and Pedrós-Alió, 2008). Their heterotrophs perform yet inestimable functional roles (Vaulot et al., 2002), whereas the autotrophs primarily form a steady background population. In terms of chlorophyll *a*, these only occasionally dominate over larger microalgae (Not et al., 2008), if they do at all (Knefelkamp et al., in prep.). Today, the picoeukaryotic community is known to perform a minimalistic way of life (Bryant, 2003) and of being able to efficiently adapt to changing environmental conditions and energy resources (Romari and Vaulot, 2004; Not et al., 2005; Vaulot et al., 2002 and 2008). Its members are an important component for the functioning of the microbial food web and the overall marine ecosystem (Vaulot et al., 2008). Their exploration by rather traditional methods, like microscopy and cultivation, is generally hampered by their very small cell size, high fragility, and morphological similarity (Massana et al., 2004a; Romari and Vaulot, 2004, Šlapeta et al., 2006). Thus, the organisms' abundances and taxonomic diversities became particularly apparent with the development of molecular approaches (Martin et al., 2005; Not et al., 2007a; Thornhill et al., 2007; Worden and Not, 2008). These also allowed comprehensive studies on the ecological importance of picoeukaryotes and exposed differences in their niche compartments (Medlin et al., 2002; Thornhill et al., 2007). For example, qualitative fingerprinting methods such as DGGE (Denaturing Gradient Gel Electrophoresis) use the separation of amplified DNA fragments on a polyacrylamide gel. The resulting sample profiles provide information about the occurrence and succession of different phylotypes, and sequencing of excised bands reveal the taxonomic composition of the organism community (Díez et al., 2001a and b; Medlin et al., 2002; Vaulot et al., 2008).

In the present study, we describe the results of our DGGE and sequencing analyses of the eukaryotic picoplankton community sampled on a weekly interval in the German Bight. We analyse its taxonomic diversity and succession over a period of 17 months, and present the identity and taxonomic relationships of single phylotypes. Furthermore, statistic approaches are used to analyse the individual responses of the picoeukaryotes towards selected environmental parameters.

METHODS

Surface samples were taken weekly at Helgoland Roads (54°11.3' N, 07° 54' E) between March 2005 and July 2006. The samples were prefiltered over 10 µm Isopore™ Membrane Filters (*Millipore*, TCTP, diameter: 47 mm) with 200 mbar at the most to prevent cell disruption. Then they were prefiltered through 3 µm Isopore™ Membrane Filters (*Millipore*, TSTP, diameter: 47 mm) and finally collected onto a 0.22 µm Sterivex™ capsule filter (*Millipore*, GP) with the pressure not exceeding 1 bar. The filters were flushed with cold STE-Buffer and stored at -80 °C.

DNA extraction DNA was extracted by chemical lysis from the Sterivex filters using a modified protocol of Doyle and Doyle (1987). Each filter was incubated with 7.5 ml CTAB Extraction-buffer (3 % cetyltrimethylammonium bromide, 100 mM TRIS-HCL (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 0.2% 2-mercaptoethanol) at 65°C for 2 hours for re-suspension of picoplankton cells. Lysates were recovered from the Sterivex units with a syringe, combined with the according STE-Buffer, and centrifuged. The top phase was further extracted using equal amounts of chloroform-isoamyl alcohol (24:1), and a phenol-chloroform-isoamyl alcohol extraction (23:24:1) was carried out to remove proteins. The nucleic acid was precipitated over night with isopropanol and washed with ethanol (80%). DNA was finally suspended in 50 µl H_2O and its integrity was checked by agarose gel electrophoresis (0.8% (w/v)) before it was stored frozen (-20 °C) until further analysis.

PCR Amplification for DGGE PCR reaction mixtures contained 10 µl 10 x Eppendorf®Taq-Buffer, 15 µl 5 x Eppendorf®Master enhancer, 3 µl 10mM dNTP Promega, 2 µl 20µM primer 516r - GC (5'- ACC AGA CTT GCC CTC C GGG GGG CAC GGG GGC GGG GCG GGC CCC GCG CGG GGC CCG C -3'; Díez et al., 2001a), 2 µl 20µM primer EUK1A (5'- CTG GTT GAT CCT GCC AG -3'; Díez et al., 2001a), 2U Eppendorf®Taq DNA Polymerase and 0.5 to 3 µl of template DNA, and were filled up to 100 µl with H_2O. The, for eukaryotes specific, primers amplified the DNA fragment between positions 4 and 563. The PCR programme used with a Mastercycler® (Eppendorf, Germany) was 3 min at 94°C, 35 cycles of 2 min at 94°C and 1 min at 54 °C, 1 min at 68°C for denaturing, annealing and extension, and a final elongation for 6 min at 68 °C. Amplification of PCR products was confirmed by electrophoresis on a 1.2% (w/v) agarose gel.

DGGE The 6% polyacrylamide gels (Díez et al., 2001a) had a denaturing gradient of 10 to 55% urea/formamide (100% was defined as 7 M urea and 40% deionised formamide). The individual volumes of PCR products were run for 16 hours at 100 V and 60°C in 0.5 x TAE buffer (20 mM Tris base, 10 mM sodium acetate, 0.5 mM EDTA), with an environmental sample as internal

marker. The gel was stained with SYBR-gold (10 ml 0.5 TAE-Buffer and 1 µl SYBR-gold) for 20 minutes, photographed and bands were excised. Digitised DGGE profiles were analysed manually.

Reamplification of excised bands and DNA sequencing Prominent DGGE bands which connected or separated samples were excised and eluted according to Sambrook et al. (1989). PCR amplification and DGGE separation were carried out as before, but for those bands initially excised from the upper parts of DGGE gels, reamplified PCR products were applied on a gel with a denaturing gradient of 10-40%. This was done to achieve a better separation of close-sized bands. Sequencing was performed by QIAGEN® GmbH (Hilden, Germany) using an ABI PRISM® 3700 DNA Analyzer (Applied Biosystems, California, USA). Sequencing primers were Euk1A and 516r without a GC-clamp.

Nucleotide sequence accession numbers The sequences reported in this paper have been deposited in the GenBank database under the accession numbers .. to .. and the according identities used in our study are displayed in Table 1.

Phylogenetic analysis Sequence data were checked for the presence of PCR amplified chimeric sequences by the CHECK_CHIMERA programme (Cole et al., 2003). The ARB software package (http://www.arb-home.de) was used for phylogenetic analysis (Ludwig et al., 2004). After addition of the sequences to the ARB 18S rDNA sequence database (release May 2005), alignment was carried out with the Fast Aligner integrated in the programme and refined by comparison of closest relatives retrieved by BLAST (http://www.ncbi.nlm.nih.gov). Newly published sequences were included and manually aligned. Sequences with more than 1300 nucleotides were used to calculate phylogenetic trees and the ARB "parsimony interactive" tool to add partial sequences to the respective trees. Taxonomic relationships were deduced by the neighbour-joining method, including the correction algorithm of Felsenstein (1993).

Statistics Multivariate analysis of fingerprints was performed by non-metric Multi-Dimensional Scaling (MDS), analysis of similarities (ANOSIM), matched similarity matrices (RELATE), and biota-environment matching (BIO-ENV) of the PRIMER software suite (version 6.1.6, Primer-E 2006). For a comparison of sample profiles, a virtual DGGE gel was designed (Figure 1) and imported into PRIMER as a binary matrix. It was analysed by the qualitative Jaccard similarity, ignoring the lack of bands in distinct samples. The extreme values were S' = 0 for no similarity of two samples and S' = 100 if two samples had all bands in common. Ordination of Jaccard similarities was performed by non-metric MDS using 25 random starting configurations of sample points. To test the profile similarities between weeks, months and seasons, analyses were conducted by the

sub-routine ANOSIM. The testing results display the degree of differences with R = 0 indicating a total similarity and R = 1 a complete separation of sample profiles.

The DGGE fingerprints were additionally analysed in relation to the environmental parameters measured at sampling time: temperature (T), secchi depth (secchi), salinity (PSU), nutrient concentrations (NH_4, NO_2, NO_3, SiO_4 and PO_4), global radiation (GR) and photosynthetic active radiation (PAR). The Jaccard similarity matrix based on DGGE sample profiles and the dissimilarity matrix based on normalised Euclidean distances of environmental variables were compared by the RELATE routine calculating a similarity matrix by using the rank correlation by Spearman. Then the best correlation between the biological community composition and one or a combination of environmental variables was tested. The comparison provides a test of significance with the matching coefficient ρ_m, which is equivalent to the Mantel's test. A ρ_m value of 0 indicates no relation between the two matrices, whereas a ρ_m near 1 indicates the ability of the measured variables to explain the community data. The sub-routine BIO-ENV of the PRIMER software compares sample profiles to all combinations of the environmental variables and identifies the best explaining variables for the similarity between DGGE fingerprints. Furthermore, CANOCO^{TM} (version 4.53, Biometris 1997-2004) was used to perform a detrended correspondence analysis (DCA) that revealed the unimodality of the profiles when using environmental factors. Thus, a canonical correspondence analysis (CCA) was carried out to test the dependency of profiles on the calculated environmental variables. Data were not transformed prior to analysis and an automated forward selection was used to analyse inter-sample distances. Statistical settings were carried out as described in detail by Sapp et al. (2007).

RESULTS

Environmental conditions Radiation values were kindly provided by the GKSS Institute for Coastal Research (Geesthacht, Germany). All other environmental conditions analysed in our study are routinely measured for the Helgoland time series. Within the sampling period, salinity ranged between 30.4 and 34.1 PSU and the water temperature between 3.2 °C in February/March and 17.4 °C between July and September. Secchi depth was lowest in December (1 m) and deepest in June (12 m). Silicate and phosphate concentrations rapidly decreased from their highest values in March (SiO_4: 8.5 µmol/L, PO_4: 1.1 µmol/L) to their lowest in May (both ≤ 0.01 µmol/L). Nitrite and nitrate were lowest (both ≤ 1 µmol/L) in July/September and September, respectively, and highest in winter (NO_2: 3.9µmol/L, NO_3: 36 µmol/L). Ammonium was lowest in July, January and February (≤ 0.01 µmol/L) and highest in September (8.2 µmol/L). At sampling

time (between 6 and 9 a.m.), the global radiation (GR) ranged between 0.4 W/m^2 and 618.2 W/m^2 and the photosynthetic active radiation (PAR) between 21.9 µmol*s/m^2 and 1564.0 µmol*s/m^2.

The RELATE analysis between the Jaccard similarity of DGGE fingerprints and the normalised Euclidean distance of environmental variables revealed a ρ_m of 0.336 (significance level 0.1%), thus indicating a small relation between the two matrices. The best explaining environmental parameters were the secchi depth, temperature, salinity, PO_4 and NO_3. Together, these parameters explained 39.4% (significance level 0.1%) of the resulting Euclidian distance between DGGE sample profiles. CCA analysis revealed temperature, salinity, PO_4 and NO_2 to be significant (for $p \leq 0.05$) and axes 1 and 2 explaining 38.9% of the variation between profiles (Table 2). Biplot scaling (Figure 2) showed winter samples being more positively correlated to NO_2 and salinity values, whereas spring samples correlated towards PO_4 and summer samples towards temperature. Nonetheless, the majority of sample profiles did rather less correlate towards abiotic conditions.

Diversity of the picoeukaryotes Within the distinct samples, we obtained between 9 and 46 amplified 18S rRNA gene fragments, being defined as band classes (Figures 1 and 3). We used these quantities to analyse the diversity of the picoeukaryotes (Figure 4). On average, it was lowest in spring 2006 (24 bands on average) and highest in autumn 2005 (32 bands on average). The greatest variability occurred in summer 2005 with the highest weekly change of 27 bands between July 19th and 26th (Figure 5), whereas the lowest variability in band quantities was detected in spring in both years.

