THE PHYTOPLANKTON CHIP - DEVELOPMENT AND ASSESSMENT OF A DNA MICROARRAY AS A RELIABLE TOOL FOR MONITORING OF PHYTOPLANKTON

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(Christine Gescher)

‘‘DAS MEER IST GROß, UND MAN KANN IHM MIT KLEINEN MITTELN NICHT BEIKOMMEN.’’

FRIEDRICH HEINCKE

1. General Introduction

1.1 Phytoplankton

Two thirds of the earth surface are covered by water. Thus, the world's oceans contain an immense diversity of life (Hardy 1965). The floating and drifting microcosmos of life in the sea was first defined by Victor Hensen in 1887 under the comprehensive term "Plankton", which includes all aquatic plants and animals that are below 200 nanometer in size and drift passively with the water current. The plant component of the plankton, termed phytoplankton, is composed of autotrophic unicellular (rarely multicellular) algae (Sournia 1978). The name comes from the Greek term, phyton or "plant" and "planktos", meaning "wanderer" or "drifter" (Thurman 1997). Most cannot be detected with the naked eye, but they contribute significantly to ocean biomass. Sometimes they can discolor the water because of the presence of chlorophyll (Vaulot 2001).

The major difference between marine and terrestrial ecosystems is that on land less than 10 % of plants are used directly while they are still alive. All other photosynthetically produced energy goes into the decomposer cycle after the annual growth cycle is completed or the plant dies. In the marine system on the other hand, most of the growing algae are consumed nearly as fast as they are produced. Consequently all produced energy is brought into the marine food web by herbivores (Steele 1974).

The majority of the organisms in the complex oceanic food webs depend on phytoplankton (both eukaryotic and prokaryotic species), because through photosynthesis CO_2 is fixed from solar energy. Furthermore, splitting of water produces oxygen that is released into the atmosphere. Marine microalgae are of great ecological significance because they provide the primary food supply for all life in the sea. The high abundance of phytoplankton compensates for their small individual biomass and consequently primary production in the sea accounts for approximately 30 % of the global annual carbon production. Thus, for example, the amount of plant life produced in a defined area in the ocean may well exceed that produced in the same area of a tropical rain forest (Bold and Wynne 1978; Falkowski 1980; Hardy 1965; Morris 1980; Round 1981; Sournia 1978).

The physical and chemical characteristics of the environment are intimately connected with the occurrence, growth and decay of phytoplankton (Round 1981). Availability of inorganic nutrients e.g., nitrogen, phosphorus, silica and iron is crucial for the structure and abundance of the phytoplankton populations (Vaulot 2001). Furthermore, the microalgae are

dependant on and limited by the influx of solar radiation into the water. Their growth is restricted to the zone penetrated by sunlight, termed the euphotic zone (Raymont 1963). This zone represents only less than 2% of the entire oceanic environment. Another much neglected influencing factor for phytoplankton growth is water movement. No water masses are stationary and the vertical mixing and controlling of availability of substances is fundamental to the structure and dynamics of oceanic phytoplankton production. Other affecting and critical factors in growth and distribution may be salinity, anthropogenic substances, water quality, predation, life-cycles and ectocrines (Falkowski 1980; Morris 1980; Round 1981).

The phytoplankton community can be divided by cell size into micro-phytoplankton (200 – 20 µm), nano- (20 – 2 µm) and picophytoplankton (2 – 0.2 µm) (Sournia 1978; Vaulot 2001). Currently there are more than 4000 species and 500 genera described in marine phytoplankton and approximately 15,000 species in freshwater taxa (Sournia et al. 1991). Two possible explanations for this difference may be that the small picoplanktonic organisms are difficult to detect and many species and genera in the marine phytoplankton have ubiquitous distributions over the world and their diversification may be limited (Vaulot 2001).

The open ocean has a relatively constant chemical composition and the phytoplankton content is remarkably constant with a relatively small number of common taxa, perhaps 100 - 200, mainly diatoms (division Bacillariophyta), dinoflagellates (division Dinophyta), haptophytes (division Haptophyta) and some other flagellates (Round 1981; Sournia 1978). The most conspicuous and numerous group are the diatoms. They feature a silica cell wall, called the frustule, and are separated by shape into pennate and centric diatoms, although this does not reflect their phylogenetic history (Medlin and Kaczmarska 2004). They occur frequently in high cell densities and few diatom species form toxic blooms (Raymont 1963; Vaulot 2001). The dinoflagellates with their flagella possess the power of movement and some of them can be temporarily mixotropic (Sournia 1978). Some of them form blooms under certain conditions, which may be toxic (Harmful Algal Blooms, in short “HABs”). Toxins can be introduced into the food chain and affect shellfish, molluscs, fish or even humans (Raymont 1963; Vaulot 2001).

The haptophytes contribute also significantly to the eukaryotic marine phytoplankton (Vaulot 2001). This group is, contrary to diatoms and dinoflagellates, mostly restricted to the marine phytoplankton fraction and a few species are widespread. They are characterized by two flagella, a thin filamentous appendix, termed the haptonema, and covering of organic scales, which may be calcified (coccolithophores). Some species also form blooms, e.g., the coccolithophore *Emiliania huxleyi*, which can be seen by satellite remote sensing,

Phaeocystis, which forms foam on coastlines (Vaulot et al. 2001) or *Chrysochromulina polylepis,* which accumulated in a massive toxic bloom in 1988 (Kaas et al. 1991).

The phytoplankton can build up a relatively high population in a short time which can be explained by their small cell size and their high division rates. These cells have a large surface to volume ratio that gives them a greater frictional resistance to the water (Raymont 1963). This will counteract their sinking and enable them to remain more easily in the euphotic zone. The second benefit of the small cell size is that the absorption of nutrients, which happens through the cell surface, will be enhanced by a greater surface area. These short diffusion lengths are of extraordinary importance in the enormous oligotrophic areas of the oceans, where nutrients may be present only in very small amounts (Hardy 1965; Raven 1998; Raven and Kübler 2002).

Phytoplankton are not evenly distributed in the ocean and the taxonomic composition of communities, the abundance and dominance of different species and algal groups undergo continuous changes and reorganization (Morris 1980). There are also seasons in the occurrence and compositions of these communities and the contrasts between them are almost as striking as those in the terrestrial vegetation. These seasonal changes certainly have a profound effect on the lives of many other organisms in the sea. In winter there is naturally a great paucity of both animals and plants in the plankton. Thereafter, in temperate waters, there is a spring bloom with sudden outburst of plant activity. Nutrient concentrations are increased because of the intense mixing of the water in winter and as solar increases, thermal stratification occurs to isolate this nutrient rich water in the euphotic layer. This spring bloom is usually started by small diatoms. From spring to summer, the abundance of the phytoplankton steadily declines, phosphates and nitrates are used up as thermal stratification increases to reduce mixing. In autumn there can be a second outburst because of increased mixing as waters cool, but this bloom is not as spectacular as the spring bloom. In spring, the diatom bloom often does not decline because of reduced nitrate and phosphate concentrations. As the phytoplankton increase, grazers, mainly copepods, will also increase. The periods of high abundances of phytoplankton and zooplankton more or less alternate with each other through the whole year (Hardy 1965; Raymont 1963).

In conclusion, the biological importance of phytoplankton are immense and there is a need to acquire an understanding of its succession and ecology by continuous research, simultaneous sampling and analysis of abiotic parameters (Sournia 1978).

1.2 Picoplankton

One particular interesting part of the phytoplankton are the so-called picoplankton, which are composed of cells between 0.2 and 2 μm and contributes enormously to the global carbon cycle, biomass and productivity in the oligotrophic waters (Campbell et al. 1994; Li 1994) and coastal zones (Courties et al. 1994; Joint et al. 1986) in the marine environment. Picoplankton are an important component of the marine ecosystem (Worden 2006; Zhu et al. 2005) and contribute up to 80 % in open ocean oligotrophic waters (Ishizaka et al. 1997) and 87 % in coastal waters (Not et al. 2004). Picoplankton can achieve periodically high abundances e.g., between 10^2 - 10^4 cells per milliliter (mL) (Countway and Caron 2006; Li 1994).

Because the recognition of the importance of picoplankton (Li et al. 1983), their physiology, ecology and distribution has been increasingly studied (Countway and Caron 2006) and hence eukaryotic picoplankton has been found to be unexpectedly diverse (Moon-Van Der Staay et al. 2001). There is evidence for many undescribed species based upon unknown sequences from natural samples in different oceanic regions (Not et al. 2004).

The photosynthetic picoplankton are comprised of three major groups. The first two are the prokaryotic cyanobacterial genera *Synechococcus* and *Prochlorococcus*. *Synechococcus* was first discovered in 1979 and is ubiquitous in relatively mesotrophic waters (Johnson and Sieburth 1979; Waterbury et al. 1979). *Prochlorococcus* is particularly remarkable because of its minute size of 0.6 μm and it is the smallest-known oxygen-evolving autotroph (Chisholm et al. 1988). *Prochlorococcus* is certainly the most abundant photosynthetic organism on earth. Its density can reach up to 100 million cells per liter (Campbell et al. 1997; Chisholm et al. 1988; DuRand et al. 2001). A remarkable number of physiological and geographical ecotypes have been found for both genera (Scanlan 2003).

The third group, the picoplanktonic eukaryotes are less well known and therefore discoveries of new groups of picoplanktonic algae are relatively frequent (Andersen et al. 2002; Andersen et al. 1993; Chrétiennot-Dinet et al. 1995; Guillou et al. 1999a; Kawachi et al. 2002; Not et al. 2007). They are much more diverse than the prokaryotes and the first eukaryotic picoplanktonic species was described only in 1952 as *Chromulina pusilla* (Butcher 1952). This initial picoplanktonic eukaryote was renamed in 1960 as *Micromonas pusilla* (Manton and Parke 1960) which is one of the most abundant and world-wide distributed picoplantkonic species (Slapeta et al. 2006).

Today, approximately 40 picoplanktonic species belonging to nine algal divisions are known: Chlorophyta, Prasinophyta, Trebouxiophyta, Haptophyta, Bolidophyta, Eustigmatophyta, Pinguiophyta, Bacillariophyta, and Pelagophyta (Not et al. 2004).

The picoplankton is now widely accepted as an important component at the basis of the pelagic food chain (Countway and Caron 2006; Díez et al. 2001b), because it serves as prey for nanoplanktonic phagotrophic protists (Caron et al. 1999). Their grazing activity provides a link to higher trophic levels (Sherr and Sherr 1991) and they are also broadly distributed in the marine environment (Derelle et al. 2006; Slapeta et al. 2006).

1.2.1 Prasinophyceae

One particular interesting group among the eukaryotic picoplankton are the Prasinophyceae which recently have been shown to be one of the key picoplankton group in marine waters (Not et al. 2004). Together with the three other classes Chlorophyceae, Trebouxiophyceae and Ulvophyceae, they belong to the Division Chlorophyta, the green algae (Bhattacharya and Medlin 1998; Turmel et al. 1999). The Chlorophyta comprise approximately 500 genera and 8000 species with representatives in almost every habitat and all harbor chlorophyll *a* and *b* (Thomsen and Buck 1998; Van Den Hoek et al. 1995). The green lineage is 1,500 million years old and evolved shortly after the primary endosymbiosis event when the photosynthetic eukaryotes evolved (Derelle et al. 2006).

The Prasinophyceae were discovered in 1951 and Manton and Parke pooled them in one group because they all feature scales (Butcher 1952; Manton and Parke 1960). Subsequently, they have been the subject of several studies (Countway and Caron 2006; Daugbjerg et al. 1995; Derelle et al. 2006; Fawley et al. 2000; Melkonian 1990; Pienaar and Sym 2002; Slapeta et al. 2006; Steinkötter et al. 1994; Sym and Pienaar 1993; Zingone et al. 2002). The group is paraphyletic, so therefore are highly diverse and heterogenous (Lemieux et al. 2000; Steinkötter et al. 1994; Turmel et al. 1999). There have been various confusions concerning the taxonomical classification of some species and the group has been under constant revisions since its first formal description (Bhattacharya and Medlin 1998; Moestrup 1991; Moestrup and Throndsen 1988; Turmel et al. 1999). One explanation is the absence of the one unifying feature within the group (Moestrup and Throndsen 1988). One challenge is the secondary loss of scales because some species are naked. Others do not possess flagella (Thomsen and Buck 1998). The Prasinophyceae are composed of at least seven distinct clades and five established orders: the Pyramimonadales, the Marmelliales, the Prasinococcales, the Pseudoscourfieldiales and the Chlorodendrales and some others containing only sequences

from clone libraries with no described species (Guillou et al. 2004). They are mainly members of marine phytoplankton, but also brackish and freshwater forms have been found (Van Den Hoek et al. 1995). Today, about 20 genera with 180 species are known within the Prasinophyceae, few have been recently described (Van Den Hoek et al. 1995; Zingone et al. 2002). The most remarkable attribute of the Prasinophyceae is their minute cell size (1 - 2,5 µm) and their simple morphology (Slapeta et al. 2006; Zingone et al. 2002). The majority have a body covered with submicroscopical scales and one to eight flagella (Guillou et al. 2004; Thomsen and Buck 1998). They do not possess a cell wall and the one chloroplast is a parietal cup (Prescott 1968).

As mentioned above, the Prasinophyceae are main components of marine phytoplankton with wide geographical ranges and high abundances in several environments (Sieburth et al. 1999; Volkman et al. 1994; Zingone et al. 1999). Detection of their diversity and distribution is hampered mainly by the absence of methods to identify reliably and monitor small cells with few morphological features (Thomsen and Buck 1998; Zingone et al. 2006).

1.2.1.1 Micromonas pusilla

One of the most known and important species within the Prasinophyceae is *Micromonas pusilla*, the only described species in the genus *Micromonas*. It is geographically wide-spread and has been detected world-wide, partially in high cell densities (Brown and Jeffrey 1992; Cochlan et al. 1990; Cottrell and Suttle 1991; Hallegraeff and Jeffrey 1984; Hoepffner and Haas 1990; Johnson and Sieburth 1982; Manton and Parke 1960; Not et al. 2004; Thomsen and Buck 1998; Throndsen 1976; Throndsen and Zingone 1994). Many representatives are present in culture collections (Slapeta et al. 2006).

It is a minute, naked, pear-shaped, solitary small green alga with a single flagellum (Cochlan and Harrison 1991; Slapeta et al. 2006). Cells usually do not exceed 2 µm, contain one mitochondrion and a single chloroplast (Slapeta et al. 2006). Two remarkable features, easily recognized with a light microscope, are the particular cell shape and the extraordinary swimming behavior. The cells of *Micromonas* float forward for some time, stop short and then turn fast around a point for a moment, and the commence forward moving (Zingone et al. 1999).

The understanding of phytoplankton succession and development of algal blooms may be closely related to the appearance and distribution of phytoplankton-infecting viruses (Sahlsten and Karlson 1998). Viruses can indirectly affect carbon and nutrient flux by lysis of

their hosts (Brussaard et al. 1999). One of the first algal viruses was found in *Micromonas pusilla*, but there is a lack of knowledge of geographical distribution or genetic diversity of phytoplankton viruses (Cottrell and Suttle 1991). Tiny and easy to culture microalgae could serve as an appropriate model for research on phytoplankton viruses and their affect on phytoplankton diversity and distribution (Cottrel and Suttle 1995). Some strains of *Micromonas pusilla* are not susceptible to viral infection. One possible explanation could be intraspecific or even cryptic diversity within the host (Zingone et al. 2006), which was found recently for *Micromonas* (Guillou et al. 2004; Slapeta et al. 2006). Cryptic or pseudo-cryptic species are known to show only minute morphological differences, but do not share genetic information. This may be explained by the relevancy of an essential particular form, size or shape (Sáez et al. 2003). Thus, an optimal phenotype is exposed to strong stabilizing selection (Sáez et al. 2003). For *Micromonas,* three independent lineages of considerably different ecotypes or even cryptic species were discovered in 2004. Recently five distinct groups have been found, which may display adaptations to different conditions and could be advantageous in the understanding of geographical distributions (Guillou et al. 2004; Slapeta et al. 2006). It seems that some or maybe all lineages are ubiquitous dispersal in the world's oceans and *Micromonas* harbors the oldest group of cryptic species known today (Slapeta et al. 2006).

1.2.1.2 Ostreococcus tauri

The smallest known autotrophic eukaryote, *Ostreococcus tauri*, was discovered in 1994, dominating the picoplankton community in Thau Lagoon, France (Chrétiennot-Dinet et al. 1995). It was first reported from Thau Lagoon, and since has been documented often from coastal to oligotrophic areas in the world's oceans, as a common member of the natural marine picophytoplankton (Caron et al. 2004; Countway and Caron 2006; Derelle et al. 2006; Díez et al. 2001b; Dupuy et al. 2000; Guillou et al. 2004; O'kelly et al. 2003; Romari and Vaulot 2004; Vaquer et al. 1996; Worden et al. 2004; Worden and Palenik 2002). *Ostreococcus tauri* is barely detectable by light microscope because of its small size of less than 1 µm. The cell body is naked without flagella and thus appears as a small ball or particle with light microscocopy. The cellular organization is very simple (Courties et al. 1998), it grows rapidly and is easily grazed (Fouilland et al. 2004; Worden et al. 2004). *Ostreococcus tauri* is the first picoplanktonic eukaryote with its genome sequenced. It has the smallest genome among free-living eukaryotes. The genome shows high diversity, unobserved levels of heterogeneity, gene fusion and extensive reduction of intergenic regions. With the minimum cellular and genomic organization necessary for a photosynthetic eukaryotic cell, it

is an excellent model in cell biology (Derelle et al. 2002; Derelle et al. 2006). The ecology of *Ostreococcus tauri* is relatively unknown, but beginning to be unraveled with the use of molecular methods (Countway and Caron 2006; O'Kelly et al. 2003).

Like *Micromonas*, four different ecotypes in *Ostreococcus* have been found (Guillou et al. 2004). Recently Rodriguez and co-workers reported nine distinct lineages (Rodriguez et al. 2005), that may represent adaptations to different environmental conditions, because the genetic distances do not reflect the geographical distribution (Rodriguez et al. 2005).

1.3 Harmful Algal Blooms

As mentioned previously, phytoplankton can occasionally form dense blooms with million cells per liter, which is visible through coloring of the sea surface, e.g., red, yellow, brown, green, blue or milky, depending on the organism involved in the bloom event. The high cell densities in these blooms can be explained by high growth rates in combination with either vertical (behavioral) or horizontal (physical) aggregation, reduced losses from viruses, sedimentation, nutrient depletion and grazing (Bratbak et al. 1996; Brussaard et al. 2005; Hallegraeff 2002; Steidinger and Garcés 2006). Bloom formation is often triggered by stratified stable conditions, high temperatures and subsequent high organic input from land after intense rainfalls (Hallegraeff 2002; Steidinger and Garcés 2006; Van Den Hoek et al. 1995; Zingone and Enevoldsen 2000). Cell concentrations can reach up to 10^4 - 10^5 cells per liter and are often dominated by one or a group of species (Masó and Garcés 2006).

Blooms can be divided in three groups, according to their potential consequences. Most blooms are non-toxic and can only cause death of marine animals by depleting oxygen. Others are non-toxic to humans, but clog fish gills or damage gill tissue (Granéli and Turner 2006a; Hallegraeff 2002; Hallegraeff 2003; Taylor and Fukuyo 1998; Van Den Hoek et al. 1995).

The “Harmful Algal Blooms” (HABs) are produced by certain species, mostly dinoflagellates. A wide range of organisms is involved in these blooms and some species have toxic effects at low cell densities, not all HABs are “algal” and not all occur as “blooms”. From approximately 5,000 known phytoplankton species, only 300 species can bloom and 97 species are know to produce toxins (Granéli and Turner 2006b; Moestrup 2004; Sournia et al. 1991; Zingone and Enevoldsen 2000). Toxic microalgae are ingested by shellfish, mussels and fishes, and are accumulated in the food web. When these fish or shellfish are consumed by humans, toxicity is transferred to humans (Hallegraeff 2002). Economic losses to fisheries, tourism, and health care can be severe (Granéli and Turner 2006b).

Many different symptoms, mainly gastro-intestinal and neurological, will develop in humans after consumption of contaminated animals. The related illnesses, which are caused by harmful algae, are divided into groups by their symptoms. They are known today as: paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), amnesic shellfish poisoning (ASP), neurotoxic shellfish poisoning (NSP), azaspiracid poisoning (AZP) and cigatuera fish poisoning (CFP) (Hallegraeff 2002).

The number and the intensity of HABs seems to be increasing in frequency and geographic distribution in the last decades. Possible explanations are more scientific interest and higher sophisticated methods for monitoring and detection, increased utilization of coastal waters for aquaculture, impact of humans by pollution, eutrophication, unusual climatological conditions, and transportation of cells and cysts worldwide through ballast water and shellfish stocks (Anderson 1998; Godhe 2002; Hallegraeff 2002; Van Den Hoek et al. 1995). Toxic and non-toxic microalgae species often co-occur in environmental phytoplankton assemblages and separation can be difficult. Nevertheless, there is an ongoing need for sensitive, high throughput methods to detect and monitor occurrence of HAB species to prevent negative effects on man and environment, but this mission is not a trivial task.

1.4 Dinophyta

The division Dinophyta contains only one class, the Dinophyceae. The name “Dino” comes from Greek, meaning “whirring” as a type of movement (Van Den Hoek et al. 1995). The majority of the Dinophyta is unicellular and possesses two flagella, one that encircles the cell and causing it to rotate and another trailing the cell, acting as a rudder. The cell is divided in an upper part, “epicone”, and a lower part, “hypocone”, by a girdle where the horizontal flagellum is located. These two sections, their relation to each other, shape, size, ornamentation and surface structure provides the fundament for the taxonomic classification (Faust and Gulledge 2002; Janson and Hayes 2006; Van Den Hoek et al. 1995). There are also some highly specialized heterotrophic dinoflagellates. Some contain unusual chloroplasts, which may have their origins in tertiary endosymbiosis. Dinoflagellates are 90 % marine and 130 genera and 2000 species are known today. They contribute enormously to phytoplankton biomass and their zygotes can be resting stages in the sediments (Van Den Hoek et al. 1995). Dinoflagellates achieve their largest size and most bizarre forms in warm waters (Dodge 1985).

1.4.1 Genus *Alexandrium*

Alexandrium belongs to the order Gonyaulacales and family Goniodomaceae. There are 29 known species within the genus and confusions and renaming of the species has been common because the species differ only in minute morphological details. The latest classification was made by Balech (1995). All members of *Alexandrium* feature the same structural characteristics in the hypocone, cingulum, sulcus, and even in the epithecal region with the exception of the 1' plate (Balech 1995). Therefore, the tabulation of the thecal plates is mainly used for distinguishing species within this genus (Janson and Hayes 2006). Furthermore, the species distinction rest on the presence of absence of the ventral pore (Van Den Hoek et al. 1995). These morphological patterns may differ only slightly and can vary with environmental factors, e.g., temperature and nutrition (Hosoi-Tanabe and Sako 2006). Not all *Alexandrium* species are known to be toxic, e.g., *Alexandrium affine, A. insuetum and A. pseudogonyaulax* are non-toxic (Janson and Hayes 2006). The toxic species of the genus produce toxins related to the PSP complex and species of the genus is perhaps the most thoroughly studied HAB species.

1.4.1.1 *Alexandrium tamarense* "species complex" (Lebour) Balech 1985

Three morpho-species compose this group: *Alexandrium catenella* (Whedon and Kofoid) Balech 1985, *A. fundyense* Balech 1985 and *A. tamarense* (Lebour) Balech 1985. *A. catenella* was the first dinoflagellate linked to PSP (Sommer and Meyer 1937; Sommer et al. 1937). The three species are morphologically difficult to distinguish (separated mainly by the presence or absence of the ventral pore and colony formation) and also share overlapping thecal characteristics. Furthermore, intermediate forms have been observed (Cembella and Taylor 1986). The cells are small (20 - 22 µm long, 25 - 32 µm wide) and often form characteristic chains of two, four, or eight cells (Hallegraeff 2002). Species belonging to this group have been detected in regions all over the world (Balech 1995; Faust and Gulledge 2002; Hallegraeff 2002; Taylor et al. 1995).

