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Cryptophyceae diversity by sequencing 18S rDNA clone libraries

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Master Thesis

First reviewer: Allan Cembella
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For my beloved Michal

Summary

Cryptophyceae are important constituents of the marine phytoplankton. However, there are difficulties in their species determination because cryptophytes lack distinct morphological features that can be seen with light microscopy. Thus, they are usually omitted in standard phytoplankton surveys or listed as bulk counts for the group. Molecular methods offer a solution to this problem of species identification. Recently, microarray technology has been applied to screen for Cryptophyceae in phytoplankton samples. In the EU project Micropad, a microarray was designed that contained probes for the most abundant classes in the marine phytoplankton, including cryptophytes. For this study, the analysis of the phytoplankton community with molecular methods was extended. Samples were collected at Helgoland Roads during the spring: in March, April and May and the structure of the plankton community assessed from partial sequences of the small subunit ribosomal RNA gene (SSU rDNA) in clone libraries. Two different approaches to minimize the number of sequencing reactions required for a group specific analysis of clone libraries were evaluated: restriction fragment length polymorphisms (RFLP) and hybridization on a reverse microarray with a class-level probe. A reverse microarray means that the clones from the clone library were spotted onto the slide and then the probe was hybridised to the slide. In a normal microarray, the probe is spotted onto the slide and the samples are hybridised to the slide.

Plankton assemblages in the clone libraries were typical for investigated time period. In March, autotrophic organisms (diatoms, dinoflagellates, chlorophytes and cryptophytes), were abundant in the clone library. With time, the heterotrophs, mainly larvae of benthic organisms, increased from 9% in March to 39% by the middle of May. In the phytoplankton, dinoflagellates were the most abundant organismsin all clone libraries. From April around 50% of dinoflagellates clones were heterotrophic. The second most abundant group were the centric diatoms. Prasinophyceae were well represented only in March. Cryptophyceae had a maximum in March but were present in the rest of samples as well. Most of the cryptophycean clones belonged to Clade 4 and formed 6 new subclades. Four clones grouped on two branches within Clade 7.The temporal changes in the community structure of Cryptophyceae are described.

The results from clone libraries sequencing supported the presence of cryptomonads as indicated by the analysis if the community with the Crypto B probe and the microarray analysis. However, when the same probe was applied to screening of clone libraries by the reverse microarray, it was non-specific and the number of clones carrying inserts from Cryptophyceae was overestimated in three out of four samples. Suggestions for improving the latter method are given.

PCR-RFLP turned out to be not well suited for screening clone libraries from complex environmental samples.

1. Introduction

The global ocean covers 70% of the Earth`s surface. In this huge system, the most important primary producers are auto- and mixotrophic planktonic, unicellular organisms referred to as phytoplankton. Phytoplankton contributes upto 50% of the global primary production. Carbon fixed by phytoplanktonic organisms in the euphotic zone (down to 200 meters depth) is the main source of organic carbon to the deep ocean. Diversity of phytoplankton is enormous, with several thousands of species described to date and new ones, especially cryptic species among the common cosmopolitan species, are continually discovered. Surveys of phytoplankton biodiversity began more than 150 years ago, but despite this, in many ways, the plankton still remains poorly characterised. The large fraction (>20 μm), containing diatoms and dinoflagellates, are usually well known because they have many discriminative morphological features and as so can be easily distinguished under the light microscopy (LM). This group has been well studied since the mid-1800s. However, other classes, including phytoflagellates like Cryptophyceae, are still poorly studied because they often lack distinctive feature that can be easily seen with LM and for this reason they are often omitted from ecological investigations (Medlin & Simon 1998).

1.1 Cryptophytes

Cryptophyta are mixotrophic flagellates ubiquitous in marine and freshwater pelagic habitats (Butcher 1967). These organisms play an important role as primary producers, bacteria consumers as well as prey for zooplankton; and can dominate the phytoplankton assemblage (Gieskes & Kraay 1983, Mura & Agusti 1998, Tang et al. 2001). Phylogenetic studies of Cryptophyceae inferred from sequence analysis of genes encoding the small subunit of the ribosomal RNA (18S rDNA) group all photosynthetic genera and non-photosynthetic, osmotrophic *Chilomonas* in seven, closely related clades (Table 1), whereas the aplastidal genus, *Goniomonas,* forms an early diverging, separate branch in the tree (Marin et al. 1998, Deane et al. 2002).

Table 1. Distribution of Cryptophyceaen genera between the clades based on 18S rRNA phylogeny (adapted from (Marin et al. 1998) and (Deane et al. 2002)).

Clade 1	Clade 2	Clade 3	Clade 4
Campylomonas	*Rhinomonas*	*Guillardia*	*Geminigera*
Chilomonas	*Rhodomonas*	*Hanusia*	*Teleaulax*
Cryptomonas	*Pyrenomonas*		*Plagioselmis*
	Storeatula		
Clade 5	Clade 6	Clade 7	
Proteomonas	*Komma*	*Falcomonas*	
	Chroomonas		
	Hemiselmis		
	Plagiomonas		

In spite of their importance, Cryptophycean diversity and biogeography is still poorly investigated and understood (Clay et al. 1999). The main reason is that identification of Cryptophycean species during routine phytoplankton surveys is virtually impossible. The determination to species and genus level involves detailed and costly studies on morphology with light microscope on living cells, scanning electron microscopy and transmission electron microscopy, as well as on the composition of phycobiliprotein pigments with high performance liquid chromatography (Klaveness 1985). Moreover, the discovery of two stages in life cycle of *Proteomonas sulcata* (haploid and diploid) with very distinct cell morphology (Hill & Wetherbee 1986) has further complicated studies on cryptophyte diversity. Molecular data also suggests that other species have dimorphic stages and that many closely related genera with different morphology are actually haploid and diploid stages of the same species (Hoef-Emden et al. 2002, Hoef-Emden & Melkonian 2003). A haplo-diplo life cycle is likely a feature of the entire group. When one adds the fact that the delicate cell structures of cryptophytes get destroyed during sample fixation, it becomes obvious that it is impossible to determine cryptophycean species based solely on LM, the most commonly used tool in ecological research. Hence, in most phytoplankton surveys, Cryptophyceae are reported at the class level, because they can be easily distinguished from other phytoflagellates on the basis of characteristic cell shape (Klaveness 1985) and orange fluoresence.

Solutions to the problems in Cryptophycean species identification could be found with the application of molecular methods.

1.2 Molecular methods in microbial ecology

Clone libraries have been successfully applied to reveal enormous biodiversity of microbial communities in many habitats (Bruemmer et al. 2004, Eslaied et al. 2004, Tananaka et al. 2004, Fuchs et al. 2005, Mills et al. 2005, Sugita et al. 2005). Similar levels of hidden biodiversity have also been reported in the pico-eukaryotic fraction (Garcia-Lopez et al. 2001, Moon-van der Staay et al. 2001, Medlin et al. in press). This approach involves the amplification and cloning of SSU rDNA from environmental samples. The SSU rRNA genes are very well suited for phylogenetic analysis, and for identification, as they are universally distributed in all known living organisms where they have the same function; carry a lot of information in their long sequence (1800 bp); contain both variable and conserved regions, and no evidence for lateral gene transfer (Woese 1987). A cloning step separates amplified 18S rDNA genes originating from different species, so each can be separately analysed by molecular techniques. This method, as applied e.g. to Cryptophyceae, should avoid overestimation of cryptophyceaen diversity resulting from assigning dimorphic stages of a single species into two different genera, as the sequence of 18S rDNA is identical in both stages. Clones can be analysed with great variety of molecular methods, most common being restriction analysis and sequencing. Recently, microarray technology has also been applied to study phytoplankton in environmental samples (Metfies & Medlin 2004, 2005). This method involves hybridization of fluorescently labelled DNA/RNA fragments from the investigated samples with probes spotted on the solid surface and is already well established in studies of gene- expression (Gershon 2005). The application of microarrays in biodiversity surveys involves spotting of phylogenetic rRNA probes on the surface of the glass slide. The community structure is investigated after amplification of 18S DNA genes with general eukaryotic primers (Metfies & Medlin 2004). The recently developed DNA microarray technology allows the simultaneous analyses of up to 250,000 probes at time. (Lockhart et al. 1996). The application of this technique is an opportunity for extremely time-efficient, automated analysis of species composition in environmental samples. This is of special interest for identification of small cells with few distinct morphological features e.g., picoplankton. The first DNA-microchip has

been developed to study samples containing nitrifying bacteria (Guschin et al. 1997). Microchips for phytoplankton have been developed in the EU-projects PICODIV (Medlin et al. 2006).

1.3 Helgoland Time Series

An important point in studies of biodiversity is the monitoring of change over a long period. A time series dataset of phytoplankton diversity is available from Helgoland Roads, where phytoplankton samples have been collected on a daily basis from the surface water at the Helgoland Road Station (Helgoland Reede, 54º 11' N, 7º 54' E) since 1962 (Figure 2). Samples are fixed using Lugol's solution and counted with the Utermöhl method to species level (Wirtz & Wiltshire 2005). The data are an invaluable source of quantitative information on ecosystem functioning but because of methodology limitations, species resolution is constrained to diatoms and dinoflagellates. It is only recently that novel molecular techniques have been applied to reveal diversity of other phytoplankton groups in the samples from Helgoland Roads (Medlin et al. 2006)

1.4 MICROPAD

This study is part of the EU-project MICROPAD to improve and facilitate cryptophyceaen recognition during routine sample investigation. To achieve this, a microarray based diversity analysis was developed (Metfies and Medlin 2005). A preliminary investigation of samples collected from Helgoland Reede during phytoplankton bloom showed that abundance of cryptophytes in nano- and pico- fraction of phytoplankton, which was not recorded in the standard microscopic investigations of samples collected with 20 μm mesh phytoplankton net (Table 2).

Table 2. Comparison of data from manual counts and microarrays on presence of Cryptophyceanin the samples (Metfies, unpublished).

	18.03.2004	15.04.2004	03.05.2004	13.05.2004
Manual counts (20 μm net)	+	+	-	-
Ratio signal CryptoA/Crypto B	5.6	5.3	4.5	5.4

1.5 Objectives

The objective of this study was to investigate plankton diversity with the main focus on Cryptophyceae by sequencing of 18S rDNA- clone libraries. These data were subsequently used to evaluate the microarray data from the MICROPAD project and evaluation of application of the reverse microarray as a tool for screening clone libraries for Cryptophyceae with class-level probe Crypto B. Additionally, restriction fragment length polymorphisms (RFLP) was evaluated as a screening method for cryptophytes and other groups of phytoplankton.

The flow scheme of my work is shown in the Figure 1.

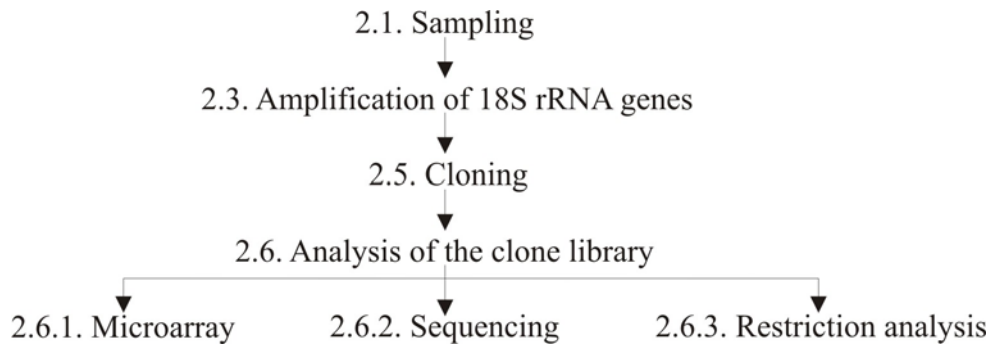


Figure 1.The flow scheme for investigating biodiversity of Cryptophyceae applied in the survey. Numbers refer to section in the text where the methodology is described in detail.

2. Material and Methods

2.1. Samples collection

Samples used in this study were collected during the spring phytoplankton bloom of 2004, on 13th March, 15th April, 3rd of May and 13th of May (He040318, He040415, He040503 and He040513, respectively) from the surface layer at the Helgoland Road Station, as a part of Helgoland time series (Prof. Karen Wiltshire group, BAH, AWI) (Figure 2). One litre of seawater collected with a bucket was filtered through 0.4 μm membrane filter (Isopore™ membrane filters, Millipore, USA) and immediately frozen at -80ºC until further processed.

Figure 2. Helgoland Road Station (54º 11' N, 7º 54' E).

2.2. Isolation of genomic DNA

Genomic DNA from the collected samples was extracted with use of DNeasy ® Plant Mini Kit (Qiagen, Germany) according to the protocol supplied by manufacturer (Appendix 1).