Multivariate analyses revealed that the DGGE fingerprints of all samples were separated to different degrees from each other with a rather random assembly. The ANOSIM analyses revealed that the monthly profiles (March 2005 to February 2006) were more dissimilar towards each other ($R = 0.437$, significance level 0.1%) than the four seasons (spring 2005 to winter 2005/2006; $R = 0.328$, significance level 0.1%). Nonetheless, a correspondence between months within distinct seasons was indicated (data not shown). However, regarding the difference between single seasons (Table 3, Figure 6), autumn and winter profiles showed the greatest discrepancy ($R = 0.501$), whereas spring and summer samples showed the lowest one ($R = 0.292$). On the original DGGE gels (Figures 1 and 5), the most visible seasonal difference was observed between summer and winter populations.

Identity, occurrence and seasonality of obtained phylotypes We were able to distinguish 154 different band classes (Figures 1 and 3). Of these, 119 (77.3%) were detected in less than a quarter of all samples throughout the sampling period. 14 band classes (9.1%) were detected in more than half of all samples taken (Figures 1 and 3). Looking on their overall seasonal occurrence (Figure

7), most of the band classes (29.9%) were detected throughout all seasons. 15.6% were found in all seasons except winter and 24.7% occurred in only a single season, primarily in spring (7.8%) and summer (9.1%), respectively. Attempts to find out which phylotypes stand behind the single band classes led to the definite identification of 35 phylotypes by sequencing, whereas the identity of 16 remained uncertain and 103 band classes could not be further identified. Of the 72 partial sequence data obtained, nine showed a 100% match to known sequences in the database (Table 1). Thus, most of our phylotypes are likely new species with maximum sequence identities ranging from 87% to 99%. Among the identified DNA fragments, most belonged to the alveolates (15 Ciliophora, 4 Dinoflagellata, 2 group I alveolates and 8 group II alveolates), but stramenopiles (19), metazoans (6 Echinodermata, 5 Appendicularia, 3 Ctenophora, 2 Ascidiae and a Copepoda), chlorophytes (3 Prasinophyceae, 1 Ulvophyceae), picobiliphytes (2) and haptophytes (1) were found as well (Table 1).

Alveolata Within the Ciliophora, 15 phylotypes were found along most of the gel gradient (Figures 3 and 8A), indicating a varying GC-content in their partial sequences. The phylotype BK328 is closely related to *Strombidium* sp. SBB99-1 (AY143565), and BK340 and BK495 to *Strombidium styliferum* (DQ631805). These latter two also show a close relationship to two uncultured marine eukaryotes isolated from a supersulfidic anoxic Norwegian fjord (DQ310195 and DQ310293) (Behnke et al., 2006). The phylotype BK271 is closely related to *Varistrombidium* sp. *kielum* (DQ811090) and BK262 is identical to a *Laboea strobila* clone previously isolated from the northwest Atlantic Ocean (AF399151) (Snoeyenbos-West et al., 2002). Two uncultured eukaryotic clones closely related to BK066 and BK282 were previously isolated from these waters as well (AY331781 and AY331785, respectively) (Savin et al., 2004). The rest of our Ciliophoran phylotypes are closely related to sequences found in diverse regions of our world's ocean, for example, in the Sargasso Sea and in 2000 m depth of the Antarctic polar front (BK457 towards AY665090 and AF290076) (Armbrust et al., published in GenBank; López-García et al., 2001), in Eastern Antarctica (BK037 and BK436 towards EU078291) (Piquet et al., 2008), and in the Pacific Ocean and the South China Sea (BK084 and BK447 towards AY129053 and AJ829840) (Yuan et al., 2004; Worden, 2006). The phylotypes BK066, BK282, BK472, BK506 and BK511 are all related to uncultured clones previously found in lakes from different origin: France (AY642703, AY642730 and AY642747) (Lefranc et al., 2005), China (EU143877) (Chen et al., 2008) and Tibet (AM709528) (Wu, published in GenBank).

It was possible to attribute 9 of the 15 Ciliophora phylotypes to distinct band classes and to analyse their seasonal occurrence. BK495 was the only phylotype showing a relationship to a further described isolate (*Strombidium styliferum*) and was found in 11 samples throughout the

year. BK436 (found in 19 samples) and BK506 (found in 11 samples) did not show a distinct seasonality, whereas BK066 (found in 12 samples) and BK282 (found in 6 samples) primarily occurred in summer. The phylotypes BK037, BK457, BK472 and BK511 occurred only sporadically throughout the entire sampling period.

Phylotypes of the dinoflagellates and the group I and II alveolates were found in the second quarter of our gels (Figure 3). The Dinoflagellata phylotype BK339 (Table 1, Figure 8B) is identical to the *Heterocapsa rotunda* strain CCCM680 (AF274267) and BK034 to the *Katodinium rotundatum* strain CCMP1542 isolated previously from the North Atlantic Ocean (DQ388464) (Lin et al., 2006). BK071 is closely related to *Gymnodinium catenatum* (AF022193) and BK500 to an uncultured eukaryotic clone of the genus *Gyrodinium* previously found in the Sargasso Sea (AY664956) (Armbrust et al., published in GenBank). The group I alveolate BK072 is identical and BK458 closely related to uncultured clones previously found at Helgoland (DQ186538 and AJ965213) (Medlin et al., 2006). Both are also closely related to isolates found in the North Atlantic Ocean (unpublished data), whereas BK458 is additionally related to an uncultured eukaryotic clone from the Norwegian Sea (DQ647527) (Massana et al., 2006). Except BK161, all of our group II Alveolata phylotypes belong to the lineage that includes *Amoebophyra* sp., parasites of dinoflagellates (Table 1, Figure 8B) (Epstein and López-García, 2008; Guillou et al., 2008). BK268, BK409 and BK416 are closely related to sequences previously found in the English Channel (DQ186526) (Romari and Vaulot, 2004), the North Atlantic Ocean (BK409 towards DQ386740 and DQ386744) (Lin et al., 2006) and at 75 m depth in the equatorial Pacific Ocean (BK268 and BK416 towards AJ402338) (Moon-van der Staay et al., 2001). The phylotype BK497 belongs to the same branch as BK409 and is closely related to a eukaryotic clone from the Mediterranean Sea (AF363213) (Díez et al., 2001b). BK022 and BK335 form a sister clade to clones previously found in the equatorial Pacific Ocean (AJ402344) (Moon-van der Staay et al., 2001) and the South China Sea (DQ674775) (Chen et al., published in GenBank). BK312 and BK161 are closely related to uncultured clones found in the Sargasso and the Mediterranean Sea (EF172942, EF172848 and DQ001446, respectively) (Marie et al., 2006; Not et al., 2007a). Regarding their seasonal occurrence we found the phylotype BK409 in 26 samples, primarily from autumn (9 samples), whereas BK312 was found in 10 samples throughout all seasons and BK416 in only one winter sample.

Stramenopiles Among the stramenopiles, 19 phylotypes were identified in the upper half of our gels (Figure 3), indicating a lower GC-content within their partial sequences. The phylotypes BK452 and BK483 are related to uncultured eukaryotes of the genus *Cryothecomonas* previously found in the convergence zone of the Greenland, Norwegian and Barents Seas (DQ314809 - DQ314811) (Lovejoy et al., 2006). BK483 is also closely related to *Cryothecomonas aestivalis* strain

1 from the German Bight (AF290539) (Kühn et al., 2000) and BK452 to an uncultured eukaryote (DQ507415). The phylotype BK322 is closely related to *Caecitellus parvulus* (AY520446) and to an uncultured eukaryote (AY789784) that can graze efficiently on bacteria growing in oil-polluted seawater (Dalby et al., 2007). Our MAST 1 phylotypes BK264, BK401, BK465 and BK466 are closely related to a sequence found at 75 m depth in the equatorial Pacific Ocean (AJ402339) (Moon-van der Staay et al., 2001) and in the Norwegian Sea (DQ121419) (Massana et al., 2006) (Figure 8C). The MAST 2 phylotype BK298 is identical to a clone from the Mediterranean Sea (AF363186) (Díez et al., 2001b), and related to a species from 3000 m depth of the Antarctic polar front (AF290083) (López-García et al., 2001) and the North Atlantic Ocean (unpublished data). MAST 3 phylotypes BK478, BK491 and BK496 are closely related to clones previously sequenced from the German Bight and the North Atlantic Ocean (AY381157 and AF363204) (Massana et al., 2004b; Groisillier et al., 2006), and BK067 (MAST 4) to sequences previously found in the Sargasso Sea (EF172982 and EF172970) (Not et al., 2007a) and the North Atlantic Ocean (unpublished data). The MAST 7 phylotypes BK004 and BK295 are closely related to uncultured clones from the Mediterranean Sea, the Pacific Ocean (AY381207 and AY129064) (Massana et al., 2004b; Worden, 2006) and the North Atlantic Ocean (unpublished data). MAST 9 phylotypes BK164 and BK181 are related to a sequence previously found in an off-axis hydrothermal vent field in the Mid-Atlantic Ocean (DQ504337) (López-García et al., 2007). MAST 12 phylotype BK197 is closely related to a clone previously found in the North Atlantic Ocean (AY885021) (Lim and Vandergrift, published in GenBank). The Labyrinthulida phylotype BK309 is identical and BK481 closely related to a clone already found in the German Bight (AY381171) (Massana et al., 2004b). Both are related to an uncultured clone from the Sargasso Sea (EF172979) (Not et al., 2007a).

The analysis of their seasonal occurrence revealed that the phylotype BK452, belonging to the genus *Cryothecomonas*, was found in 30 samples primarily from winter (10 samples), whereas BK181 (MAST 9) was found in 39, BK298 (MAST 2) in 22, BK496 (MAST 3) in 9, BK466 (MAST 1) in 7 and BK478 (MAST 3) in 6 samples from all seasons. BK067 (MAST 4) occurred in 37 and BK164 (MAST 9) in 19 samples, both primarily in summer (11 and 9 samples, respectively) and autumn 2005 (11 and 7 samples, respectively). The MAST 7 phylotypes BK004 and BK295 were found in 18 and 16 samples, respectively, primarily from spring 2005 (9 and 8 samples, respectively), whereas phylotype BK197 (MAST 12) was only found in 3 samples.

Others Phylotypes of the Prasinophyceae, Ulvophyceae, haptophytes and picobiliphytes were found in the middle of our gels (Figure 3), indicating a moderate GC-content in their partial sequences. The prasinophycean phylotypes BK359 and BK477 are closely related to *Micromonas pusilla* RCC434 previously isolated from the Mediterranean Sea (AY425316) (Guillou et al., 2004),

but also to sequences isolated from the Pacific (BK359 towards AF525854) (Worden, 2006) and North Atlantic Ocean (BK477 towards AY955008) (Šlapeta et al., 2006). Regarding their seasonal occurrence BK359 was found in 62 of the 70 samples taken. BK454 belongs to clade VII B of the Prasinophyceae. Its relatives were previously found in 75 m depth of the equatorial Pacific Ocean (AJ402358) (Moon-van der Staay et al., 2001) and the North Atlantic Ocean (unpublished data). The ulvophycean phylotype BK310 is closely related to *Enteromorpha* sp. WA4-20b (AY303587) and was found in 32 samples, especially from autumn 2005 (10 samples). The only phylotype of the Haptophyta is BK433. It forms a single clade in our phylogenetic tree (Figure 8D), is closely related to *Isochrysis* sp. MBIC10557 (AB183617) and was found in 9 samples throughout all seasons. BK060 and BK307 are identical phylotypes of the picobiliphytes (Figure 8C) and are closely related to a clone found in the English Channel (DQ222878) (Not et al., 2007b). Both were detected throughout different seasons with BK307 in 53 and BK060 in 14 samples.