Recently it was found that these three "morphospecies" are closely related to each other (Janson and Hayes 2006; Scholin et al. 1994). They are related geographically rather than by morphology (Cembella et al. 1987; Cembella et al. 1988; Medlin et al. 1998; Scholin et al. 1995) and several phylogenetic studies of the complex have been carried out (Adachi et al. 1996a; Higman et al. 2001; Medlin et al. 1998; Scholin et al. 1994). The geographic areas correspond to six different "ribotypes", based on the D1/D2 region of the 28S rRNA level. The three toxic types are: the North American (NA), the Temperate Asian (TA) and the

Tropic Asian (TROP); the other three are non-toxic: the Tasmanian (TASM), the Western European (WE) (Scholin et al. 1995) and the recently described Mediterranean (ME) clade (John et al. 2003b). Thus, it seems that the morphology may underestimate and belie the true underlying genetic diversity within this group (Janson and Hayes 2006; John et al. 2003b; Scholin 1998b).

Molecular methods have the possibility to improve the differentiation of the morphologically similar *Alexandrium tamarense* "species complex" and, to improve the understanding of the biogeography and genetic diversity of populations of this important group of organisms.

1.4.1.2 *Alexandrium ostenfeldii* (Paulsen) Balech & Tangen 1985

This species was first described as *Goniodoma* by Paulsen and is relatively easy to distinguish from the other members of this genus (Paulsen 1904). The cells are large (40-56 µm long, 40-50 µm transdiameter), nearly spherical, globose and non-chained (Balech 1995). The most distinctive feature is the shape of the first apical pore. The toxicity of *Alexandrium ostenfeldii* is usually low, it is known as the least toxic species of the genus (Cembella et al. 1987; Cembella et al. 1988). Occasionally there are also high toxic strains found and some strains produce both saxitoxins and the spirolide shellfish toxins (Hallegraeff 2002). The species is worldly distributed in cold waters (Balech 1995; Faust and Gulledge 2002; Hallegraeff 2002; Lundholm and Moestrup 2006; Taylor et al. 1995).

1.4.1.3 *Alexandrium minutum* Halim 1960

This species was first described from the harbor of Alexandria, Egypt (Halim 1960). The cells of *Alexandrium minutum* are small, inconspicuous and spherical. They are rarely in pairs, often solitary. The cells range from 15 - 30 µm in length and 13 - 24 µm in width (Faust and Gulledge 2002). The identification of the species is rather difficult only made by minute details of the apical tabulation (Hallegraeff 2002; Taylor et al. 1995). Another discriminative feature is the characteristic ventral pore (Faust and Gulledge 2002), but there have also been reports on strains lacking ventral pores, thus it has been confused with other species of the genus (Taylor et al. 1995; Vila et al. 2005). Possible explanations of the morphological variations may be different environmental conditions (Lilly et al. 2005) and recently, it has been shown that strains with and without ventral pore do not differ in their 28S rRNA sequences (Nascimento et al. 2005). *Alexandrium minutum* is a producer of PSP toxins and

cause red tides. The distribution is world wide, mainly in coastal areas (Faust and Gulledge 2002; Hallegraeff 2002; Taylor et al. 1995).

1.5 Methods for detection and monitoring of phytoplankton

In the past, regular monitoring of phytoplankton has been hampered mainly by the lack of reliable features in some groups of species. Even with the introduction of electron microscopy, it is difficult to make the correct classification, especially in picoplanktonic taxa or with hidden genetic diversity in morphological indistinguishable species (Janson and Hayes 2006; Massana et al. 2002; Scholin 1998b; Zingone et al. 1999). Consequently, the complexity of the phytoplanktonic ecosystems, the distribution and the diversity of species is still unknown. It is the stated aim of modern research to assess the abundance of phytoplankton at different spatial and temporal scales in order to estimate their importance to the marine ecosystem (Díez et al. 2001b; Not et al. 2004; Zhu et al. 2005).

Below, I review the methods mainly used for phytoplankton classification, detection and monitoring and their advantages and drawbacks.

1.5.1 Traditional and classical methods

Culturing

Phytoplankton can also be investigated by culturing and separating cells, however this approach is selective and there is no security that the species in culture are dominant or even important in the community (Díez et al. 2001b; Guillou et al. 1999b; Lim et al. 1999). Furthermore, there is also evidence that the culturing bias is even larger than the bias introduced with the use of molecular methods (Massana et al. 2004), because many groups seem to be resistant to cultivation (Sieburth et al. 1999).

Microscopy

For a long time, microscopy has been the traditional method for detection, identification and monitoring of phytoplankton. Taxonomic classification of phytoplankton by light microscopy demands extensive time for sample preparation, counting and determination of size and furthermore extensive taxonomic expertise of the examiner. Statistically valid counts of groups or species with few morphological markers and of less abundant taxa (e.g., picoplanktonic groups) are exceptionally time-consuming and demanding (Mackey et al. 2002). Furthermore, many species are sensitive to sample fixation (Gieskes and Kraay 1983) and some possess different life stages with varying morphological properties (Partensky et al.

1988). The experience of the scientist may also affect the identification (Bornet et al. 2005; Godhe et al. 2007). Furthermore, this method can hardly clarify the simplest morphological characteristics of many species, even at the class level (Marie et al. 2006; Murphy and Haugen 1985).

With the introduction of transmission and scanning electron microscopy, a better identification of phytoplanktonic species was achieved and several groups indistinguishable with light microscopy could be identified (Andersen et al. 1996; Johnson and Sieburth 1982). It is mainly possible to detect the cells at class level (Andersen et al. 1996), but most species do not possess enough ultrastructural features for identification at lower taxonomic levels (Potter et al. 1997). Consequently, the major drawbacks of this method are surely the uncertainty of classification of several groups, low sample through-put, fragility of cells, difficulty and time of sample treatment, which combined makes it nearly impossible for long-time monitoring (Andersen et al. 1996; Mackey et al. 2002).

Pigments (e.g., chlorophyll, phycobilin, carotenoids) of phytoplankton can be detected by epifluorescent microscopy. The pigments are excited with fluorescent light of certain wavelengths. This method is quite tedious and it is not possible to distinguish species (Moon-Van Der Staay et al. 2000). The aid of other methods is required (Zhu et al. 2005).

Pigment analysis

The different pigments in phytoplankton cells and also PSP toxins can be detected and analyzed by high performance liquid chromatography (HPLC). This method is based on column chromatography and the pigments of organisms from different algal classes, which have different diagnostic markers, can be separated (Díez et al. 2001b). It is a fast and objective way of estimating the major classes of the phytoplankton community (Guillou et al. 1999b). However, taxonomic resolution is limited, because many groups may lack specific diagnostic pigments or may contain pigments, which are marker pigments for other groups (Massana et al. 2002). Consequently, one major drawback is the possibility of overlooking important groups with no or overlapping pigments (Breton et al. 2000; Schlüter and Møhlenberg 2003). This problem was only realized after groups were cultured in the laboratory and their pigments extracted and identified (Marie et al. 2006; Simon et al. 2000). The pigment composition of a species can change with environmental conditions (Massana et al. 2002). Furthermore, cells are not examined directly with this method and the detection is influenced by optical settings of the device and species composition in the sample (Andersen et al. 1996; Díez et al. 2001b). The analysis and interpretation of complex pigment patterns

acquires application of algorithms, which makes this method cumbersome and time-consuming (Díez et al. 2001b; Letelier et al. 1993), but HPLC is useful for the characterization of newly described strains and species (Letelier et al. 1993).

1.5.2 Molecular Methods

Within the last decade, molecular methods, mainly used previously for studying prokaryotes, have been applied to eukaryotes to study phytoplankton biodiversity and abundance in the ocean (Díez et al. 2001a; Moon-Van Der Staay et al. 2001; Not et al. 2002). Molecular methods make it is possible to determine composition and distribution of the phytoplankton without having to observe or cultivate it (Countway and Caron 2006; Gentry et al. 2006). The introduction of molecular biological methods brings a variety of alternative methods for detection and monitoring of phytoplankton, although it has to be kept in mind that every method has its own promises and pitfalls (Moon-Van Der Staay et al. 2000).

Antibodies

The utilization of monoclonal or polyclonal antibodies is possible for detection of individual species or ecotypes and is particularly helpful for the detection of phytoplankton cells without morphological markers (Shapiro and Campbell 1998; Zhu et al. 2005). The antibodies are produced against the cell surface or intracellular antigens in a host (e.g., rabbit) in the presence of the foreign molecule and therefore, the culture or the antigen is needed before (Shapiro and Campbell 1998). The advantages of this method are that the cells are not destroyed and, it can be a quantitativemethod (Godhe 2002) which therefore has been applied on microalgae (Scholin et al. 2003; Vrieling and Anderson 1996). But the disadvantages are their expenses, the non-specificity of the polyclonal antibodies (Kamikawa et al. 2007), that only a small percentage of species in a field probe is detected, that that the reactivity may be affected by different growth and life cycle stages and they can not be applied to higher taxonomic levels (Adachi et al. 1994; Adachi et al. 1996b; Anderson et al. 1999; Shapiro and Campbell 1998; Zhu et al. 2005).

DGGE and TGGE

Denaturant and temperature gradient gel electrophoresis (DGGE and TGGE) are fingerprinting methods that provide quick analytical tools to study and compare the composition and molecular ecology of microbial communities (Díez et al. 2001a; Van Hannen et al. 1998). These methods can discern stains, that differ by only a few DNA base

pairs, by performing first a PCR, followed by a polyacrylamide gel electrophoresis with a denaturant or temperature gradient (Coyne et al. 2001; Etscheid and Riesner 1998; Godhe 2002; Muyzer et al. 1993). The method is based on the melting behavior of DNA sequences in the gel with increasing concentrations of denaturing substances or increasing temperature (Etscheid and Riesner 1998; Van Hannen et al. 1998). The benefits are obviously the rapidness, the high sensitivity, specificity, and good resolution to study a population structure, whereas the drawbacks are the non-quantitative detection due to the PCR and the utilization of neurotoxin acrylamide (Biegala et al. 2003; Godhe 2002; Van Hannen et al. 1998).

DNA Sequencing and clone libraries

The use and comparison of coding and non-coding DNA sequences is helpful to reveal questions at all taxonomic levels concerning evolutionary history of organisms and their relationships. These molecular methods rose with the development of the Polymerase Chain Reaction (PCR) in the 1980s (Medlin et al. 1988; Mullis and Faloona 1987; Mullis et al. 1986). In particular, the ribosomal genes have been determined as good markers to examine these questions, because of their special abilities (Medlin and Simon 1998). These genes arose very early in evolution, are large in size and possess highly variable, moderately variable and highly conserved regions (Medlin and Simon 1998; Woese 1987). In the last decades, the databases with ribosomal RNA (rRNA) sequences have increased rapidly (Metfies et al. 2006). This large set of comparable sequences has facilitated research in this field, reshaped the view of evolutionary relationships among organisms and opened new avenues in microbial taxonomy (Countway and Caron 2006; Medlin and Simon 1998; Simon et al. 2000; Woese 1987; Zhu et al. 2005).

For obtaining the sequences, the most convenient method is the processing of fragments of these genes. Fur this purpose a clone library is constructed and the DNA fragments are cloned into vectors (e.g., plasmids or bacteriophages). The term library means that the entire genome of an organism or a collection of organisms or a field sample can be found in the vectors. It is also possible to use messenger RNA (mRNA) and translate it back into DNA by reverse transcription. The so-called complementary DNA (cDNA) library only consist of transcribed genes (Zehr and Hiorns 1998). Nevertheless, extracting DNA, amplifying a gene, possible constructing of a library and sequencing afterwards demands time and is time-consuming and cost-intensive (Valentin et al. 2005).

Molecular Probes

The term molecular probe characterizes a certain DNA sequence that matches a complementary region in a target gene. The probe hybridizes to its complement, governed by hydrogen bonds between nucleic acids and forms double stranded helices (Scholin 1998a). The application of probes is possible using a wealth of different methods and the duplex formation can be detected by different labels placed at the end of the probe, e.g., radioactively, fluorescently or enzyme labeled (Groben et al. 2004; Groben and Medlin 2005; Medlin and Simon 1998; Scholin 1998a). The probes can be designed from several genes but the most widely used marker gene for design of molecular oligonucleotide probes is the ribosomal RNA (Metfies et al. 2006; Simon et al. 1995). As previously mentioned, the gene has a mosaic organization consisting of conserved and variable regions and the transcribed RNAs are abundant in the cell with several thousands of copies (Medlin and Simon 1998; Simon et al. 2000). The rRNA probes are much more flexible than antibodies and they can target any demanded taxonomic level from the ecotype to division (Zhu et al. 2005). Afterwards, the specific probe can be utilized as a phylogenetic marker at a variety of taxonomic levels in phytoplankton from kingdoms down to species or strains using whole-cell and cell-free formats (John et al. 2003a; John et al. 2005; Metfies and Medlin 2004; Metfies et al. 2006; Scholin et al. 2003). Furthermore, the application of hierarchical probes can be advantageous, because they allow the validation of one probe signal at different hierarchical levels (Metfies et al. 2006). The expansion of the known sequences in databases provides a good basis for the development of oligonucleotide probes, but the drawback may be that the developed probes must be checked regularly against new sequences to prevent unspecific binding or cross-hybridization. Another disadvantages is that, in some cases it is difficult to find a probe for a specific target and even impossible for polyphyletic groups (Medlin and Simon 1998).

Fluorescence-in-situ-hybridization (FISH)

This method is based on the hybridization of the whole cell with a labeled oligonucleotide probe and was pioneered with bacteria (DeLong et al. 1989; Devereux et al. 1992). For the successful detection it is necessary to fix the cells on a filter or in a culture for preservation and to permeabilize the membrane before the application of probes (Medlin and Simon 1998). The advantages are definitely the rapid quantitative detection and visualization of algal species in a mixed field sample and the discrimination of closely related species or strains with even similar appearance (Godhe 2002; Groben et al. 2004; John et al. 2003a; John

et al. 2005; Litaker and Tester 2006; Massana et al. 2002; Metfies et al. 2006). Furthermore the morphology of the cells is conserved and thus also different external life stages can be recognized (Godhe 2002). One drawback of this method is that cellular rRNA content may vary under different environmental conditions and could have an impact on the fluorescence signal. Further challenges are the autofluorecence of photosynthetic cells, non-specific binding of probes, difficulties with penetration of thick cell walls of resting cysts and non-stability of rRNA molecules (DeLong 1998; Garcés et al. 1998; Godhe 2002; Medlin and Simon 1998; Rice et al. 1997). The enhancement of signals 10 - 20 fold higher in comparison to conventional protocols can be achieved by utilization of amplification methods like the TSA- (Tyramide Signal Amplification) or CARD-FISH (Catalyzed Reporter Deposition) protocol (Amann et al. 1990; Biegala et al. 2003; DeLong et al. 1989; Massana et al. 2002; Metfies et al. 2006). The enhancement is accomplished with probes labeled with the enzyme horseradish peroxidase and there is a subsequent deposition of additional tyramide-bound fluorochromes adjacent to hybridized probes (Biegala et al. 2003; Metfies et al. 2006). Nevertheless, the processing and quantitative analysis of samples by microscopy can be tedious, slow, demanding and even statistically inadequate. Thererfore, the interpretation of samples with automated devices, like solid-phase chromatography (ChemScan) or flow cytometers can be a great improvement in ecosystem investigation (Godhe 2002; Metfies et al. 2006; Rice et al. 1997; Töbe et al. 2006; Zhu et al. 2005).

Flow Cytometry

Flow cytometry was originally used to distinguish certain cells in liquid suspension without probes and now offers excellent counting statistics (Mackey et al. 2002; Olson et al. 1989; Veldhuis and Kraay 1990; Veldhuis and Kraay 2000). Identification of a cell is based on its visual characteristics. For photosynthetic picoplankton the size (“side scatter”) and the natural chlorophyll cell fluorescence is used (Metfies et al. 2006; Simon et al. 1995). However, the sorting here can only be done on higher taxonomic levels. (Campbell et al. 1994; Jacquet et al. 1998; Marie et al. 2005; Worden et al. 2004). The application of molecular probes and FISH technique in combination with flow cytometry greatly increased the detection and monitoring of phytoplankton communities, especially in picoplankton, which is too small to be counted by conventional microscopy (Mackey et al. 2002; Metfies et al. 2006; Rice et al. 1997). The coactions of both methods resulted in high resolution for taxonomic identification and rapid, sensitive and statistically proven automated cell counting, which makes it even possible to study picoplankton dynamics (Biegala et al. 2003; Mackey et

al. 2002). The adjustment of the TSA-FISH protocol further refined the detection, it greatly enhanced the signal intensity (Metfies et al. 2006), and has been applied to detect and monitor phytoplankton (Biegala et al. 2003; Not et al. 2004; Not et al. 2002). Low signal-to-background-ratios, coincidence of particles, interference from fluorescent detritus, cell losses and cell clumps during sample preservation can be the difficulties that arise when using this device (Biegala et al. 2003; Mackey et al. 2002).

Real-Time PCR

Real-Time PCR or Quantitative PCR (QPCR) is a modification of conventional PCR protocols and allows the product formation in the PCR reaction to be monitored *in-situ* by fluorescence (Marie et al. 2006). This PCR approach can be used to determine the abundance of specific groups. The simple approach is the utilization of nucleic acid dyes (e.g., SYBR Green or Ethidiumbromide), which bind to the newly synthesized double-stranded DNA as soon as it is formed during PCR. The incorporation is measured in a special thermocycler device and compared to a standard. The major drawback is that the dye will unspecifically bind to all double-stranded DNA including any unexpected PCR products, which may lead to errors in the calculation. In contrast, the Taqman approach to QPCR uses an oligonucleotide probe with a fluorochrome and a quencher is added to the usual primer pair in a conventional PCR. The sequence of the probe is complementary to a region in the target and is incorporated in each cycle. The quencher initially blocks the fluorescent signal, but is released by the 5' to 3' exonuclease activity of the polymerase as the probe binds specific to the target sequence in the PCR product and when excited, the fluorescent dye emits light. The relative fluorescence is related to the number of free fluorescent molecules in the solution that originated in PCR product formation (Scholin et al. 2003; Zhu et al. 2005). The method provides all benefits of traditional non-quantitative PCR, e.g., the sensitivity and specificity (Godhe 2002), but the main advantage are the detection accuracies over a large dynamic range, the fast analysis and the large sample throughput (Countway and Caron 2006; Johnson et al. 2006; Zhu et al. 2005). However, it destroys cells, and the equipment and components are expensive (Godhe 2002). It requires sophisticated controls and calibrations (Johnson et al. 2006). QPCR has been used for detection and identification of dinophytes, raphidophytes and prasinophytes in field samples (Bowers et al. 2000; Countway and Caron 2006; Dyhrman et al. 2006; Galluzzi et al. 2005; Galluzzi et al. 2004; Handy et al. 2006; Marie et al. 2006; Tengs et al. 2001; Zhu et al. 2005).

Microarray

DNA microarrays or so-called DNA chips are one of the most powerful innovations in microbiology. Microarrays were introduced in the mid 1990s primarily for the detection and monitoring of gene expression (Schena et al. 1995; Schena et al. 1996). The application of sequences onto the surface of a glass slide with special surface properties in an ordered array is based on a minimized, but high throughput form of a dot-blot (Gentry et al. 2006; Ye et al. 2001). Cell-free systems with utilization of nucleic acids have the unparalleled potential to facilitate the analysis of thousands of targets from one sample in a single experiment (DeRisi et al. 1997; Gentry et al. 2006; Lockhart et al. 1996; Lockhart and Winzeler 2000; Metfies et al. 2006; Schena et al. 1995; Ye et al. 2001). The DNA microarray experiment is performed by chip production, sample isolation and preparation, hybridization and data analysis. There are two possible approaches of microarray fabrication; the first is the *in-situ* synthesis on the chip by adding nucleotides sequentially to an initial oligonucleotide, which is immobilized to the glass slides as in the Affymetrix or Agilent system (Ye et al. 2001). The alternative is the printing of presynthesized probes with direct surface contact by fine-pointed pins and high-speed robots or non-contact based on piezoelectric technology (Graves 1999; Schena et al. 1998; Ye et al. 2001). Prior to the hybridization, the target nucleic acid is labeled with a fluorescent dye, which can be incorporated directly to the nucleic acid or via indirect labeling of other substances (Cheung et al. 1999; Metfies et al. 2006; Southern et al. 1999). The hybridization pattern is captured via fluorescent excitation in a special device, the microarray scanner (Ye et al. 2001). One of the major drawbacks of this method is a possible cross-reaction from unspecific binding. The probes are designed to be specific to known sequences but there are a high number of unknown environmental sequences. As a consequence, species without a probe on the chip can also be overlooked (Gentry et al. 2006). Furthermore, the development of a functional chip is time-consuming, expensive and all probes on one chip need to work specifically under the same hybridization conditions (Boireau et al. 2005; Feriotto et al. 2002; Metfies et al. 2006).

However, for a comprehensive understanding of the complexity of the marine ecosystems and their ecology it is indispensable to detect and monitor the abundance and dynamics of different contributors simultaneously (Gentry et al. 2006). Therefore, a further aim of microarray research is to refine and expand the technology into microbial ecology with the application of a different kind of microarray, the so-called “Phylochip”. This term is used for a DNA microarray, designed with probes from a conserved marker, e.g., the ribosomal RNA. The rRNA gene is an excellent marker gene with the huge number of available

sequences in public databases, which are steadily increasing (Gentry et al. 2006). Each probe on the microarray represents a different taxon, from kingdom to strain. The application of hierarchical probes at different taxonomic levels can enhance the accuracy of a Phylochip, because the detection of species is assessed by more than one probe (Metfies et al. 2006).

This microarray format is most commonly used with prokaryotes (Gentry et al. 2006; Lehner et al. 2005; Loy et al. 2002; Loy et al. 2005; Peplies et al. 2004a; Peplies et al. 2006; Peplies et al. 2004b). Recently, there has been a Phylochip based on the plastid 16S rRNA gene to detect photosynthetic eukaryotic picoplankton (Fuller et al. 2006a; Fuller et al. 2006b) and there are a few publications on the successful detection and monitoring of harmful algae (Ki and Han 2006) and marine microalgae (Medlin et al. 2006; Metfies and Medlin 2004).

1.6 Helgoland Roads Time-series

The island of Helgoland is situated approximately 60 km off the mainland in the North Sea, has a high diversity of marine life and features many different habitats (Franke et al. 2004). There has been a long history of scientific research on the island since the first data was collected in 1873. In 1962, a milestone in aquatic long-term monitoring series was set with the startup of the Helgoland Roads time-series station (Hickel 1998; Wiltshire 2004). The monitoring program is regarded today as one of the most important and valuable marine data sets in the world and it is especially inimitable with the sampling length, frequency and numbers of parameters measured (Franke et al. 2004; Wiltshire 2004). One of the main objectives of the program on North Sea ecosystem research is the determination of ecological dynamics in the German Bight with special reference to trophic interconnections (Franke et al. 2004). On a daily basis, sampling is completed in a narrow channel at an anchorage area between the two islands of Helgoland, the Roads (54°11.3’ N, 07°54.0’E). Furthermore, physico-chemical parameters (temperature, salinity, concentration of dissolved inorganic nutrients, such as nitrate, nitrite, ammonium and silicate), and biological parameters (qualitative and quantitative date on phytoplankton, microorganisms and since 1974, particular groups of zooplankton) are measured (Franke et al. 2004; Hickel 1998; Wiltshire 2004).