The DNeasy ® Plant Mini Kit yields pure, free of polymerase chain reaction (PCR) inhibitors DNA from the cells. Cells were first lysed by addition of RNase containing lysis buffer and incubated at 65ºC for ten minutes. RNase digested RNA, whereas

proteins and polysaccharides were salt precipitated and subsequently removed by centrifugation through the QIAshredder column provided with the kit. Cleared lysate was transferred to the DNeasy Mini Spin column where DNA bound to the DNeasy membrane in the presence of chaotropic salts and ethanol. Two washing steps efficiently removed all contaminants. DNA was eluted in low-salt buffer.

2.3. Amplification of the 18S rDNA

The extracted genomic DNA served as a template for amplification of an 800 base pair (bp) fragment of 18S rDNA (primers 82F (5' GAA ACT GCG AAT GGT TCA TTA AAT CAG 3') and 690R-58 (5' CAG AGG TGA AAT TCT 3')). The PCR reaction was performed in an Eppendorf gradient MasterCycler (Eppendorf, Germany) with Eppendorf Taq polymerase (2U per 50 µl of a reaction) and the following program: 5 minutes 94°C, 45 cycles of one minute 94°C, two minutes 54°C, two minutes 72°C, followed by 10 minutes of final extension at 72°C.

The success of the PCR reactions was monitored by running 5 µl of PCR reaction mixed with 3 µl of loading buffer (75 mM EDTA, 3% glycerol, 0.02% bromophenol blue) on the 1.5 % agarose (Invitrogen™) in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA) gel (ethidium bromide concentration 0.1 µg per 1 ml of gel) with 80 V applied current. DNA bands on the gel were visualized under UV light (Vilber Lourmat, France) with BioCapt 11.02 for Windows software.

2.4. Purification of PCR-Products

The PCR products were purified from the primers, Taq polymerase and deoxynucleotides with the QIAquick® PCR Purification Kit (Qiagen, Germany). The samples were processed according to the protocol supplied by manufacturer (Appendix 1). The kit is based on the following principle: DNA was first absorbed to the silica-membrane of the spin column in the presence of high salt concentration (PB Buffer), whereas the contaminants passed through during centrifugation. Salts were resolved and then washed away quantitatively with ethanol-containing buffer PE. Afterwards, DNA was eluted with 50 µl of autoclaved MilliQ water. Concentration and quality of the purified products was monitored with NanoDrop ND-1000 spectrophotometer (PeqLab).

2.5. Cloning

2.5.1. Description of the cloning vector

The clone libraries were created with the pCR®4-TOPO® vector (Figure 3). This vector is a plasmid that carries genes for ampicillin and kanamycin resistance so that the cells that took up the plasmid can be easily selected. TOPO® cloning site is in the region of LacZα-ccdB gene fusion. The product of the ccdB gene is a potent poison of gyrase and is lethal to *Escherichia coli* cells (Bernard et al. 1994). Ligation of a PCR product disturbs the fused genes permitting growth of positive recombinants only. The vector supplied with the kit (TOPO TA Cloning® Kit for Sequencing; Invitrogen™) is linearized and has sticky end with single 3' deoxythymidine, introduced by topoisomerase I from *Vaccina* virus (Shuman 1994). Because *Taq* polymerase adds a single deoxyadenosine to the 3' ends of the products, the PCR product can be efficiently ligated into the vector.

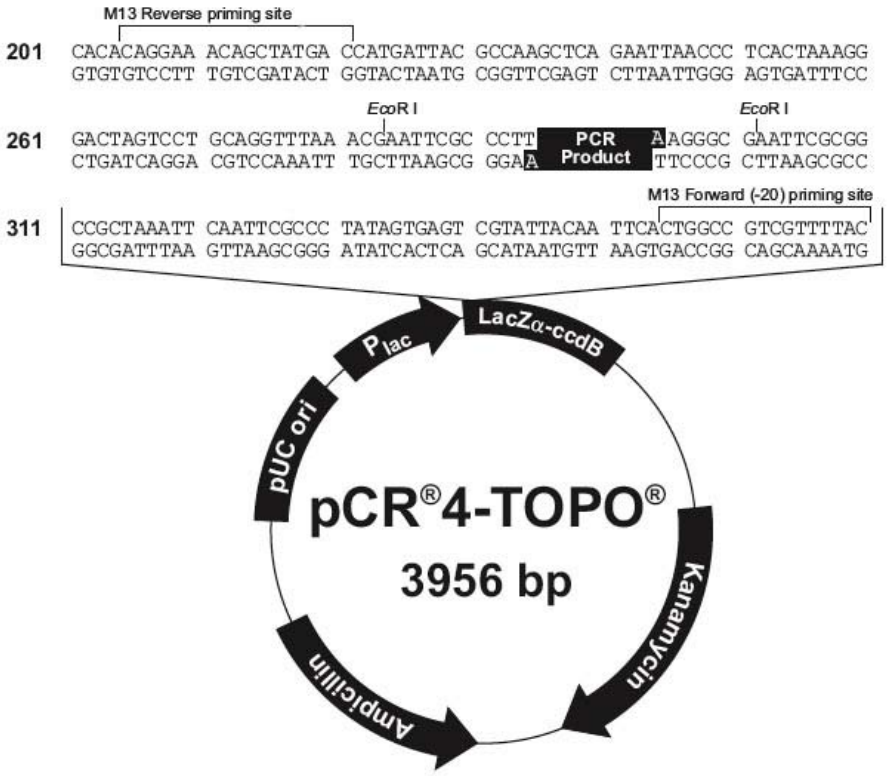


Figure 3. pCR®4-TOPO® vector. The site of an insert ligation is shown in detail. Restriction and priming sites are shown only for the enzyme and the primers used in analyses. For other explanations refer to text. (adapted from the user's manual to TOPO TA Cloning® Kit for Sequencing, Invitrogen™).

2.5.2. Cloning reaction

The freshly purified PCR product was ligated into the vector (molar ratio 5:1) in the presence of salt (200mM NaCl, 10mM $MgCl_2$). The ligation reaction was carried out overnight at 15°C. The complete reaction volume (6 µl) was added into a vial with TOP 10 chemically competent One Shot® cells (*Escherichia coli* strain F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G). After 30 minutes incubation on ice, cells were heat shocked for 30 seconds at 42°C and then incubated for an hour in S.O.C. medium (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5 mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose; provided with the kit) at 37°C. After the incubation, small volumes (20 and 150 µl) were spread on pre-warmed plates with selective LB agar medium (1% tryptone, 0.5% yeast extract, 1% NaCl, 15% agar, pH 7.0, kanamycin 50 µg per ml) and cells were allowed to grow overnight at 37°C. For each of

the samples, 96 single colonies were picked up and grown in liquid selective LB medium (without agar) for 24 hours at 37°C. Afterwards plasmids were isolated.

2.5.3. Isolation of plasmids

Before the environmental 18S rDNA genes could be analysed, the plasmids containing the insert had to be isolated from the bacterial cells. After 24 hours incubation of the clones in liquid LB medium, cells were pelleted and the medium discarded. After resuspension, cells were lysed in NaOH and SDS solution in the presence of RNase. SDS lysed the cells by solubilizing the phospholipids and proteins of the cell membrane, whereas the alkaline conditions denatured proteins as well as chromosomal and plasmid DNA. The lysate was then neutralized and adjusted to high-salt binding conditions that caused denatured proteins, chromosomal DNA and cellular debris to precipitate. Plasmids were separated from chromosomal DNA based on co-precipitation of the chromosomal DNA bounded to the cell wall with insoluble complexes containing salt, detergent and proteins. Plasmids, which remained in the solution, were precipitated with 100% isopropanol, washed with 70% ethanol, resuspended in the sterile water by overnight incubation at 4°C and stored at -20°C. This procedure was facilitated by use of R.E.A.L. Prep 96 Plasmid Kit (detailed protocol in Appendix 1). Isolated plasmids were screened for an insert by digestion with an *EcoRI* restriction enzyme (8U per 20 µl of reaction; New England BioLabs) at 37°C for 2h. The presence and size of the cut insert were monitored with gel electrophoresis, as described above.

2.6. Analysis of the clone library

2.6.1. Microarray analysis

A microarray analysis with a biotinylated Crypto B probe (5'biotin- ACG GCC CCA ACT GTC CCT 3') was performed in order to screen for plasmids containing insert with 18S rDNA from cryptophytes. Biotin-labelled 82F primer was used as a general eukaryotic probe to evaluate the amount of 18SrDNA PCR-fragment immobilized on the slide-surface.

The inserts were amplified with M 13 primers (forward and reverse, Figure 3). 1U per 50 µl of a reaction of Eppendorf polymerase and the following program: 7 minutes 94°C, 35 cycles of 2 minute 94°C, 4 minutes 54°C, 2 minutes 72°C, followed by 10 minutes of final extension at 72°C. The success of the PCR reaction was monitored by gel electrophoresis as described in section 2.3. The PCR products were purified as described in section 2.4 and measurements of DNA concentration were performed. 5 µl of each purified PCR product (average concentration 70 ng/µl, range from 37 ng/µl to 290 ng/µl) were transferred onto 384-well plate, mixed with 5 µl of QMT Spotting Solution I (Quantifoil Micro Tools GmbH, Jena, Germany) and spotted with Microarray Spotter Vers Array ChipWriter™ ProSystem (BioRad) on epoxy-coated glass slides (Qantifoil Micro Tools GmbH, Jena, Germany). On each of the microscopic slides, two arrays were spotted. Each clone was spotted in two replicates that were placed in the same column, one under the other. Subsequently to spotting, chips were incubated at 60°C for 30 minutes and store at -20°C.

Prior to the hybridization, the DNA on the chip was denatured for three minutes in MilliQ water at 96°C. After this step, DNA on the chip was single stranded, which facilitated the binding of the probe. The hybridization was preceded by one hour prehybridization at 58°C in 1x STT (1M NaCl, 10 mM Tris pH 8, 0.005% Triton x-100) and 0.5 mg/ml of Bovine Serum Albumin (BSA) buffer to provide the right conditions on the chip. The slide was washed in RT MilliQ water and dried by centrifugation at 3000 rpm for 2 minutes. For the hybridization the 30 µl of hybridization mixture (15 µl of hybridization buffer (0.5 mg/ml BSA, 0.1 µg/µl herring sperm-DNA, 1x STT-Buffer), 2 µl of 250 bp fragment of TATA-box binding protein gene of yeast *Saccharomyces cerevisiae* (29 ng/µl, positive control for the hybridization reaction), 3 µl of the probe (final concentration 10 µM) and 10 µl of autoclaved MilliQ water) was applied to the microarray covered with a cover-slip.

The slide was then incubated in a wet chamber for an hour at 58°C. Hybridization with each probe was done in four replicates or eight replicates for a clone. Unbound probes were removed in three 5 minutes washing steps performed at room temperature. With each step, stringency of buffers increased (1st buffer – 2xSSC (2 M NaCl, 20 mM Tris, pH 8.0, 0.01% Triton x-100), 10 mM EDTA, 0.05% SDS; 2nd buffer – 1xSSC, 10 mM EDTA; 3rd buffer – 0.2xSSC, 10 mM EDTA). The last buffers did not contained SDS, because residual SDS would generate high background intensi-

ties. The washed chip was afterwards stained for 30 minutes at room temperature with 30 µl of Streptavidin-Cy5 solution (0.1 µg/ml in 1x hybridization buffer) and washed again. The microarray was dried by centrifugation for two minutes at 3000 rpm after the prehybridization and subsequently to washing steps. Dried microarrays were scanned with GenePix™ 4000B scanner (Axon Instuments, USA) with 635 nm wavelength and analyzed with GenePix Pro 6.0 software (Axon Instruments, USA).

GenePix software has been designed to enable analysis of microarray images. First each individual spot was assigned a circular feature indicator with name of the clone. Subsequently, mean value of intensity of all pixels within the single spot was computed, with exclusion of any pixels that contacted the feature boundary. The background fluorescence for each individual spot was calculated using a circular region with diameter three times the diameter of the feature indicator and centred on the spot. All pixels inside the circle and outside of feature-indicators were used to calculate the mean value of the background that was subtracted from the mean value of intensity of the spot. Calculated this way the true measure of the fluorescence of a spot (signal intensity for the spot) was used in further analyses.

To compare the results between hybridizations, normalization of the signal intensity for the spot, for each hybridization had to be performed. The normalization factor was calculated on the basis of the signal intensity from the positive controls spots (Figure 4).