Metazoa Metazoan sequences were found in different melting zones on our gels (Figure 3). The five Appendicularia phylotypes (Figure 8E) were closely related to *Oikopleura* sp. cf. *dioica* (AY116613), and the two Ascidiae phylotypes to *Chelyosoma siboja* (AB104872) and *Megalodicopia hians* (AB075543). Our Copepoda phylotype is identical to an uncultured isolate (AY145104), whereas the three Ctenophora phylotypes are closely related to *Mnemiopsis leidyi* isolated from the Western Atlantic Ocean (AF293700) (Podar et al., 2001). The obtained Echinodermata phylotypes are identical or closely related to *Plexechinus planus* (AY957468) and *Spatangus raschi* (DQ073794). The phylotype BK269 (Ctenophora) was found in 14 samples throughout all seasons, BK080 (Ascidiae) sporadically in spring both years, BK384 (Appendicularia) primarily in spring and summer, and the Echinodermata primarily in summer (*Plexechinus planus*) or summer and autumn (*Spatangus raschi*).

DISCUSSION

Picoplankton diversity The high number of separated band classes in our samples represents an overall striking taxonomic diversity among the picoeukaryotes in the German Bight. DGGE fingerprints revealed highest average phylotype diversities in autumn (Figure 4) with the community structure being as uniform as in spring and summer (data not shown). In contrast, winter samples showed a lower and more heterogeneous diversity. This is congruent with previous findings by Medlin et al. (2006), although they obtained highest diversities in spring (April) and summer (August). The discrepancy towards our findings in respect of picoplankton diversity in summer is expected to result from the lower sampling frequency used by Medlin et al.

(2006). It may hide the high variations in diversity in summer, as it was revealed by our results, thus leading to a lower seasonal average.

Regarding the proportion of the picophototrophs to the picoheterotrophs, Vaulot et al. (2008) mentioned that 70% of compiled 18S rRNA gene sequences generally belong to heterotrophic organisms, primarily alveolates, stramenopiles and metazoans. Medlin et al. (2006) found nonphotosynthetic groups dominating clone library investigations of the waters around Helgoland as well (~66%), but with a dominance of group II alveolates (45%) and ciliophores (42%). Although heterotrophs made up 90.3% of our identified phylotypes and 69.5% of the detected and identified band classes (Figure 9), we found a different composition: stramenopiles dominated the identified phylotypes with 26.4%, whereas ciliophores made up 20.8% and group II alveolates 11.1%. Referring to the detected and identified band classes, stramenopiles made up 53%, metazoans 20%, ciliophores 18% and group II alveolates 9.5%. Phototrophs on the other hand made up only 9.7% of the identified phylotypes, but accounted for 30.5% of the detected and identified band classes. Of these, 39.4% belonged to the Picobiliphyta, 36.4% to *Micromonas pusilla*, 18.8 % to the Ulvophyceae and 5.3% to the Haptophyta. However, disagreeing results on the picoplankton community composition obtained by the distinct studies may rely on methodological differences between clone library investigations and sequencing attempts by the use of DGGE.

Picoplankton occurrence and distribution Overall, the amount of phylotypes not identical to previously sequenced isolates but related to known lineages, highlights the still incomplete discovery of existing species and clades among the picoeukaryotes (Massana and Pedrós-Alió, 2008). Nonetheless, our phylotypes are closely related to species and uncultured clones previously found in diverse regions of our world's ocean and at various environmental conditions. This overall widespread spatial occurrence of picoeukaryotes represents their evolutionary spreading around the world, enhanced by their minimal niche requirements and their ability to efficiently adapt to diverse environmental conditions (Šlapeta et al., 2006, Viprey et al., 2008; Simon et al., 2009; Worden et al., 2009). Even though, an anthropogenic influence on the species' distribution, for example, by ballast water exchange, cannot be excluded.

Picoplankton ecology and succession The occurrence and composition of the picoeukaryotic community changed significantly within 7 days, though some phylotypes were of rather permanent occurrence (Figures 1, 3 and 7). Although we found 19.5% of the detected band classes to occur only once, we also found a *Micromonas pusilla* phylotype (BK359) to be present over a period of 55 weeks. This represents the parallel occurrence of sporadic and steady community members. Regarding the annual succession of the phylotypes within our samples, the phototrophic

ones were found in equal proportions throughout all seasons, whereas 24% of the heterotrophs occurred in spring, 31% in summer, 30% in autumn and 15% in winter. Because the heterotrophic phylotypes primarily occurred when larger microalgae exposed highest abundances (data not shown), and because most of the heterotrophic sequences belong to organisms larger than 3 µm, we suggest them to be grazers of larger microalgae. For example, the highly diverse fraction of the group II alveolates is known to consist of partially or entirely intracellular parasites. These are truly heterotrophic and specific for a reduced number of larger sized hosts (Groisillier et al., 2006). They do parasite dinoflagellates, ciliophores, Acantharians and other parasites, and are able to produce mobile cells (dinospores) (Groisillier et al., 2006). We observed their rare occurrence in spring and summer, and their frequent occurrence in autumn (43%) and winter (36%), those two seasons when larger microalgae, for example, dinoflagellates (data not shown), are less abundant. We therefore assume the parasites were integrated within their larger-sized hosts in spring and summer, thus being excluded from DNA analyses by prefiltration. In autumn and winter they might have been present as dinospores or parasites of other parasites or members of the picoeukaryotes, thus being included in the analyses of pico-sized DNA. In comparison to the differing seasonal abundance of group II alveolates, heterotrophic stramenopile phylotypes showed a rather equal seasonal occurrence. This is possibly caused by their lesser restriction by energy resources and their role of being the primary grazers of the constantly occurring picoplankton organisms (Vaulot et al., 2002; Massana et al., 2004b; Worden and Not, 2008).

Our data also support the suggestion of an annual reassembling of the picoplankton community (Medlin et al., 2006), because 56.4% of our phylotypes occurring only in spring and 49.2% of those occurring only in summer, were present in both years (Figure 10). Furthermore, 78.4% of those phylotypes occurring in spring and summer were found in both years, though, 75% of them were also found in autumn and winter. To identify the underlying factors of the temporal occurrence of picoeukaryotes, we analysed the influence of a set of environmental parameters. Our analyses revealed that 39.4% of the similarity between the DGGE sample profiles can be explained by the secchi depth, temperature, salinity, PO_4 and NO_3. CCA analysis identified temperature, salinity, PO_4 and NO_2 to be the only significant factors explaining the differences between sample profiles. Furthermore, these parameters showed dissimilar effects on seasonal samples: PO_4 exposed the highest correspondence towards DGGE fingerprints from spring, whereas the temperature corresponded most to summer profiles, and NO_2 as well as salinity values to winter profiles (Figure 2). Because the picoplankton has minimal niche requirements and is highly adaptable to environmental conditions, its growth is fairly independent from abiotic factors, allowing a steady occurrence (Fogg, 1991; Bryant, 2003; Knefelkamp et al., in prep.). The correlation between DGGE sample profiles and environmental parameters is rather caused by the dominance of larger-sized

heterotrophs in our PCR products. Their occurrence depends on the abundance of prey and host organisms, the larger microalgae, which in turn depend on environmental parameters. Therefore, we assume an indirectly induced correlation of our DGGE sample profiles towards abiotic factors.

Methodological biases of the DGGE method In the present study, we combined visible DNA fragments occurring in the same melting zone of different samples to a common band class because they were supposed to have the same nucleotide composition (Medlin et al., 2006). Nonetheless, in some cases we found different sequences in the same band class and identical sequences in different band classes, even within the same sample. Moreover, it was recognised that excised bands, particularly those from the top of the initial gels, resulted in a whole sample profile after re-amplification. These biases are described elsewhere and can be subscribed to the co-migration of different partial sequences, 18S operon heterogeneity and the incomplete strand synthesis during PCR (Sekiguchi et al., 2001; Speknijder et al., 2001; Nikolausz et al., 2005).

The high number of sequences belonging to heterotrophic organisms larger than 3 µm in comparison to the small number of picoautotrophs may primarily be caused by slim or small nano-sized representatives (Medlin et al., 2006; Lin et al., 2006), cell fragments (Díez et al., 2001b) or sperm cells (Marie et al., 2006) that slipped through the preparation filters (Knefelkamp et al., in prep.). Furthermore, it is possible that extracellular DNA had an effect on our analyses or that the extraction of phototrophic DNA was hampered (Burton, 1996; Medlin et al., 2002 and 2006; Vaulot et al., 2008; Viprey et al., 2008; Not et al., 2009). However, the higher amount of 18S rDNA copy numbers in heterotrophs and/or their preference in PCR amplification enhances their influence on the DGGE profiles even more (Burton, 1996; Medlin et al., 2002 and 2006; Zhu et al., 2005; Vaulot et al., 2008). The reduced matching of universal primers, the occurrence of heteroduplex formations or chimeric sequences, and the shortness and variable quality of analysed sequences are able to affect DGGE analyses as well (Amann et al., 1995; Burton, 1996; Díez et al., 2001a; Medlin et al., 2002 and 2006; Vaulot et al., 2008). We therefore agree with Nikolausz et al. (2005) that the majority of picoeukaryotic DNA fragments, especially those of picophototrophs, somehow get lost during preparation. These observations of previously described methodological biases support the urgent need for methodological improvements, especially regarding the specificity of picoeukaryotic primers. This is also encouraged by the insignificant correlations of our DGGE sample profiles towards fluorescence *in situ* hybridisation and analyses of pigment profiles of the same samples (data not shown) (Knefelkamp et al., in prep.).

CONCLUSIONS

Although the high number of still undiscovered and undescribed picoeukaryotes does not allow a reliable estimation of their actual diversity, abundance and ecology (Not et al., 2004, Medlin et al., 2006), our approach has led to a better understanding of their occurrence in the German Bight. In the present study, we identified picoeukaryotic phylotypes and analysed their occurrence on a weekly interval over 17 months. Although a high taxonomic diversity was already observed around Helgoland (Medlin et al., 2006), our detection of new and only distantly related phylotypes supports the suggestion of a higher picoplankton diversity than the one known today (Biegala et al., 2003; Masquelier and Vaulot, 2008). We found seasonal differences in the taxonomic diversity and phylotype composition of the community, though in our approach this was largely dominated by heterotrophs, primarily alveolates (especially ciliophores and group II alveolates) and marine stramenopiles (MAST). Some phylotypes that we found in the German Bight were of sporadic occurrence, whereas others exhibited rather a continuous presence. Overall, we were able to complement findings by Medlin et al. (2006) and to improve the understanding of previous results. This was mainly possible by our high sampling interval in combination with the long-term approach, and the in-depth analysis of the ecological occurrences of individual phylotypes. Nonetheless, our results also confirmed previously described methodological biases. These were primarily related to the differentiation of the DNA belonging to distinct size classes and nutritional groups, and do support the need for methodological improvements.

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Figure 5: DGGE gels showing changing phylotype diversity in summer and winter 2005, and the weekly change between July 19th and 26th 2005. The internal marker is an environmental sample taken on August 9th 2005.

Figure 6: ANOSIM analysis between average DGGE sample profiles of distinct months between March 2005 and February 2006. Discrepancies between months accounted for R = 0.437 (significance level 0.1%).

Figure 7: Seasonal occurrences of our 154 detected band classes. Spring and summer samples, respectively, were combined for both years.

Figure 8: Phylogeny of the (A) ciliophores, (B) dinoflagellates, group I and II alveolates, (C) stramenopiles and picobiliphytes, (D) chlorophytes, and (E) metazoans showing the distribution of their DGGE sequences found in our study (red) among known sequences obtained by neighbour-joining analysis. Bootstrap values in nodes indicate posterior probabilities. Sequences obtained from the North Atlantic Ocean (unpublished data) are shown in bold and start with the prefix VN.