The time-series has been running for over 40 years and has provided a multitude of data (Franke et al. 2004), that have often been cited and used in scientific papers, in lectures on marine ecology and for the parameterisation and validation of mathematical ecosystem models (Wiltshire 2004; Wiltshire and Dürselen 2004). This treasury of data is a fingerprint of history and represents an excellent basis for analyzing past changes, evaluating the current

status of the ecosystem and predicting future changes of our aquatic system (Franke et al. 2004; Wiltshire 2004). Such conclusions are indispensable for understanding the long-term effects of climate changes and anthropogenic inputs (Reid et al. 1990). In 2004 the first indication in the field of climate change was made utilizing data from the Helgoland Roads time-series, observing an obvious warming of 1.1 °C of the water temperature since 1962, which has resulted in a shift in the diatom spring bloom (Wiltshire and Manly 2004). This shift will affect other members of the food web that are dependant on the microalgae as a food resource (Wiltshire and Dürselen 2004).

The Helgoland Roads time-series is one of the longest data series for phytoplankton where species composition has been identified. The method used did not vary over the time, a water sample was taken with a bucket, mixed, and phytoplankton cells were preserved in Lugol's iodine. The Utermöhl method was used to settle one liter to 25 mL and afterwards the cells were counted with an inverted microscope. The collected data offer the possibility of examination of phytoplankton succession against the backdrop of the climate change (Wiltshire and Dürselen 2004), however one of the greatest concerns of the time series is to ensure the credibility and comparability of data over time (Wiltshire 2004). This is mainly hampered by frequent change in counting staff over time and this bias cannot be eliminated completely, because taxonomic expertise takes many years to acquire (Franke et al. 2004; Reid et al. 1990; Wiltshire and Dürselen 2004). A further drawback is that for some taxa, microscopy alone appears to be insufficient. Cells do not possess enough discriminative morphological markers, or, especially small cells are easily overlooked in samples containing particles or aggregates. Other groups, such as Cryptophyceae and Prasinophyceae, do not preserves well although they are known to be common in the North Sea (Gieskes and Kraay 1984; Reid et al. 1990).

Because of these difficulties, there is neither identification nor enumeration of the picoplanktonic fraction in the Helgoland Roads time-series (Medlin et al. 2006). But accurate identification of algal species is indispensable for further phytoplankton research (Reid et al. 1990) and therefore the demand for continuity should not exclude the design and application of advanced methods in order to achieve efficient characterization of the community on finer temporal and spatial scales (Franke et al. 2004). The picophytoplankton dominate the photosynthetic biomass in many marine ecosystems and molecular methods are useful for understanding and description of their diversity. Medlin et al. (2006) compared and evaluated picoeukaryotic diversity in samples from Helgoland using three different molecular methods: 1.) sequencing of cloned eukaryotic 18S rRNA genes in libraries, 2.) a fingerprinting

technique using the single-strand conformational polymorphisms, and 3.) a DNA microarray with class-level oligonucleotide probes. The results indicated high variances in species composition on a weekly basis, but a comparison of yearly samples showed a high congruence and indicated a seasonality in the picoplanktonic fraction (Medlin et al. 2006). The microarray results agreed quite well with the picoeukaryotic plankton composition of the clone libraries, however that microarray is a prototype with only class-level identification. The next step is to extend this first-generation microarray with the design and assessment of a multiplicity of new probes with deeper hierarchical probes and even down to species level. With the high throughput format of the microarray, there is the opportunity for a quick, reliable and profound investigation, which therefore overcomes the labor intensive task of other traditional and molecular methods. The analysis of complex environmental samples of picoplanktonic communities and detection of changes in their composition through time would be a great improvement in microbial ecology.

1.7 Aim of thesis

As mentioned above, phytoplankton play an important role in the marine environment as the basis of the food web, as a producer of oxygen and as a carbon sink. The overall understanding of marine phytoplanktonic ecosystems is hampered mainly by the challenges in classification and enumeration of morphologically quite similar species. Thus, there is a need of trustworthy devices based on molecular methods. Oligonucleotide probes of the ribosomal RNA and DNA microarrays as robust and high throughput hybridization methods in combination could serve as a reliable and fast tool to detect and to unravel phytoplankton community structure. Therefore, the improvement of the DNA microarray as a method to study phytoplankton biodiversity was the main objective of my thesis. The PHYTOPLANKTON CHIP was established by developing probes for certain taxonomic groups of microalgae and adapting these probes and probes designed for other probe-based methods to one optimal hybridization protocol. The utilization of this device enables the rapid and reliable detection and differentiation of toxic algae and furthermore of the morphological indistinguishable prasinophytes. Finally, the analysis software was developed and subsequently the PHYTOPLANKTON CHIP was used to examine field samples from the Helgoland Time Series.

1.8 Outline of thesis

1.8.1 Assessment of a rRNA hybridization protocol for quantification of cell densities

Progress in classification and enumerating phytoplankton species is hampered by absence of reliable and exact monitoring methods. Even more important, if possible, are defined results of cell numbers for the detection of species causing HABs, because they are threatening the coastlines all over the world and are an enormous risk to humans, animals and the environment. Their monitoring is based on governmental regulations for a rapid response of changing conditions and protection. When cell densities of some harmful species increase, aquaculturists can bring their cages out of the affected region or interrupt their harvest to prevent the possible consumption of contaminated fish and seafood. Therefore, it is highly desirable to accurately count any toxic species in a water sample.

The DNA microarray used with rRNA probes and hybridized PCR fragments offers a robust, reliable and fast opportunity to rapidly detect and to qualify microalgae in pure cultures and environmental samples. Nevertheless, as shown by several studies (Kanagawa 2003; Medlin et al. 2006; Polz and Cavanaugh 1998), the utilization of the PCR method can introduce biases to the approach by different target amplification and prevent quantifying of exact cell numbers. To accurately relate cell densities to signal intensities, it is necessary to avoid the PCR step and to isolate rRNA for direct hybridization on the microarray.

Publication I was devoted to the development and assessment of a hybridization protocol with rRNA for the exact determination of cell densities. This was achieved by the evaluation of rRNA isolation and direct labeling of the nucleic acid with a commercial kit. Furthermore, it was necessary to correlate cell numbers to signal intensities. The entire method and equipment needed was described and illustrated, based on the protocol shown by Metfies et al. (2004), and advantages, drawbacks and possible pitfalls were also discussed, because this publication will be part of a book with manuals and guidelines for phytoplankton detection and the information provided could be useful for other scientists.

1.8.2 Design and refinement of a software for the analysis of hierarchical microarrays

The probable most challenging concern with the utilization of ribosomal RNA probes and DNA microarrays for investigation of microbial communities and their ecology is the number of unknown environmental sequences. The number of sequences deposited in public databases is growing every day, but the uncollected organisms could still lead to biases in the hybridization of probes and false positive signals. With the application of hierarchical probes

at different taxonomical levels, a signal for a species at the bottom of a taxonomic hierarchy can be validated by the obligatory positive signals for the probes in the hierarchy above. Hence, the analysis and reliability of a microarray hybridization could be greatly improved by the application of a hierarchical approach, therefore an automated validation of all probes in a taxonomic hierarchy of species with a computer based program is highly needed.

In **Publication II** a software program for the analysis of a microarray format with hierarchical probes was designed, evaluated and improved. The "PhylochipAnalyzer" program has facilitated the analysis and interpretation of data sets from the PHYTOPLANKTON CHIP. The aim was to develop a procedure with following steps included establishment of a hierarchical tree of probes, uploading data files from a conventional scanner format and automated analysis of signal intensities of probes according to the hierarchical tree. Applicability and adaptability of the software was tested with a PCR fragment of *Micromonas pusilla*.

1.8.3 Validation of probe modification for improvement of signal intensities

For the accurate detection of phytoplankton biodiversity and dynamics with microarrays, their sensitivity is an essential component, in particular for covering low abundant taxa and species with small cell size and little cytoplasmic content. Several studies have shown that the application of oligonucleotide probes with Locked Nucleic Acids (LNA), bicyclic RNA analogs, can enhance sensitivity, specificity and mismatch discrimination (Silahtaroglu et al. 2003; Ugozzoli et al. 2004; Vester and Wengel 2004). LNAs are easily implemented in the sequence of conventional probes, obey Watson-Crick base pairing and can be used with standard reagents and protocols (Braasch and Corey 2001; Koshkin et al. 1998). Furthermore, it was stated that they can be used in any hybridization assay as a modified probe or primer to increase specificity and reproducibility (Kongsbak 2002).

Publication III thematized the enhancement and improvement of probes with low sensitivity by implantation with LNAs. The redesign of probes for specific taxonomic groups is often impossible because of highly conserved regions in the rRNA gene that differ only in a few base pairs. In the microarray section of the study, five conventional and five LNA-modified probes were hybridized with specific PCR fragments to verify the potential of the LNA probes in order to increase the microarray signal. Furthermore, the hybridization of unspecific fragments to the microarray was conducted to explore the specificity and discriminative potential of the modified probes.

1.8.4 Development and adaptation of probes for the PHYTOPLANKTON CHIP

HABs are often caused by dinoflagellates, particularly members of the genus *Alexandrium* are often involved. The species of this genus belong to the most potent PSP toxin producers (Hallegraeff 2003; Nascimento et al. 2005). However, not all species are toxic; hence discrimination demands electron microscopy and trained experts. It may also happen that two species of the genus co-occur in one bloom (John et al. 2003a). Even more challenging is the *Alexandrium tamarense* "species complex", with the three "morphospecies" and to date six known "ribospecies" of which only three are toxic. Species belonging to *Alexandrium* are world-wide distributed and their reliable detection is therefore highly desirable.

Furthermore among the phytoplankton, picoplanktonic groups can contribute significantly to biomass and oxygen production in all areas of the ocean, even in the oligotrophic areas. The prasinophytes are an important part of the picoplankton and their reliable and high resolution classification and enumeration by conventional light microscopical methods is not feasible. The utilization of ribosomal probes and a promising molecular technique, such as the DNA microarray, offers great possibilities to overcome the difficulties that hamper reliable identification of phytoplankton groups.

In **Pulication IV** a probe set for the detection of members of the genus *Alexandrium* was evaluated and adapted to the microarray. Probes designed for different other probe-based methods showed good discriminative potential and promising hybridization results (John et al. 2003a; John et al. 2005; Metfies et al. 2005) and were successfully adapted to the microarray. One new probe was designed for the species *Alexandrium minutum* and specificity and sensitivity on the microarray were tested by hybridization of 18S and 28S rRNA PCR fragments from several target and non-target members of the genus and analyzed with the "PhylochipAnalyzer" software.

The **Publication V** concerned with the design of a probe set that recognizes different important members of the Prasinophyceae at class, order, clade or species level with a microarray hybridization format. One subset of probes was previously developed for other purposes (Not et al. 2004); the other probes were developed with the ARB software package (Ludwig et al. 2004) according to the prasinophyte clades shown by Guillou et al. (2004). A selection of several species of this group was amplified, analyzed and evaluated with the microarray in combination with the "PhylochipAnalyzer" program.

1.8.5 Expanded phytoplankton detection in field samples with the PHYTOPLANKTON CHIP

Detection and monitoring of organism of the North Sea and their ecology have a long history in marine research with the Helgoland Roads time-series. The sampling and data analysis on the physico-chemical environment and the organisms in the ocean (pelagic bacteria, microalgae, zooplankton, macroalgae, macrozoobenthos) and their classification can be tracked back to 1962. The series is described as a highly important marine time-series, because it is unprecedented in length, sampling intervals and obtained data (Franke et al. 2004). Especially for the monitoring of phytoplankton biodiversity, the Helgoland Roads time-series contains one of the longest data series in the world. The sampling has been on a daily basis and microalgae were counted and, if possible, identified down to the species level (Wiltshire and Dürselen 2004).

Nevertheless, the identification of some groups on a regularly basis can be very rough. Possible explanations are limited resources and lack of time and knowledge of personnel. Therefore, the potential, prospect and effort of the time-series demands the development and application of innovative advanced technology (Franke et al. 2004). With the utilization of the new developed PHYTOPLANKTON CHIP for the Helgoland Roads time-series phytoplankton sampling can improve and enhance the data obtained by this important and historically established long time sampling series to an extremely high degree. For example the time intervals can be shortened, and data from more phytoplankton taxa can be obtained. The data will possess a greater reliability and the taxonomic resolution will be more profound and precise.

Publication VI demonstrates the applicability and reliability of the PHYTOPLANKTON CHIP by evaluating with the analysis of field samples from three annual cycles of the Helgoland Roads time-series. To evaluate the species composition, DNA was extracted from the filters of environmental samples and PCR fragments were amplified and labeled. Subsequently, the nucleic acids were applied onto the microarray, analyzed and interpreted with the "PhylochipAnalyzer" software. The aim of the development of the PHYTOPLANKTON CHIP was to study the seasonal distribution and abundances of the North Sea phytoplankton community. Picoplanktonic groups that cannot be distinguished by light microscopy have also been detected. Consequently this analysis of complex environmental samples from the North Sea with picoplanktonic determination and the examination of the succession of three years will contribute highly to microbial ecology.

2. Publications

2.1 List of publications

This doctorial thesis is based on the following publications:

I. CHRISTINE GESCHER, KATJA METFIES AND LINDA K. MEDLIN
Microarray hybridization for quantification of microalgae
Manual and Guides: Microscopic and molecular methods for quantitative phytoplankton analysis, submitted

II. KATJA METFIES, PHILIPP BORSUTZKI, CHRISTINE GESCHER, LINDA K. MEDLIN AND STEPHAN FRICKENHAUS
PhylochipAnalyzer - A program for analyzing hierarchical probe-sets
Molecular Ecology Notes, accepted

III. SONJA DIERCKS AND CHRISTINE GESCHER, KATJA METFIES, LINDA K. MEDLIN
Evaluation of Locked Nucleic Acids for signal enhancement of oligonucleotide probes for microalgae immobilized on solid surfaces
Limnology and Oceanography: Methods, submitted

IV. CHRISTINE GESCHER, KATJA METFIES AND LINDA K. MEDLIN
THE ALEX CHIP - Development of a DNA chip for identification and monitoring of *Alexandrium*
Harmful Algae, to be submitted

V. CHRISTINE GESCHER, KATJA METFIES AND LINDA K. MEDLIN
Development and assessment of a DNA microarray for the identification of Prasinophytes
Applied and Environmental Microbiology, to be submitted

VI. CHRISTINE GESCHER, KATJA METFIES, STEPHAN FRICKENHAUS, KAREN H. WILTSHIRE AND LINDA K. MEDLIN
Assessment of phytoplankton dynamics over three annual cycles at Helgoland Roads
Molecular Ecology, to be submitted

Other publications prepared with contribution of the candidate from the period of time:

GODHE, A., AND OTHERS (2007)
Intercalibration of classical and molecular techniques for identification of *Alexandrium fundyense* (Dinophyceae) and estimation of cell densities
Harmful Algae, 6: 56-72.

GESCHER, C., METFIES, K. AND MEDLIN, L. K. (2005)
Development of a DNA Microchip as a standard analytical tool for the identification of phytoplankton.
Phycologia, 44: 37.

2.2 Statement of my contribution to the publications

Publication I

The experiments were planned together with L. K. Medlin and K. Metfies and performed by myself. I wrote the manuscript.

Publication II

The experiments were planned together with K. Metfies, L.K Medlin and S. Frickehaus. I did the experiments and analyzed the data. The PhylochipAnalyzer was programmed by P. Borsutzki and S. Frickenhaus. The manuscript was written by K. Metfies.

Publication III

The experiments were planned together with K. Metfies, L. K. Medlin and S. Diercks and carried out from S. Diercks and myself. The manuscript was written equally with S. Diercks.

Publication IV

The experiments were planned together with K. Metfies and L. K. Medlin. The experiments were carried out by myself and analyzed by myself. The manuscript was written by myself.

Publication V

The experiments were planned together with K. Metfies and L. K. Medlin. The experiments were carried out by myself. I have analyzed the data and wrote the manuscript.

Publication VI

The experiments were planned together with K. Metfies and L. K. Medlin. The experiments were carried out and analyzed by myself and K. Metfies. Graphical presentation of data and clusteranalysis was done by S. Frickenhaus. The manuscript was written by myself.

2.3 Publication I:

MICROARRAY HYBRIDIZATION FOR QUANTIFICATION OF MICROALGAE

CHRISTINE GESCHER, KATJA METFIES AND LINDA K. MEDLIN

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Manual and Guides: Microscopic and molecular methods for quantitative phytoplankton analysis, submitted

Introduction

The introduction of DNA microarray technology in 1995 is one of the latest and most powerful innovations in microbiology. Because of true parallelism and miniaturization, the acquisition of many data with reduced consumption of reagents and time is accomplished with this technique (Schena et al. 1995). It is a completely new experimental approach in molecular biology (Blohm and Guiseppi-Elie 2001), which offers the possibility to analyze a large number of samples to different probes in parallel under a diverse spectrum of applications (Ye et al. 2001).

DNA microarrays consist of glass microscope slides with particular surface properties (Metfies and Medlin 2005b). Probes are immobilized as spots on the glass slide in a defined pattern. Each spot consists of many copies of oligonucleotide probes that are complementary to a specific target DNA sequence (Graves 1999) and the targets (RNAs or DNAs) hybridize to the capture oligonucleotide probes on the microarray. The hybridization is detected via a fluorescent label that is attached to the target during PCR or directly to the rRNA (Metfies and Medlin 2004).

Microarray technology was launched with a publication concerning gene expression (Schena et al. 1995). Many functional genomic methods profit from microarrays, such as genome expression profiling, single nucleotide polymorphism detection and DNA resequencing (Al-Shahrour et al. 2005; Broet et al. 2006; Gamberoni et al. 2006; Ji and Tan 2004; Kauppinen 2003; Lipshutz et al. 1999; Yap et al. 2004). Thus, DNA microarrays are a powerful and innovative tool that can facilitate surveying and monitoring of any organism, especially those in the marine environment tracking changes in biodiversity and ecosystem functioning.

The application of DNA microarrays for the identification of marine organisms is a relatively new and innovative field of research. It provides the possibility to analyze a large number of targets (species or taxa) in one experiment (Ye et al. 2001), but they are not yet widely applied to marine biodiversity and ecosystem science. For the use of microarray technology as a standard tool with fast and simple routinely handling, further research into methodical optimizations has to be done (Peplies et al. 2003b).

Some European groups already utilize DNA microarrays for the identification of marine organisms, the so called "Phylochip", e.g., phytoplankton (Ki and Han 2006; Medlin et al. 2006; Metfies and Medlin 2004) and bacteria (Lehner et al. 2005; Loy et al. 2002; Loy et al. 2005; Peplies et al. 2003b; Peplies et al. 2004b; Peplies et al. 2006b; Peplies et al. 2004c), as well as fishes (Kappel et al. 2003). At the Alfred Wegner Institute, specific probes that were developed for other hybridization techniques have successfully adapted for microarray hybridization. Specific signals of high intensities demonstrate their potential for phytoplankton identification.

Materials

Laboratory facilities

For just detecting algae with microarrays it is possible to use rDNA from a PCR reaction and hybridize the PCR-fragments to the probes, but for quantification of cell counts, it is necessary to work with rRNA. The PCR will likely introduce a bias and if it is essential to relate signals to cell counts, then the RNA of the cells must be used. This requires a molecular laboratory with security level one. Additionally, a clean fume hood should be available because of β-mercaptoethanol.

Equipment

Microarray production

For the taxonomic determination of microorganisms with a DNA microarray, it is necessary either to design new probes or to choose probes from other applications that are specific for the target taxonomic group or species. If using the 18S rRNA gene, probes should only be designed from the first 1000 base pairs of the gene because of inhibitory secondary structures in the latter part of the real molecule (Metfies and Medlin, unpublished).

The probes can be ordered from a commercial supplier and are spotted onto a glass slide. Fig. 1 shows a light microscope picture of spots on a glass slide. Spotting services are also commercially available, even though it is more flexible and convenient to have a spotter

in the laboratory. However, a spotter is a big investment of approximately 50,000-100,000 € and at the beginning, outsourcing of spotting is a better choice.

RNA isolation

RNA isolation requires the following devices: a Mini-Beadbeater (e.g., BioCold Scientific Inc., Fenton, USA) to homogenize the algal cells with glass-beads and a conventional Mini-Centrifuge for small Eppendorf tubes. For hybridization, a thermoheater (Fig.2), an incubator, a bellydancer or shaker (Fig. 3) and a microarray scanner (Fig. 4) with software (e.g., GenePix 4000B device and GenePix Pro.6.0 software from Molecular Devices Corporation, Sunnyvale, USA) are needed.

Chemicals and consumables

RNA preparation

For RNA isolation from microalgae, we recommend the RNeasy Plant Mini Kit (Qiagen Inc., Valencia, USA). Labeling of RNA should be done with the Biotin-ULS-Kit (Fermentas Inc., Hanover, USA) and purification of labeled RNA with the RNeasy MinElute Cleanup Kit (Qiagen Inc., Valencia, USA). For general cleaning of the fume hood, pipettes and any other labware to remove RNAse we recommend RNaseZap (Ambion Inc., Austin, USA)

Methods

RNA Isolation with the RNeasy Plant Mini Kit (Qiagen)

General remarks on handling RNA

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Because RNases are difficult to inactivate, even minute amounts are sufficient to destroy RNA. Do not use any plastic ware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the isolation procedure.

General handling

Always wear latex or vinyl gloves when handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep isolated RNA on ice while aliquots are pipetted for downstream applications.

Disposable plastic ware

The use of sterile, disposable polypropylene tubes is recommended throughout. These tubes are generally RNase-free and do not require pre-treatment to inactivate RNases.

Glassware

Glassware used for RNA work should be cleaned with a detergent, thoroughly rinsed, and oven baked at 240 °C for four or more hours before use.

Determining the correct amount of starting material

It is essential to begin with the correct amount of algal material in order to obtain optimal RNA yield and purity with RNeasy columns. This depends on the target species. A maximum of 100 mg plant material or 1 x 10^7 cells can generally be processed with RNeasy mini columns.

Fresh or frozen tissue can be used. To freeze tissue for long-term storage, flash-freeze it in liquid nitrogen and immediately transfer to –70 °C. Tissue can be stored for several months at –70° C. To process, do not allow tissue to thaw during weighing or handling prior to disruption in Buffer RLT. Homogenized lysates (in Buffer RLT) can also be stored at –70 °C for several months. To process frozen lysates, thaw samples and incubate for 15 – 20 min at 37 °C in a water bath to dissolve salts.

Important notes before starting

- β-Mercaptoethanol (β-ME) must be added to Buffer RLT before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 10 µL β-ME per 1 mL Buffer RLT. Buffer RLT is stable for 1 month after addition of β-ME.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 44 mL of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
- All steps of the RNeasy protocol should be performed at room temperature. During the procedure, work quickly.

- All centrifugation steps are performed at 20 – 25 °C in a standard microcentrifuge.

Harvesting of cells

The harvesting of cells can be done by centrifugation or filtration; the supernatant is discarded and the cell pellet processed.