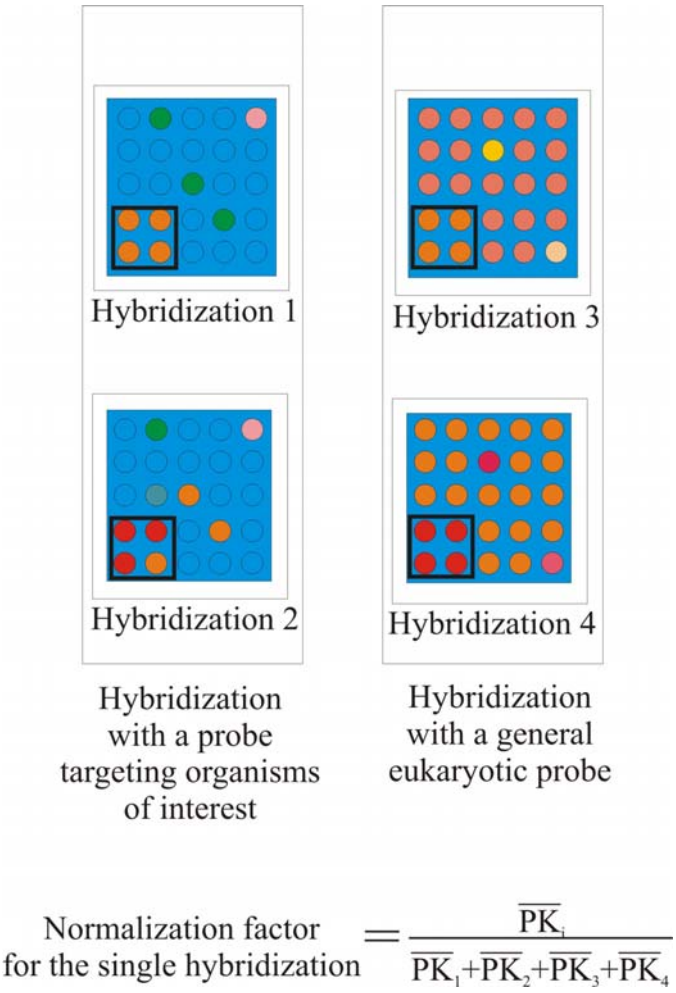


Figure 4. Calculation of normalization factor for four hybridizations. PK_i – mean of the intensity signals of positive control spots (in the bold square) for a single hybridization

First, a mean signal intensity value from all of the positive control spots from all slides to be compared was calculated. Then, the mean signal intensity value from all of the positive control spots from single hybridization was calculated. The normalization factor was calculated by dividing the mean value calculated for the single hybridization by the mean signal intensity value of all positive controls (Figure 4). Signal intensity for every spot was then multiplied by the normalization factor. Spots that were considered positive were those that resulted in normalized signal value higher than 500 arbitrary units from at least four replicates for both of the probes.

The results from the analyses were compared with the picture from the slide and only those were accepted that had given strong, clear signal on the microarray.

2.6.2. Restriction analysis

A restriction analysis of clones was performed to assess the numbers of unique clones. 5U of Hae III enzyme (Promega, recognition sequence 5'GG-CC 3') per 20µl reaction was used. The amount of DNA in the reaction varied from 371 ng to 2900ng To sustain enzyme activity in the presence of possible contaminants, acetylated BSA to final concentration 0.1 mg/ml was added to the reaction. The reaction mixture was incubated at 37°C for two hours and after that time immediately frozen at -20°C. All the reaction volume was mixed with 5 µl of loading buffer and run on the 2% agarose gel for two hours at 100V and was analysed with BioCapt software (see section 2.3).

2.6.3. Sequencing

Clone libraries from all four samples were sequenced with M13 reverse primer. Per 10 µl of reaction, 1 µl of Big Dye Sequencing RR100 (contains Polymerase, MgCl2, dNTPs and fluorescently labelled ddNTPs) was added. The cycle sequencing program used was as follows: 96°C for one minute, 24 cycles of 96°C for ten seconds, 50°C for five seconds, 60°C for four minutes. The products were purified with Dye Ex 96 Kit (Qiagen, Germany) according to the protocol supplied by manufacturer (Appendix 1). DNA fragments were separated from the dye terminators using method derived from gel filtration chromatography that separates molecules on the basis of molecular weight. Dye terminator was stopped in the pores of the gels, whereas DNA fragments were excluded and recovered in flow-through.

Purified reactions were mixed with 10 µl of Hi-Di formamide (Applied Biosystem), denatured for 3 minutes at 93°C and analysed with 3130*xl* Genetic Analyzer (Applied Biosystem Hitachi) capillary electrophoresis sequencer. The quality of the sequences was monitored on the basis of a chromatogram with SeqMan™II 5.07© software (DNASTAR Inc.) and stored in fasta file format. Sequences longer than 400 bp were compared against GenBank database with use of Washington University Basic Local Alignment Search Tool Version 2.0 (WU-Blast2, http://www.ebi.ac.uk/blast2/nucleotide.html). Those clones that were identified as Cryptophycean were

sequenced as described above but with M13 forward primer and searched against GenBank again to obtain more accurate information on taxonomic affiliation.

3. Results

3.1. Amplification of 18S rDNA genes from environmental samples

The amplification of 18S DNA genes with the primer-set 82F/690-58R from environmental samples resulted in ~800 bp fragments visualized in gel electrophoresis (Figure 5). Besides the 800 bp fragments, there was also small amount of DNA ~2000 bp long, but restriction analysis with *EcoRI* enzyme that cuts in the multicloning site at both sites of the insert (Figure 3) showed that these fragments were not incorporated into the vector (Figure 6A). Also the amplification of the inserts with M13 primers, priming the insert from both sites (Figure 3) confirmed that only 800 base pair long fragment had been incorporated into the vector (Figure 6B).

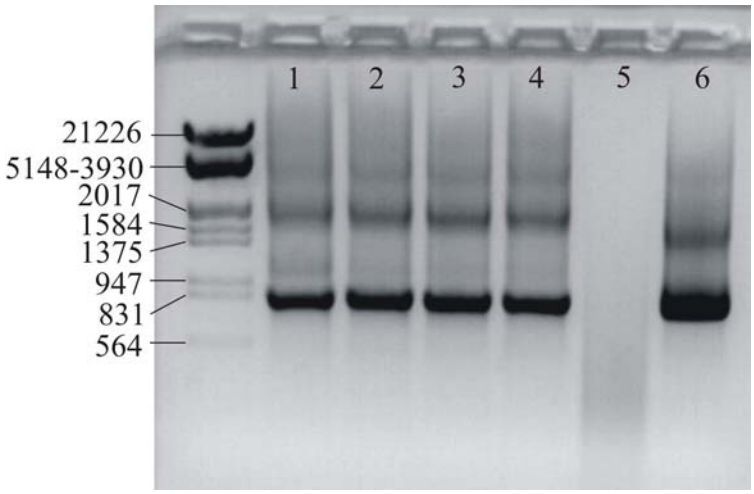
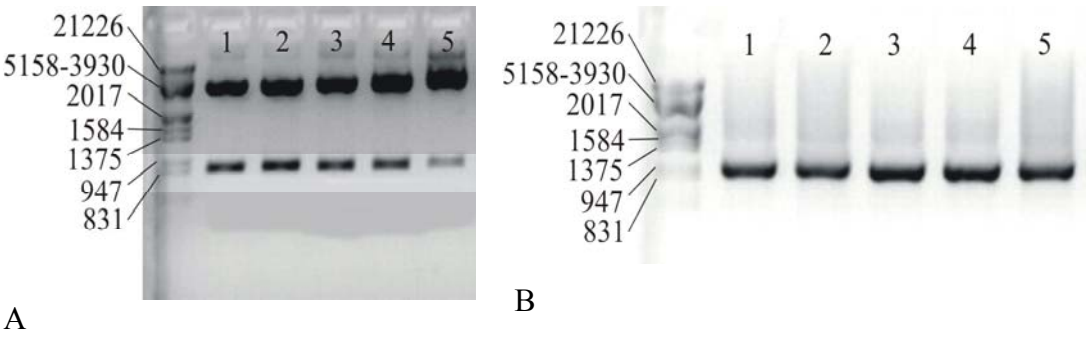


Figure 5. PCR products obtained with primers 82F and 690R on genomic DNA extracted from environmental samples. 800 bp long 18S rDNA product is visible as a bold bands, whereas 2000 bp products gave very weak bands. Lane 1 – He040318; lane 2 – He040415; lane 3 – He040503; lane 4 – He040513; lane 5 –water for molecular biology, Fluka, Germany (negative control); lane 6 – *Camplylomonas reflexa* (positive control).

The purification of the PCR reaction yielded pure DNA (260/230 ratio > 2.1, 260/280 ratio > 1.85) in concentrations ranging from 72 to 94 ng/µl. The cloning reaction was considered successful because hundreds of colonies grew on each plate. Isolated plasmids were pure (260/230 ratio > 2.1, 260/280 ratio > 1.4), but some of

them have very low concentrations (data not shown). Nevertheless, it was possible to amplify the insert from all the plasmids with M13 primers (Figure 6B). The purification of the amplified insert yielded DNA in an average concentration above 70 ng/µl.



A B

Figure 6. Restriction analysis of plasmids with *EcoRI* enzyme (A) and PCR products on plasmids with M13 primers (B). 800 bp 18S rDNA products gave a strong, distinct band in both analyses. Products around 4000 bp long in the panel A are plasmids. 2000 bp product did not appear in any of the clones. This figure displays a representative subset of the complete analysis. Panel a: lane 1 – clone He040513_7H; lane 2 – clone He040503_4D; lane 3 – clone He040415_10G; lane 4 – clone He040318_1C; lane 5 – clone He040513_10B. Panel b: lane 1 – clone He040503_12G; lane 2 – clone He040318_5B; lane 3 – clone He040415_6F; lane 4 – clone He040513 10D; lane 5 – clone He040503 6A.

3.2. Plankton community structure by sequencing of the clone library

96 clones from each sample were sequenced, resulting in a total number of 384 sequences. For 34 clones, the quality of the chromatogram was not good enough to obtain minimum of 400 bp for analysis. These low quality sequences were excluded from BLAST analysis. For the analysed sequences the closest relative with sequence identity above 80% was found. This allowed to assign each of sequences to a class.

3.2.1. General information on plankton composition

Many groups of planktonic organisms were represented in the clone libraries, both phyto- and zooplankton. In all the samples, most sequences represented phytoplanktonic organisms (including all dinoflagellates) (273 clones), but an increase in the proportion of heterotrophic organisms with time was noticeable (Figure 7). In sample

He040318, only eight clones (9%) contained fragments from heterotrophic organisms. This number increased to 20 (24%) and 17 (19%) in samples He040415 and He040503, respectively. By the middle of May, as many as 34 clones (39%) carried a fragment of 18S rDNA from heterotrophs.

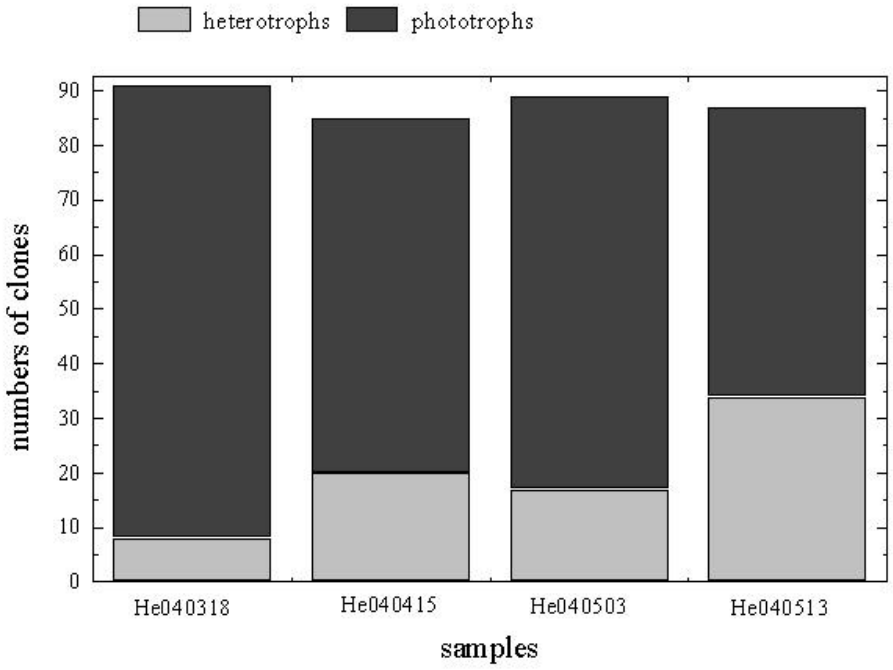


Figure 7. Ratio between clones carrying insert from heterotrophic and phototrophic organisms from investigated samples. All dinoflagellates have been arbitrary assigned to phytoplankton (phototrophs).