Figure 9: Percentage seasonal time of occurrence of all obtained heterotrophic and autotrophic phylotypes identifying distinct band classes.

Figure 10: Reassembling of phylotypes between spring and/or summer of both years.

Table 1

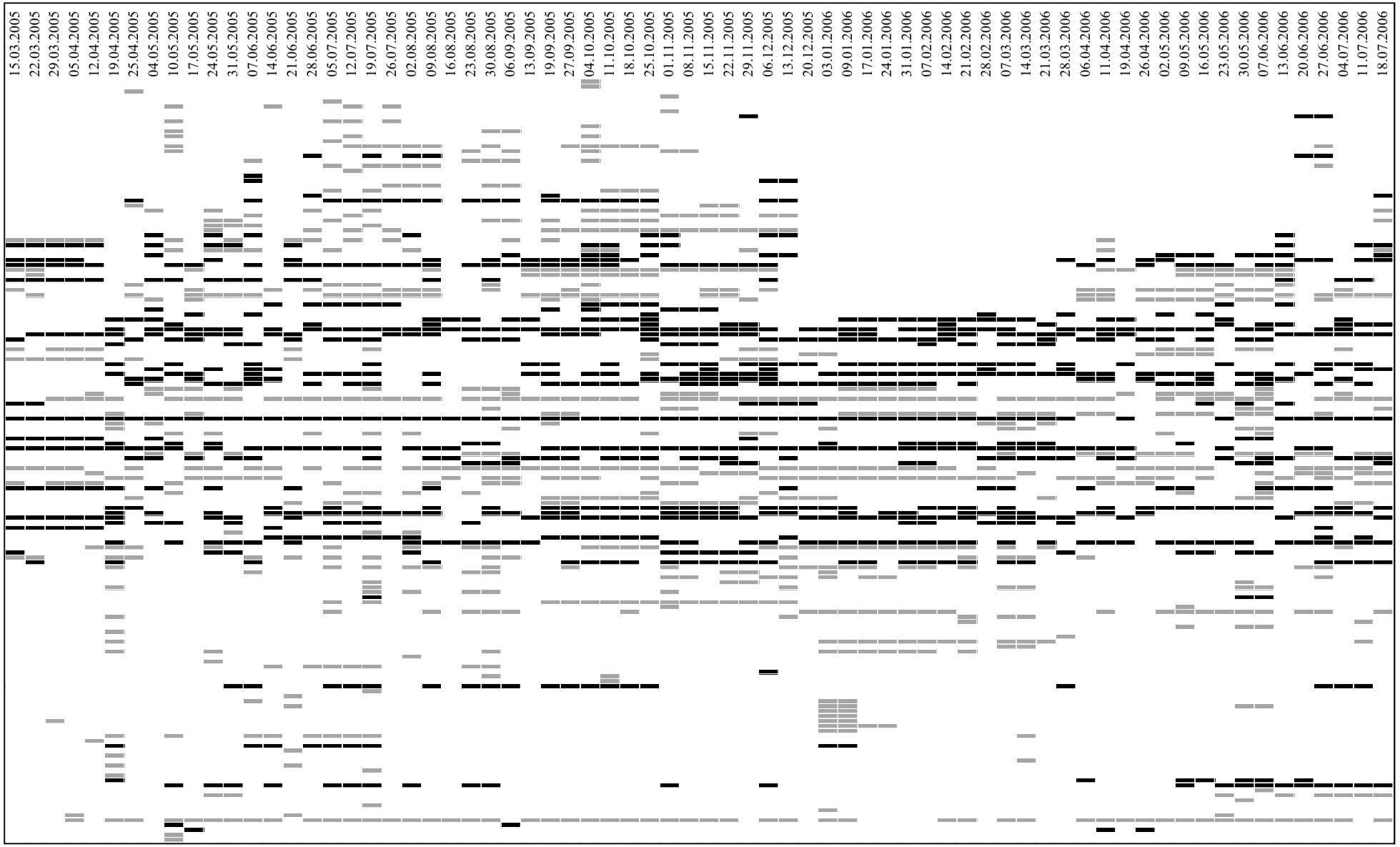
Related group	Phylotype	Acc. number	Sequenced from sample taken on	Closest relative	Acc. number	Similarity
Alveolata group I	BK072 [11]		13/06/2006	Uncultured alveolate clone HE010218_87	DQ186538	100%
	BK458 [12]		19/04/2006	Uncultured eukaryote clone CD8S.30	DQ647527	99%
Alveolata group II	BK022 [13]		27/06/2006	Eukaryote clone OLI11055	AJ402344	97%
	BK161 [11]		03/01/2006	Uncultured eukaryote clone SSRPB36	EF172848	94%
	BK268 [8]		13/06/2006	Uncultured alveolate clone RA000609.21	DQ186526	96%
	BK312		29/11/2005	Uncultured eukaryote clone SSRPD58	EF172942	97%
	BK335 [10]		15/11/2005	Eukaryote clone OLI11055	AJ402344	99%
	BK409		06/12/2005	Uncultured alveolate clone RA000609.21	DQ186526	98%
	BK416		06/12/2005	Uncultured alveolate clone RA000609.21	DQ186526	93%
	BK497 [8]		09/08/2005	Eukaryote clone ME1-10	AF363213	97%
Ciliophora	BK037		10/05/2005	Uncultured eukaryote clone E2-22	EU078291	95%
	BK066		13/06/2006	Uncultured eukaryote clone j34	AY331781	98%
	BK084 [1]		13/06/2006	Uncultured eukaryote clone UEPACAIp5	AY129053	99%
	BK262 [2]		23/05/2006	*Laboea strobila* clone	AF399151	100%
	BK271 [4]		28/03/2006	*Varistrombidium* sp. *kielum*	DQ811090	95%
	BK282		27/06/2006	Uncultured eukaryote clone j58	AY331785	98%
	BK328 [2]		04/05/2005	*Strombidium* sp. SBB99-1	AY143565	98%
	BK340 [4]		10/05/2005	*Strombidium styliferum*	DQ631805	98%
	BK436		07/06/2006	Uncultured eukaryote clone E2-22	EU078291	98%
	BK447 [5]		02/05/2006	Uncultured eukaryote clone NS51D157	AJ829840	96%
	BK457		27/06/2006	Uncultured eukaryote clone SCM16C45	AY665090	87%
	BK472		07/06/2005	Uncultured eukaryote Namocuo-5-2	AM709528	99%
	BK495		14/06/2005	*Strombidium styliferum*	DQ631805	97%
	BK506		08/11/2005	Uncultured ciliate clone WLB46.77	EU143877	94%
	BK511		06/12/2005	Uncultured eukaryote clone P34.44	AY642703	97%
Dinoflagellata	BK034 [7]		04/05/2005	*Katodinium rotundatum* strain CCMP1542	DQ388464	100%
	BK071 [12]		28/03/2006	*Gymnodinium catenatum*	AF022193	98%
	BK339 [7]		15/03/2005	*Heterocapsa rotundata* strain CCCM680	AF274267	100%
	BK500 [9]		01/11/2005	Uncultured eukaryote clone SCM38C58	AY664956	98%
Stramenopiles	BK004 [a]		04/07/2006	Uncultured eukaryote clone BL010320.6	AY381207	99%
	BK067		23/05/2006	Uncultured eukaryote clone Q2H12N10	EF172982	96%
	BK164 [b]		30/08/2005	Uncultured heterokont clone LC23_5EP_40	DQ504337	99%
	BK181 [b]		31/01/2006	Uncultured heterokont clone LC23_5EP_40	DQ504337	99%
	BK197		28/06/2005	Uncultured eukaryote clone BB01_67	AY885021	95%
	BK264 [c, 2]		19/04/2006	Eukaryote clone OLI11026	AJ402339	99%
	BK295 [a]		04/05/2005	Uncultured eukaryote clone BL010320.6	AY381207	99%
	BK298		20/06/2006	Eukaryote clone ME1-17	AF363186	100%
	BK309 [6]		04/10/2005	Uncultured eukaryote clone HE001005_112	AY381171	100%
	BK322 [14]		09/08/2005	*Caecitellus parvulus*	AY520446	99%
	BK401 [c, 3]		16/05/2006	Eukaryote clone OLI11026	AJ402339	99%
	BK452		06/04/2006	Uncultured eukaryote isolate	DQ507415	99%
	BK465 [c, 3]		07/02/2006	Eukaryote clone OLI11026	AJ402339	99%
	BK466 [c]		16/05/2006	Eukaryote clone OLI11026	AJ402339	99%
	BK478		01/11/2005	Uncultured eukaryote clone HE000427_21	AY381157	95%
	BK481 [6]		06/12/2005	Uncultured eukaryote clone HE001005_112	AY381171	99%
	BK483 [3]		11/10/2005	*Cryothecomonas aestivalis* strain 1	AF290539	98%
	BK491 [1]		06/12/2005	Uncultured eukaryote clone HE000427_21	AY381157	99%
	BK496		06/12/2005	Uncultured eukaryote clone HE000427_21	AY381157	94%
Appendicularia	BK050 [d, 14]		04/07/2006	*Oikopleura* sp. cf. *dioica*-TTGS-2	AY116613	99%
	BK318 [d, 10]		22/11/2005	*Oikopleura* sp. cf. *dioica*-TTGS-2	AY116613	99%
	BK329 [d, 13]		12/07/2005	*Oikopleura* sp. cf. *dioica*-TTGS-2	AY116613	99%
	BK360 [d, 14]		27/06/2006	*Oikopleura* sp. cf. *dioica*-TTGS-2	AY116613	99%
	BK384 [d]		15/03/2005	*Oikopleura* sp. cf. *dioica*-TTGS-2	AY116613	99%
Ascidiae	BK080		11/04/2006	*Chelyosoma siboja*	AB104872	95%
	BK153 [15]		02/08/2005	*Chelyosoma siboja*	AB104872	96%
Copepoda	BK316 [15]		29/11/2005	Uncultured eukaryote isolate JPeuk-27	AY145104	100%
Ctenophora	BK092 [e, 3]		28/06/2005	*Mnemiopsis leidyi*	AF293700	99%
	BK269 [e]		23/05/2006	*Mnemiopsis leidyi*	AF293700	99%
	BK473 [e, 5]		05/07/2005	*Mnemiopsis leidyi*	AF293700	99%
Echinodermata	BK025 [f]		14/06/2005	*Plexechinus planus*	AY957468	98%
	BK029		30/05/2006	*Plexechinus planus*	AY957468	99%
	BK047		11/07/2006	*Spatangus raschi*	DQ073794	100%
	BK114 [16]		09/05/2006	*Plexechinus planus*	AY957468	100%
	BK137 [f]		09/05/2006	*Plexechinus planus*	AY957468	99%
	BK459 [16]		07/06/2006	*Spatangus raschi*	DQ073794	99%
Prasinophyceae	BK359		20/06/2006	*Micromonas pusilla*	AY425316	98%
	BK454 [9]		30/05/2006	Eukaryote clone OLI11305	AJ402358	99%
	BK477 [5]		22/11/2005	*Micromonas pusilla*	AY425316	99%
Ulvophyceae	BK310		18/10/2005	*Enteromorpha* sp. WA4-20b	AY303587	97%
Haptophyta	BK433		16/05/2006	*Isochrysis* sp. MBIC10557	AB183617	93%
Picobiliphyta	BK060		03/01/2006	Uncultured eukaryote clone RA001219.38	DQ222878	98%
	BK307		22/11/2005	Uncultured eukaryote clone RA001219.38	DQ222878	99%

Table 2

	DCA	CCA, inter-species distances, VIF < 20, p < 0.05			
	Length of gradient	Eigenvalue (explained variance by axis)	Species-environment correlation of axis	explained variance of species data [%]	explained variance of species - environment relation [%]
Axis 1		0.258	0.909	6.5	21.1
Axis 2	2.685 2.523	0.218	0.922	11.9	38.9
Axis 3	1.944 1.657	0.165	0.894	16.1	52.4
Axis 4		0.131	0.919	19.4	63.2