Processing of RNA-Isolation

1. Add 450 µL Buffer RLT with β-ME to the cell pellet
2. Pipette the lysate to glass beads (212 µm - 300 µm and 312 - 600 µm) and shred the lysate in a bead beater for 20 seconds
3. Pipette the lysate directly onto a QIAshredder spin column (lilac color) placed in 2 mL collection tube, and centrifuge for 2 min at maximum speed. Carefully transfer the supernatant of the flow-through fraction to a new microcentrifuge tube without disturbing the cell debris pellet in the collection tube. Use only this supernatant in subsequent steps.
4. Add 0.5 volume (usually 225 µL) ethanol (96 – 100 %) to the clear lysate, and mix immediately by pipetting. Do not centrifuge. Continue without delay.
5. Apply sample (usually 650 µL), including any precipitate that may have formed, to an RNeasy mini column (pink color) placed in a 2 mL collection tube. Close the tube gently, and centrifuge for 15 s at ≥ 8,000 x *g* (≥ 10,000 rpm). Discard the flow-through.
   Reuse the collection tube in the next step.
6. Add 700 µL Buffer RW1 to the RNeasy column. Close the tube gently, and centrifuge for 15 s at ≥ 8,000 x *g* (≥ 10,000 rpm) to wash the column. Discard the flow-through and collection tube.
7. Transfer the RNeasy column into a new 2 mL collection tube (supplied in kit). Pipette 500 µL Buffer RPE onto the RNeasy column. Close the tube gently, and centrifuge for 15 s at ≥ 8,000 x *g* (≥ 10,000 rpm) to wash the column. Discard the flow-through. Reuse the collection tube in step 9.
8. Add another 500 µL Buffer RPE to the RNeasy column. Close the tube gently, and centrifuge for 2 min at ≥ 8,000 x *g* (≥ 10,000 rpm) to dry the RNeasy silica gel membrane.
9. To elute, transfer the RNeasy column to a new 1.5 mL collection tube. Pipette 30 – 50 µL RNase-free water directly onto the RNeasy silica gel membrane. Close the tube gently, and centrifuge for 1 min at ≥ 8,000 x *g* (≥ 10,000 rpm) to elute.

10. To obtain a higher total RNA concentration, a second elution step may be performed by using the first eluate (from step 9). Pipette the eluate back on the column and centrifuge for 1 min at ≥ 8,000 x *g* (≥ 10,000 rpm) to elute once again.
11. The concentration of the RNA was measured with a Nanodrop Spectrophotometer (Peqlab, Erlangen, Germany).

Labeling of RNA with the Biotin-ULS-Kit (Fermentas)

Description

The labeling kit uses the Universal Linkage System (ULS) technique, which is based on the stable coordinative binding of a platinum complex to nucleic acids. The platinum complex acts as a linker between a detectable marker (label) molecule, i.e., fluorescein or biotin, and DNA or RNA. The marker is coupled directly to nucleic acid without significant interfering or altering it. ULS consists of a Pt complex stabilized by a chelating diamine and has two binding sites, one of which is used to bind a marker. The other binding site is used to link the complex to the aromatic nitrogen atoms of nucleobases and one nitrogen atom of guanine is strongly preferred. The resultant Pt-N bond is very stable both chemically and thermally (see Fig. 5).

Features

- One-step reaction.
- Fast - only 30min to label the target.
- Universal - any nucleic acid, independent of size or structure can be labeled.
- Easy to scale up and down. It allows labeling of as little as 25 ng or as much as 10 µg of nucleic acid in a single reaction

Protocol

1. Add 1 µL (= ½ U) of Biotin ULS reagent to 500 ng of nucleic acid template.
2. Adjust volume with labeling solution to 20 µL and mix well.
3. Incubate for 30 minutes at 85 °C.
4. Add 5 µL Stop solution and mix well.
5. Incubate for 10 minutes at room temperature.
6. Purify the solution with the RNeasy MinElute Cleanup Kit before hybridization.

Purification of labeled RNA with the RNeasy MinElute Cleanup Kit (Qiagen)

Protocol

1. Adjust sample to a volume of 100 µL with RNase-free water. Add 350 µL of Buffer RLT, and mix thoroughly.
2. Add 250 µL of 96 – 100 % ethanol to the diluted RNA, and mix thoroughly by pipetting. Do not centrifuge. Continue immediately with step 3.
3. Apply 700 µL of the sample to an RNeasy MinElute Spin Column in a 2 mL collection tube. Close the tube gently, and centrifuge for 15 s at ≥ 8,000 x g (≥ 10,000 rpm). Discard the flow-through.
4. Transfer the spin column into a new 2 mL collection tube. Pipette 500 µl Buffer RPE onto the spin column. Close the tube gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the column. Discard the flow-through. Reuse the collection tube in step 5.
5. Add 500 µL of 80 % ethanol to the RNeasy MinElute Spin Column. Close the tube gently, and centrifuge for 2 min at ≥ 8,000 x g (≥ 10,000 rpm) to dry the silica gel membrane. Discard the flow through and collection tube.
6. Transfer the RNeasy MinElute Spin Column into a new 2 mL collection tube. Open the cap of the spin column, and centrifuge in a microcentrifuge at full speed for 5 min. Discard the flow through and collection tube.
7. To elute, transfer the spin column to a new 1.5 mL collection tube. Pipette 14 µL RNase-free water directly onto the center of the silica-gel membrane. Close the tube gently, and centrifuge for 1 min at maximum speed to elute.
8. To obtain a higher total RNA concentration, a second elution step may be performed by using the first eluate (from step 7).
9. The concentration of the RNA was measured with a Nanodrop Spectrophotometer (Peqlab, Erlangen, Germany).

Microarray Hybridization

For a general overview of amicroarray hybridization experiment and the steps necessary see Fig. 6.

Positive and negative control

The positive control in the microarray hybridization experiments is a probe (ATGGCCGATGAGGAACGT) specific for a 250 basepair (bp) fragment of the TATA-box

binding-protein (TBP) gene of *Saccharomyces cerevisiae* (Metfies and Medlin 2004). The gene is amplified with the primers TBP-F (5'-ATG GCC GAT GAG GAA CGT TTA A-3') and TBP-R-Biotin (5'-TTT TCA GAT CTA ACC TGC ACC C- 3') and is added to the hybridization solution. As a negative control, a probe (TCCCCCGGGTATGGCCGC) is used that has no match to any sequence found in the NCBI database (Metfies and Medlin 2004).

Pre-Hybridization

The microarrays are incubated in a microarray box in ~ 20 mL pre-hybridization buffer [1x STT, 1 mg/mL BSA] for 60 min at hybridization temperature (58 °C). Subsequent to the pre-hybridization, the microarrays are washed briefly in deionised water and dried by centrifugation.

Hybridization Solution

The hybridization solution has a total volume of 40 µL, but only 30 µL are applied to the microarray. It contains labeled target nucleic acid dissolved in hybridization buffer. The final concentration of the target nucleic acid should be ~10 ng/µL. It also contains the positive control, the 250 bp PCR-fragment TBP from *S. cerevisiae* with biotin-labeled primers in a final concentration of 4.7ng/µL.

Hybridization

The hybridization solution is incubated for 5 min at 94 °C in a thermoheater (Fig. 2)to denature the target nucleic acid. We recommend the use of a special kind of cover slip, the Lifter Slip cover slip (Implen, München, Germany) to ensure even dispersal of the hybridization mixture on the microarray. The cover slips are placed on the slide and 30 µL of the hybridization mixture is pipetted under the cover slip (Fig. 7). The hybridization is carried out at 58 °C for 1 hour in a humid chamber. A 50 mL Falcon-tube with a wet Whatman-paper functions very well as a moisture chamber. Apply approximately 1 mL of hybridization buffer onto the Whatman-paper to obtain enough humidity in the chamber.

Washing of the microarrays

After completion of the hybridization, excessive target nucleic acid and unspecific bindings have to be removed by stringent washing of the microarrays. The cover slips are removed from the array and the microarray is put into a 50 mL Falcon-tube (Fig. 3). In total, two washing steps are carried out for 10 min and another one for 5 min with increasing

stringency. The first washing buffer contains SDS. However, it is recommended that the last washing buffers avoid SDS, because residual SDS will generate high background intensities on the microarray.

Wash-buffer 1: 2x SSC, 10 mM EDTA, 0.05 % SDS

Wash-buffer 2: 1x SSC, 10 mM EDTA

Wash-buffer 3: 0.2x SSC, 10 mM EDTA

The microarrays are dried by centrifugation subsequent to the washing steps.

Fluorescent staining of the microarrays

Hybridized biotinylated target nucleic acids are visualized by staining the microarray for 30 min at room temperature in a wet chamber with 50 µL Streptavidin-Cy5 [0.1 µg/mL Streptavidin / 1x hybridization buffer].

Removal of excessive Streptavidin-Cy5

Excessive stain is removed by washing the microarrays twice for 5 min with wash-buffer 1 and once for 5 min with wash-buffer 2. The washing takes place at room temperature in a 50 mL Falkon-tube. Following the washing procedure, the microarrays are dried by centrifugation.

Analysis

The microarray is scanned with the GenePix Axon 4000B scanner (Fig. 4) at 635 nm and the obtained signal intensities are analyzed with the GenePix 6.0 software. The signal to noise-ratios are calculated according to (Loy et al. 2002) and all calculated ratios are normalized to the signal of the TBP positive control. An schematic picture of the excitation is shown in Fig. 8.

Preservation and storage

It is possible to store the hybridized microarrays for at least one year at -20 C, but it does not seem necessary to keep them once scanned. We have no experience in reusing Phylochips for new hybridizations, but in expression analysis, it has already been tested and the microarrays are reused 5 times (Bao et al. 2002; Dolan et al. 2001).

Discussion

Using microarrays for detection and monitoring of marine algae is still rather new and under development. There are already working microarrays for the phytoplankton identification with 18S rDNA probes at the class level (Medlin et al. 2006; Metfies and Medlin 2004). Ki and Han showed the specificity of 28S rDNA probes for the detection of harmful algae at the species level (Ki and Han 2006).

Advantages

With the utilization of molecular methods, it is theoretically possible to answer ecological questions addressed to all levels of biodiversity. Phytoplankton identification, especially of harmful algal species, is important from an ecological and economic point of view. Microorganisms dominate global biological diversity in terms of their species numbers, but their small size and the few morphological features makes it difficult to assess their overall biodiversity. With the application of microarrays to answer these ecological and biodiversity questions, they offer the possibility to analyze samples to a large number of different targets (species or taxa) in parallel (Ye et al. 2001). Once developed, the microarray is a cost-effective, trusted and efficient tool to detect microalgae and can be very useful to monitor biodiversity of phytoplankton on long-time scales (Medlin et al. 2006).

Drawbacks

It is estimated that the known rRNA sequence database is only a small fraction of the total biodiversity and the number of 18S rRNA sequences in public databases is constantly increasing. Therefore, it is very important that every working probe be regularly checked against all known sequences. Developing and evaluating microarrays is time-consuming and costly, and cross-reactions to unknown species are always possible. The detection limit of the microarray depends on the sensitivity of the chosen probes. In general, a high sampling volume up to several liters is necessary especially if the cell counts are expected to be low. The manual isolation of RNA is currently the limiting factor of the analysis. Different users could possibly isolate different amounts of rRNA from the same number of algal cells. This could result in different signal intensities that can not be compared to cells counts. An automatic rRNA isolation device would be a good solution to overcome this problem. The isolation of a sufficient rRNA is very important because the target rRNA presents only a small fraction of the isolated rRNA. For each probe used to monitor an algal species, it is also necessary to develop a calibration curve and to convert the signal to cell counts. A good

correlation of cell counts and RNA concentration per cell with signal intensity is prerequisite for a reliable analysis of field samples.

Acknowledgements

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References

AL-SHAHROUR, F., R. DIAZ-URIARTE, and J. DOPAZO. 2005. Discovering molecular functions significantly related to phenotypes by combining gene expression data and biological information. Bioinformatics **21:** 2988-2993.

BAO, Z., M. WENLI, S. RONG, L. LING, G. QUIYE, and Z. WENLING. 2002. Re-use of a stripped cDNA microarray. Brit. J. Biomed. Sci. **59:** 118-119.

BLOHM, D. H., and A. GUISEPPI-ELIE. 2001. New developments in microarray technology. Curr. Opin. Biotech **12:** 41-47.

BROET, P., V. A. KUZNETSOV, J. BERGH, E. T. LIU, and L. D. MILLER. 2006. Identifying gene expression changes in breast cancer that distinguish early and late relapse among uncured patients. Bioinformatics **22:** 1477-1485.

DOLAN, P. L., Y. WU, L. K. ISTA, R. L. METZENBERG, M. A. NELSON, and G. P. LOPEZ. 2001. Robust and efficient synthetic method for forming DNA microarrays. Nucleic Acids Res **29:** e107.

GAMBERONI, G., S. STORARI, and S. VOLINIA. 2006. Finding biological process modifications in cancer tissues by mining gene expression correlations. BMC Bioinformatics **7:** 6.

GRAVES, D. J. 1999. Powerful tools for genetic analysis come of age. Trends Biotechnol. **17:** 127-134.

JI, L., and K.-L. TAN. 2004. Mining gene expression data for positive and negative co-regulated gene clusters. Bioinformatics **20:** 2711-2718.

KAPPEL, K., H. V. WESTERNHAGEN, and D. H. BLOHM. 2003. Microarray-based identification of eggs and larvae from fish species common in the North Sea, Dechema Chip-Technology Meeting, Frankfurt, Germany.

KAUPPINEN, S. and others 2003. LNA microarrays in genomics. Pharma Genomics**:** 24-34.

KI, J.-S., and M.-S. HAN. 2006. A low-density oligonucleotide array study for parallel detection of harmful algal species using hybridization of consensus PCR products of LSU rDNA D2 domain. Biosens. Bioelectron **21:** 1812-1821.

LEHNER, A. and others 2005. Oligonucleotide microarray for identification of *Enterococcus* species. FEMS Microbiol. Lett. **246:** 133-142.

LIPSHUTZ, R. J., S. P. FODOR, T. R. GINGERAS, and D. J. LOCKHART. 1999. High density synthetic oligonucleotide arrays. Nature Genet. **21:** 20 - 24.

LOY, A. and others 2002. Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Appl. Environ. Microbiol. **68:** 5064-5081.

---. 2005. 16S rRNA gene-based oligonucleotide microarray for environmental monitoring of the betaproteobacterial order "Rhodocyclales". Appl. Environ. Microbiol. **71:** 1373-1386.

MEDLIN, L. K., K. METFIES, H. MEHL, K. H. WILTSHIRE, and K. VALENTIN. 2006. Picoeukaryotic plankton diversity at the Helgoland Time Series Site as assessed by three molecular methods. Microbial Ecol. **52:** 53-71.

METFIES, K., and L. K. MEDLIN. 2004. DNA microchips for phytoplankton: The fluorescent wave of the future. Nova Hedwigia **79:** 321-327.

---. 2005. Ribosomal RNA probes and microarrays: Their potential use in assessing microbial biodiversity, p. 258-278. *In* a. E. H. R. Elizabeth A. Zimmer [ed.], Methods in Enzymology, Molecular Evolution: Producing the Biochemical Data. Elsevier Academic Press, San Diego, USA.

PEPLIES, J., F. O. GLÖCKNER, and R. AMANN. 2003. Optimization strategies for DNA microarray-based detection of bacteria with 16S rRNA-targeting oligonucleotide probes. Appl. Environ. Microbiol. **69:** 1397-1407.

PEPLIES, J., F. O. GLÖCKNER, R. AMANN, and W. LUDWIG. 2004a. Comparative sequence analysis and oligonucleotide probe design based on 23S rRNA genes of Alphaproteobacteria from North Sea bacterioplankton. Syst. Appl. Microbiol. **27:** 573-580.

PEPLIES, J., C. LACHMUND, F. O. GLÖCKNER, and W. MANZ. 2006. A DNA microarray platform based on direct detection of rRNA for characterization of freshwater sediment-related prokaryotic communities. Appl. Environ. Microbiol. **72:** 4829-4838.

PEPLIES, J., S. C. K. LAU, J. PERNTHALER, R. AMANN, and F. O. GLÖCKNER. 2004b. Application and validation of DNA microarrays for the 16S rRNA-based analysis of marine bacterioplankton. Environ. Microbiol **6:** 638-645.

SCHENA, M., D. SHALON, R. W. DAVIS, and P. O. BROWN. 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science **270:** 467-470.

YAP, Y., X. ZHANG, M. LING, X. WANG, Y. WONG, and A. DANCHIN. 2004. Classification between normal and tumor tissues based on the pair-wise gene expression ratio. BMC Cancer **4:** 72.

YE, R. W., T. WANG, L. BEDZYK, and K. M. CROKER. 2001. Applications of DNA microarrays in microbial systems. J. Microbiol. Meth. **47:** 257-272.

Table 1. Chemicals and suppliers

Chemical	Supplier
EDTA	Sigma, München, Germany
Citric Acid	MERCK KGaA, Darmstadt, Germany
Sodium chloride	Sigma, München, Germany
SDS	MERCK KGaA, Darmstadt, Germany
Bovine Serum Albumin	Sigma, München, Germany
Trizma Base	Sigma, München, Germany
Triton x-100	MERCK KGaA, Darmstadt, Germany
Ethanol	MERCK KGaA, Darmstadt, Germany
Hering-Sperm DNA	Roche, Grenzach-Wyhlen, Germany
Streptavidin-CY5	KPL, Gaithersburg, USA
Probes	Thermo Electron, Karlsruhe, Germany

Table 2. Hybridization buffers and staining solution

2x STT-Buffer (Pre-Hybridization buffer)

	Final Concentration	**500 mL**
NaCl	2 M	200 mL [5 M]
Tris pH 8	20 mM	10 mL [1 M]
Triton x-100	0.01 %	1 mL [10 %]
Fill up to 500 mL with pure H_2O.		

Hybridization buffer

	Final Concentration	**50 mL**
BSA	0,5 mg/mL	2,5 mL [10 µg/µL Stock]
Heringsperm DNA	0,1 µg/µL	500 µL [10 µg/µL Stock]
2x STT-Buffer	1x	25 mL [2x STT]
Fill up to 50 mL with pure H_2O.		

Staining solution

	Final Concentration	**400 µL**
Hybridization Buffer	1x	200 µL [2x Stock]
Streptavidin-Cy5	0,1 µg/mL	4 µL [10 µg/mL]
Fill up to 400 µL with pure H_2O.		

Table 3. Wash buffer

20x SSC

	Final Concentration	**1000 mL**
NaCl	3 M	175.3 g
Citric Acid	0.3 M	88.2 g

Dissolve both in 800 mL pure H_2O, adjust pH with HCl (14 N) to 7.0 and fill up to 1000 mL with pure H_2O. Then autoclave the buffer.

2x SSC

	Final Concentration	**800 mL**
20x SSC	2x	80 mL
EDTA (0.5 M, pH 8)	10 mM	16 mL
10% SDS (w/v)	0.05 %	4 mL

Put in the 20x SSC first, then fill up to 600 mL with pure H_2O and afterwards add the EDTA, SDS and fill up to 800 mL with pure H_2O.

1x SSC

	Final Concentration	**800 mL**
20x SSC	1x	40 mL
EDTA (0.5 M, pH 8)	10 mM	16 mL

Put in the 20x SSC first, then fill up to 600 mL with pure H_2O and afterwards add the EDTA and fill up to 800 mL with pure H_2O.

0.2x SSC

	Final Concentration	**400 mL**
20x SSC	0.2x	4 mL
EDTA (0.5 M, pH 8)	10 mM	8 mL

Put in the 20xSSC first, then fill up to 250 mL with pure H_2O and afterwards add the EDTA and fill up to 400 mL with pure H_2O.

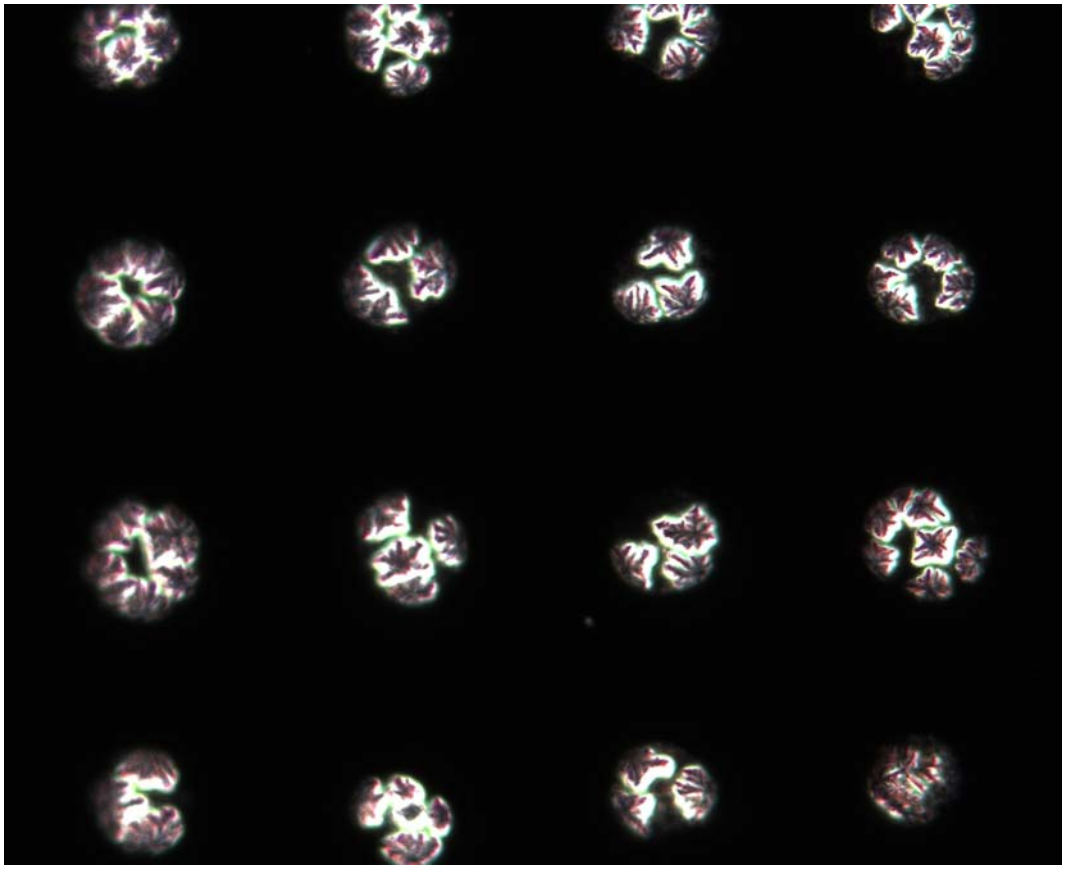

Figure 1. Spots of probes on the microarray, detected by light microscopy

Figure 2. Thermoheater for fragmentation of nucleic acids prior hybridization

Figure 3. Washing of microarrays by shaking on the bellydancer

Figure 4. GenePix 4000B microarray scanner

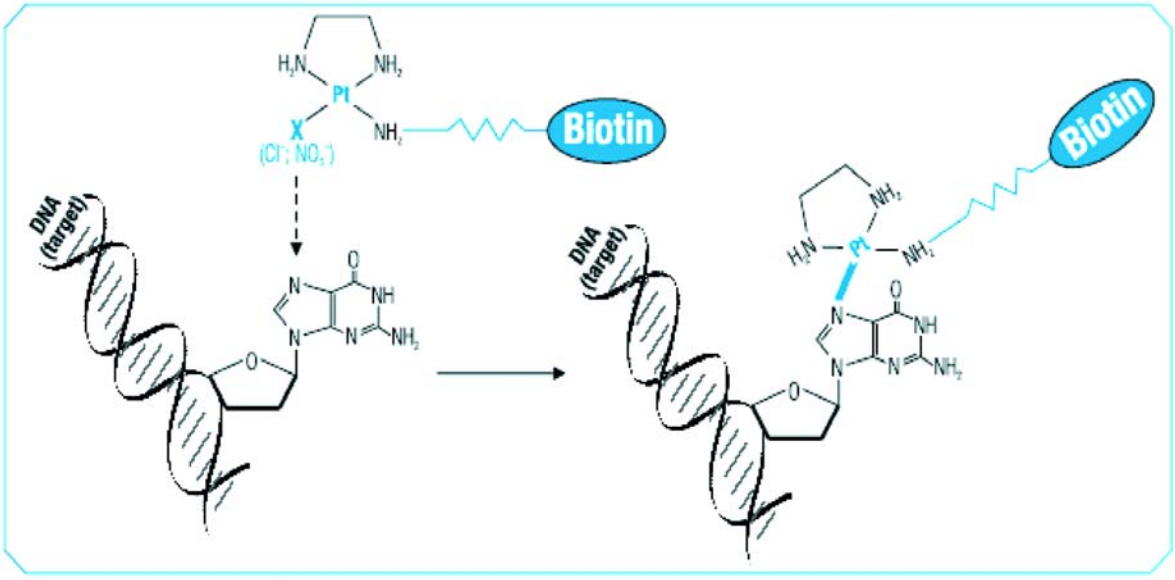

Figure 5. Labeling of nucleic acids with Biotin-ULS (from www.fermentas.com)