3.2.2. Composition of heterotrophic organisms

Heterotrophic sequences originate mainly from benthic animals with pelagic larvae – meroplankton – such as Polychaeta (22 clones), Echinodermata (18 clones) and Porifera (3 clones). Meroplanktonic sequences were most abundant in samples from April and the beginning of May (Polychaeta) and in the middle of May (Echinodermata and Polychaeta) (Figure 8). Five clones belonged to jelly-fish (Cnidaria and Hydrozoa). Hydrozoan sequences were present in the sample He040318. Cnidaria

sequences appeared in samples He040318 (one clone), He040415 (two clones) and He040503 (one clone). Tunicates (Chordata) clones were present in samples He040318 (one clone) and He040513 (five clones) (Figure 8). The rest of the clones (20 clones) belonged to heterotrophic groups of protists (not including heterotrophic dinoflagellates). In the sample from March, these were *Ancyromonas* (one clone), Cercozoa (one clone) and Ciliophora (two clones) (Figure 8). In April heterotrophic protists were represented in the clone library by Choanoflagellates (two clones), Ciliophora (five clones) and Cryothecomonas (one clone) (Figure 8). In May, Bicosoecida appeared (one clone in sample He040503), as did Cercozoa (one clone in each of the samples), Choanoflagellates (two clones in He040503 and one in He040513), Ciliophora (one clone in each of the samples) and *Cryothecomonas* (one clone in sample He040513) (Figure 8).

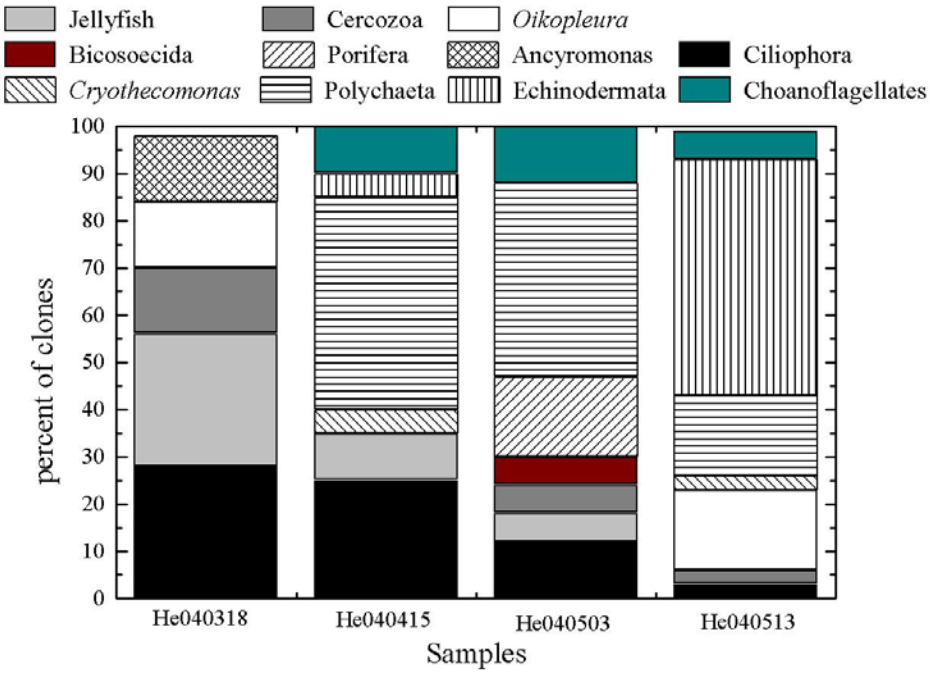


Figure 8. Proportion of different groups of heterotrophs in the samples as derived from the total number of clones containing 18S rDNA insert from heterotrophic organisms.

3.2.3. Composition of phototrophic organisms

Amongst the phytoplankton, dinoflagellates dominated in all the samples (116 clones all together). In March, they made 36 % of phytoplankton clones, in April 43%, in May 36% at the beginning and as much as 60% in the middle (Figure 9). Starting from April around half of the dinoflagellates in each sample were heterotrophic (> 90% of sequence identity to *Gyrodinium* or *Nocticluca*).

The second most abundant group in the clone library were centric diatoms (64 clones). In sample He040318, 18% (15 clones) had an insert from diatoms. In sample He040415, 23 sequences (35%) originated from diatoms. In May, diatoms made up 22 % (16 clones, sample He040503) and 19% (10 clones, sample He040513) of autotrophs (Figure 9). In each clone library, except of the one from He040513, there was a clone carrying the insert originating from a pennate diatom.

At the beginning of May (He040503), Dictyophyceae constituted to 8% of phytoplankton, but it was the only sample in which they were present. All of the sequences had more than 94% identity to sequence of a Dictyophyceaen species.

Nanophytoplankton was represented mainly by clones originating from Prasinophyceae and Cryptophyceae. Prasinophyceae contributed to 23% of clones in March (He040318), but afterwards their share decreased to 5% in April (He040415), 3% at the beginning of May (He040503). In the middle of May, there were no clones in the library with Prasinophyceaen insert. Cryptophyceae made almost 20% of phototrophs in March (14 clones), then their abundance decreased to around 10% (6 clones) (Figure 9).

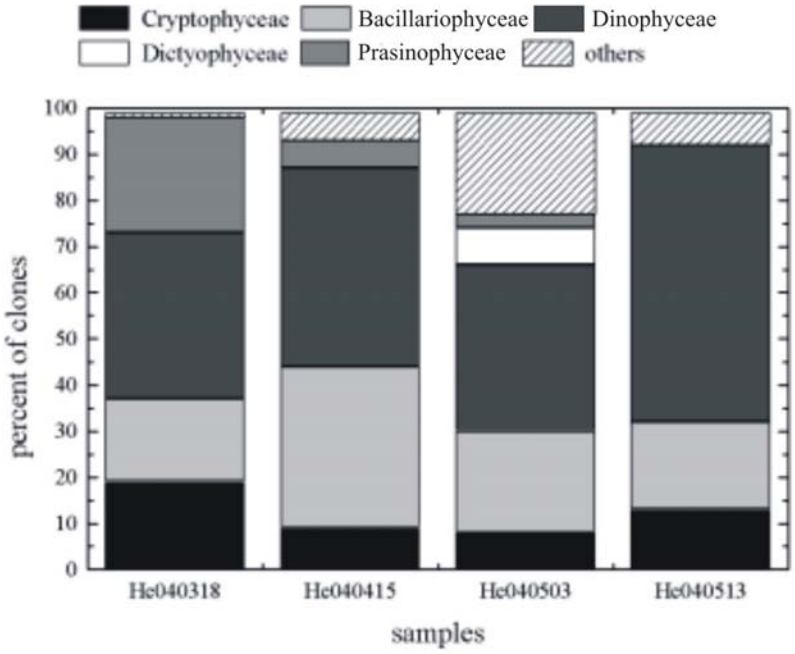


Figure 9. Portion of the most abundant phytoplankton classes in the clone library. Others compromise Chrysophyceae, Bolidophyceae, Dictyophyceae, Eustigmatophyceae, Katablepharidophyta and Stramenopiles.

3.2.4. Composition of Cryptophyceae

Clones identified in the BLAST search as Cryptophyceae were sequenced with both forward and reverse M13 primers to give a final length of these sequences of approximately 830 base pair. The sequences were aligned in the ARB program and a phylogenetic tree was calculated to reveal the taxonomic position of the new sequences (Figure 10).

Out of 31 clones assigned as Cryptophyceae on the basis of BLAST search, 30 were placed in the nuclear branch and one in the nucleomorph. The nucleomorph sequence was amplified from the April sample (clone He040415_10B). Its closest relative was the plastid from the *Geminigera cryophila* but the clone did not represent this species as the branch was relatively long (Figure 10).

Most of the new sequences (27 out of 31) grouped in Clade 4 (Figure 10). They were placed in two separate branches within this clade. Eight clones formed a branch within *Geminigera* branch (Figure 10). One of these clones (He040415_9A) was indeed the *Geminigera cryophila* but the others were distantly related and grouped in separate branch. These clones had been present during the whole investigated period, from March to the mid-May.

The remaining 19 clones from Clade 4 grouped in the *Plagioselmis-Teleaulax* branch (Figure 10). 7 clones were most probably *Plagioselmis prolonga*. These clones were present in all samples except of in April. 11 formed four separate branches very distantly related to *Plagiosemis* and one clone (He040503_12F) formed a sister branch to other new clones (Figure 10).

There were two branches that had been formed only by clones from the one sample. Three very closely related clones representing most probably a new, unknown species (clones He040318_12D, He040318_2D and He040318_5H, Figure 10) formed the first branch. The other sample-specific branch was formed by the clones collected in the middle of May (clones He040513_6A and He040513_7E). Clones within this branch were also very closely related and represented an unknown species.

The third of the *Plagioselmis-Teleaulax* branches was formed by four clones from all the libraries except of He040513 (Figure 10). This branch represented three, distantly related to other species in *Plagioselmis-Teleaulax* branch and to environmental clones from this study, species (clones He040318_4C and He040503_10B were the same species).

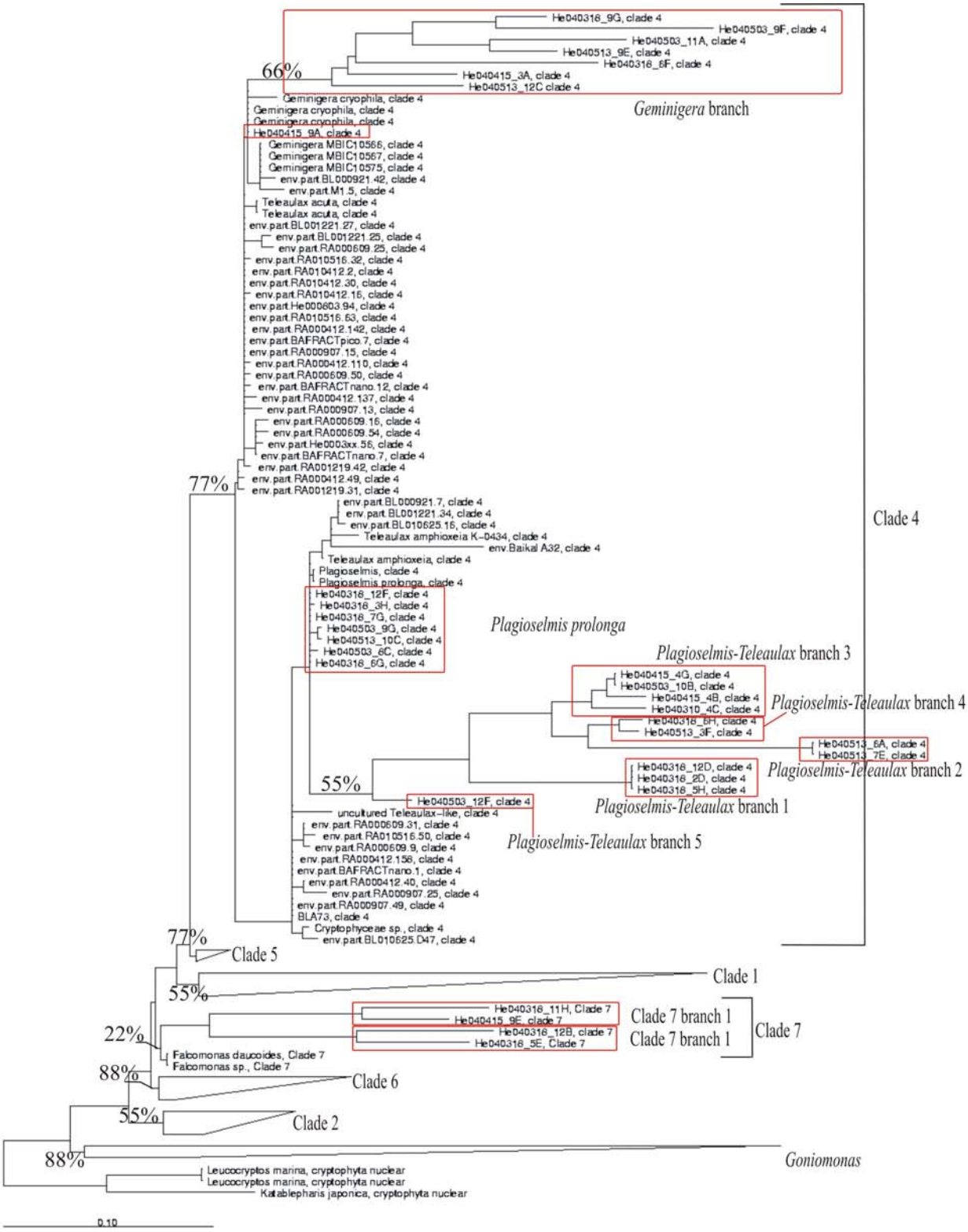


Figure 10. 18S rDNA cryptophytes phylogenetic tree showing the position of the sequences obtained from Helgoland (He) clone libraries. The topology of the tree was obtained by neighbourhood-joining analysis of the sequences into which partial sequences were added using the ARB maximum parsimony tool. Bootstrap values (100 replicates) for the main branches are shown. *Leucocryptos marina* and *Katablepharis japonica* were used as outgroup. The bar indicates 10% sequence divergence.