Table 3

	spring	summer	autumn
summer	0.292 (0.4)		
autumn	0.307 (0.1)	0.314 (0.1)	
winter	0.336 (0.3)	0.400 (0.1)	0.501 (0.1)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22.03.2005
29.03.2005
05.04.2005
12.04.2005
19.04.2005
25.04.2005
04.05.2005
10.05.2005
17.05.2005
24.05.2005
31.05.2005
07.06.2005
14.06.2005
21.06.2005
28.06.2005
05.07.2005
12.07.2005
19.07.2005
26.07.2005
02.08.2005
09.08.2005
16.08.2005
23.08.2005
30.08.2005
06.09.2005
13.09.2005
19.09.2005
27.09.2005
04.10.2005
11.10.2005
18.10.2005
25.10.2005
01.11.2005
08.11.2005
15.11.2005
22.11.2005
29.11.2005
06.12.2005
13.12.2005
20.12.2005
03.01.2006
09.01.2006
17.01.2006
24.01.2006
31.01.2006
07.02.2006
14.02.2006
21.02.2006
28.02.2006
07.03.2006
14.03.2006
21.03.2006
28.03.2006
06.04.2006
11.04.2006
19.04.2006
26.04.2006
02.05.2006
09.05.2006
16.05.2006
23.05.2006
30.05.2006
07.06.2006
13.06.2006
20.06.2006
27.06.2006
04.07.2006
11.07.2006
18.07.2006

Figure 1

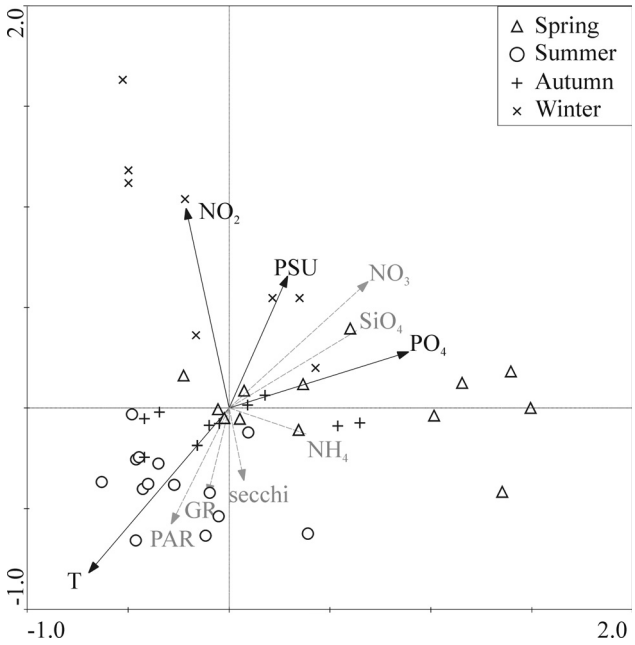

Spring
Summer
Autumn
Winter
NO2
PSU
NO3
SiO4
PO4
NH4
secchi
GR
PAR
T
2.0
-1.0
-1.0
2.0

Figure 2

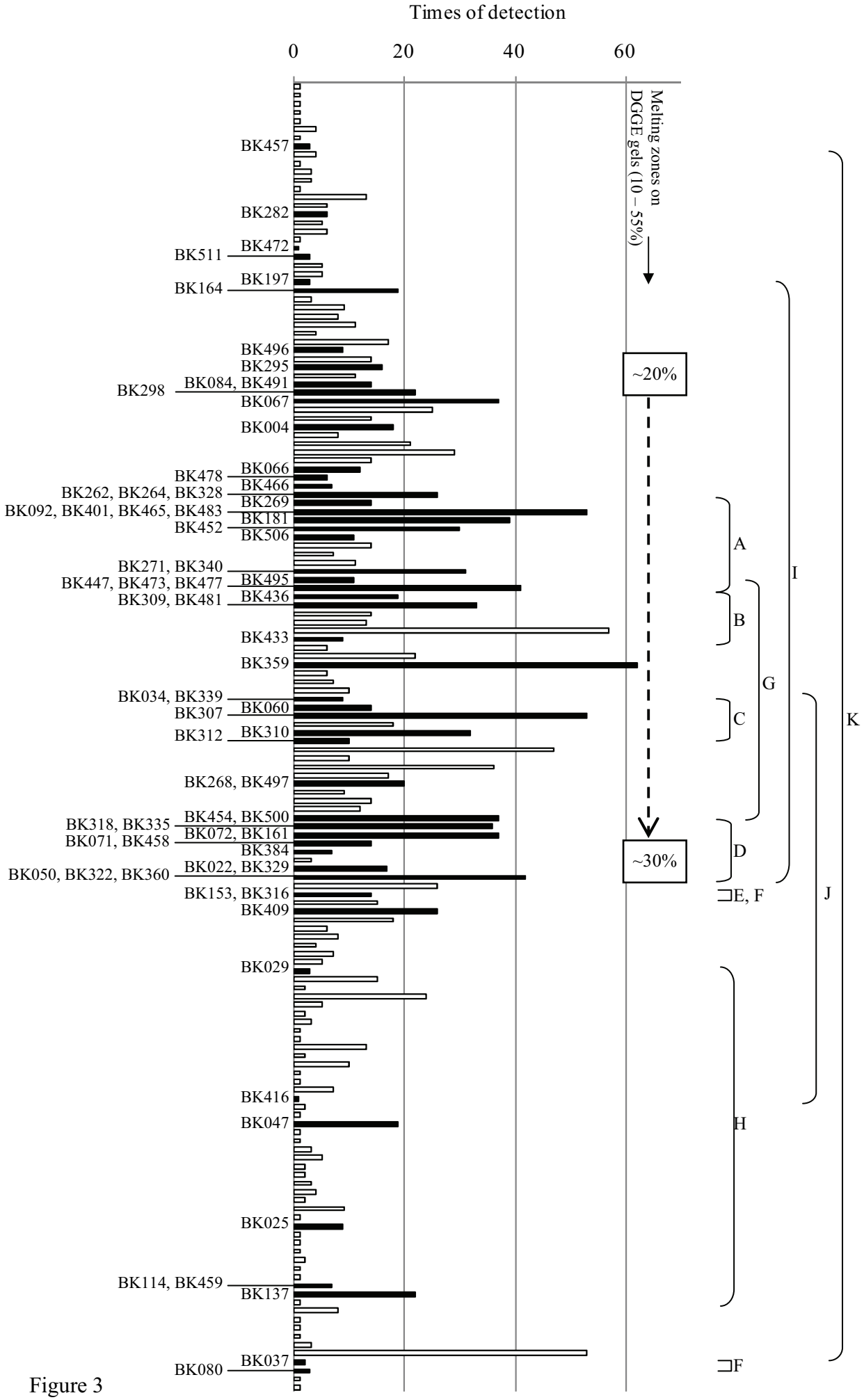

Times of detection
0
20
40
60
Melting zones on DGGE gels (10 – 55%)
~20%
~30%
BK457
BK282
BK472
BK511
BK197
BK164
BK496
BK295
BK298
BK084, BK491
BK067
BK004
BK066
BK478
BK466
BK262, BK264, BK328
BK269
BK092, BK401, BK465, BK483
BK181
BK452
BK506
BK271, BK340
BK495
BK447, BK473, BK477
BK436
BK309, BK481
BK433
BK359
BK034, BK339
BK060
BK307
BK310
BK312
BK268, BK497
BK454, BK500
BK318, BK335
BK072, BK161
BK071, BK458
BK384
BK022, BK329
BK050, BK322, BK360
BK153, BK316
BK409
BK029
BK416
BK047
BK025
BK114, BK459
BK137
BK037
BK080
A
B
C
D
E, F
F
G
H
I
J
K