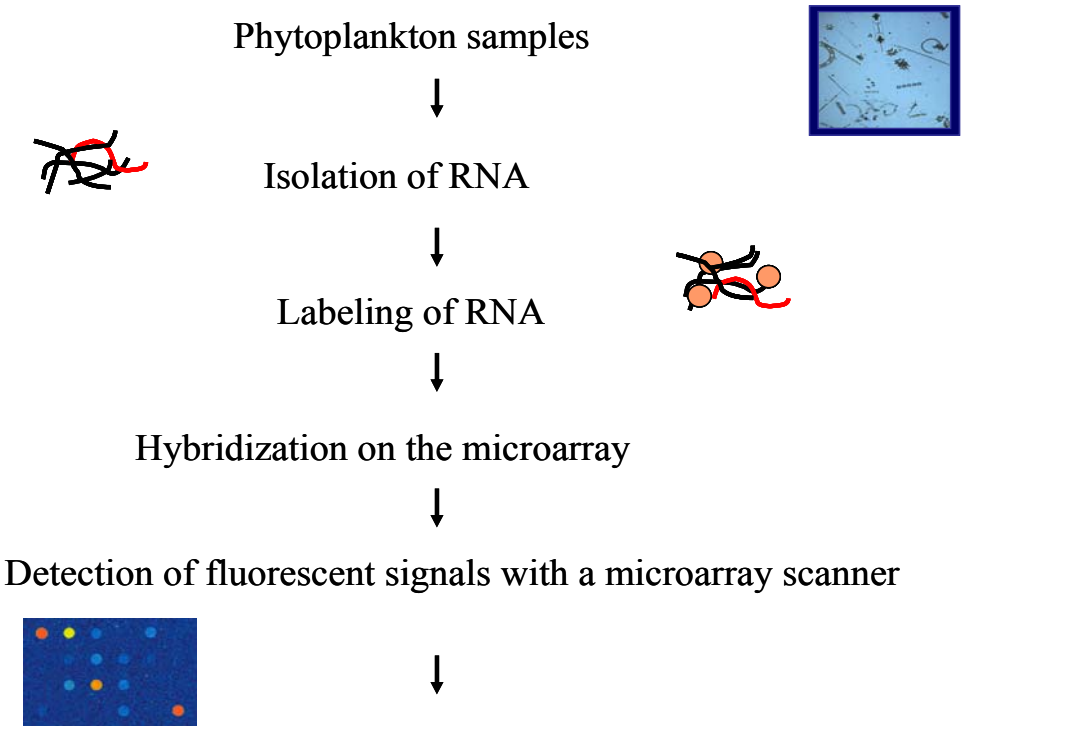

Figure 6. Workflow of sample handling and hybridization

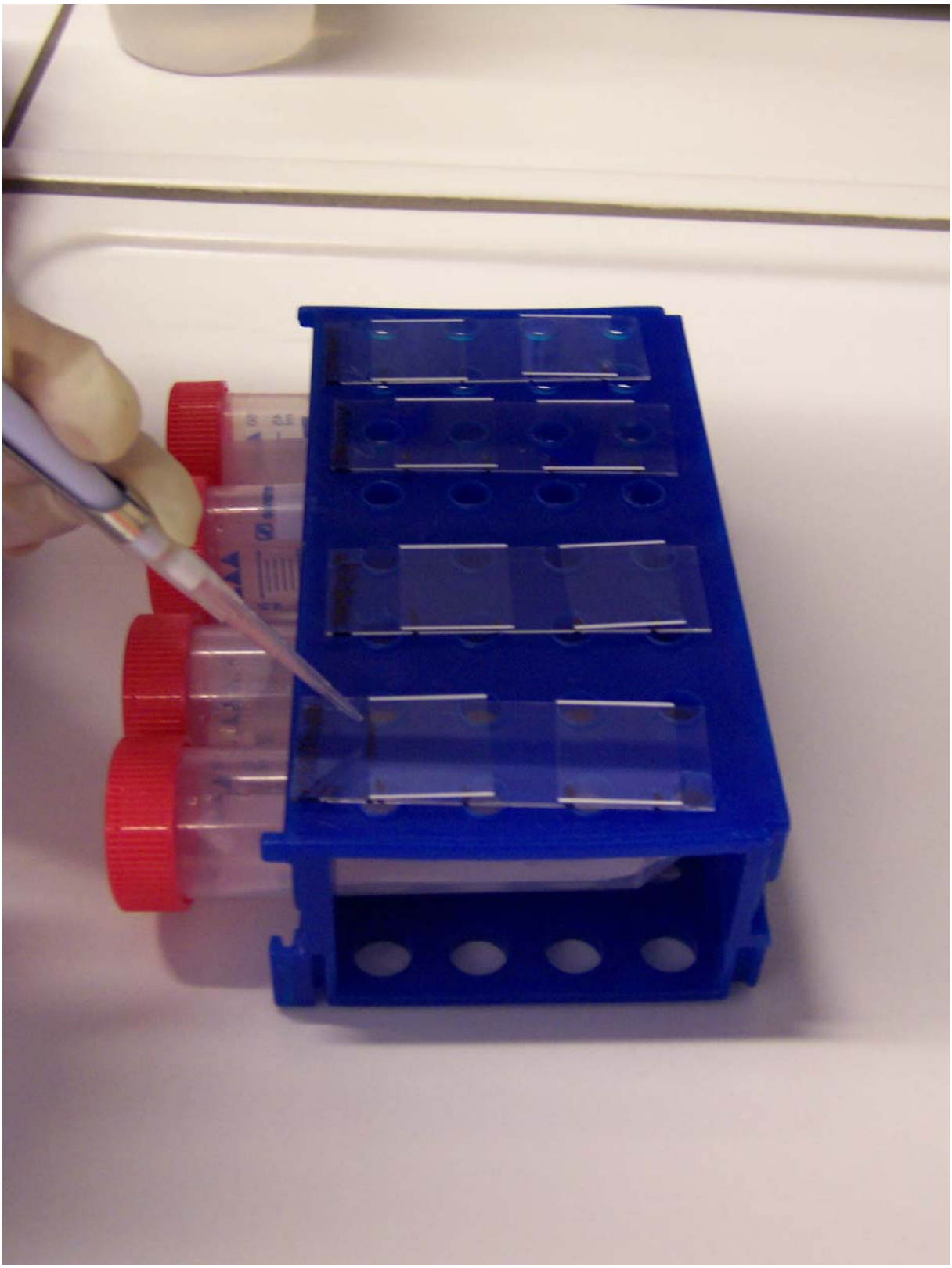

Figure 7. Pipetting of hybridization solution to the microarray

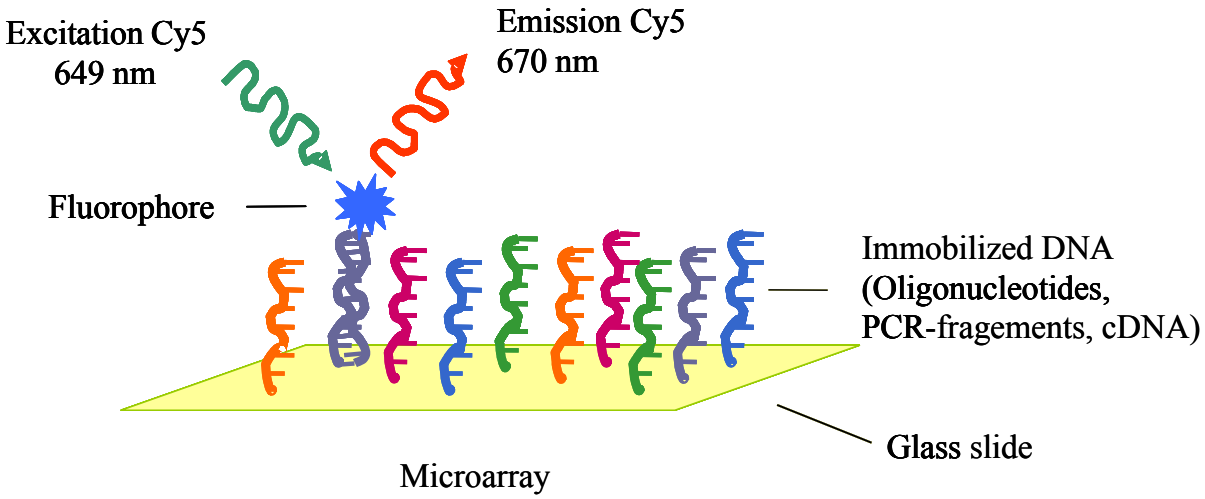

Figure 8. Microarray detection via fluorescent excitation

2.4 Publication II

PHYLOCHIPANALYZER - A PROGRAM FOR ANALYZING HIERARCHICAL PROBE-SETS

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Molecular Ecology Notes, submitted

Abstract

The recent introduction of phylochips that contain molecular probes facilitates environmental microbial identification in a single experiment without previous cultivation. A set of probes recognizing species at different taxonomic levels is denoted as a hierarchical set. Application of hierarchical probe sets on a DNA microarray allows the assessment of biodiversity with different resolutions. It significantly increases the robustness of the results retrieved from phylochip experiments because of the possible consistency checks of hybridization across different taxonomic levels. Here, we present a computer program, PhylochipAnalyzer, for the hierarchy editing and the evaluation of phylochip data generated from hierarchical probe-sets.

Basic rationale

Recently, more and more publications describe the application of DNA microarrays for species identification (phylochips) from environmental samples (Call 2005; Guschin et al. 1997; Loy et al. 2002; Medlin et al. 2006; Metfies and Medlin 2004). Phylochips are DNA-microarrays containing molecular probes that bind to unique sequences in a target. The target sequence is usually part of marker genes, e.g., the ribosomal RNA gene. Ribosomal RNA-genes are particularly well suited for phylochip- and phylogenetic analysis, because they are universal, found in all cellular organisms, are of relatively large size; and contain both highly conserved and variable regions with no evidence for lateral gene transfer (Woese 1987). The large number of published 18S rDNA-sequences, e.g., RDP (Maidak et al. 2001) makes it possible to design hierarchical probe sets that specifically target the 18S rDNA from higher taxa down to species level (Groben et al. 2004; Guillou et al. 1999; Lange et al. 1996). Phylochips provide a promising tool to identify large numbers of microbial species in complex environmental samples quickly without a cultivation step. Our phylochip contains a hierarchical set of molecular probes, which target phytoplankton species at different taxonomic levels (Medlin et al. 2006; Metfies and Medlin 2004). In a hierarchical probe-set, a target species is only considered present, if all hierarchical probes for each species result in a positive signal. Therefore, hierarchical probes add to the accuracy of molecular probe based identification approaches.

In spite of the growing number of applications for phylochips, they represent only a small proportion of all DNA-microarray related work. Most publications describe expression studies (Csako 2006; Lockhart and Winzeler 2000; Rensink and Buell 2005; Stoughton 2005). Consequently, the majority of protocols are optimized for applications related to expression analysis. However, the application of phylochips for species identification in environmental samples presents technical challenges that are not encountered in gene expression studies of laboratory samples (Call 2005; Medlin et al. 2006; Peplies et al. 2003). There are numerous commercial and non-commercial programs for the analysis of expression studies (Dondrup et al. 2003; Vaquerizas et al. 2005), but few programs exist for phylochip analysis. One example is the Unix-based program ChipChecker (Loy et al. 2002), which is dedicated to data interpretation from phylochips. It calibrates signal to noise ratios to a set threshold determined by the user and finds positive signals with respect to that threshold based on the fact that a positive signal can only be located where there is a fully complementary probe to its target. However, in a hierarchical probe set, a signal is only considered truly positive, if all probes in the hierarchy are positive. Therefore, the analysis of hierarchically organized phylochips

requires an additional step in comparison to the functions provided by ChipChecker. The positive signals must be tested for their robustness in relation to the hierarchy on the phylochip. In summary, a program for the analysis of hierarchically organized phylochips has to provide an algorithm for the calculation of a signal to noise-value and a tool that allows to set positive signals in relation to the hierarchy inherent in the design of the probe-set. Here we present the program, PhylochipAnalyzer that implements the calculation of signal to noise ratios and the evaluation of phylochip data with respect to probe hierarchy.

Functionality and Implementation aspects of the program

PhylochipAnalyzer is a GUI-based Windows-program, developed under Borland-Delphi. The program combines two strongly interconnected functions: hierarchy editing and data analysis. The user starts editing interactively and graphically the hierarchy that is inherent in the chip/probe design process. Editing is started by loading a spot description file in GAL-format generated by the GenePix- software (Axon Instruments Inc., USA). A procedure to convert other formats is described in the software documentation. Spot entries are shifted manually so that a hierarchically structured tree-like layout appears, in correspondence to the hierarchical probe design of the chip seen in Fig. 2A, upper part. The hierarchy is then saved as an XML-file that is used later for data analysis. Whereas the XML-file stores the pure hierarchy information of the chip, spot-intensity data are read from files with externally defined format, such as tab-delimited tables. The user may include the probe sequence in the comment field. The hierarchy can be exported as a tree file in Newick-format.

The second mode of operation is for the analysis of processed scanner data, i.e., tables with data for foreground and background intensities of the individual spots. The presence or absence of a hybridization signal is checked by a threshold criterion. The foreground-background intensity contrasts are normalized with respect to intensities of the negative control spots (Loy et al. 2002). Here intensity data of multiple copies (blocks) of the spots on each chip are evaluated and means and standard deviations are computed. The results for the blocks on the chip are shown independently (Fig. 2A, bottom right) such that entire blocks can be excluded from the analysis. It is assumed that if some spots in a certain block are identified as outliers or if positive controls fail, the user should exclude the whole block from evaluation because of the questionable quality of hybridization. A false positive signal on a higher hierarchical level has consequences for the validity of lower levels, down to the species level: PhylochipAnalyzer marks all positive signals that are below the hierarchy level of a spot showing a negative signal, i.e., corrected lines are crossed out. The user may export

the evaluation results directly to an Excel-graph (Fig. 2B) in which the signals are given as bars, labeled with the probe identifier. The size of a bar indicates the quality above the threshold, i.e., the longer the bar, the stronger the evidence for a positive signal. All data are shown with error bars of the mean due to the variance over the different blocks.

Validation

The PhylochipAnalyzer was used to analyze data retrieved from a hybridization of *Micromonas pusilla* 18S rDNA to a phylochip that contained 44 probes, including a hierarchical probe-set for the Prasinophyte genus *Micromonas*. The hierarchical probe-set consisted of six probes that bind, respectively, at the level of Kingdom (Euk1209, Euk328), Class (Chlo01, Chlo02), Clade or Order (Pras04) and Genus (Micro01) to *Micromonas pusilla*. The additional probes on the chip identified other phytoplankton taxa, a negative control, and two positive controls. Fluorescence images of the hybridized phylochips were taken with the Genepix 4000B Scanner (Axon Instruments Inc. USA). The signal intensities were quantified using the GenePix 6.0 software (Axon Instruments Inc. USA). Raw data were saved as a GPR-file and imported to the PhylochipAnalyzer-program. The computation of the raw data with the PhylochipAnalyzer-program identified only positive signals for the perfectly matching probes. For those probes, a signal/noise ratio was calculated that was above the threshold. The complete hierarchical probe set resulted in positive signals, therefore the signal for Micro01 can be considered truly positive (see Fig. 1 and Fig. 2B).

Discussion

The program simplifies tremendously the time consuming tasks of data processing of results from hierarchical phylochips. This is from particular interest, if high-throughput data are analyzed. The program is flexible with respect to configuration because the user can influence the threshold criterion by modifying the code that is implemented as a Delphi-script. This allows arbitrary modifications of the basic formula of data processing. Other formats of intensity description can easily be converted into appropriate GAL-format. On screen, the user may change the threshold value (default 2) interactively for sensitivity studies and recalculation. We plan to extend the program for quantitative analysis, i.e., spots from higher hierarchical levels are expected to show stronger signals than the lower hierarchical spots because they target more individuals. Multi-chip comparative analysis (e.g., clustering) for time-series analysis is also a desirable feature. The proposed XML-format for hierarchy representation can be seen as a prototype for standardization in phylochip hierarchy

description. It is now necessary to introduce community standards for the representation of both, chip description and data-processing details. For gene-expression analysis by means of DNA-microarrays guidelines already exist (Brazma et al. 2001). Standards for phylochip design and processing description are considered to be a prerequisite for permanent archiving of publication supplemental data accompanied by catalogues of metadata in repositories.

Acknowlegement

The work was partly funded by the EU-project FISH&CHIPS (GOCE-CT-2003-505491). We thank the group of F.O. Glöckner for providing a data file in Perkin-Elmer format to verify the applicability of the program.

Copyright, Download

The copyright is specified by the author of the software (PB). The use of Phylochip-Analyzer is free of charge. For software and supplemental material see this webpage: http://www.awi.de/en/go/phylochipanalyzer.

References

BRAZMA, A. and others 2001. Minimum information about a microarray experiment (MIAME) - toward standards for microarray data. Nat. Genet. **29:** 365 - 371.

CALL, D. R. 2005. Challenges and opportunities for pathogen detection using DNA microarrays. Crit. Rev. Microbiol. **31:** 91-99.

CSAKO, G. 2006. Present and future of rapid and/or high-throughut methods for nulcleic acid testing. Clin. Chem. Ac. **363:** 6-31.

DONDRUP, M. and others 2003. EMMA: a platform for consistent storage and efficient analysis of microarray data. J. Biotechnol. **106:** 135-146.

GROBEN, R., U. JOHN, G. ELLER, M. LANGE, and L. K. MEDLIN. 2004. Using fluorescently-labelled rRNA probes for hierarchical estimation of phytoplankton diversity – a mini-review. Nova Hedwigia **79:** 313-320.

GUILLOU, L., S.-Y. MOON-VAN DER STAAY, H. CLAUSTRE, F. PARTENSKY, and D. VAULOT. 1999. Diversity and abundance of Bolidophyceae (Heterokonta) in two oceanic regions. Appl. Environ. Microbiol. **65:** 4528-4536.

GUSCHIN, D. Y., B. K. MOBARRY, D. PROUDNIKOV, D. A. STAHL, B. E. RITTMANN, and A. D. MIRZABEKOV. 1997. Oligonucleotide microchips as genosensors for determinative and environmental studies in microbiology. Appl. Environ. Microbiol. **63:** 2397-2402.

LANGE, M. and others 1996. Identification of the class Prymnesiophyceae and the Genus *Phaeocystis* with ribosomal RNA-targeted nucleic acid probes detected by flow cytometry. J. Phycol. **32:** 858-868.

LOCKHART, D. J., and E. A. WINZELER. 2000. Genomics, gene expression and DNA arrays. Nature **405:** 827-836.

LOY, A. and others 2002. Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Appl. Environ. Microbiol. **68:** 5064-5081.

MAIDAK, B. L. and others 2001. The RDP-II (Ribosomal Database Project). Nucleic Acids Res. **29:** 173-174.

MEDLIN, L. K., K. METFIES, H. MEHL, K. H. WILTSHIRE, and K. VALENTIN. 2006. Picoeukaryotic plankton diversity at the Helgoland Time Series Site as assessed by three molecular methods. Microbial Ecol. **52:** 53-71.

METFIES, K., and L. K. MEDLIN. 2004. DNA microchips for phytoplankton: The fluorescent wave of the future. Nova Hedwigia **79:** 321-327.

PEPLIES, J., F. O. GLÖCKNER, and R. AMANN. 2003. Optimization strategies for DNA microarray-based detection of bacteria with 16S rRNA-targeting oligonucleotide probes. Appl. Environ. Microbiol. **69:** 1397-1407.

RENSINK, W. A., and C. R. BUELL. 2005. Microarray expression profiling resources for plant genomics. Trends Plant Sci. **10:** 603-609.

STOUGHTON, R. B. 2005. Applications of DNA Microarrays in Biology. Ann. Rev. Biochem. **74:** 53-82.

VAQUERIZAS, J. M. and others 2005. GEPAS, an experiment-oriented pipeline for the analysis of microarray gene expression data. Nucleic Acids Res. **33:** W616-620.

WOESE, C. 1987. Bacterial evolution. Microbiol. Rev. **51:** 221-271.

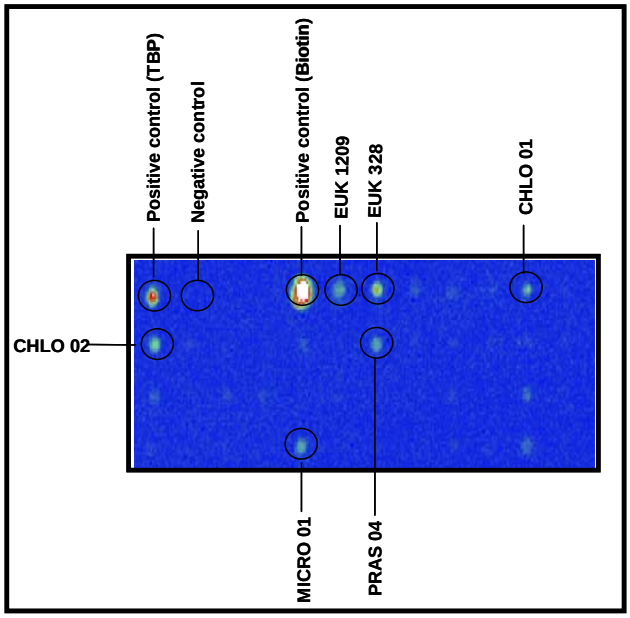
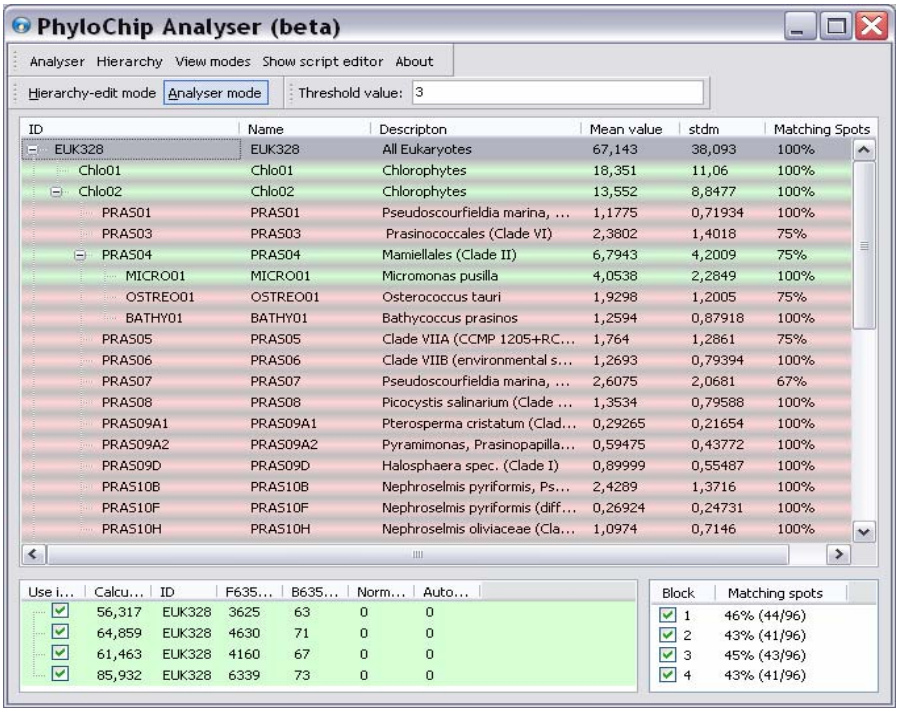


Figure 1. The 18S rDNA of *Micromonas pusilla* was hybridized to a set of 44 probes. The set of probes contained a hierarchical set that binds to the 18S rDNA of *M. pusilla* at four different taxonomic levels (Euk1209, Euk328, Chlo01, Chlo02, Pras04 and Micro01).