The fourth of the new branches within the *Plagioselmis-Teleaulax* branch of Clade 4 was formed by two clones from library He040318 and He040513 (Figure 10). These clones were distantly related to other clones and species in the *Plagioselmis-Teleaulax* branch as well as to themselves.

In samples from March and April clones representing Clade 7 were sequenced. These clones formed two distantly related branches within the clade and most probably represented new, unknown genera (Figure 10). One branch was formed by two clones from the March and the other by a clone from March and a clone from April (Figure 10). The clones within the branches were also distantly related and each represented different species or even genus.

Based on the tree (Figure 10) temporal changes in the Cryptophyceae composition could be observed (Table 3). Although Clade 4 was present in all clone libraries, changes could be observed with the sequences that appeared over time. Species that were related to *Geminigera* were present in all clone libraries. Almost the same could be said about the clones that were related to *Plagioselmis prolonga*, which were present in all the samples except for the one from April (Table 4). Also *Plagioselmis-Teleaulax* branch 3 was present in all clones libraries except He040513. The remaining *Plagioselmis-Teleaulax* affiliations were rather specific for one or two samples. *Plagioselmis-Teleaulax* branch 1, representing a single species, was found only in the sample from March. Another single-species branch (*Plagioselmis-Teleaulax* branch 2) was present only in the middle of May. *Plagioselmis-Teleaulax* branch 4 was formed by two clones, one from March and one form the mid-May (Table 4).

The occurrence of Clade 7was restricted to March and April (Table 4). Clones that represented this clade formed two distinct branches. One of the branches was formed only by clones that had occurred only in March. The other branch was formed by clones that were present in clone libraries from March (He040318) and April (H040415) (Table 4).

Table 3. Number of clones sequenced in this study representing the branch in each clone library. The branches typical for some of the samples can be identified what shows the temporal transition in the species composition of Cryptophyceae.

Branch in the tree (Figure 10)	He040318	He040415	He040503	He040513
Geminigera branch	2	2	1	2
Plagioselmis prolonga	4	0	2	1
Plagioselmis-Teleaulax branch 1	3	0	0	0
Plagioselmis-Teleaulax branch 2	0	0	0	2
Plagioselmis-Teleaulax branch 3	1	2	1	0
Plagioselmis-Teleaulax branch 4	1	0	0	1
Plagioselmis-Teleaulax branch 5	0	0	1	0
Clade 7 branch 1	1	1	0	0
Clade 7 branch 2	2	0	0	0

3.3. Restriction analysis patterns

Results from sequencing were compared with patterns obtained from restriction analysis to screen for patterns that would indicate redundancy among the clones or that were typical for Cryptophyceae as well as other groups of algae. In total, 36 different patterns were identified in the sample He040318, 39 in the sample He040415, 41 in the sample He040503 and 36 in the sample He040513. In theory, that meant that around 40% of clones in every clone library were redundant (identical). But sequencing revealed that the clones that shared a restriction-pattern mostly did not share the same sequence or belonged to a common group of organisms. Seven patterns were shared by more than two clones that were assigned to the same classes. Nevertheless, these patterns were not shared by all clones of the corresponding classes. For these seven patterns one was specific for Cryptophyceae, three were specific for diatoms, and three for dinoflagellates (Figure 11).

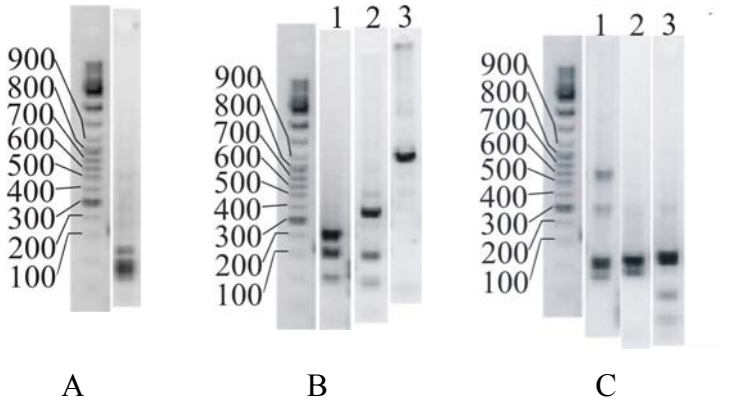


Figure 11. Restriction patterns specific for Cryptophyceae (A), Bacillariophyceae (B) and Dinophyceae (C).

The Crytpophycean pattern (Figure 11 A) was specific for seven clones out of 32 that were present in the all clone libraries. All these clones were affiliated as *Plagioselmis prolonga* (Figure 10) Other Cryptophycean clones gave patterns that were also quite common in other groups.

Of the diatom-clones 34 out of 64 shared one of patterns identified for this group of algae (Figure 11). The most abundant diatom patterns were those in the lanes 1 and 2 (Figure 11 B). These patterns were common to 12 and 19 clones, respectively. Clones that gave these patterns appeared in all the samples (Table 4). The pattern in lane 3 was observed for only three clones in the sample He040513. For the rest of clones carrying diatom-insert observed pattern was indistinguishable from patterns appearing in other classes.

Table 3. Distribution of band patterns amongst the clone libraries for each class of organisms. Minus (-) means that no clones gave the pattern in the sample.

Class	patterns (Figure 11)	He040318	He040415	He040503	He040513
Cryptophyceae	A	3	-	2	-
Bacillariophyceae	B lane 1	4	4	3	1
	B lane 2	2	9	5	3
	B lane 3	-	-	-	3
Dinophyceae	C lane 1	4	-	-	-
	C lane 2	10	14	12	2
	C lane 3	-	2	6	5

Of the dinoflagellate-clones 45 out of 116 shared one of patterns identified for this group of algae (Figure 11). For dinoflagellates, the most abundant pattern was that in the lane 2 (Figure 11 C), which occurred in as many as 38 clones (Table 4). The pattern in the lane 1 appeared only four times in the sample He040318. The pattern in the lane 3 appeared in 13 clones in all samples except of He040318 (Table 4). None of the dinoflagellates patterns was specific for either hetero- or phototrophs.

3.4. Screening the clone library with reverse microarray

The length of the PCR products spotted on the slide was ~950bp (Figure 6 B). The DNA fragment was 164 bp longer from the PCR products amplified from environmental samples. This was caused by the use of M13 primers that are located 89 bp (M13 reverse) and 75 bp (M13 Forward) from the cloning site (Figure 3). The probe did not match in the vector.

The quality of the hybridization was monitored with a positive control (PK). The positive control consisted of a target sequence amplified from the gene of the TATA-box binding-protein from *S. cerevisiae*. In all cases the PK spots gave very strong signal. This indicated that hybridization, washing and staining processes were performed correctly. Positive controls were used to calculate the normalization factor that enabled results to be compared between hybridizations. Negative control (NK), water and empty spots gave only very weak or no signal, this meant that non-specific

binding was very low (Figure 12, Figure 13). With this knowledge, it was possible to interpret our data.

Results from the microarray hybridizations are shown in Figure 12 and Figure 13. The biotin-labelled 82F primer proved to be a very good eukaryotic probe because it bound to and gave strong signal with 99 % of clones (Figure 12). The probe also did not bind unspecifically, either to DNA in negative control, or to the slide surface (spots with water and empty). Hybridization with the 82F probe showed that DNA had been spotted equally on the slide surface.

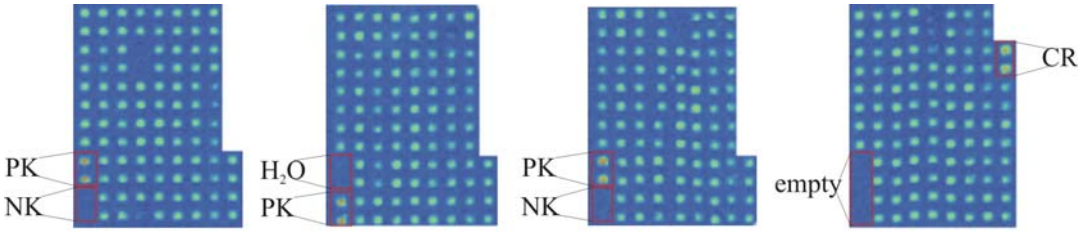


Figure 12. Example of scanned microarray image hybridized with general eukaryotic 82F probe. PK – positive control; NK – negative control; CR- control for Cryptophycean (PCR product from *Campylomonas reflexa*); H20 – water had been spotted; empty – nothing had been spotted.

Table 5. Estimation of number of Cryptophyceae clones derived from microarray and sequencing.

Sample	Microarray	Sequencing	Microarray & sequencing
He040318	15	14	11
He040415	28	6	3
He040503	24	6	5
He040513	22	7	7

Under the tested conditions, the probe Crypto B estimated correctly the number of Cryptophycean clones in sample He040318 (Table 5). However, for the rest of the samples the probe was not specific because it bound to many other clones in addition to Cryptophycean clones (Figure 13). It especially bound to diatoms, but also to few dinoflagellates, Ciliophora and even to jelly-fish (Cnidaria). On the other hand, some of the Cryptophyceae did not give a signal stronger than negative controls (Figure

13). The numbers of cryptophycean clones estimated on the basis of microarray was severely overestimated by 310 to 460 % when compared to estimations derived from sequencing (Table 5). But still, some cryptophyceab clones did not hybridised very well with the probe, up to 50% in sample He040415 (Table 5).

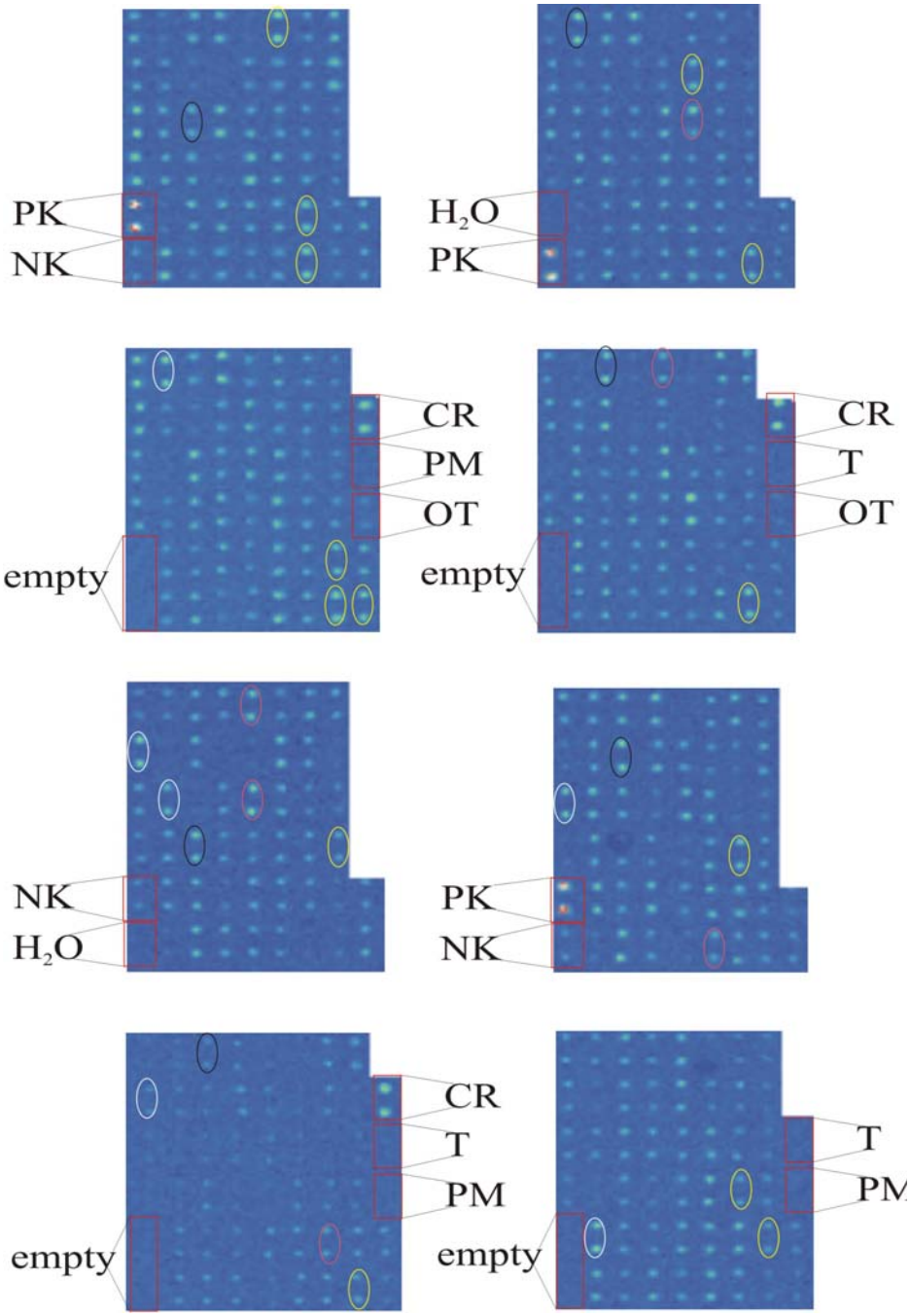


Figure 13. Image of microarray hybridized with cryptophycean specific probe. T - control for diatoms (PCR product from *Thalassiosira* xm 53); PM - control for dinoflagellates (PCR product from *Prorocentrum minimum* BAH ME 152); OT - control for Prymnesiophyceae (PCR product from *Ostreococcus taurii* RCC 344). All PCR reactions were performed with 82F/690R-58 primer set. For explanation of other shortcuts refer to Figure 12. Spots that contained PCR fragments of species belonging to cryptophytes are encircled: yellow ellipses – He040318; pink ellipses – He040415; black ellipses – H040503; white ellipses – He040513.