Figure 3

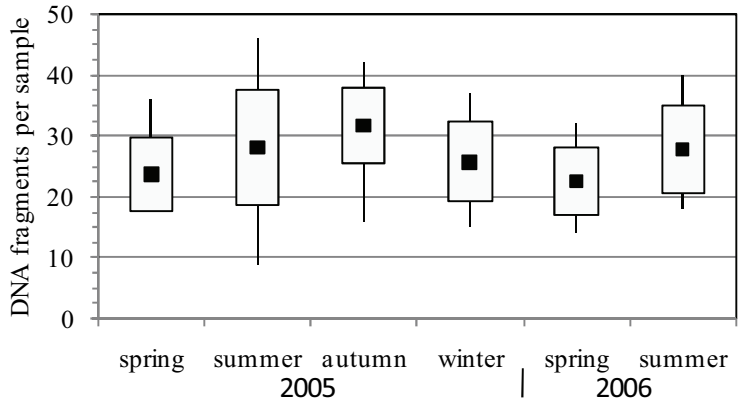


Figure 4

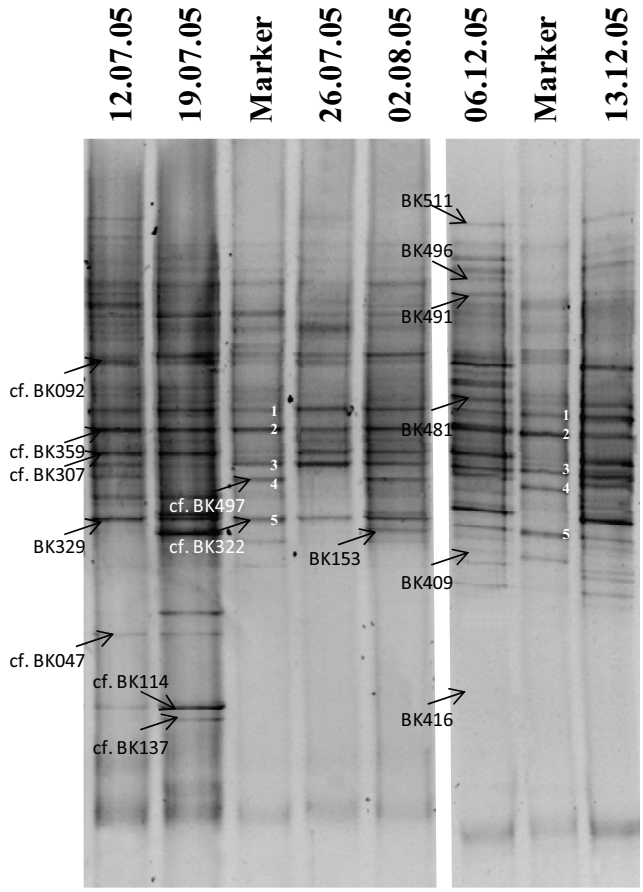


Figure 5

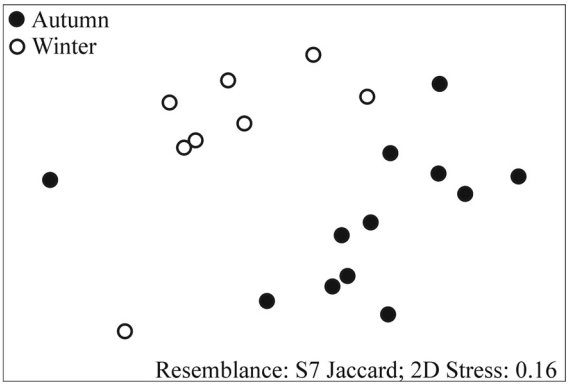
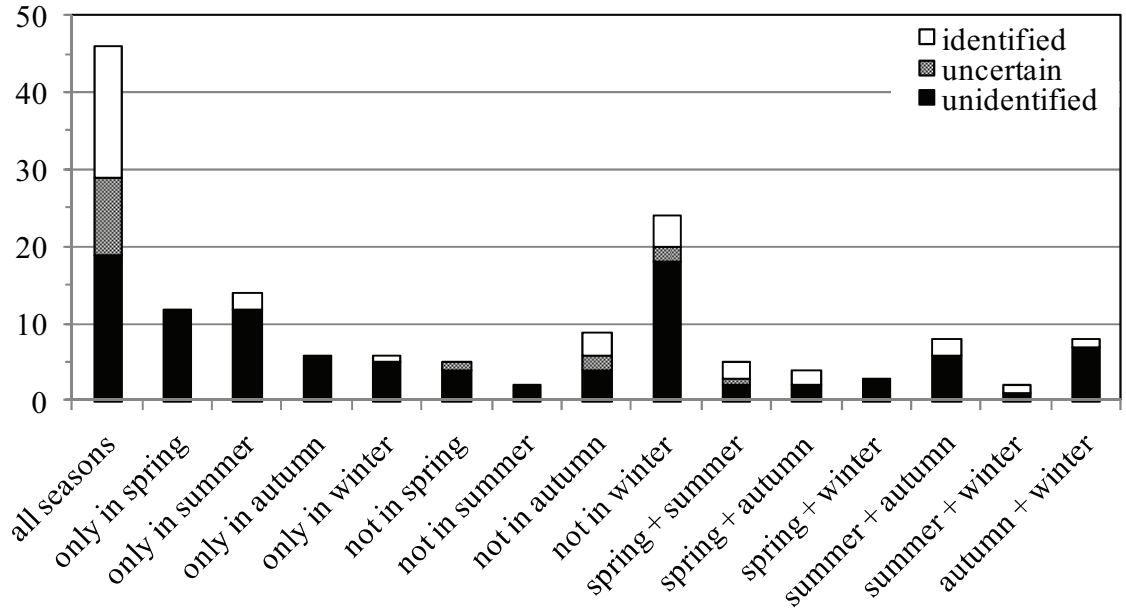
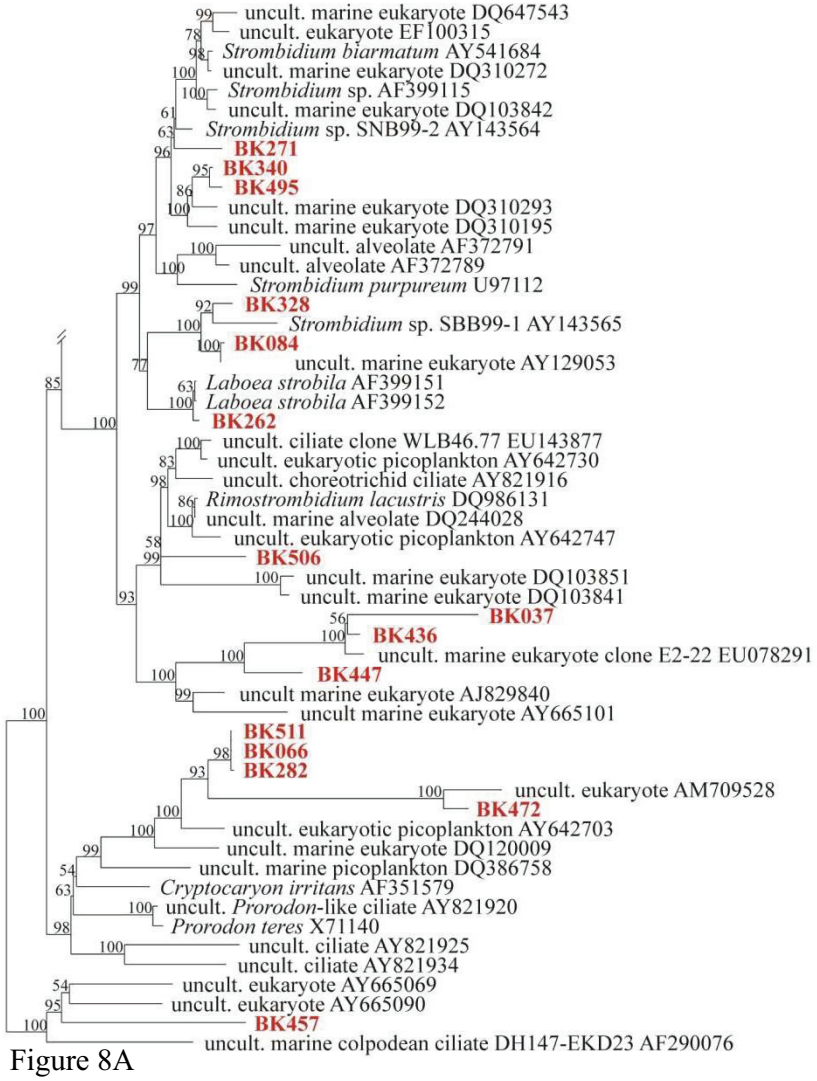


Figure 6

Figure 7

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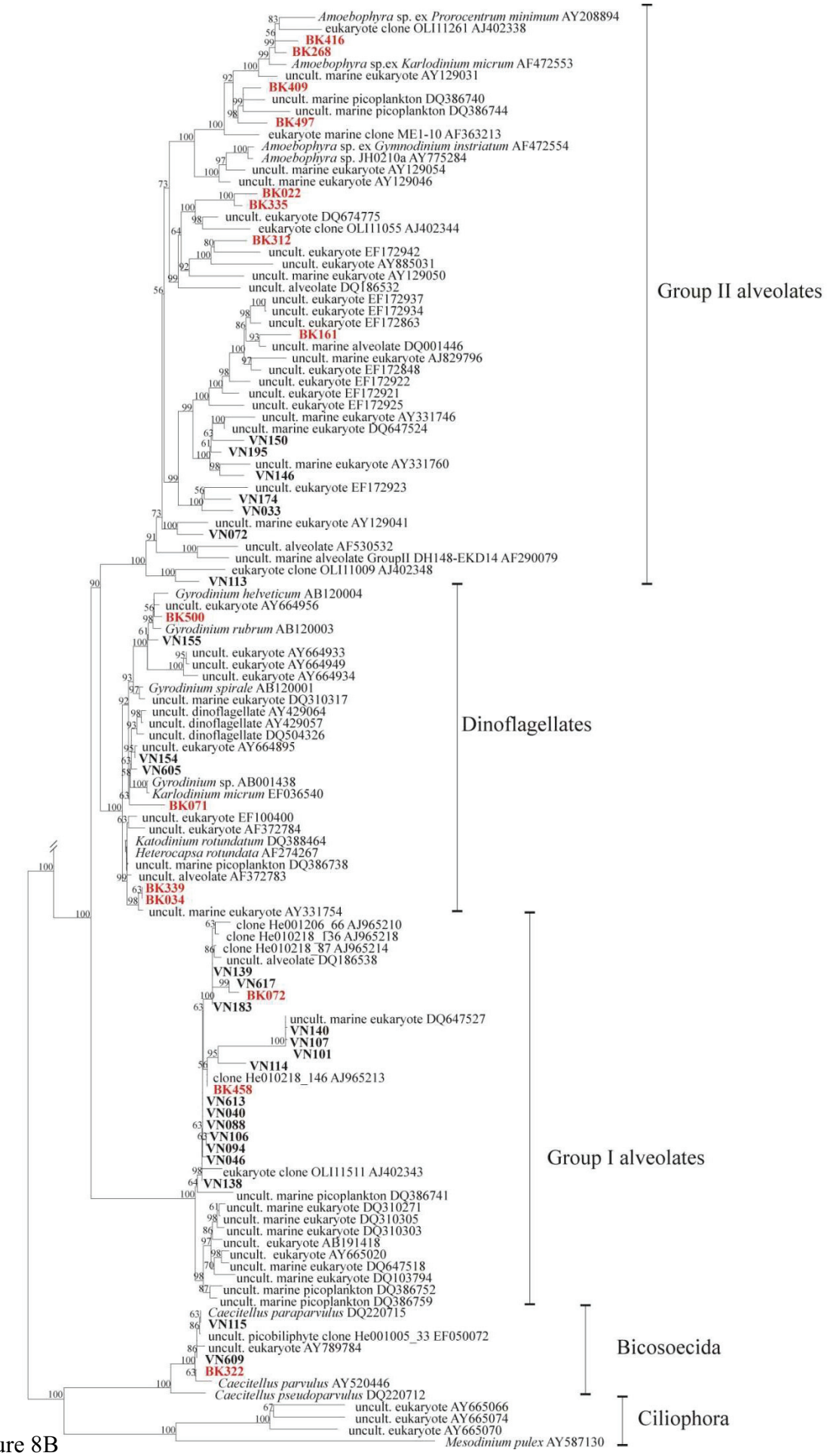
uncult. eukaryote EF100315
Strombidium biarmatum AY541684
uncult. marine eukaryote DQ310272
Strombidium sp. AF399115
uncult. marine eukaryote DQ103842
Strombidium sp. SNB99-2 AY143564
BK271
BK340
BK495
uncult. marine eukaryote DQ310293
uncult. marine eukaryote DQ310195
uncult. alveolate AF372791
uncult. alveolate AF372789
Strombidium purpureum U97112
BK328
Strombidium sp. SBB99-1 AY143565
BK084
uncult. marine eukaryote AY129053
Laboea strobila AF399151
Laboea strobila AF399152
BK262
uncult. ciliate clone WLB46.77 EU143877
uncult. eukaryotic picoplankton AY642730
uncult. choreotrichid ciliate AY821916
Rimostrombidium lacustris DQ986131
uncult. marine alveolate DQ244028
uncult. eukaryotic picoplankton AY642747
BK506
uncult. marine eukaryote DQ103851
uncult. marine eukaryote DQ103841
BK037
BK436
uncult. marine eukaryote clone E2-22 EU078291
BK447
uncult marine eukaryote AJ829840
uncult marine eukaryote AY665101
BK511
BK066
BK282
uncult. eukaryote AM709528
BK472
uncult. eukaryotic picoplankton AY642703
uncult. marine eukaryote DQ120009
uncult. marine picoplankton DQ386758
Cryptocaryon irritans AF351579
uncult. Prorodon-like ciliate AY821920
Prorodon teres X71140
uncult. ciliate AY821925
uncult. ciliate AY821934
uncult. eukaryote AY665069
uncult. eukaryote AY665090
BK457
uncult. marine colpodean ciliate DH147-EKD23 AF290076