A



B

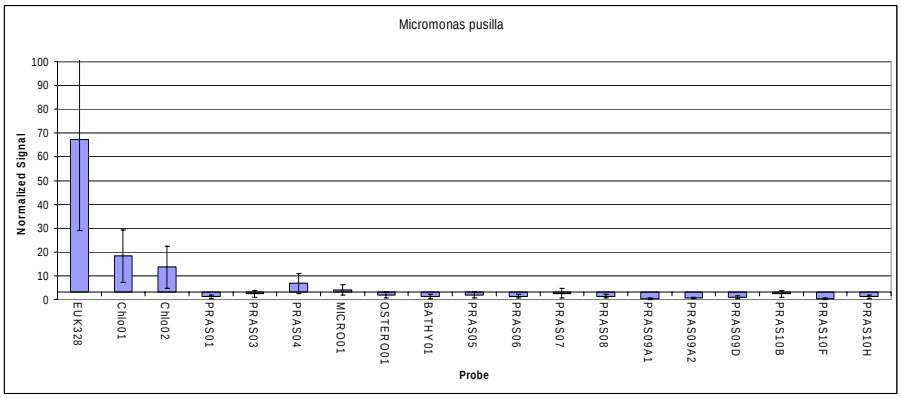


Figure 2. A: Screenshot of the analyzer-mode. Any set of molecular probes can be organized as a user defined Phylogenetic tree by a drag and drop function in editor-mode. The screenshot displays a tree of probes that bind to Prasinophytes at different hierarchical levels. The bottom part shows an individual probe result for the selected probe (Euk328, top part). **B:** Output of signal-noise values in graphical form.

2.5 Publication III

EVALUATION OF LOCKED NUCLEIC ACIDS FOR SIGNAL ENHANCEMENT OF OLIGONUCLEOTIDE PROBES FOR MICROALGAE IMMOBILIZED ON SOLID SURFACES

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Limnology and Oceanography: Methods, submitted

Abstract

Biosensors and microarrays are powerful tools for species detection and monitoring of microorganisms, e.g., phytoplankton. A reliable identification of microbial species with probe-based methods requires highly specific and sensitive probes. The introduction of LNA (locked nucleic acid) probe technology promises an enhancement of both specificity and sensitivity of molecular probes. In this study, we compared the specificity and sensitivity of conventional molecular probes and LNA modified probes in two different solid phase hybridization methods; sandwich hybridization on biosensors and on DNA-microarrays. In combination with the DNA-microarrays, the LNA-probes displayed an enhancement of sensitivity, but also more false-positive signals. In combination with the biosensor, the LNA probes could show neither signal enhancement nor discrimination of only one mismatch. In all examined cases, the conventional DNA probes showed equal or better results than the LNA probes. In conclusion, the LNA technology may have great potential in methods that use probes in suspension and possible in gene expressions studies, but under certain solid surface-hybridization applications they do not improve signal intensity.

Introduction

LNA (locked nucleic acids) were first presented by Wengel (Koshkin et al. 1998a; Koshkin et al. 1998b) and Imanishi (Obika et al. 1998) and their co-workers. They are a class of bicyclic RNA analogs with exceptionally high affinities and specificities toward their complementary DNA and RNA target molecules (Koshkin et al. 1998b; Singh et al. 1998). They can be substituted for any conventional nucleic acid in any synthetic oligonucleotide. It is possible to enhance the Tm of conventional oligonucleotides by replacing any of the conventional nucleic acid in the oligonucleotides with a LNA (Singh et al. 1998). Thus, the use of LNAs could significantly increased mismatch discrimination (Kauppinen et al. 2003). In modified nucleic acids, a methylene bridge connects the 2'-oxygen and the 4'-carbon (Parekh-Olmedo et al. 2002) and consequently produces higher conformational determination of the ribose and increased local organization of the phosphate backbone in a 3P-endo conformation (Braasch and Corey 2001). Furthermore, LNAs obey Watson-Crick base pairing (Koshkin et al. 1998b) and thus, are easy to implement into standard oligonucleotide synthesis chemistry (Kauppinen et al. 2003). LNAs offer new potentials for use in DNA/RNA oligo recognition based methods because of certain enhanced properties over normal nucleic acids. According to (Kongsbak 2002), they could be used in any hybridization assay as a modified probe or primer to increase specificity and reproducibility. They are used with standard reagents and protocols, have the same solubility as DNA or RNA, low toxicity, can make chimeras with DNA or RNA, are obtainable from industrial companies (Braasch and Corey 2001) and are not affected by nucleases (Vester and Wengel 2004). The only disadvantage is that they are much more expensive than conventional nucleic acids. Because of these enhanced properties, LNAs have been used in many applications since their first introduction, e.g., gene expression profiling (Nielsen and Kauppinen 2002), genotyping assays (Jacobsen et al. 2002a; Jacobsen et al. 2002b), fluorescence-*in-situ* hybridization (Kloosterman et al. 2006; Kubota et al. 2006; Silahtaroglu et al. 2004; Silahtaroglu et al. 2003; Wienholds et al. 2005), real-time PCR (Hummelshoj et al. 2005; Sun et al. 2007; Ugozzoli et al. 2004)DNAzymes (Vester et al. 2006; Vester et al. 2004) and other methods.

Because of these successful applications of LNA-modified probes, their use in species identification in sandwich hybridization and microarray assays should be evaluated. LNA modified probes could possibly overcome problems of low hybridization efficiency and cross hybridization of probes to closely related non-target species, often separated from the target species by a single base mismatch.

Molecular probes are widely applied for the identification of micro-organisms, e.g., toxic algae. They are applied in combination with a variety of detection techniques: Fluorescence-*in-situ*-hybridization or FISH (Kim and Sako 2005; Scholin et al. 1996; Scholin et al. 1997; Simon et al. 2000; Smit et al. 2004), sandwich hybridization assays or SHA (Metfies et al. 2005; Scholin et al. 1996) and DNA microarrays (Metfies and Medlin 2004). The small and the large subunit ribosomal RNA genes are the usual targets for molecular probes, because there is a high target number in the cell and they contain more or less conserved regions, making it possible to develop probes that are specific at different taxonomic levels (Groben et al. 2004). Probe specificity is dependent on the number of sequences of the targeted gene available in databases. Cross-reactions can occur with unknown non-targeted species if the target sequence of the probe is designed from a low number of sequences or the group is relatively unknown or unculturable and there are many non-targeted species whose sequences have not yet been determined. Even when a probe is designed from a large database, it is necessary to revise probe sequences frequently because new sequences are added almost daily to databases. Genetic variability has been documented among geographically dispersed strains of the same species (Scholin et al. 1994), making specific probes design even more challenging if global strains have not be sampled. One important problem in probe design and construction is to choose the best sequence from several possibilities that could theoretically identify the target. Excellent in-situ hybridization results of any probe does not always appear to correlate well with in-silico parameters, such as G–C content or melting temperature (Graves 1999). It is not possible to predict which probes will work well under all hybridization conditions. Sometimes probes that work well in dot-blot and FISH formats do not work at all in a microarray format (Metfies and Medlin, unpublished).

The identification of phytoplankton, especially of harmful algae species, is important from an ecological and economic point of view. Certain harmful algae have the potential to produce toxins that have the capability to seriously harm, or even kill, other organisms or even humans if intermediaries in the food chain, such as mussels, are consumed. Numerous monitoring programs are established along all coastlines around the world for the detection of harmful algae. The European Union demands the monitoring of the coastlines for toxin-producing phytoplankton and toxins in mussels by the member states (Directive 91/492d/EC and Commission Decision 2002/225/EC). Cell detection methodology based on light microscopy can be tedious and time-consuming when large numbers of samples need to be processed routinely, and identification of some species may require highly trained personnel

and expensive equipment (Tyrrell et al. 2002). Reliable species identification and long-term monitoring are difficult to achieve by traditional methods, because unicellular algae are taxonomically challenging with toxic and non-toxic strains belonging to the same species. In the past decade, a variety of molecular methods have been adapted for the identification of microbial species, which are often lacking in distinct morphological features. Molecular identification is a very useful alternative in the study of natural phytoplankton populations (Guillou et al. 1999). In our lab, we are working on the development of a molecular probe-based biosensor and a DNA-microarray for the detection of harmful algae and for estimating hidden biodiversity. In particular, we focus on those species that have the potential to harm the environment by the production of potent toxins.

The two solid-phase methods described here: DNA microarrays for phylogenetic analyses and an rRNA-biosensor, are used to measure the abundance of algal species using target specific probes bound to a surface.

rRNA biosensor - The detection method using a rRNA-biosensor was successfully introduced by (Metfies et al. 2005) as a molecular method for the detection and identification of the toxic dinoflagellate *Alexandrium ostenfeldii*. It utilizes sandwich hybridization (SHA) with a capture probe that binds to the target RNA or DNA and a second signal probe that carries the signal moiety and binds near the binding site of the capture probe. A third additional probe, the so-called helper probe, binds near the binding site of the two other probes to modify the secondary structure of the molecule so that the signal probe can easily form its heteroduplex. This region usually consists of approximately 50 bps leaving little for probe manipulation should the probes not work properly. The search for suitable probes is complicated by the relative conservation of the 18S gene at the species level (Gagnon et al. 1996; Ki and Han 2006). More variable genes have not been rigorously evaluated because only hyper-variable regions have been sequenced leaving the majority of the gene unknown and open for non-specific binding. The detection is measured electrochemically by the PalmSens instrument and its PSLite software (Palm Instruments, Houten, Netherlands) and was adapted from the original biosensor presented by Metfies et al. (2005).

Probes for the rRNA biosensor (Table 1) - AOST1 (the signal probe), AOST2 (the capture probe), and their helper oligonucleotide, H3, are 18S-rRNA probes designed by (Metfies et al. 2005) and were tested for specificity with dot-blot and SHA. Although normalized signals for *A. ostenfeldii* are significantly higher than the signals from all non-target organisms, there is a low cross hybridization to *A. minutum,* which has 2 mismatches to the capture probe. An improved protocol for the isolation of algal RNA with the Qiagen

RNeasy Plant Mini Kit, Hilden only enhances this cross reaction. The recently described non-toxic *Alexandrium tamutum* (Montresor et al. 2004) presents a single mismatch to the capture probe for *A. ostenfeldii*, thus challenging the limits of specificity of this probe.

DNA-Microarray - A DNA-microarray consists of a glass-slide with special surface properties (Niemeyer and Blohm 1999) and many copies of nucleic acids, e.g., oligonucleotides, cDNAs or PCR-fragments spotted on it (Graves 1999) in a specific pattern . It is a widely used routine tool in many applications because it offers the possibility to analyze a large number of up to 250,000 different targets in parallel without a cultivation step (Graves 1999; Lockhart et al. 1996; Ye et al. 2001). Nucleic acids are fluorescently labelled before hybridization and they are detected afterwards with a microarray scanner (DeRisi et al. 1997). Many functional genomic methods benefit from this technology, such as genome expression profiling, single nucleotide polymorphism detection and DNA resequencing (Al-Shahrour et al. 2005; Broet et al. 2006; Gamberoni et al. 2006; Ji and Tan 2004; Kauppinen et al. 2003; Lipshutz et al. 1999; Yap et al. 2004). DNA-microarray technology is also used to differentiate microalgae (Ki and Han 2006; Metfies et al. 2006; Metfies and Medlin 2004, Medlin et al. 2006), fish (Kappel et al. 2003) and bacteria (Lehner et al. 2005; Loy et al. 2002; Loy et al. 2005; Peplies et al. 2003; Peplies et al. 2004a; Peplies et al. 2006; Peplies et al. 2004b).

Probes for the DNA-microarray – Four out of five probes used here (Table 2) were previously evaluated on the DNA-microarray (Metfies and Medlin 2004). The fifth probe, Crypto B, recognizes all pigmented cryptomonad algae. It could be shown that these probes work specifically with microarrays, but there was potential for enhancement of the signal-to-noise-ratios because these probes gave low signals and thus were good candidates for signal-enhancement with LNAs.

Materials and Procedures

Culture conditions - All algal strains were cultured under sterile conditions in seawater-based media (Eppley et al. 1967; Keller et al. 1987) at 15 °C and 150 µEinstein – 200 µEinstein with a light: dark cycle of 14:10 hours (Table 1).

RNA-extraction - Total RNA was isolated from all algal cultures with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) with modifications of the protocol to enhance the quality of the RNA. This involved a centrifugation of 15 minutes instead of two minutes to achieve an improved separation of supernatant and cell debris. Buffer RW1 was applied two

times to the RNeasy column, incubated for one minute and then centrifuged. The first wash step with buffer RPE was repeated. RNA concentration was measured with a Nanodrop Spectrophotometer (Peqlab, Erlangen, Germany). All of these changes increased the removal of polysaccharides and proteins to improve quality and quantity of the rRNA extracted.

DNA-extraction - The template DNA from the environmental clones was isolated from bacteria by using the Plasmid Mini Kit (Qiagen, Hilden, Germany). DNA from the algal strains was extracted from pure cultures with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

PCR Amplification of 18S rRNA - The entire 18S gene (1800 bp) from the target DNA was amplified with universal specific PCR primers 1F (5'-AAC CTG GTT GAT CCT GCC AGT- 3') and 1528R (5'- TGA TCC TTC TGC AGG TTC ACC TAC- 3') without the polylinkers (Medlin et al. 1988). The PCR protocol was: 5 min 94 °C, 2 min 94 °C, 4 min 54 °C, 2 min 72 °C, 29 cycles and 7 min 72 °C. All PCR experiments were done in a Mastercycler (Eppendorf, Hamburg, Germany). A 250 bp fragment of the TATA-box binding protein-gene (TBP) of *Saccharomyces cerevisiae* was amplified with the primers TBP-F (5'-ATG GCC GAT GAG GAA CGT TTA A-3') and TBP-R-Biotin (5'-TTT TCA GAT CTA ACC TGC ACC C- 3') and used as a positive control in the microarray hybridization experiments. The TBP amplification protocol was: 5 min 94 °C, 1 min 94 °C,1 min 52 °C, 1 min 72 °C, 35 cycles, 10 min 72 °C. All PCR-fragments were purified with the QIAquick PCR purification (Qiagen, Hilden, Germany) with modifications of the protocol to enhance the quantity of the PCR-fragments. The elution with the elution buffer EB (Step 8) was performed twice with the same buffer. The concentration of the DNA was measured with a Nanodrop Spectrophotometer (Peqlab, Erlangen, Germany).

Biotin-Labeling of the purified PCR- fragments - For the enhancement of signal intensities the Biotin DecaLabel DNA Labeling Kit (Fementas, St. Leon-Rot, Germany) was used. Labeling of 200 ng of purified PCR-fragment was carried out over night (17 to 20 hours) to maximize biotin incorporation into the PCR-fragments. After labelling the purification was done with the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) with modifications of the protocol to enhance the quantity of the PCR-fragments as above. Concentration of the DNA was measured with a Nanodrop Spectrophotometer (Peqlab, Erlangen, Germany).

Probe synthesis –All probes and helper oligonucleotide probes and positive and negative controls were synthesized from Thermo Electron Corporation, Ulm, Germany. The locked nucleic acids were synthesized from Exiqon (Vedbaek, Denmark). The position of the LNA-residues within the sequence is proprietary information from Exiqon but they were regularly interspersed among normal nucleic acids.

rRNA biosensor

Probe set – A set of two specific 18S-rRNA probes (AOST1 and AOST2, Table 2) was used to assess the impact of LNA-probes on the specificity of probes with the biosensor. The sequence of capture probe AOST2 was redesigned from Exiqon with locked nucleic acids as a shorter oligonucleotide to maintain the identical melting temperature as the conventional probe AOST2. Three different probes, LNA 65, LNA 66 and LNA 67, were synthesized with a biotin-label and were used as signal probes in combination with AOST1. Probe AOST2 has a melting temperature (Tm) of 66 °C, AOST1 of 64.3 °C, LNA 65 and LNA 66 of 65 °C and LNA 67 of 60 °C. The positive control was not modified with LNAs.

Algal strains and templates - The specificity of the LNA probes using the rRNA biosensor was tested with the target strain *Alexandrium ostenfeldii* (Table 1) and the non-target strains, *Alexandrium minutum* AL3T and *Alexandrium tamutum* SZNB029.

Immobilization of the probes on the sensor chip - The biotinylated capture probes (AOST2, LNA 65, LNA 66; LNA 67) were immobilized on the sensor chips as described by (Metfies et al. 2005). The working electrode was pretreated with Carbonate buffer (50 mM $NaHCO_3$, pH 9.6) following which incubation with NeutrAvidin [0.5 mg/mL] (Pierce Biotechnology, Rockford, USA) for at least 4.5 hours at 4 °C was carried out. Excessive NeutrAvidin was removed from the working electrode by washing the sensor with PBS (BupH phosphate saline pack, Pierce Biotechnology, USA). Subsequently, the working electrode was blocked with 3 % [w/v] casein in PBS for 1 hour at room temperature and afterwards washed in PBS. The probes were dissolved at a concentration of 10 µM in bead buffer (0.3 M NaCl, 0.1 M Tris, pH 7.6) prior to immobilization on the electrodes for 30 minutes at room temperature. All incubation steps were carried out in a moisture chamber to avoid evaporation. Unbound probe was removed from the electrode by washing with hybridization buffer (75 mM NaCl, 20 mM Tris, pH 8.0, 0.04 % SDS).

Hybridization - Prior to hybridization the total rRNA was fragmented in fragmentation buffer (40 mM Tris, pH 8.0, 100 mM KOAc, 30 mM MgOAc) for 5 minutes at 94 °C. The hybridization mixture for the detection of rRNA contained 1x hybridization buffer (75 mM NaCl, 20mM Tris, pH 8.0, 0.04 % SDS), 0.25 µg/µL herring sperm DNA, 0.1 pmol/µL dig-labeled probe AOST1 and rRNA at different concentrations. Negative control and positive controls contain water and Test-DNA, respectively, instead of rRNA. Incubation for 4 minutes at 94 °C of the hybridization mixture was carried out to denature the target nucleic acid. Subsequently 2 µL of the mixture was applied to the working electrode and the sensor was incubated for 30 minutes at 46 °C. The hybridization was accomplished in a moisture chamber to avoid evaporation. Afterwards, the sensor chips were washed with POP buffer (50 mM $NaH_2PO4 \times H_2O$, pH 7.6, 100 mM NaCl).

Detection - The sensor chip was incubated for 30 minutes at room temperature with an antibody-enzyme complex directed against the digoxigenin coupled to horseradish-peroxidase (Anti-DIG-POD). 1.5 µL of the antibody-enzyme solution (7.5 U/mL in PBS, pH 7.6, 0.1 % BSA [w/v], 0.05 % Tween 20 [v/v]) was added onto the electrode. Excessive enzyme was removed by washing the sensor with POP buffer; subsequently the sensor chip was inserted into the PalmSens (Palm Instruments BV, Houten, Netherlands), 20 µL of the substrate solution (4-aminophenylamine hydrochloride [44 µg/mL]/0.44 % ethanol [v/v], 0.048 % H_2O_2 [v/v], 50mM $NaH_2PO_4 \times H_2O$, 100 mM NaCl) was applied to the working electrode and an electrochemical signal was generated that was directly measured for 10 seconds at a potential of -147 millivolt (versus Ag/AgCl) after 8 seconds of equilibration.

Experimental setup - The LNA probe and the AOST probe experiments were carried at four different temperatures: 46 °C (normal hybridization temperature), 55 °C, 60 °C and 65 °C. Each LNA probe and the AOST2 probe were tested using the rRNA of the target and non-target species at each temperature. A hybridization experiment contained three replicates for detection of target RNA, and a negative and positive control. Unclear results were repeated to verify the data. The mean value of the signals was calculated and the standard derivation was determined with the following formula:

$$\frac{\sqrt{\frac{n\sum x^2 - (\sum x)^2}{n(n-1)}}}{\sqrt{n}}$$

Microarray

Probe set DNA microarray - The five probes evaluated in this publication target the 18S-rRNA: one for the super kingdom of Eukarya and one for each of these four major phyla of algae: the Chlorophyta, Bolidophyta, Prymnesiophyta and Cryptophyta. The probe lengths of the conventional probes varied from 16-20 base pairs (Table 3). Euk1209, Chlo 02, Boli 02, Prym 02 and Crypto B were processed by Exiqon with two different locked nucleic acid modifications, LNA2 or LNA3 varying in the number of LNAs/probe and the length and in the methylation of Cytosine. The positive control was not modified with LNAs.

Algal strains and templates - The tests of the LNA probes using the microarray-format were carried out with PCR-fragments amplified from two uncultured, environmental clones and two algal strains (Table 3) as target strains. Four strains from the genus *Alexandrium* (*A. catenella* BAHME217, *A. ostenfeldii* BAHME 136, *A. ostenfeldii* AOSH1 and *A. minutum* Nantes) were used as non-target strains.

Microarray production - The probes for the microarray had a C6/MMT aminolink at the 5'-end of the molecule and were spotted onto epoxy-coated "Nexterion Slide A" slides (Peqlab Biotechnologie GMBH, Erlangen, Germany). The oligonucleotides were diluted to a final concentration of 1 µM in 3x saline sodium citrate buffer and printed onto the slides with the pin printer VersArray ChipWriter Pro (Bio-Rad Laboratories GmbH, München, Germany) and split pins (Point Technologies, Inc., Colorado, USA). The probes were immobilized on the slides with a baking procedure of 30 min at 60 °C and stored at -20 °C.

Standard hybridization protocol - The hybridization solution contained a hybridization buffer (1 M NaCl, 10 mM Tris, pH 8, 0.005 % Triton X-100, 1 mg/mL BSA, 0.1 µg/µL HS-DNA), the biotin-labeled PCR-fragment in a final concentration of 11.25 ng DNA per µL and the positive control, the 250 bp PCR-fragment TBP from *S. cerevisiae* with biotin-labeled primers in a final concentration of 4.7 ng DNA per µL. First, one hour pre-hybridization was carried out at 58 °C with 2x STT buffer. The hybridization solution was denatured for 5 min at 94 °C and for even dispersal of hybridization solution between the chip and the coverslip, a volume of 30 µL was injected under a Lifter Slip cover slip (Implen, München, Germany). The slides were hybridized as follows: 1 hour hybridization in a humid chamber with the hybridization solution at a hybridization temperature of 58 °C, washing afterwards with 2x and 1x saline sodium citrate (2x SSC, 10 mM EDTA, 0.05 % SDS; 1x SSC, 10 mM EDTA)

for 15 min each. In all microarray hybridization experiments, the chip contained four replicates of each probe in four individual arrays. These hybridizations were done four times with the perfectly matched targets. For the non-target hybridizations, the hybridizations were repeated twice.

Staining - The bound PCR-fragments were stained subsequently with Streptavidin-CY5 (Amersham Biosciences, Stadt, Germany) in hybridization buffer at a final concentration of 100 ng /mL. The staining took place for 30 min at room temperature in a humid chamber. Excess staining moieties were removed by washing twice with 2x saline sodium citrate for 5 min and once with 1x saline sodium citrate for 5 min.

Scanning and quantification of Microarrays - The fluorescent signals of the microarrays were scanned with a GenePix 4000B scanner (Molecular Devices Cooperation, Sunnyvale USA) and the obtained signal intensities were analyzed with the GenePix 6.0 software (Molecular Devices Coperation, Sunnyvale USA). The signal to noise-ratio was calculated with a formula according to (Loy et al. 2002) and all ratios were normalized on the signal of the TBP positive control. The mean value of the signal-to-noise-ratios was calculated as above.