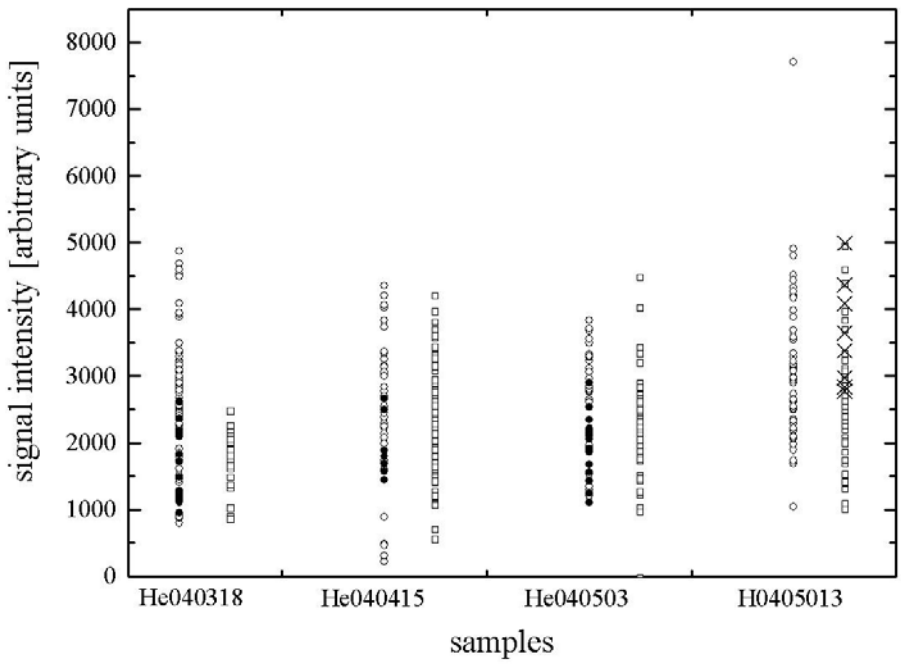


Figure 14. Normalized hybridization intensities of cryptophycean clones (circles) and those non-cryptophytes that could be accepted as cryptophytes on the basis of microarray (squares). For each of the clones values from eight replicates are shown. All non-cryptophytes had at least two mismatches except for clone He040513_10F (crosses). The intensities for cryptophytes containing mismatches to Crypto B probe are showed in solid circles.

The signal intensity for cryptophytes after normalization ranged from 214 to 7702 arbitrary units. However, signal intensity for others, non-cryptophyceaen clones were approximately in the same range (Figure 14). All clones that were not cryptophytes, except He040513_10F, had at least two mismatches to the Crypto B probe. The diatoms, which gave strong signal more often than any other group, had three mismatches starting from the second base at the 5' end. However, two of these mismatches were to guanine, that actually pairs with any other base – a so-called weak mismatch (Stahl & Amann 1991). Dinoflagellates that gave the signal also had three mismatches, two of which were to guanine. Still, some clones with the same number and kind of mismatches did not give the signal on the microarray.

There were three cryptophycean clones that had mismatches to the CryptoB probe. Two of them carried the insert from the nucleomorph. Two clones had two mismatches and at least one of them was the weak mismatch. Third clone had seven mismatches and was not assigned to Cryptophyceae on the basis of microarray analysis. On the other hand, five out of 27 Cryptophycean clones with perfect match to the probe also did not give the signal on the microarray.

Two clones (He040503_11A and He040318_12B, Clade 4 and Clade 7, respectivley) that had double mismatches and gave strong signal on the microarray had their mismatches at 5'end (5'-•C•G…)(• indicates perfect match at this position; … indicates perfect match to/from the end of the probe). The rest of the clones that had mismatches did not give a signal on the microarray. Third of clones (He040318_5E, Clade 4) had 7 mismatches to Crypto B probe (5'-AC••CC•••A••••••CT-3').

There was also a clone that carried the nucleomorph insert (Figure 10). This clone had four mismatches to Crypto B probe and did not give the signal on the microarray.

4. Discussion

4.1. PCR on environmental samples

The polymerase chain reaction was performed on genomic DNA from environmental samples to amplify 800 bp fragments of 18SrRNA genes from all eukaryotic organisms. These fragments were subsequently used for the creation of the clone libraries. The results from PCR of genomic DNA were not perfect because in addition to target fragment other, ~2000 bp long fragments also had been amplified (Figure 5). The best solution for these would have been purification of the target PCR product directly from the gel. However, according to manufacturer of the TOPO TA cloning kit (Invitrogen™, USA) shorter fragments ligate preferentially into the vector. The special kit has been even designed for cloning of longer fragments. The assumption that only shorter fragments would ligate was also supported by the fact that this fragment was in higher concentration and, albeit risky, proved to be correct, as showed by restriction with *EcoRI* enzyme and amplification with M13 primers (Figure 6).

4.2. Sequencing of the clone library.

Sequencing of small subunit rRNA genes (16S rDNA for Prokaryota and 18S rDNA for Eukarya) from clone libraries is well-established method for investigation of diversity of microbial communities (Madigan & Martinko 2006). Although the best option is to sequence the entire gene, in many research only a partial sequence has been used (Hallett et al. 2003, Harris et al. 2004, Valentin et al. 2005). In this study only partial sequences were also used. The selected 800 bp fragments between 82 and 900 bp is the most variable fragment in 18S rDNA (Neefs et al. 1993). As the focus of this study was to obtain information on clade and genus level of Cryptophyceae, this variable region was well suited for the purpose of the survey. The amount of information on 400 bp long sequence is enough for a BLAST search because this software creates, in pairwise fashion, local alignments that include only the most similar local region or regions, and does not force alignments of a partial sequence to full-length sequence. Sequences assigned in BLAST search as Cryptophyceae were sequenced with forward primer so the final length of cryptophyte sequences was at

least 740 bp. These sequences were aligned in ARB to obtain higher taxonomic resolution.

Another reason for using only partial sequences was that only the first part of 18S rRNA gene had been used in a microarray format. The reason for this is that the formation of secondary structures in fragments longer than first 900 base pairs hampers binding of probes to target sequence (Metfies & Medlin submitted).

Finally, obtaining a full-length sequence of Cryptophycean 18S rDNA gene is also a methodological problem (Klaus Valentin, personal communication). The bias against Cryptophyceae when amplifying full-length 18S rDNA gene could be so strong that it could lead to severe underestimation of cryptophycean abundance (derived from the number of clones in the library). As the main focus has been on this group of phytoplankton, it would be too hazardous to try to obtain full length sequences with general eukaryotic primers from the samples as the possible result could have been abscence of any sequences from Cryptophyceae.

4.2.1. Plankton composition

Helgoland Roads time series is one of the most extensive ecological data sets currently available. The series for phytoplankton and nutrients was started in 1962 (Hickel 1998) and for meso- and macrozooplankton in 1974 (Greve et al. 2004). This gives a great opportunity for testing hypotheses about changes in the environment caused by global warming, eutrophication on the German Bight ecosystem as well as accuracy of ecological models (Wirtz & Wiltshire 2005).

In temperate regions seasonality in pelagic ecosystems is very strong. The spring phytoplankton bloom, dominated by diatoms, is followed by dinoflagellates and flagellates dominance. The peak of abundance of herbivorous (Copepoda) and carnivorous (fish larvae, larvae of benthic animals) zooplankton follows phytoplankton bloom, too. In summer, the energy flows usually through a microbial loop. The most important eukaryotes in the microbial loop are heterotrophic nanoflagellates. This very general pattern was mirrored the clone libraries analysed in this study. In March, most clones originated from autotrophs and heterotrophs constituted only 9% (Figure 7). However, two months later (sample He040513) sequences from heterotrophs accounted for 39% of the library. These sequences originate mainly from meroplankton (larvae of benthic animals) (Figure 8). Heterotrophic protists (except

for heterotrophic dinoflagellates), on the other hand, were not represented abundantly in the clone library. The reason for that might be that the last sample was collected still in spring season when heterotrophic flagellates are not so important in the plankton.

The largest source of phytoplankton variability is caused by the seasonal cycles of diatoms and flagellates, two most important components. They follow the pattern typical for temperate regions (Sathyendranath & Platt 2001). Diatoms bloom intensively in spring, whereas flagellates, dominated by large dinoflagellates, in summer (Hickel 1998). Biomass of nanoflagellates at Helogland Road Station is 10-20 μg C dm^{-3} through a year (Hickel 1998). Indirect and direct measurements indicate that these flagellates are mainly heterotrophic (van Duyl et al. 1990, Hickel 1998).

Described above general annual pattern of phytoplankton, was, to some extent, reflected by the numbers of clones originating form different groups. The increase number of clones carrying the diatom insert in sample He040415 could have reflected the spring bloom of the diatoms that usually occurs at this time (Hickel 1998). Still, the most abundant in all the samples were the large dinoflagellates (Figure 9). The dominance of dinoflagellates usually starts in July, but also may happen in May (Hickel 1998). On the other hand, large number of clones carrying the insert from dinoflagellates might also have been caused by preferential amplification because of larger amount of template, as dinoflagellates contains enormous amount of DNA in the nucleus as compared to other planktonic algae (Rizzo 1987).

Nano- and picophytoplankton (fraction 3 – 20 μm and < 3 μm, respectively) are not included in the Helgoland Road time series. In March, nanoplanktonic prasinophytes and cryptophytes were quite common (25% and 19% of clones in the clone library, respectively) but then their proportion decreased. The possible reason for this could be that in March it was the pre-bloom period, dominated by nanoflagellates (Hickel 1998).

The results from the clone libraries are congruent with manual counting for the samples performed as a part of Helgoland Road time-series (Appendix 3). The number of dinoflagellate species increased in the investigated period. The increase of propotrion of heterotrophic species with time was also observed in manual counting. Diatoms in all samples were mostly centrics but there was more species of pennate diatoms than revealed in the clone libraries. The number of diatom species decreased

with time. Dictyophyceae, present in the clone library from the sample He040503, were also revealed in this sample with manual counting. However, Dictyophyceae were also present in other samples, but not recorded by sequencing of corresponding clone libraries. Nanophytoplankton, like Cryptophyceae and Prasinophyceae, is not reported in detail in manual counting. Cryptophyceae were present in samples He040318 and He040415 whereas Prasinophyceae were reported only from sample He040503 (*Pterosperma polygonum*). This could indicate that the cryptophyte-species changed from species with a size >20µm to nanoplanktonic species at <20µm

Methods based on PCR-step on multi-template samples are exposed to template-to-products ratio bias (Polz & Cavanaugh 1998). The main force driving PCR bias is PCR selection that favours the amplification of certain genes because of their properties (Wagner et al. 1994), but there is many other factors that may influence the final ratio of products (Kanagawa 2003). Most of this influence may be removed by decreasing the number the cycles to minimum (Kanagawa 2003). The initial amplification of 18S rRNA genes involved 45 cycles. Nevertheless, for microplankton (species > 20µm), the comparison of the clone library-analysis with manual counting indicates that the bias of the PCR was not very pronounced. The importance of centric diatoms and phototrophic and heterotrophic dinoflagellates in the phytoplankton could be concluded with both methods (see section 3.2.3). This indicates that molecular methods are very well suited to improve the comprehensiveness of microbial field studies.

Data from sequencing the clone libraries were also used to evaluate results from the EU-project MICROPAD. Results from sequencing the clone libraries has confirmed the data generated by microarray analysis (MICROPAD), that indicate a presence of the Cryptophyceae in < 20 µm fraction of the phytoplankton in the samples of May 2004 (Table 2). This fraction was not reported by manual counting of samples collected with 20 µm phytoplankton net and examined under an inverted microscope.