Figure 8A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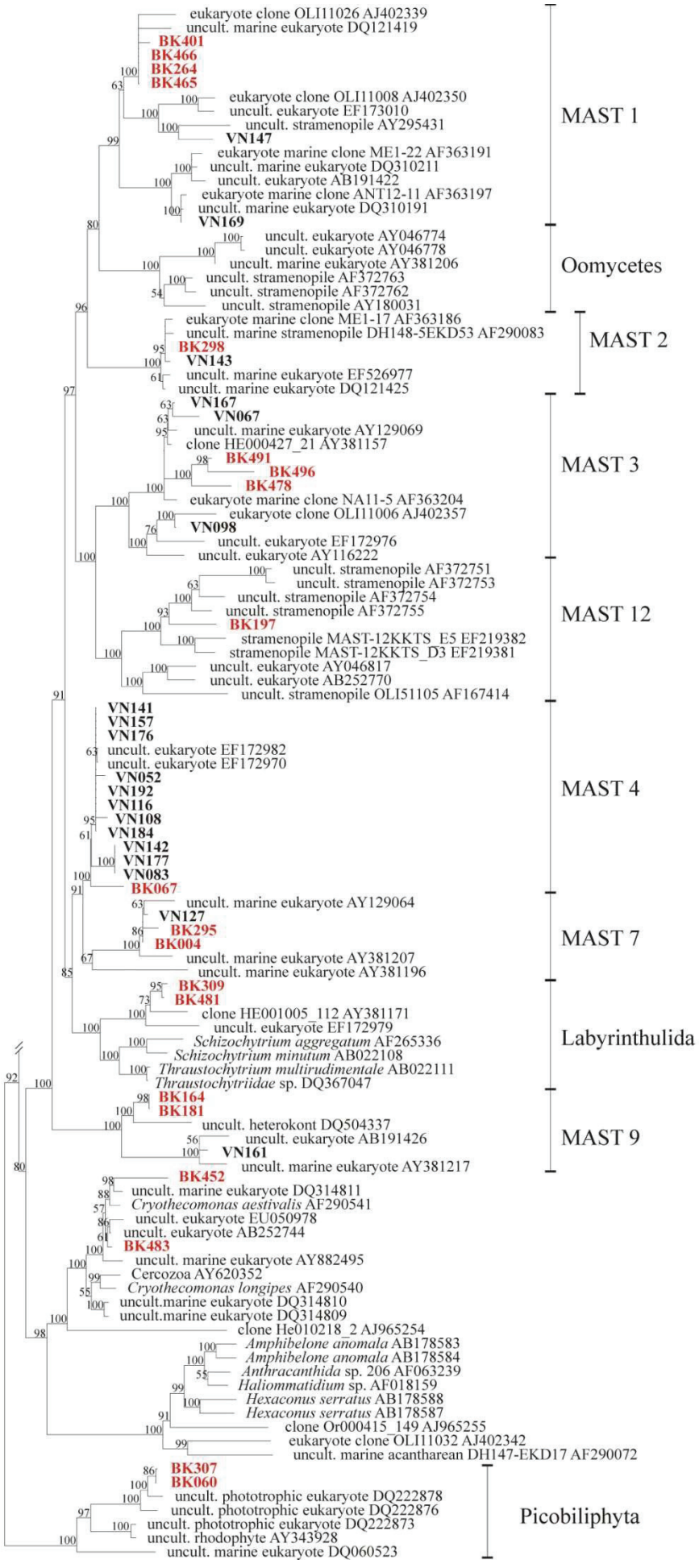
BK268
Amoebophyra sp.ex Karlodinium micrum AF472553
uncult. marine eukaryote AY129031
BK409
uncult. marine picoplankton DQ386740
uncult. marine picoplankton DQ386744
BK497
eukaryote marine clone ME1-10 AF363213
Amoebophyra sp. ex Gymnodinium instriatum AF472554
Amoebophyra sp. JH0210a AY775284
uncult. marine eukaryote AY129054
uncult. marine eukaryote AY129046
BK022
BK335
uncult. eukaryote DQ674775
eukaryote clone OLI11055 AJ402344
BK312
uncult. eukaryote EF172942
uncult. eukaryote AY885031
uncult. marine eukaryote AY129050
uncult. alveolate DQ186532
uncult. eukaryote EF172937
uncult. eukaryote EF172934
uncult. eukaryote EF172863
BK161
uncult. marine alveolate DQ001446
uncult. marine eukaryote AJ829796
uncult. eukaryote EF172848
uncult. eukaryote EF172922
uncult. eukaryote EF172921
uncult. eukaryote EF172925
uncult. marine eukaryote AY331746
uncult. marine eukaryote DQ647524
VN150
VN195
uncult. marine eukaryote AY331760
VN146
uncult. eukaryote EF172923
VN174
VN033
uncult. marine eukaryote AY129041
VN072
uncult. alveolate AF530532
uncult. marine alveolate GroupII DH148-EKD14 AF290079
eukaryote clone OLI11009 AJ402348
VN113
Gyrodinium helveticum AB120004
uncult. eukaryote AY664956
BK500
Gyrodinium rubrum AB120003
VN155
uncult. eukaryote AY664933
uncult. eukaryote AY664949
uncult. eukaryote AY664934
Gyrodinium spirale AB120001
uncult. marine eukaryote DQ310317
uncult. dinoflagellate AY429064
uncult. dinoflagellate AY429057
uncult. dinoflagellate DQ504326
uncult. eukaryote AY664895
VN154
VN605
Gyrodinium sp. AB001438
Karlodinium micrum EF036540
BK071
uncult. eukaryote EF100400
uncult. eukaryote AF372784
Katodinium rotundatum DQ388464
Heterocapsa rotundata AF274267
uncult. marine picoplankton DQ386738
uncult. alveolate AF372783
BK339
BK034
uncult. marine eukaryote AY331754
clone He001206_66 AJ965210
clone He010218_136 AJ965218
clone He010218_87 AJ965214
uncult. alveolate DQ186538
VN139
VN617
BK072
VN183
uncult. marine eukaryote DQ647527
VN140
VN107
VN101
VN114
clone He010218_146 AJ965213
BK458
VN613
VN040
VN088
VN106
VN094
VN046
eukaryote clone OLI11511 AJ402343
VN138
uncult. marine picoplankton DQ386741
uncult. marine eukaryote DQ310271
uncult. marine eukaryote DQ310305
uncult. marine eukaryote DQ310303
uncult. eukaryote AB191418
uncult. eukaryote AY665020
uncult. marine eukaryote DQ647518
uncult. marine eukaryote DQ103794
uncult. marine picoplankton DQ386752
uncult. marine picoplankton DQ386759
Caecitellus paraparvulus DQ220715
VN115
uncult. picobiliphyte clone He001005_33 EF050072
uncult. eukaryote AY789784
VN609
BK322
Caecitellus parvulus AY520446
Caecitellus pseudoparvulus DQ220712
uncult. eukaryote AY665066
uncult. eukaryote AY665074
uncult. eukaryote AY665070
Mesodinium pulex AY587130
Group II alveolates
Dinoflagellates
Group I alveolates
Bicosoecida
Ciliophora

Figure 8B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uncult. marine eukaryote DQ121419
BK401
BK466
BK264
BK465
eukaryote clone OLI11008 AJ402350
uncult. eukaryote EF173010
uncult. stramenopile AY295431
VN147
eukaryote marine clone ME1-22 AF363191
uncult. marine eukaryote DQ310211
uncult. eukaryote AB191422
eukaryote marine clone ANT12-11 AF363197
uncult. marine eukaryote DQ310191
VN169
uncult. eukaryote AY046774
uncult. eukaryote AY046778
uncult. marine eukaryote AY381206
uncult. stramenopile AF372763
uncult. stramenopile AF372762
uncult. stramenopile AY180031
MAST 1
Oomycetes
eukaryote marine clone ME1-17 AF363186
uncult. marine stramenopile DH148-5EKD53 AF290083
BK298
VN143
uncult. marine eukaryote EF526977
uncult. marine eukaryote DQ121425
MAST 2
VN167
VN067
uncult. marine eukaryote AY129069
clone HE000427_21 AY381157
BK491
BK496
BK478
eukaryote marine clone NA11-5 AF363204
eukaryote clone OLI11006 AJ402357
VN098
uncult. eukaryote EF172976
uncult. eukaryote AY116222
MAST 3
uncult. stramenopile AF372751
uncult. stramenopile AF372753
uncult. stramenopile AF372754
uncult. stramenopile AF372755
BK197
stramenopile MAST-12KKTS_E5 EF219382
stramenopile MAST-12KKTS_D3 EF219381
uncult. eukaryote AY046817
uncult. eukaryote AB252770
uncult. stramenopile OLI51105 AF167414
MAST 12
VN141
VN157
VN176
uncult. eukaryote EF172982
uncult. eukaryote EF172970
VN052
VN192
VN116
VN108
VN184
VN142
VN177
VN083
BK067
uncult. marine eukaryote AY129064
VN127
BK295
BK004
uncult. marine eukaryote AY381207
uncult. marine eukaryote AY381196
MAST 4
MAST 7
BK309
BK481
clone HE001005_112 AY381171
uncult. eukaryote EF172979
Schizochytrium aggregatum AF265336
Schizochytrium minutum AB022108
Thraustochytrium multirudimentale AB022111
Thraustochytriidae sp. DQ367047
Labyrinthulida
BK164
BK181
uncult. heterokont DQ504337
uncult. eukaryote AB191426
VN161
uncult. marine eukaryote AY381217
MAST 9
BK452
uncult. marine eukaryote DQ314811
Cryothecomonas aestivalis AF290541
uncult. eukaryote EU050978
uncult. eukaryote AB252744
BK483
uncult. marine eukaryote AY882495
Cercozoa AY620352
Cryothecomonas longipes AF290540
uncult.marine eukaryote DQ314810
uncult.marine eukaryote DQ314809
clone He010218_2 AJ965254
Amphibelone anomala AB178583
Amphibelone anomala AB178584
Anthracanthida sp. 206 AF063239
Haliommatidium sp. AF018159
Hexaconus serratus AB178588
Hexaconus serratus AB178587
clone Or000415_149 AJ965255
eukaryote clone OLI11032 AJ402342
uncult. marine acantharean DH147-EKD17 AF290072
BK307
BK060
uncult. phototrophic eukaryote DQ222878
uncult. phototrophic eukaryote DQ222876
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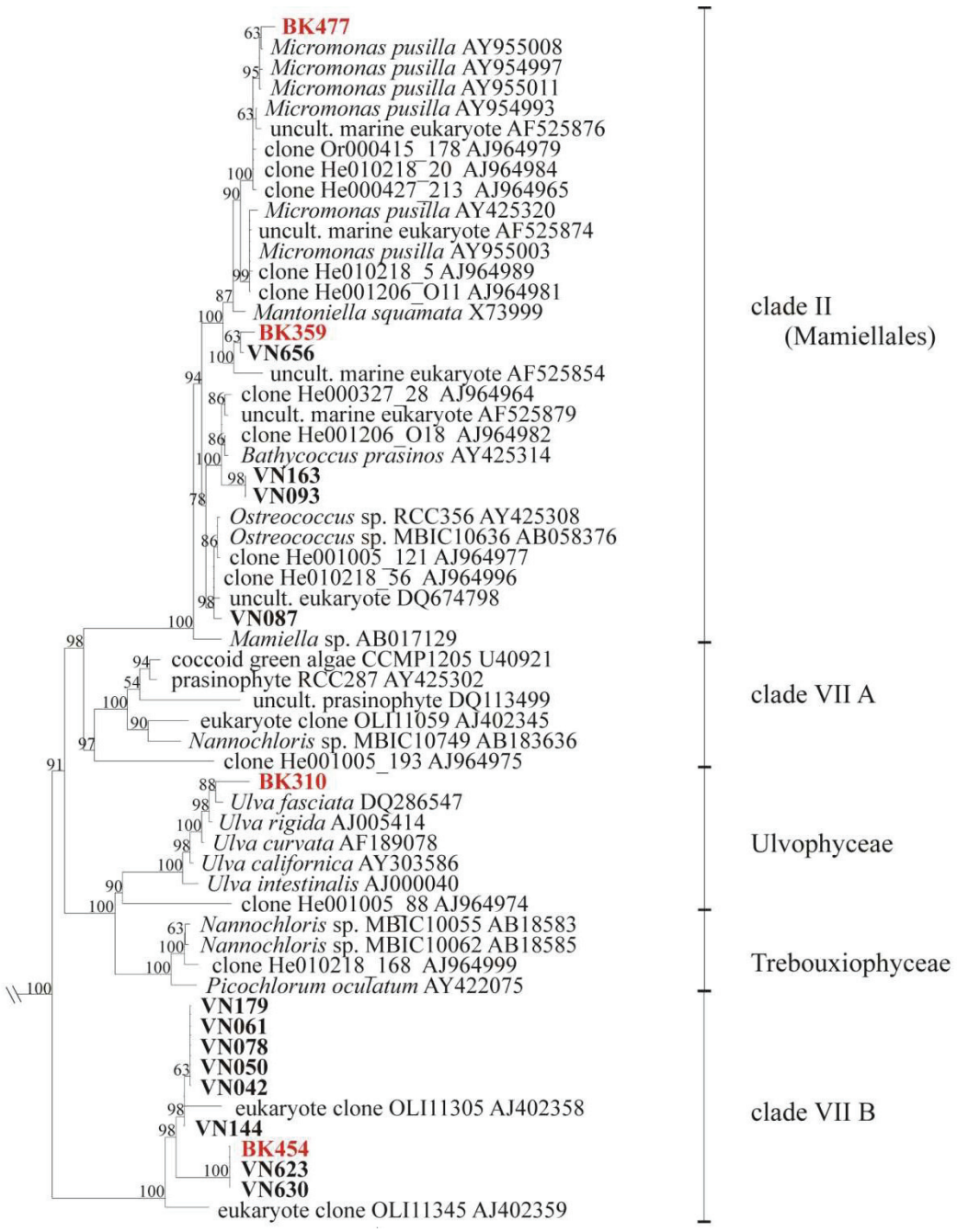
uncult. rhodophyte AY343928
uncult. marine eukaryote DQ060523
Picobiliphyta