Assessment

rRNA Biosensor - The PalmSens was adapted for the biosensors using a control chip with a fixed resistance of 2682 nanoampere (nA). In this study, an amperometric detection technique was used with measurement duration of 10 seconds. At the recommendation of Palm Instruments, the time equilibration of 8 seconds was programmed into measuring method, which means a total measurement duration of 18 seconds, 8 seconds longer than with the Inventus Biotec GmbH potentiostat used by Metfies et al (2005). The redox-reaction goes to completion and then signals decrease over the measurement time because of the limited substrate amount. Consequently, the signal intensity is lower after 18 seconds than after 10 seconds. Compared to the signals measured by Metfies et al (2005), all the signals presented in this study are about 600 nA lower for the AOST probes than those in Metfies et al. (2005).

The hybridization temperature for both *Alexandrium ostenfeldii* probes was optimized in the present assay to 46 °C (Figure 1A). This is around 20 °C below the calculated Tm of AOST probes. Hybridization reactions can be carried out at a Tm 25 °C below its theoretical calculation because the rate of DNA annealing is maximal at 20 - 25 °C below its melting

temperature. Hybrids formed from completely homologous nucleic acids will be thermally stable under these conditions (Howley et al. 1979). However, if hybridizations are performed at temperatures significantly below the theoretical Tm, the probes could cross hybridize to non-target nucleic acids. The AOST probes gave a signal for *Alexandrium ostenfeldii* of 680 nA and also showed high cross hybridization signals for *A. minutum* at 605 nA. However, *A. tamutum*, having only one mismatch to AOST2 was not detected by the AOST probes, thus it is possible to discriminate target from non-target with a single base pair mismatch. All three LNA probes showed almost no signals at 46 °C for the different species (Figure 1A). Only LNA 66 showed a weak signal for *A. ostenfeldii*. Also the positive control signals were about twofold lower for LNA 65 and about 2.7 x lower for LNA 66 and LNA 67 than for the AOST probes, which can be explained by the suboptimal hybridization temperature for the LNA probes and their melting temperature. It seems that LNA probes do not have the same hybridization properties as conventional probes in this method.

Metfies et al 2005 showed that a temperature of 55 °C results in higher hybridization signals than at 46 °C but at this temperature, all probes were non-specific (Figure 1B). Only LNA 67 gave very low signals for all species similar to the signals at a hybridization temperature of 46 °C. Probes AOST1/AOST2, LNA 65 and LNA 66 have a Tm of about 65 °C; LNA 67 has a Tm of 60 °C. A hybridization temperature of 55 °C should be the optimum temperature for the first three probes. We maintained uniform temperatures and salt concentrations in the washing buffers in order to compare the performance of the LNAs against optimal conditions for the unmodified probes. At hybridization temperature of 60 °C (Figure 1C) the AOST probes were specific for *A. ostenfeldii* and showed no signals for the other species, but the signal intensity was lower than at 46 °C. All three LNA probes detected *A. ostenfeldii* and *A. minutum*. The AOST probes detected all three species at a hybridization temperature of 65 °C (Figure 1D), but the signals for *A. ostenfeldii* and *A. minutum* were quite low and there was a high signal for *A. tamutum* similar to the signals obtained at 55 °C. LNA probe 65 was specific at 65 °C and detected only *A. ostenfeldii*. This was the only specific signal that we detected. LNA probes 66 and 67 showed only low signals for *A. ostenfeldii* and *A. minutum* but high signals for *A. tamutum*. The properties of the LNA probes should enhance the signal intensity at higher temperatures and discriminate the mismatches but we obtained exactly the opposite results. All three LNA probes show non-specific signals at 46 °C, 55 °C and 60 °C for *A. ostenfeldii*.

For the use on an rRNA biosensor the probes were also tested for long term stability (data not shown). Probes without LNAs are stable over a year. During the experiments with

LNA probes on the biosensors, it was observed that the LNA probes were unstable after immobilization after only a few weeks of storage.

Microarray

Probe development/design - For this hybridization study, previously published and microarray tested probes were used. They all target higher taxonomic levels, so it is challenging to design probes to achieve better specificity and sensitivity that can recognize all taxa belonging to the target group. The selected probes are working moderately well but do not show sufficient sensitivity for use in routine applications and monitoring of phytoplankton because cell counts in field samples are often not high and taxonomic groups with low abundance cannot be detectable.

Validation of results in the hybridization protocol - The results of the microarray hybridization (Figure 2) with specific PCR-fragments indicated that both LNA probes showed increased signal intensity. LNA2 performed the best, except for CryptoB, the probe for the Cryptophyceae, where LNA3 had the highest result. Signal enhancement varied from approx. 4.5-fold higher results in the Cryptophyceae and Bolidophyceae to 8.5-fold higher signals in the Chlorophyceae.

Validation of results using non- target hybridizations - In comparison to the above results, signals of the hybridization of the conventional and LNA-modified probes with non-target algae species (Figure 3) demonstrated that the conventional probes worked specifically with weak cross hybridization with non-related species. All probes, both conventional and LNAs, showed positive enhanced signals with the Eukaryotic probe, as they should but there was no pattern to the enhancement and these data are not presented. All LNA probes showed cross hybridization signals with non-target DNA. Hybridizations with 27 other *Alexandrium* strains all showed the same tendency (data not shown).

Increase of hybridization temperature to enhance the discriminative potential of the LNAs was already tested with the biosensor and the LNA modified probes did not perform as conventional oligonucleotides. Thus the microarray protocol was not modified any further. Even though the results from the hybridizations with target DNA using standard protocols are promising with increase in signal-to-noise-ratios, in the hybridization with non target DNA, the LNA probes show an unacceptable lack of pecificity. For further clarification, the mismatches of the probes to the sequences of the four *Alexandrium* strains are shown in Table

4. The differences span from 2 to 9 base pairs. Theoretically, it is impossible for these DNAs to bind to these probes.

Discussion

In this study, we tested and evaluated the use of LNA probes in two solid-phase hybridization methods. Although there have been many publications on enhancement of probe or hybridization signals with LNA modified probes, there has been no rigorous testing of these probes using known target sequences. We found that LNA probes showed no signal enhancement in the sandwich hybridization method using the rRNA biosensor. Only one of the tested LNA probes showed specific signals at a hybridization temperature of 65 °C. Using the microarray, the LNA probes could enhance the sensitivity and gave higher signals than the conventional probes using only target DNA but unfortunately, unspecific binding with non-target DNA also was enhanced. These results were surprising because in other methods the LNA modified probes show great potential and an ability to enhance the signals and to improve specificity, accuracy and sensitivity in the whole method (Kloosterman et al. 2006; Kubota et al. 2006; Silahtaroglu et al. 2004; Sun et al. 2007; Wienholds et al. 2005). Results from other methods using LNA probes cannot be easily compared to the results presented in this study, because of the different experimental setups, such as *in-situ* hybridizations in tissues (fluorescence-*in-situ*-hybridization (FISH), *in-situ* hybridization). In FISH experiments, the LNA probes using human-specific repetitive elements were very efficient (Silahtaroglu et al. 2004; Silahtaroglu et al. 2003). To evaluate the potential possibilities and abilities of LNA probes, more experiments with more methods are necessary. A comprehensive and ultimate evaluation of the potential of LNA probes cannot be conducted here because only a small subset of probes were tested in two different solid phase based hybridization techniques with the use of our standard hybridization protocols. It is likely that the increased signals seen in these studies result from non-sepcific binding which cannot be documented because the target and non-target sequences are unknown. The standard protocols developed for our unmodified probes on multiprobe chips at specific hybridization temperatures are appropriate for monitoring of phytoplankton. By choosing other salt concentrations in combination with other hybridization temperatures, the signals of the LNA probes could be different. Further optimization experiments are only appropriate for the use of only one LNA probe at a time, because different LNA probes can have different hybridization temperature optima. Additionally to unspecific binding, other problems occurred using LNA probes. For example, the biosensors for the monitoring of the toxic algae are prepared in

advance of application. Because of this, the probes on the biosensors have to be stable and need to give the same signals after several months of storage. With the LNA probes, this application was not possible.

Signal enhancement of both methods, biosensors and microarrays, has been achieved by changing substrate concentration for the biosensor and by reducing the background noise with the help of other blocking solutions. In the case of the microarrays, signal enhancement can be accomplished by using labelling kits that incorporate multiple labels to a target.

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References

AL-SHAHROUR, F., R. DIAZ-URIARTE, and J. DOPAZO. 2005. Discovering molecular functions significantly related to phenotypes by combining gene expression data and biological information. Bioinformatics **21:** 2988-2993.

BRAASCH, D. A., and D. R. COREY. 2001. Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA. Chem. Biol. **8:** 1-7.

BROET, P., V. A. KUZNETSOV, J. BERGH, E. T. LIU, and L. D. MILLER. 2006. Identifying gene expression changes in breast cancer that distinguish early and late relapse among uncured patients. Bioinformatics **22:** 1477-1485.

DERISI, J. L., V. R. IYER, and P. O. BROWN. 1997. Exploring the metabolic and genetic control of gene expression on a genomic scale. Science **278:** 680-686.

EPPLEY, R. W., R. W. HOLMES, and J. D. H. STRICKLAND. 1967. Sinking rates of marine phytoplankton measured with a fluorometer. J. Exp. Mar. Biol. Ecol. **1:** 191-208.

GAGNON, S., D. BOURBEAU, and R. C. LEVESQUE. 1996. Secondary structures and features of the 18S, 5.8S and 26S ribosomal RNAs from the Apicomplexan parasite *Toxoplasma gondii*. Gene **173:** 129-135.

GAMBERONI, G., S. STORARI, and S. VOLINIA. 2006. Finding biological process modifications in cancer tissues by mining gene expression correlations. BMC Bioinformatics **7:** 6.

GRAVES, D. J. 1999. Powerful tools for genetic analysis come of age. Trends Biotechnol. **17:** 127-134.

GROBEN, R., U. JOHN, G. ELLER, M. LANGE, and L. K. MEDLIN. 2004. Using fluorescently-labelled rRNA probes for hierarchical estimation of phytoplankton diversity – a mini-review. Nova Hedwigia **79:** 313-320.

GUILLOU, L., S.-Y. MOON-VAN DER STAAY, H. CLAUSTRE, F. PARTENSKY, and D. VAULOT. 1999. Diversity and Abundance of Bolidophyceae (Heterokonta) in Two Oceanic Regions. Appl. Environ. Microbiol. **65:** 4528-4536.

HOWLEY, P. M., M. A. ISRAEL, M. LAW, and M. A. MARTIN. 1979. A rapid method for detecting and mapping homology between heterologous DNAs. J. Biol. Chem. **254:** 4876-4883.

HUMMELSHOJ, L., L. P. RYDER, H. O. MADSEN, and L. K. POULSEN. 2005. Locked nucleic acid inhibits amplification of contaminating DNA in real-time PCR. Biotechniques **38:** 605-610.

JACOBSEN, N. and others 2002a. LNA-enhanced detection of single nucleotide polymorphisms in the apolipoprotein E. Nucleic Acids Res. **30:** e100.

---. 2002b. Genotyping of the apolipoprotein B R3500Q mutation using immobilized Locked Nucleic Acid capture probes. Clin. Chem. **48:** 657-660.

JI, L., and K.-L. TAN. 2004. Mining gene expression data for positive and negative co-regulated gene clusters. Bioinformatics **20:** 2711-2718.

KAPPEL, K., H. V. WESTERNHAGEN, and D. H. BLOHM. 2003. Microarray-based identification of eggs and larvae from fish species common in the North Sea, Dechema Chip-Technology Meeting, Frankfurt, Germany.

KAUPPINEN, S. and others 2003. LNA microarrays in genomics. Pharma Genomics**:** 24-34.

KELLER, M. D., R. C. SELVIN, W. CLAUS, and R. R. L. GUILLARD. 1987. Media for the culture of oceanic ultraphytoplankton. J. Phycol. **23:** 633-338.

KI, J.-S., and M.-S. HAN. 2006. A low-density oligonucleotide array study for parallel detection of harmful algal species using hybridization of consensus PCR products of LSU rDNA D2 domain. Biosens. Bioelectron. **21:** 1812-1821.

KIM, C.-J., and Y. SAKO. 2005. Molecular identification of toxic *Alexandrium tamiyavanichii* (Dinophyceae) using two DNA probes. Harmful Algae **4:** 984-991.

KLOOSTERMAN, W. P., E. WIENHOLDS, E. DE BRUIJN, S. KAUPPINEN, and R. H. A. PLASTERK. 2006. *In situ* detection of miRNAs in animal embryos using LNA-modified oligonucleotide probes. Nature Methods **3:** 27-29.

KONGSBAK, L. 2002. LNA: Fine-tuning of primers and probes. LNA **01:** 1.

KOSHKIN, A. A., V. K. RAJWANSHI, and J. WENGEL. 1998a. Novel convenient syntheses of LNA [2.2.1] bicyclo nucleosides. Tetrahedron Lett. **39:** 4381-4384.

KOSHKIN, A. A. and others 1998b. LNA (Locked Nucleic Acids): Synthesis of the adenine, cytosine, guanine, 5-methylcytosine, thymine and uracil bicyclonucleoside monomers, oligomerisation, and unprecedented nucleic acid recognition. Tetrahedron **54:** 3607-3630.

KUBOTA, K., A. OHASHI, H. IMACHI, and H. HARADA. 2006. Improved *in-situ* hybridization efficiency with Locked-Nucleic-Acid-incorporated DNA probes. Appl. Environ. Microbiol. **72:** 5311-5317.

LEHNER, A. and others 2005. Oligonucleotide microarray for identification of *Enterococcus* species. FEMS Microbiol. Lett. **246:** 133-142.

LIPSHUTZ, R. J., S. P. FODOR, T. R. GINGERAS, and D. J. LOCKHART. 1999. High density synthetic oligonucleotide arrays. Nature Genet. **21:** 20 - 24.

LOCKHART, D. J. and others 1996. Expression monitoring by hybridization to high-density oligonucleotide arrays. Nat. Biotechnol. **14:** 1675-1680.

LOY, A. and others 2002. Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Appl. Environ. Microbiol. **68:** 5064-5081.

---. 2005. 16S rRNA gene-based oligonucleotide microarray for environmental monitoring of the betaproteobacterial order "Rhodocyclales". Appl. Environ. Microbiol. **71:** 1373-1386.

MEDLIN, L. K., H. J. ELWOOD, S. STICKEL, and M. L. SOGIN. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene **71:** 491-499.

MEDLIN, L. K., K. METFIES, H. MEHL, K. H. WILTSHIRE, and K. VALENTIN. 2006. Picoeukaryotic plankton diversity at the Helgoland Time Series Site as assessed by three molecular methods. Microbial Ecol. **52:** 53-71.

METFIES, K., M. BERZANO, C. GUALERZI, G. MUYZER, and L. K. MEDLIN. 2006. Meeting Report: Molecular Ecology Workshop. Detection of microbial biodiversity in environmental samples, Camerino, Italy, September 19-21, 2005. Protist **157:** 247-250.

METFIES, K., S. HULJIC, M. LANGE, and L. K. MEDLIN. 2005. Electrochemical detection of the toxic dinoflagellate *Alexandrium ostenfeldii* with a DNA-biosensor. Biosens. Bioelectron. **20:** 1349-1357.

METFIES, K., and L. K. MEDLIN. 2005. Ribosomal RNA probes and microarrays: their potential use in assessing microbial biodiversity, p. 258-278. *In* E. A. Zimmer and E. H. Roalson [eds.], Methods in Enzymology, Molecular Evolution: Producing the Biochemical Data. Elsevier Academic Press, San Diego, USA.

---. 2004. DNA microchips for phytoplankton: The fluorescent wave of the future. Nova Hedwigia **79:** 321-327.

MONTRESOR, M., U. JOHN, A. BERAN, and L. K. MEDLIN. 2004. *Alexandrium tamutum* sp. nov. (Dinophyceae): A new nontoxic species in the genus *Alexandrium*. J. Phycol **40:** 398-411.

NIELSEN, P. S., and S. KAUPPINEN. 2002. The use of LNA Oligonucleotide Microarrays Provides Superior Sensitivity and Specificity in Expression Profiling. LNA **17:** 1-3.

NIEMEYER, C. M., and D. BLOHM. 1999. DNA microarrays. Angew. Chem. Int. Edit. **38:** 2865-2869.

OBIKA, S. and others 1998. Stability and structural features of the duplexes containing nucleoside analogues with a fixed N-type conformation, 2'-O,4'-C-methyleneribonucleosides. Tetrahedron Lett. **39:** 5401-5404.

PAREKH-OLMEDO, H., M. DRURY, and E. B. KMIEC. 2002. Targeted nucleotide exchange in *Saccharomyces cerevisiae* directed by short oligonucleotides containing Locked Nucleic Acids. Chem. Biol. **9:** 1073-1084.

PEPLIES, J., F. O. GLÖCKNER, and R. AMANN. 2003. Optimization strategies for DNA microarray-based detection of bacteria with 16S rRNA-targeting oligonucleotide probes. Appl. Environ. Microbiol. **69:** 1397-1407.

PEPLIES, J., F. O. GLÖCKNER, R. AMANN, and W. LUDWIG. 2004a. Comparative sequence analysis and oligonucleotide probe design based on 23S rRNA genes of Alphaproteobacteria from North Sea bacterioplankton. Syst. Appl. Microbiol. **27:** 573-580.

PEPLIES, J., C. LACHMUND, F. O. GLÖCKNER, and W. MANZ. 2006. A DNA microarray platform based on direct detection of rRNA for characterization of freshwater sediment-related prokaryotic communities. Appl. Environ. Microbiol. **72:** 4829-4838.

PEPLIES, J., S. C. K. LAU, J. PERNTHALER, R. AMANN, and F. O. GLÖCKNER. 2004b. Application and validation of DNA microarrays for the 16S rRNA-based analysis of marine bacterioplankton. Environ. Microbiol. **6:** 638-645.

SCHOLIN, C. A., K. R. BUCK, T. BRITSCHGI, G. CANGELOSI, and F. P. CHAVEZ. 1996. Identification of *Pseudo-nitzschia australis* (Bacillariophyceae) using rRNA-targeted probes in whole cell and sandwich hybridization formats. Phycologia **35:** 190-197.

SCHOLIN, C. A., M. HERZOG, M. SOGIN, and D. M. ANDERSON. 1994. Identification of group- and strain-specific genetic markers for the globally distributed *Alexandrium* (Dinophyceae). II. Sequence analysis of a fragment of the LSU rRNA gene. J. Phycol. **30:** 999-1011.

SCHOLIN, C. A. and others 1997. Detection and quantification of *Pseudo-nitzschia australis* in cultured and natural populations using LNA rRNA-targeted probes. Limnol. Oceanogr. **42:** 1265-1272.

SILAHTAROGLU, A. N., H. M. PFUNDHELLER, A. A. KOSHKIN, N. TOMMERUP, and S. KAUPPINEN. 2004. LNA-modified oligonucleotides are highly efficient as FISH probes. Cytogen. Gen. Res. **107:** 32-37.

SILAHTAROGLU, A. N., N. TOMMERUP, and H. VISSING. 2003. FISHing with locked nucleic acids (LNA): evaluation of different LNA/DNA mixmers. Mol. Cell. Probe **17:** 165-169.

SIMON, N. and others 2000. Oligonucleotide probes for the identification of three algal groups by dot-blot and fluorescent whole-cell hybridization. J. Eukaryot. Microbiol. **47:** 76-84.

SINGH, S. K., A. A. KOSHKIN, J. WENGEL, and P. NIELSEN. 1998. LNA (locked nucleic acids): synthesis and high-affinity nucleic acid recognition. Chem. Commun.**:** 455 - 456.

SMIT, E. and others 2004. Molecular tools and approaches in eukaryotic microbial ecology. Bios Scientific Publishers, Oxford, UK.

SUN, Z., L. ZHOU, H. ZENG, Z. CHEN, and H. ZHU. 2007. Multiplex locked nucleic acid probes for analysis of hepatitis B virus mutants using real-time PCR. Genomics **89:** 151-159.

TYRRELL, J. V., L. B. CONNELL, and C. A. SCHOLIN. 2002. Monitoring for *Heterosigma akashiwo* using a sandwich hybridization assay. Harmful Algae **1:** 205-214.

UGOZZOLI, L. A., D. LATORRA, R. PUCKET, K. ARAR, and K. HAMBY. 2004. Real-time genotyping with oligonucleotide probes containing locked nucleic acids. Anal. Biochem. **324:** 143-152.

VESTER, B. and others 2006. Locked nucleoside analogues expand the potential of DNAzymes to cleave structured RNA targets. BMC Mol. Biol. **7:** 19.

VESTER, B., L. B. LUNDBERG, M. D. SØRENSEN, B. R. BABU, S. DOUTHWAITE, and J. WENGEL. 2004. Improved RNA cleavage by LNAzyme derivatives of DNAzymes. Biochem. Soc. T. **32:** 37-40.

VESTER, B., and J. WENGEL. 2004. LNA (Locked Nucleic Acid): High-affinity targeting of complementary RNA and DNA. Biochemistry USA **43:** 13233-13241.

WIENHOLDS, E. and others 2005. MicroRNA expression in zebrafish embryonic development. Science **309:** 310-311.

YAP, Y., X. ZHANG, M. LING, X. WANG, Y. WONG, and A. DANCHIN. 2004. Classification between normal and tumor tissues based on the pair-wise gene expression ratio. BMC Cancer **4:** 72.

YE, R. W., T. WANG, L. BEDZYK, and K. M. CROKER. 2001. Applications of DNA microarrays in microbial systems. J. Microbiol. Meth. **47:** 257-272.