Peaks in the zooplankton abundance follow the phytoplankton bloom. First herbivorous animals occur. The peak of herbivores abundance is followed by the peak of carnivorous to omnivorous species that prey on herbivorous ones; and, finally, by detritivorous zooplanktoners (Greve et al. 2004). The herbivores are usually represented by calanoids, carnivores by cyclopoids and detritivores by harpacticoids (Greve et al. 2004). However, these functions can be also fulfilled by herbivorous

appendicularians and larvae of echinoderms and polychaetes, carnivorous larvae of decapods and partially detritivorous mysids (Greve et al. 2004).

In the clone library this pattern is only partially reflected. Namely, only herbivorous consumers are present. These are not represented by copepods but by larvae of polychaetes and echinoderms (Figure 8). The presence of only herbivorous zooplankton indicates that samples were collected during and shortly after the phytoplankton bloom. It is likely that an analysis of clone libraries from further samples taken later in the year, would identify sequences of carnivore species.

The fact that in the sample from March (He 040318) only protists were present in the heterotrophic fraction supports, like the presence of nanophytoplankton, the fact that the samples were collected in the pre-bloom time.

4.2.2. Cryptophyceae diversity

This study is the first one that describes Cryptophyceae diversity and its changes with time in the environmental samples. Among the identified cryptophyceaen clones most were affiliated to Clade 4. One clone was *Geminigera cryophila* and seven were *Plagioselmis prolonga*. Other clones grouped in six new branches within Clade 4 (Figure 10). Five of the branches grouped inside the *Plagioselmis-Teleaulax* branch and one in the *Geminigera* branch. As the majority of environmental clones from the PICODIV project also grouped in the Clade 4 (Medlin, personal communication), it might be concluded that the new branches discovered in this study are picoplanktonic too. This would explain the lack of the Cryptophyceae in the manual counting for the samples from May (Table 2). What is more, the environmental sequences are the major portion of Clade 4 (Medlin, personal communication). From this it can be concluded that marine picoplanktonic Cryptophyceae form separate branches from the nanoplanktonic ones. So far there have been not a single species of pico-Cryptophyceae cultured, but because they grouped within the photosynthetic clades, they can be considered as primary producers. These picoplanktonic Cryptophyceae can be responsible for dominance of the Cryptophyceae in the phytoplankton assemblages in the North Sea when investigated with indirect methods (Gieskes & Kraay 1983).

Two new branches were also found within Clade 7. This clade is represented by a single known species that could not be placed in any other clade (Deane et al. 2002). Clones that formed the new branches were distantly related and each of them most

probably is a new, unknown species and even genus. As the information on Clade 7 is scarce, nothing can be concluded about the new species/genera except that they are phototrophs.

Pronounced temporal changes of Cryptophyceae species composition could be observed in the clone libraries (Table 4). These changes were not observed in the surveys in which presence of the Cryptophyceae was reported only on class level (). At the clade level, the changes could be observed only for the clade 7 that was present in early spring, in March and April. Clade 4 was present in all the samples. However, the deeper insight revealed changes on the generic-level. Nanoplanktonic species *Plagioselmis prolonga* and *Geminigera* sp. were present in all the samples. The changes occurred in the picoplanktonic cryptoflagellates. Three branches (*Plagioselmis-Teleaulax* branch 1, 2 and 5) were restricted to only one samples which indicates that these species are somehow limited in other periods. One scenario might be that early spring-branches, namely *Plagioselmis-Teleaulax* branch 1 and new branches in Clade 7, are less sensitive to low temperatures than species that were present in May (*Plagioselmis-Teleaulax* branch 2 and 5) but are out-competed for nutrients when water gets warmer and the nutrients are used up by others planktoners. Early spring appearances may also have higher light demand and are able to grow only in relatively clear water, whereas May appearance *Plagioselmis-Teleaulax* branches 2 and 5 tolerate shadowing in more turbid bloom-waters.

Plagioselmis-Teleaulax branches 3 and 4 were not specific to a single sample but also did not appeared in all clone libraries (Table 4). However, this results from the fact that only few Cryptophyceaen clones were found in each of the libraries rather than from disappearance in the middle of the sampling period and reoccurrence after some time. So, this species cannot be regarded as restricted to any season and, similarly like nanoplanktonic species, are capable of growth under wide environmental conditions.

4.3. Restriction analysis

By comparing results from the sequencing of the clone library with results of restriction analysis, it was aimed to find patterns that could be typical for groups or species of phytoplankton organisms. Fingerprinting with restriction analysis, termed restriction fragment length polymorphisms (RFLP), can be also used for general assessment of comparative biodiversity with a large number of samples (Medlin et al.

2002). However, the main application of the method is species identification in clone cultures on the basis of the pattern and screening clone libraries for unique clones (Hansen et al. 1998, Madigan & Martinko 2006). The main aim in screening clone libraries is to identify redundant (identical) clones to reduce number of clones for sequencing. PCR-RFLP analysis for partial 16S rDNA (fragment ~900 bp) with tetrameric restriction enzyme have been used to discriminate bacterial species in cultures mixture (Hansen et al. 1998).

The restriction analysis performed to screen the clone libraries in this study revealed 21% of redundant clones. There might have been three reasons for low resolution of the restriction analysis. Firstly, digestion of the short fragment of DNA restricted the possible numbers of patterns that could be visualised. Moreover, if one clone had been cut in three pieces of length, let say, 200, 100 and 50 bp, and the other one in four pieces of 200, 100, 50 and 50 bp, the pattern on the gel would have been identical. Very short fragments, for example 25 bp, give only very weak band, if at all. It might have happened that some of such short fragments on the gel were overseen. One possible way to visualise these difference would be to selectively stain the fragments with a dye that intercalates with certain nucleotides, such as HA (Hansa Analysis) red or yellow so that similar sized fragments can be differentiated by their base composition (Medlin et al. 2006).

In the analysis *HaeIII* enzyme was used. This enzyme have been proved to be useful in screening picoplankon clone libraries (Diez et al. 2001). This enzyme is a frequent cutter with a four base pairs recognition site. Hence, the situation described in the previous paragraph was likely to occur. On the other hand, use of the restriction enzyme recognizing six base pairs could result in many uncut fragments and a number of redundant clones would be overestimated, too.

Finally, the resolution of agarose gel is not very high. Many fragments of similar length could have not been separated very well on the gel what would have resulted in a single band instead of multiple. Especially, it might have been the case for very thick bands (e.g. Figure 10 A).

Nevertheless, described patterns can be useful to identify, to some extent, inserts originating from Cryptophyceae, diatoms and dinoflagellates in clone libraries and reduced the number of clones for sequencing. In the present study the number of sequenced clones would have been reduced to 315 clones (18% less) if the redundant ones had been excluded.

4.4. Screening the clone library with reversed microarray.

The screening the clone libraries for Cryprophyceae with reverse microarray analysis, turned out to be an approach that requires further optimisation (Figure 12). Nevertheless, it has the potential to be very useful for screening clone libraries, as shown by hybridization with probe 82F (Figure 11). Possible reasons for the sub-optimal results of the method with Crypto B probe are discussed below.

The length of DNA fragments that can be spotted on microarray surface ranges from short oligonucleotides, like for instance probes (15-20 bp), to 5000 bp fragments (Service 1998). The fragments spotted on the microarray in this study were around 900 bp long (Figure 6 B), so there is no reason to assume that incorrect hybridizations resulted from an inappropriate length of DNA fragment spotted on the microarray.

Although most of the clones hybridized very well with the 82F probe, five of them did not give any signal. The reason for this was not insufficient amount of DNA, because they all have concentrations above 70 ng/µl. The lack of the hybridization was not specific for any group of organisms. Three of the clones belonged to dinoflagellates, one to Cercozoa and one to Dictyophyceae. Therefore, it seems that in this case the cause was unsuccessful spotting of these clones on the chip. This speculation is also supported by the fact that all these clones were spotted in the 3rd and 4th rows, regardless of the block and column. None of these clones gave the signal with Crypto B, either.

Hybridization with Crypto B probe was much less successful than with 82F probe because limited specificity of the Crypto B probe under the hybridization conditions applied in this study. In contrast to the results generated with the reverse microarrays, Crypto B was specific when it was used as an immobilized probe on a DNA-microarray. It has been shown previously that molecular probes behave differently depending on the method that they are combined with, e.g., probes that have been shown to work very well in combination with Dot-blots did not work as well, if they were used on the microarray (Metfies & Medlin submitted). Discrimination between perfect match and mismatch is essential in all experiments involving use of probes. Washing is a key step in discrimination between perfect match duplexes and mismatch duplexes (Liu et al. 2001). To establish the best washing conditions, temperature-depended dissociation curves at different salt concentration need to be known

(Guschin et al. 1997, Liu et al. 2001, Loy et al. 2002). Non-specific hybridization is more likely to occur at lower temperatures (Guschin et al. 1997). In the experiment the washing was performed at room temperature (15-20ºC). The washing temperatures in other microarray experiments were 55ºC (Loy et al. 2002) and around 35 ºC (Liu et al. 2001). Rough calculation of dissociation temperature for the probe Crypto B (T_d = 2ºC×AT + 4ºC×GC) gives 60 ºC. For the probe with the same theoretical T_d Liu et al. (2001) have found experimentally that dissociation temperature is ≈ 41ºC, which was 15ºC higher than its non-target species. If the same parameters had been correct also for Crypo B, washing at room temperature (15-20ºC) would have not provided conditions stringent enough to differentiate mismatch from perfect match. High hybridization temperature in the experiment (58ºC), even if prevent formation of mismatching duplexes, was inefficient in discriminating mismatches from perfect matches in the whole experiment.

The stringency during washing step may be also increased by lowering salt concentration in washing buffers. Concentration of salt is a key washing parameter. Lower salt concentration destabilized mismatching duplexes much more than perfectly matching ones (Liu et al. 2001). This allows for a better discrimination of mismatches from perfect matches. In order to optimise the washing conditions, a series of experiments combining both parameters, namely washing in buffers with different salt concentrations and at different temperatures, should be performed. Such experiments lead to obtaining set of melting (dissociation) curves that allow choosing the conditions providing best discrimination between perfect matches and mismatches (Liu et al. 2001). The discrimination is also improved when substances which equalize the stability of A•T and G•T pairs, like tetramethylammonium chloride (Maskos & Southern 1992) or betaine (Rees et al. 1993) are present in the washing buffer.

The formation of secondary structures involving the region with the mismatches was possible, reducing the three bp mismatch to one bp mismatch (Figure 15). The formation of secondary structures in ssDNA is reduced when organic solvents, such as dimethyl sulfoxide (DMSO) or glycelor, are added to the solution (Pomp & Medrano 1991). The organic solvents have a general property to destabilize DNA in solution and for this reason are used to enhance PCR amplification and reduce PCR bias (Pomp & Medrano 1991, Hansen et al. 1998).

Figure 15. Possible secondary structure that may have been formed by the Crypto B probe in the hybridization solution. Formation of these structures would result in decrease of number of mismatches to diatoms from three to one. Mismatching bases are encircled.

In addition, discrimination of mismatches from perfect matches is better when mismatch to non-target sequences is in the middle of the probe (Stahl & Amann 1991). In most cases occurrence of one stronger mismatch (not with G) in the middle of the probe was enough to prevent signal from mismatching duplexes.

The most important thing in using phylogenetic rDNA probes is that the probe should be perfectly matching only to target organisms sequence and mismatching to all the others. This limits the use of probes to organisms from which at least 18S rDNA sequence is known. Design of a good probe is also limited by the quality of database used. Many sequences deposited in GenBank contain ambiguities. In result, most of designed probes do not cover all the target organisms and, at the same time, covers some non-target organisms. Moreover, new sequences are continuously added to databases so that the probes specificity needs to frequently re-checked. Three of the clones that had mismatches were distantly related to the known species of Cryptophyceae (sequence identity < 90%). It might have happen that these sequences belong to yet unknown Cryptophycean species. On the other hand, differences in the sequence might have been caused by the errors that had been introduced in amplification and sequencing steps (Kanagawa 2003).

Additional consideration is required for phytoplankton groups that acquired chloroplast in the secondary endosymbiosis event, like the Cryptophyceae. The plastid of Cryptophyceae contains a nucleomorph that is remnant of the nucleus of the eukaryotic endosymbiont from the secondary endosymbiosis (Marin et al. 1998). In the genome of the nucleomorph 18S rRNA genes that can be amplified with general eukaryotic primers are also present (Hoef-Emden et al. 2002). Although phylogenetic trees derived from 18S rDNA sequences from nucleus and nucleomorph are congru-

ent (Hoef-Emden et al. 2002), the probes designed on the basis of nuclear 18S rDNA sequence will not hybridized with nucleomorph 18S rDNA fragments. There was one clone that carried insert originating from the nucleomorph (He040415_10B). This clones had four mismatches to the probe and, as expected, did not give the signal on the microarray (see section 3.4).