Figure 8C


BK477
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Micromonas pusilla AY954997
Micromonas pusilla AY955011
Micromonas pusilla AY954993
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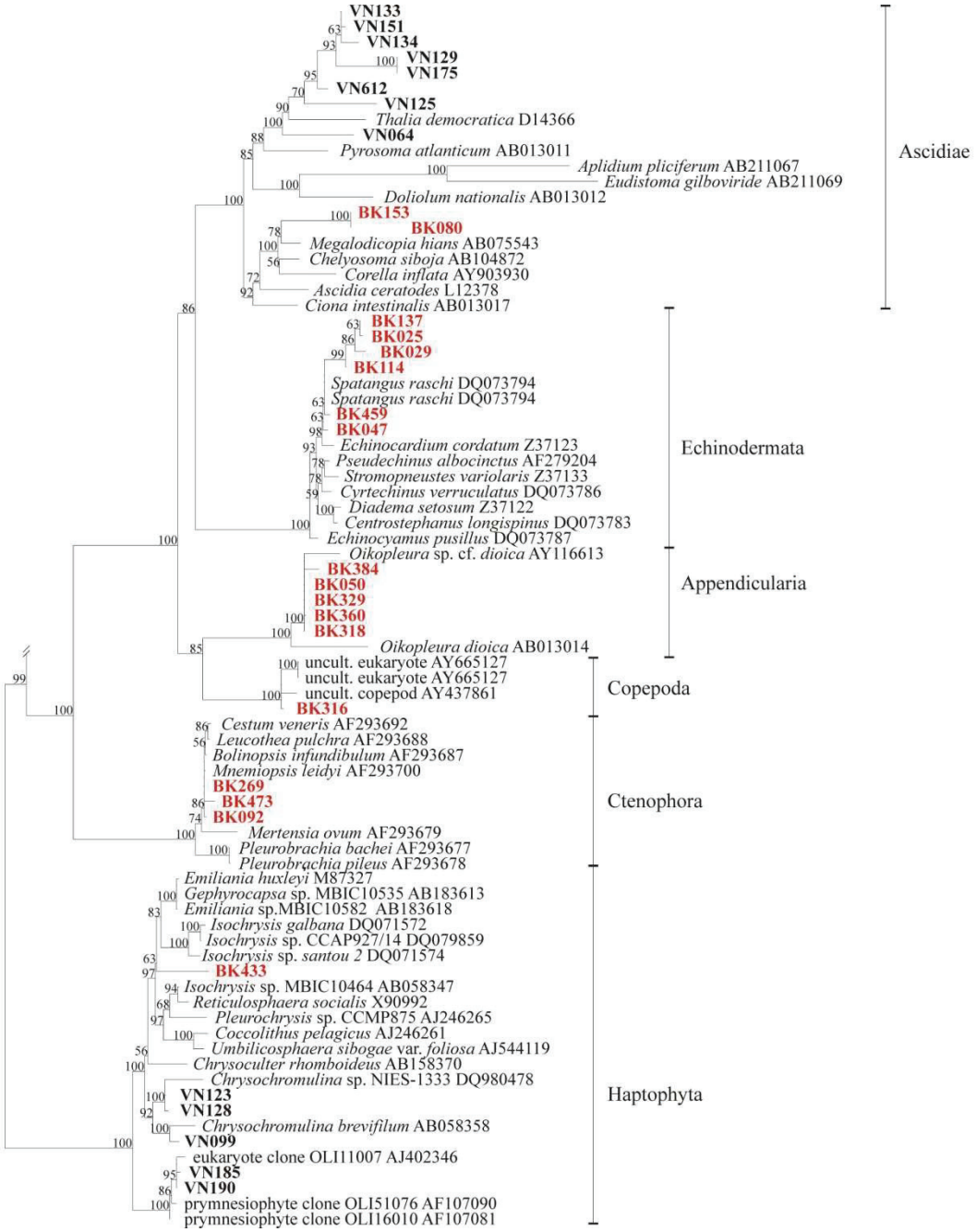
clone Or000415_178 AJ964979
clone He010218_20 AJ964984
clone He000427_213 AJ964965
Micromonas pusilla AY425320
uncult. marine eukaryote AF525874
Micromonas pusilla AY955003
clone He010218_5 AJ964989
clone He001206_O11 AJ964981
Mantoniella squamata X73999
BK359
VN656
uncult. marine eukaryote AF525854
clone He000327_28 AJ964964
uncult. marine eukaryote AF525879
clone He001206_O18 AJ964982
Bathycoccus prasinos AY425314
VN163
VN093
Ostreococcus sp. RCC356 AY425308
Ostreococcus sp. MBIC10636 AB058376
clone He001005_121 AJ964977
clone He010218_56 AJ964996
uncult. eukaryote DQ674798
VN087
Mamiella sp. AB017129
coccoid green algae CCMP1205 U40921
prasinophyte RCC287 AY425302
uncult. prasinophyte DQ113499
eukaryote clone OLI11059 AJ402345
Nannochloris sp. MBIC10749 AB183636
clone He001005_193 AJ964975
BK310
Ulva fasciata DQ286547
Ulva rigida AJ005414
Ulva curvata AF189078
Ulva californica AY303586
Ulva intestinalis AJ000040
clone He001005_88 AJ964974
Nannochloris sp. MBIC10055 AB18583
Nannochloris sp. MBIC10062 AB18585
clone He010218_168 AJ964999
Picochlorum oculatum AY422075
VN179
VN061
VN078
VN050
VN042
eukaryote clone OLI11305 AJ402358
VN144
BK454
VN623
VN630
eukaryote clone OLI11345 AJ402359
clade II
(Mamiellales)
clade VII A
Ulvophyceae
Trebouxiophyceae
clade VII B

Figure 8D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VN134
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VN125
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VN064
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Aplidium pliciferum AB211067
Eudistoma gilboviride AB211069
Doliolum nationalis AB013012
BK153
BK080
Megalodicopia hians AB075543
Chelyosoma siboja AB104872
Corella inflata AY903930
Ascidia ceratodes L12378
Ciona intestinalis AB013017
Ascidiae
BK137
BK025
BK029
BK114
Spatangus raschi DQ073794
Spatangus raschi DQ073794
BK459
BK047
Echinocardium cordatum Z37123
Pseudechinus albocinctus AF279204
Stromopneustes variolaris Z37133
Cyrtechinus verruculatus DQ073786
Diadema setosum Z37122
Centrostephanus longispinus DQ073783
Echinocyamus pusillus DQ073787
Echinodermata
Oikopleura sp. cf. dioica AY116613
BK384
BK050
BK329
BK360
BK318
Oikopleura dioica AB013014
Appendicularia
uncult. eukaryote AY665127
uncult. eukaryote AY665127
uncult. copepod AY437861
BK316
Copepoda
Cestum veneris AF293692
Leucothea pulchra AF293688
Bolinopsis infundibulum AF293687
Mnemiopsis leidyi AF293700
BK269
BK473
BK092
Mertensia ovum AF293679
Pleurobrachia bachei AF293677
Pleurobrachia pileus AF293678
Ctenophora
Emiliania huxleyi M87327
Gephyrocapsa sp. MBIC10535 AB183613
Emiliania sp.MBIC10582 AB183618
Isochrysis galbana DQ071572
Isochrysis sp. CCAP927/14 DQ079859
Isochrysis sp. santou 2 DQ071574
BK433
Isochrysis sp. MBIC10464 AB058347
Reticulosphaera socialis X90992
Pleurochrysis sp. CCMP875 AJ246265
Coccolithus pelagicus AJ246261
Umbilicosphaera sibogae var. foliosa AJ544119
Chrysoculter rhomboideus AB158370
Chrysochromulina sp. NIES-1333 DQ980478
VN123
VN128
Chrysochromulina brevifilum AB058358
VN099
eukaryote clone OLI11007 AJ402346
VN185
VN190
prymnesiophyte clone OLI51076 AF107090
prymnesiophyte clone OLI16010 AF107081
Haptophyta

Figure 8E

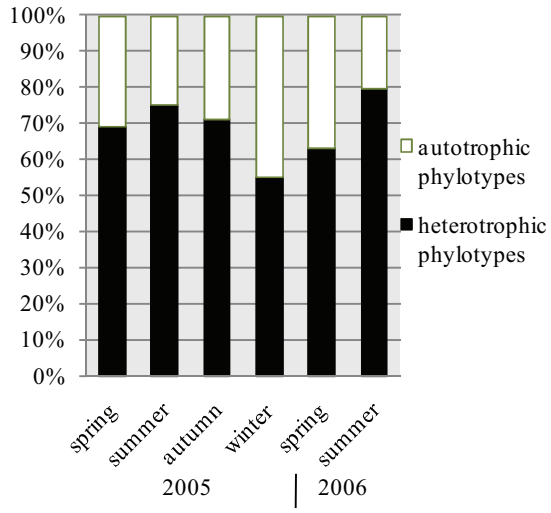

Figure 9

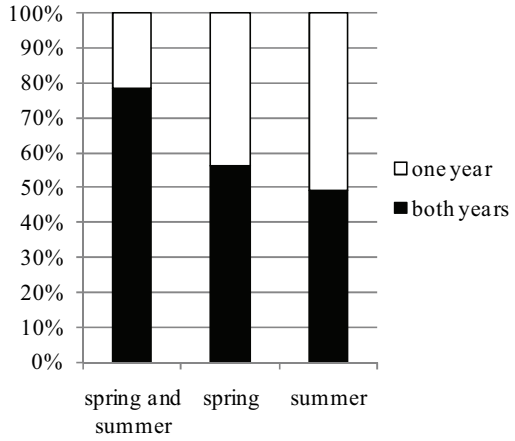

Figure 10

Information about the author

Britta Knefelkamp

contact: b.knefelkamp@gmx.de

Current manuscripts and publications

Knefelkamp, B., K. Carstens, K.H. Wiltshire: HPLC determination of picophytoplankton pigments from the North Sea; Journal of Experimental Marine Biology and Ecology, submitted

Knefelkamp, B., D. Vaulot, N. Simon, W. Eikrem, E. Foulon, S. Masquelier, K.H. Wiltshire, J. Throndsen, R.I. Amann, G. Gerdts: Assessment of picoplankton abundance and dynamics in the German North Sea by FISH, flow cytometry, fluorometry, HPLC and electron microscopy; Aquatic Microbial Ecology, submitted

Knefelkamp, B., G. Gerdts, R. Niestroj, K.H. Wiltshire, A. Wichels: Feasibility to assess the diversity, occurrence and ecology of picoplanktonic eukaryotes by DGGE and sequencing; Aquatic Microbial Ecology, submitted

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„As every microbiologist inherently knows, little things can be the cause of much greater things that are often of the utmost importance, and this is especially true for marine picophytoplankton.“

(Donald A. Bryant, 2003)

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Bremen, den

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Britta Knefelkamp

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