One of the clones (He040513_10F), although not a Cryptophyte, had perfect match to the Crypto B probe. This sequence had 87% identity to *Chlorokybus atmophyticus*, a charophyte, which is a class within plants (Viriplantae, Streptophyta). However, an alignment of the 18S rDNA sequence *of Chlorokybus atmophyticus* with Crypto B reveals that there are two mismatches and a Blast-search with Crypto B shows that the probe does have a perfect match to Cryptophyte sequences. There are no other known sequences in the database that have a perfect match to Crypto B. Therefore the clone with the 87% identity to *Chlorokybus atmophyticus* is a sequence of a not yet known species. Charophyceae are, in general, freshwater algae and their presence in North Sea must have been accidental (Szweykowska & Szweykowski 2001), or the clone is a representative of a marine species from this class. The clone is an example that shows the weak point of molecular-probe applications for the analysis of field samples. The specificity of a probe strongly relies on the comprehensiveness of the database that was used for the probe-development. It is estimated that currently the majority of the global species, in particular microbial and marine species is yet unknown. Moreover, the amount of sequence information is even smaller, because not all known species are sequenced. Therefore, for the analysis of cryptophytes in field-samples with Crypto B one must keep in mind that a hybridization signal on the microarray might originate from the described clone. The set of probes on the DNA-microarray should be extended by a probe, which is specific for this clone. The probe would contribute to the accuracy of the analysis.

5. Conclusions

The analysis of four clone libraries from samples taken in spring 2004 at Helgoland identified new photosynthetic, nanoplanktonic branches of Cryptophyceae tree. Six of new branches formed subclades within Clade 4 and are most probably picoplanktonic. This clade proves to be most important in the marine waters. Two new subclades were placed within Clade 7. Temporal changes could be observed on the subclade level, with branches specific to early spring (March and April) and May.

Analysis of the clone libraries revealed typical pre-bloom, bloom and post bloom plankton assemblages in Helgoland Road. Because of the dominance of autotrophic groups, the high proportion of clones from pico-phytoplankton (Cryptophyceae and Prasinophyceae) and the presence of exclusively protist heterotrophs, we concluded that in the middle of March (sample He040318) a pre-bloom situation still existed. The bloom assemblages could be characterized by high proportion of diatoms and other autotrophs with increasing abundance of metazoan heterotrophs that appeared in samples from April and beginning of May. High abundances of larvae of benthic organisms and dinoflagellates and a decrease in the proportion of diatoms in the sample He040513 indicated that by the middle of the May, the main bloom event was already over.

Data on Cryptophycean presence in the samples revealed by microarray analysis and sequencing of the clone library were congruent. Thus, the microarray class-level probes developed in the EU-project MICROPAD (Crypto A and Crypto B) have proved to be useful for application in analysis in environmental samples with microarray analysis.

Screening of the clone libraries for cryptophytes with reverse microarray technique (clones spotted on the slide surface) needs further optimization. In addition to washing condition, which were not sufficiently stringent, hybridization conditions could be optimised to prevent formation of secondary structures on the probe, which could be cause for the low specificity.

PCR-RFLP, although commonly used in assessing diversity (Medlin & Simon 1998, Medlin et al. 2002), proved to have too low a resolution in case of very complex plankton samples. Nevertheless, one pattern for Cryptophyceae, three for diatoms and three for dinoflagellates were found. These patterns could be used in future

to screen for Cryptophyceae, dinoflagellates and diatoms in clone libraries and avoid sequencing of redundant clones when class-level information is required.

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APPENDIX I

RESULTS FROM MANUAL COUNTING

thanks of courtesy of prof. Karen Wiltshire

Responsible scientist Mona Hoppenrath & Karen Wiltshire

Diatoms

Achnanthes longipes

Actinocyclus octonarius

Actinoptychus senarius

Asterionellopsis glacialis

Asteroplanus karianus

Biddulphia altemans

Brockmanniella brockmannii

Chaetoceros affinis

Chaetoceros borealis

Chaetoceros curvisetus

Chaetoceros danicus

Chaetoceros debilis

Chaetoceros densus

Chaetoceros diadema

Chaetoceros teres

Coscinodiscus granii

Coscinodiscus wailesii

Cylindrotheca closterium

Delphineis surirella

Ditylum brightwellii

Grammatohora marina

Grammatophora sp.

Gyrosigma sp.

Helicotheca tamesis

Leptocylindrus danicus

Licmophora sp.

Melosira moniliformis

Odontella aurita

Odontella aurita var. minima

Odontella granulata

Odontella obtusa

Odontella regia

Odontella rhombus

Odontella sinensis

Paralia marina

Plagiogrammopsis vanheurckii

Podosira stelliger

Porosira glacialis

Rhaphoneis amphiceros

Rhizosolenia imbricata

Rhizosotenia setigera

Roperia tesselata

Skeletonema costatum

Meuniera (Statiropsis) membranacea

Thalassionema frauenteldii

Thalassionema nitzschioides

Thalassiosira aestivalis/concavluscuia

Thalassiosira angulata

Thalassiosira eccentrica

Thalassiosira minima

Thalassiosira nordenskioeldii

Thqlassiosira punctigera

Thalassiosira rotula

Thalassiosira sp.

Thalassiosira tenera

Dinoflagellates

Ceratium fusus

Ceratium horridum

Ceratium longipes

Dinophysis rotundata

Diplopsalis-group spp.

Gymnodinium chlorophorum

Gyrodinium calyptoglyphe

Heterocapsa rotundata

Mesoporus perforates

Nematodinium armatum

Noctiluca scintilians

Prorocentrum micans

Protoperidinium bipes

Protoperidinium brevipes

Protoperidinium conicum

Protoperidinium marielebouriae

Scripsiella sp.

Prymesiophyceae

Coccolithus pelagicus

Phaeocystis globosa

Other Plankton

Cryptophyceae sp.

Scenedesmus sp.

15.04.2004

Diatoms

Actinocyclus octonarius

Actinoptychus senarius

Asterionellopsis glacialis

Asteroplanus karianus

Bellerochea/Helicotheca sp.

Brockmanniella brockmannii

Cerataulina pelagica

Chaetoceros curvisetus

Chaetoceros danicus

Chaetoceros densus

Chaetoceros diadema

Chaetoceros socialis

Chaetoceros teres

Coscinodiscus concinnus

Coscinodiscus granii

Coscinodiscus radiatus

Coscinodiscus wailesii

Cylindrotheca closterium

Detphineis surirella

Ditylum brightwellii

Grammatophora sp.

Guinardia delicatula

Guinardia ilaccida

Gyrosigma sp.

Helicotheca tamesis

Navicula sp.

Odontella aurita

Odontella regia

Odontella rhombus

Odontella sinensis

Paralia marina

Podosira stelliger

Rhaphoneis amphiceros

Rhizosolenia setigera

Roperia tesselata

Skeletonema costatum

Stephanopyxis turns

Thalassionema frauenfeldii

Thalassionema nitzschioides

Thalassiosira aestivalis/concaviuscula

Thalassiosira angulata

Thalassiosira anguste-lineata

Thalassiosira curviseriata/tealata

Thalassiosira delicatula

Thalassiosira eccentrica

Thalassiosira minima

Thalassiosira nordenskioeldii

Thalassiosira punctigera

Thalassiosira rotula

Thalassiosira tenera

Dinofiagellates

Ceratium fusus

Ceratium horridum

Dinophysis acuminata

Dinophysis rotundata

Diplopelta bomba

Diplopsalis-group spp.

Dissodinium pseudolunula

Gyrodinium calyptoglyphe

Gyrodinium sp.

Heterocapsa rotundata

Nematodinium armatum

Prorocentrum micans

Protoperidinium achromaticum

Protoperidinium bipes

Protoperidinium brevipes

Protoperidinium conicum

Protoperidinium denticulatum

Protoperidinium sp.

Protoperidinium subinerme

Protoperidinium thorianium

Warnowia sp.

Prymesiophyceae

Phaeocystis globosa

Other Plankton

Cryptophyceae sp.

Dictyocha speculum

03.05.2004

Diatoms

Actinocyclus octonarius

Actinoptychus senarius

Asterionellopsis glacialis

Brockmanniella brockmannii

Cerataulina pelagica

Chaetoceros curvisetus

Chaetoceros danicus

Chaetoceros densus

Chaetoceros eibenii

Chaetoceros socialis

Chaetoceros teres

Coreihron hystrix

Coscinodiscus granii

Coscinodiscus radiatus

Coscinodiscus wailesii

Cylindrotheca closterium

Delphineis surirella

Detonula pumila

Ditylum brightwellii

Eucampia zodiacus

Grammatophora sp.

Guinardia delicatula

Guinardia flaccida

Guinardia striata

Gyrosigma sp.

Lauderia annulata

Licmophora sp.

Navicula sp.

Nitzschia sp.

Odontella aurita

Odontella aurita var. *minima*

Odontella regia

Odontella rhombus

Odontella sinensis

Paralia marina

Podosira stelliger

Pseudo-nitzschia pungens

Rhizosolenia imbricata

Rhizosolenia pungens

Rhizosolenia setigera

Roperia tesselata

Stephanopyxis turris

Thalassionema frauenfeldii

Thalassionema nitzschioides

Thalassiosira aestivalis/concaviuscula

Thalassiosira angulata

Thalassiosira anguste-lineata

Thalassiosira delicatula

Thalassiosira eccentrica

Thalassiosira nordenskioeldii

Thalassiosira punctigera

Thalassiosira rotula

Dinoflagellates

Ceratium fusus

Ceratium lineatum

Dinophysis acuminata

Dinophysis rotundata

Diplopsalis-group spp.

Dissodinium pseudocalani

Dissodinium pseudolunula

Fragilidium subglobosum

Gonyaulax digitate

Gonyaulax spinifera

Gyrodinium calyptoglyphe

Gyrodinium sp.

Gyrodinium spirale

Nematodinium armatum

Noctiluca scintillans

Prorocentrum micans

Protoperidinium bipes

Protoperidinium brevipes

Protoperidinium conicum

Protoperidinium denticulatum

Protoperidinium depressum

Protoperidinium leonis

Protoperidinium ovatum

Protoperidinium pallidum

Protoperidinium pellucidum

Protoperidinium sp.

Protoperidinium subinerme

Scripsiella sp.

Torodinium robustum

Prymesiophyceae

Phaeocystis globosa

Other Plankton

Chattonella veruculosa

Dictyocha speculum

Pterosperma polygonum

13.05.2004

Diatoms

Actinocyclus octonarius

Actinoptychus senanus

Asterionellopsis glacialis

Biddulphia alternans

Brockmanniella brockmannii

Cerataulina pelagica

Chaetoceros curvisetus

Chaetoceros danicus

Chaetoceros densus

Chaetoceros didymus

Chaetoceros socialis

Corethron hystrix

Coscinodiscus concinnus

Coscinodiscus radiatus

Coscinodiscus wailesii

Cylindrotheca clostenum

Delphineis surirella

Ditylum brightwellii

Eucampia zodiacus

Fragilaria sp.

Guinardia delicatula

Guinardia flaccida

Guinardia striata

Gyrosigma sp.

Lauderia annulata

Odontella aurita

Odontella regia

Odontella rhombus

Odontella sinensis

Paralia marina

Podosira stelliger

Pseudo-nitzschia fraudulenta

Pseudo-nitzschia pungens

Rhizosolenia imbricata

Rhizosolenia pungens

Rhizosolenia setigera

Rhizosolenia styliformis

Roperia tesselata

Stephanopyxis turris

Thalassionema frauenfeldii

Thalassionema nitzschioides

Thalassiosira angulata

Thalassiosira nordenskioeldii

Thalassiosira punctigera

Thalassiosira rotula

Dinoflagellates

Ceratium furca

Ceratium fusus

Ceratium horridum

Dinophysis acuminata

Dinophysis acuta

Dinophysis rotundata

Diplopsalis-group spp.

Dissodinium pseudocalani

Dissodinium pseudolunula

Fragilidium subglobosum

Gonyaulax digitate

Gyrodinium calyptoglyphe

Gyrodinium sp.

Gyrodinium spirale

Nematodinium armatum

Noctiluca scintillans

Polykrikos kofoidii

Prorocentrum micans

Protoperidinium bipes

Protoperidinium conicum

Protoperidinium depressum

Protoperidinium oblongum

Protoperidinium pallidum

Protoperidinium pellucidum

Proloperidinium sp.

Protoperidinium steinii

Protoperidinium subinerme

Protoperidinium thorianium

Scripsielta sp.

Spatolodinium pseudonoctiluca

Torodinium robustum

Prymesiophyceae

Phaeocystis globosa

Phaeocystis pouchetii

Other Plankton

Chattonella verrucosa