Table 1. Algae cultures

Species	Strain	Culture medium	Temperature	Origin
Alexandrium minutum	AL3T	K	15 °C	Gulf of Trieste, Italy, A. Beran
Alexandrium minutum	Nantes	K	15 °C	Atlantic Ocean, France
Alexandrium tamutum	SZNB029	K	15 °C	Gulf of Naples, Italy, M. Montresor
Alexandrium ostenfeldii	AOSH 1	K	15 °C	Ship Harbour, Nova Scotia, Canada, A. Cembella
Alexandrium ostenfeldii	CCMP 1773	K	15 °C	Limfjordan, Denmark, Hansen
Alexandrium ostenfeldii	BAH ME 136	K	15 °C	Biologische Anstalt Helgoland, Germany
Prymnesium parvum f. patelliferum	PLY 527	K	15 °C	Plymouth Culture Collection, UK
Rhinomonas reticulate	PLY 358	IMR	15 °C	Plymouth Culture Collection, UK
Alexandrium catenella	BAH ME 217	IMR	15 °C	Biologische Anstalt Helgoland, Germany

Table 2. Sequences of the probes, the helper oligonucleotide H3, positive and negative control for the biosensor

Probe name	Probe sequence	Target	Source
Signal probe: AOST1	CAA CCC TTC CCA ATA GTC AGG T	*Alexandrium ostenfeldii* CCMP 1773	(Metfies et al. 2005)
Capture probe: AOST2	GAA TCA CCA AGG TTC CAA GCA G	*Alexandrium ostenfeldii* CCMP 1773	(Metfies et al. 2005)
Capture probe: LNA 65	AAT CAC CAA GGT TCAA	*Alexandrium ostenfeldii* CCMP 1773	Exiqon
Capture probe: LNA 66	AGG TTC CAA GCAG	*Alexandrium ostenfeldii* CCMP 1773	Exiqon
Capture probe: LNA 67	CCA AGG TTC CAAG	*Alexandrium ostenfeldii* CCMP 1773	Exiqon
Helper oligonucleotide: H3	GCA TAT GAC TAC TGG CAG GAT C	*Alexandrium ostenfeldii* CCMP 1773	(Metfies et al. 2005)
Test DNA (positive control	CTGC TTG GAA CCT TGG TGA TTC ACCT GAC TAT TGG GAA GGG TTG		(Metfies et al. 2005)

Table 3. Probe Sequences for the microarray

Probe name	Probe sequence	Target	Source
Euk1209	GGGCATCACAGACCTG	All Eukaryotes 18S	(Lim et al. 1993)
Chlo02	CTTCGAGCCCCCAACTTT	Chlorophytceae HE001005.53*	(Simon et al. 2000)
Boli02	TACCTAGGTACGCAAACC	Bolidophyceae HE001005.51*	(Guillou et al. 1999a)
Prym02	GGAATACGAGTGCCCCTGAC	*Prymnesium parvum* f. *patelliferum* PLY 527**	(Simon et al. 2000)
CryptoB	ACGGCCCCAACTGTCCCT	*Rhinomonas reticulata* PLY 358**	(Medlin, unpublished)
Positive control (PC)	ATGGCCGATGAGGAACGT	*S. cerevisiae*, TBP	(Metfies and Medlin 2004)
Negative control (NC)	TCCCCCGGGTATGGCCGC		(Metfies and Medlin 2004)

*Environmental clone from EU FP5- Project PICODIV, ** Plymouth Culture Collection, UK

Table 4. Mismatches of the probes to the *Alexandrium* strains in base pairs (bp)

	A. catenella BAHME217	***A .ostenfeldii BAHME136***	***A.ostenfeldii AOSH1***	***A. minutum Nantes***
Chlo02	3 bp	2 bp	2 bp	2 bp
Boli02	9 bp	8 bp	8 bp	5 bp
Prym02	5 bp	5 bp	5 bp	5 bp
CryptoB	3 bp	3 bp	3 bp	3 bp

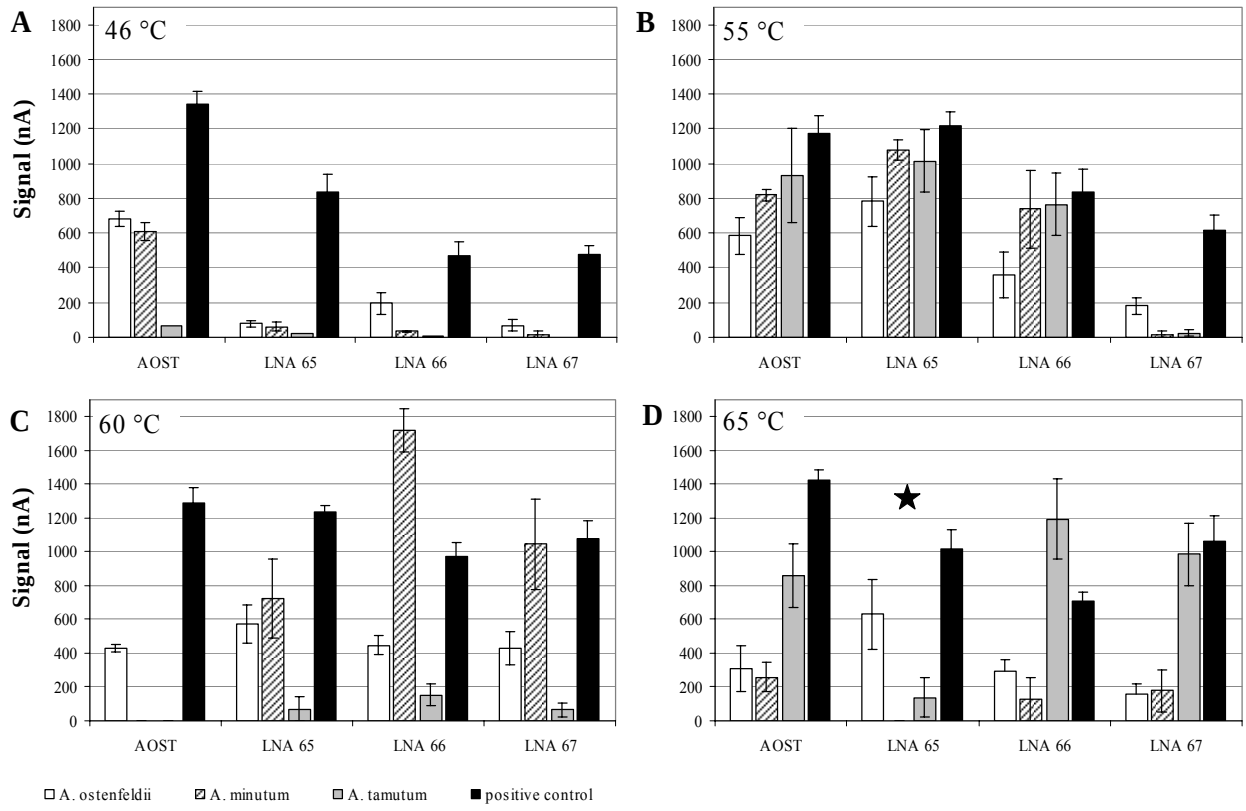

Figure 1. Signal intensity of the rRNA-biosensor. Four different probes were tested at four different hybridization temperatures and with three different species. (A) 46 °C, (B) 55 °C, (C) 60 °C, (D) 65 °C. The concentration of the rRNA for all tested species was 450 ng/µL. The asterisk marks the only specific LNA probe.

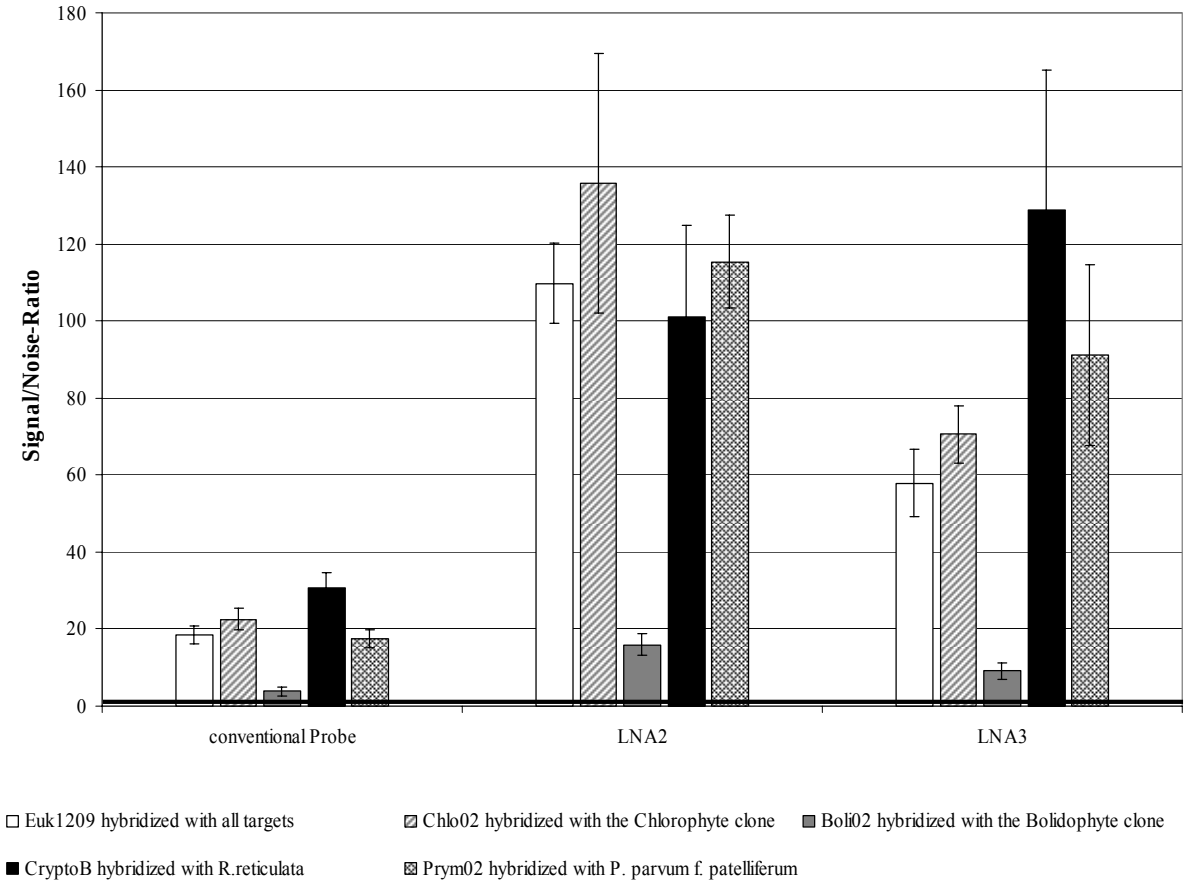

Figure 2. Signal/Noise-Ratios of all fifteen probes in comparison from hybridization with specific PCR-fragments for each set of probes. The black line represents the value of 2 for the signal-to-noise ratio, defining the threshold for a true signal.

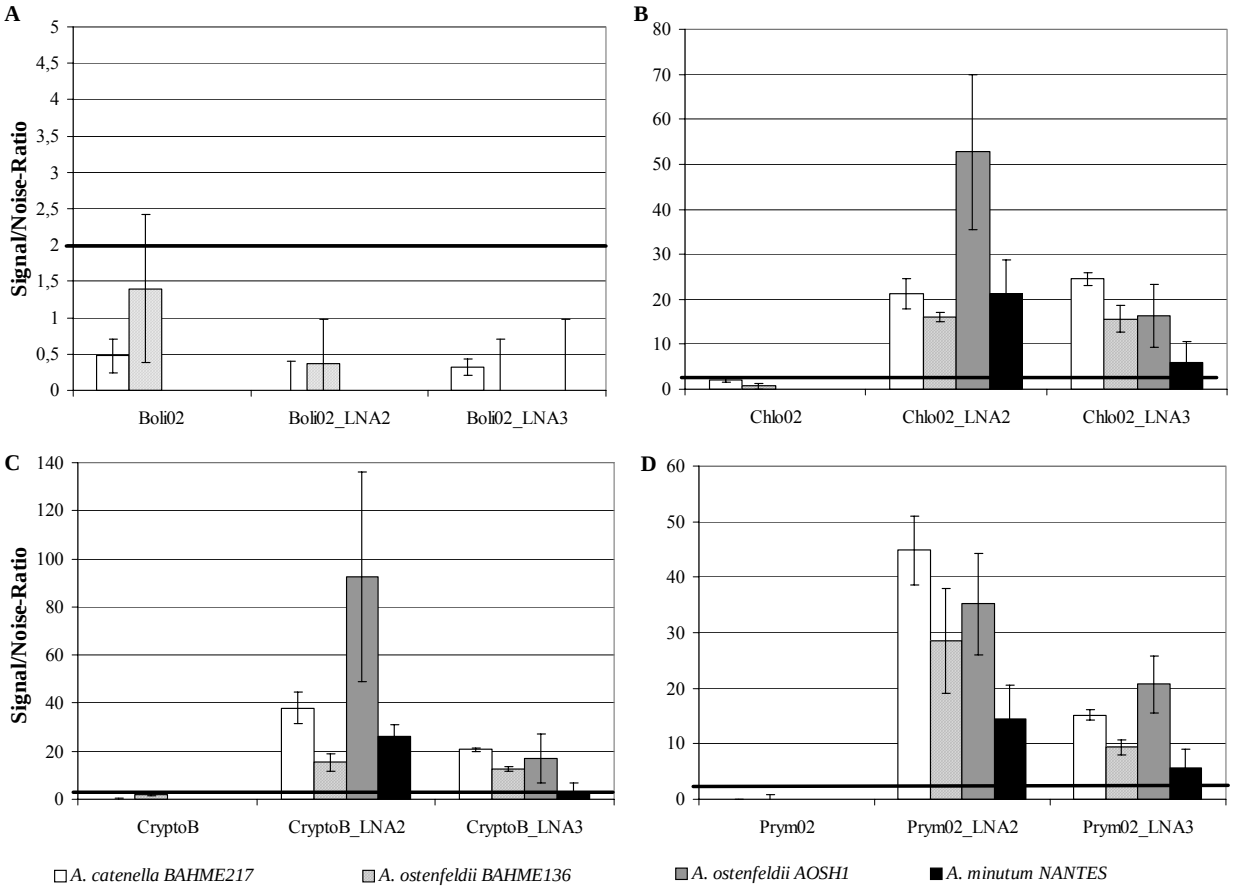

Figure 3. (A) Signal/Noise-Ratios of the set of three Boli02 probes in comparison from hybridization with unspecific PCR-fragments from the genus *Alexandrium.* (B) Signal/Noise-Ratios of the set of three Chlo02 probes in comparison with hybridization with unspecific PCR-fragments from the genus *Alexandrium.* (C) Signal/Noise-Ratios of the set of three CryptoB probes in comparison with hybridization with unspecific PCR-fragments from the genus *Alexandrium.* (D) Signal/Noise-Ratios of the set of three Prym02 probes in comparison with hybridization with unspecific PCR-fragments from the genus *Alexandrium.* The black line represents the value of 2 for the signal-to-noise ratio, defining the threshold for a true signal.

2.6 Publication IV

THE ALEX CHIP - DEVELOPMENT OF A DNA CHIP FOR IDENTIFICATION AND MONITORING OF *ALEXANDRIUM*

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Harmful Algae, to be submitted

Abstract

Harmful Algae Blooms (HABs) threaten humans, ecosystems, fishery, tourism, and aquaculture, and the occurrence of single cells in mixed phytoplankton assemblages is often difficult to detect. The genus *Alexandrium* has undergone steady taxonomic revision since its first description, and identification of its species has been confused because of overlapping morphological features and minute differences. The design of molecular probes from the 18S and 28S rDNA has shown great potential for distinguishing of species or even clades, but using these probes in a whole-cell hybridization format is tedious and time-consuming. Solid-phase methods, such as DNA microarrays, offer the potential to analyze multiple targets in a single experiment. This study describes the development of a DNA microarray for detection of several species belonging to the genus *Alexandrium*. Nine probes from other hybridization methods (fluorescence-*in-situ*-hybridization [FISH] and sandwich hybridization assay [SHA]) were tested on the microarray, and one new probe was developed for *A. minutum*. The specificity of the probes was tested by hybridization with 18S and 28S PCR-fragments from pure cultures and by analysis of spiked field samples from the Weser estuary (German Bight). A hybridization protocol was established, and the subset of the best performing probes for each species or clade was determined and recommended for classification and monitoring of field samples in the high throughput microarray format .

Introduction

Harmful Algae Blooms (HABs) have a serious impact on public health and on the economic stability in many areas (Hallegraeff 1993; Hallegraeff 2003; Hoagland et al. 2002; Van Dolah 2000). They threaten humans, complex ecosystems, and important economic areas with fishery, tourism, and aquaculture. Their frequency, intensity, duration, and geographic distribution seem to have increased in the last decades (Godhe 2002; Hallegraeff 2002; Scholin et al. 1994). HABs can introduce several illnesses, and one of the worst is Paralytic Shellfish Poisoning (PSP), which is caused by a group of neurotoxins, mainly saxitoxins (Hallegraeff 1993). When contaminated fish or shellfish are ingested, these neurotoxins block the neural sodium channels in the human body (Taylor and Fukuyo 1998). Some of the most important and thoroughly investigated PSP toxin producers can be found within the dinoflagellate genus *Alexandrium* (Balech 1995; Cembella 1998; Taylor and Fukuyo 1998), although some non-toxic species are also present (Janson and Hayes 2006).

The differentiation of *Alexandrium* species is difficult and tedious, because it mainly depends on minute morphological characteristics, e.g., fine thecal tabulation, chain formation, and cell shape (Balech 1995). Furthermore, the different taxonomic patterns can vary with environmental conditions, and also morphological intermediate forms have been observed (Cembella and Taylor 1985; Hallegraeff 2003; Hosoi-Tanabe and Sako 2005; John et al. 2005). Therefore, the exact species determination requires time and taxonomic expertise. The three species *A. tamarense* (Lebour) Balech, *A. catenella* (Whedon and Kofoid) Balech, and *A. fundyense* Balech are particularly demanding to differentiate because they are separated mainly by the presence or absence of a ventral pore and colony formation. They also share overlapping thecal characteristics. It has been shown that the strains of these species are related by geographic origin rather than by morphology and therefore, they are often referred to as the *Alexandrium tamarense* "species complex" (Cembella et al. 1987; Cembella et al. 1988; Medlin et al. 1998; Scholin et al. 1995). The phylogeny of this species complex has been studied intensively (Adachi et al. 1996; Higman et al. 2001; Medlin et al. 1998; Scholin et al. 1994) and the geographic areas correspond to six different genetically distinct "ribotypes", based on the D1/D2 region of the 28S rRNA gene. The three toxic ribotypes are: the North American (NA), the Temperate Asian (TA) and the Tropic Asian (TROP) (John et al. 2005); members of the NA clade also have been found in Asian waters (Scholin et al. 1994), at the Orkney Islands (Medlin et al. 1998) and in South America (Persich et al. 2006). The non-toxic strains are: the Tasmanian (TASM), the Western European (WE) (Scholin et al. 1995) and the recently described Mediterranean (ME) clade (John et al. 2003b). The most

characteristic feature for identification of *Alexandrium ostenfeldii* (Paulsen) Balech & Tangen is the shape of its first apical plate and its large ventral pore (Balech 1995). It is the least toxic of the toxic species in *Alexandrium* (Cembella et al. 1987; Cembella et al. 1988; Hansen et al. 1992), but highly toxic strains have also been found, and they produced both PSP toxins (MacKenzie et al. 1996) and spirolides (Cembella et al. 2001; Cembella et al. 2000; Hallegraeff 2002). For the identification of *Alexandrium minutum* Halim, minimal details of the apical tabulation (Hallegraeff 2002; Taylor et al. 1995) and the characteristic ventral pore (Faust and Gulledge 2002) are used, but also strains without ventral pores have been reported (Taylor et al. 1995; Vila et al. 2005). *A. minutum* also produces PSP toxins and other toxins (Chen and Chou 2002; Nascimento et al. 2005; Taylor and Fukuyo 1998).

All these toxic *Alexandrium* species are distributed world-wide, mainly in coastal areas (Balech 1995; Faust and Gulledge 2002; Hallegraeff 2002; Lundholm and Moestrup 2006; Taylor et al. 1995) and co-occur with non-toxic species. Therefore a reliable detection of these species is highly desirable. Traditional microscope based techniques are tedious and time-consuming, but molecular methods, especially the utilization of molecular probes, have shown great potential for *Alexandrium* species identification. One great advantage is that they are based on genetic features rather than on morphological characteristics (Anderson et al. 2005; John et al. 2003a; John et al. 2005; Metfies et al. 2005). A further promising molecular approach has been presented by DNA microarrays, which are applied generally for gene expression (Schena et al. 1995; Schena et al. 1996), but have also been used with oligonulceotide probes of conserved genes for species identification at all taxonomic levels (Ki and Han 2006; Loy et al. 2005; Medlin et al. 2006a; Metfies and Medlin 2004; Peplies et al. 2006). The technique is based on a minimized, but high throughput form of a dot-blot through application of sequences or probes in an ordered array on the chip. The chip is made of glass and has special surface properties. The microarray offers the potential to facilitate the analysis of multiple targets from one sample in a one experiment (DeRisi et al. 1997; Gentry et al. 2006; Lockhart et al. 1996; Lockhart and Winzeler 2000; Metfies et al. 2006; Schena et al. 1995; Ye et al. 2001). A combination of molecular probes and DNA microarrays could serve as a rapid and reliable tool for detection of toxic microalgae and is not affected by environmental conditions or cell physiology.

This study evaluated and assessed the application of previously published probes to a DNA microarray. The probes target species of the genus *Alexandrium* and are specific in other methods. We developed a microarray (ALEX CHIP) to detect species of the genus

Alexandrium in pure cultures and field samples spiked with *Alexandrium* cells. The experiments showed that the microarray is a valid tool for monitoring of toxic microalgae.

Materials and Methods

Culture conditions – The algal strains listed in Table 1 were cultivated under sterile conditions in seawater-based F2-media for *A. fundyense* (Guillard and Ryther 1962), IMR-media for *A. tamarense* GTLI21, BAHME182, SZNB01, 08, 19 and 21 (Eppley et al. 1967) and K-media for all other species (Keller et al. 1987) at 15 °C and with an irradiance of 150 – 200 µEinsteins and a light: dark cycle of 14:10 hours.

Spiked field samples – We prepared simulated field samples with a natural phytoplankton background from the river Weser in North Germany and different cultures of *Alexandrium* to prove the specificity of the probes on the ALEX CHIP. For this purpose, aliquots of the cultures *A. ostenfeldii* CCMP1773, *A. minutum* AL3T, *A. tamarense* CA 28, 31/9, and SZNB01 were used. Cell densities were determined with a Multisizer 3 Coulter Counter (Beckman Coulter GmbH Diagnostics, Krefeld, Germany). Each culture aliquot contained 100,000 cells, except for the SZNB01, where 60,000 cells were taken. The water sample taken from the estuary of the Weser River with a bucket on the 18.12.2006 was pre-filtered twice with a 180 µm and a 10 µm polycarbonate filter before being finally collected onto a 5 µm polycarbonate filter (Millipore, Billerica, USA). The simulated field sample contained 500 mL of the pre-filtered water and cells of one *Alexandrium* culture. For each strain, triplicate spiked samples were prepared.

DNA extraction – DNA was extracted from the pure cultures and spiked field samples with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with one minor modification. The elution with buffer AE was repeated once with the same eluate. Concentration of the extracted DNA was determined with a Nanodrop Spectrophotometer (Peqlab, Erlangen, Germany).

PCR Amplification of rDNA-genes – PCR products were used to test probe specificity on the microarray. PCR amplification was performed for the entire 18S gene with the universal specific primer-pair 1F-1528R without the polylinkers (Medlin et al. 1988). For the D1/D2 region of the 28S rDNA gene, the primer-pair DIR1F-DIR2CR from Scholin et al. (1994) was used. The PCR was carried out in a Mastercycler (Eppendorf, Hamburg,

Germany) and protocols described by Medlin et al. (1988) and Scholin et al. (1994) were used. The positive control for the hybridization procedure, a 250 bp fragment of the TATA-box binding protein-gene (TBP) of *Saccharomyces cerevisiae* was produced with the specific yeast primers TBP-F (5'-ATG GCC GAT GAG GAA CGT TTA A-3') and TBP-R-Biotin (5'-TTT TCA GAT CTA ACC TGC ACC C- 3'). The reaction was accomplished as follows 5 min 94 °C, 1 min 94 °C, 1 min 52 °C, 1 min 72 °C, for 35 cycles and for 10 min at 72 °C extension. Amplification success was verified by agarose gel electrophoresis, and the PCR-fragments were purified with the QIAquick PCR purification Kit (Qiagen, Hilden, Germany) according to the manufactures instructions with one modification. An improvement in the yield was achieved as follows: step 8 (elution with elution buffer EB) was repeated once with the same eluate. Concentration of amplified fragments was measured with a Nanodrop Spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany). One additional probe, which has no positive match in GenBank (Altschul et al. 1990), was used as a negative control to measure unspecific binding.

Labeling of PCR-fragments – For hybridization, the purified PCR-fragments were labeled with biotin using the DecaLabel DNA Labeling Kit (Fementas, St. Leon-Rot, Germany). The incorporated biotin can bind to Streptavidin-CY5 and this allows the fluorescent detection of hybridized PCR-fragments. One labeling reaction contained 200 ng of PCR-fragments and was incubated at 37 °C overnight (17 to 20 hours) to achieve the best biotin incorporation. Labeled PCR-fragments were purified with the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) with protocol modifications to enhance the yield of the PCR-fragments as described above. Concentration of obtained labeled fragments was measured as described above.

Probe synthesis – Probes used in this study were synthesized from Thermo Electron Corporation (Ulm, Germany) with a C6/MMT aminolink at the 5'-end of the molecule (Table 2).

Microarray production – We used epoxy-coated slides (Nexterion Slide E from Peqlab, Erlangen, Germany) to produce the ALEX CHIP. All probes were diluted to a final concentration of 1 µM in 3x saline sodium citrate buffer and were printed onto the slides. A VersArray ChipWriter Pro (Bio-Rad Laboratories GmbH, München, Germany) and split pins (Point Technologies, Inc., Colorado, USA) were utilized. All chips contained four replicates

of each probe in four individual arrays. The cross-linking of probes with the amino group on the slide took place in an immobilization reaction at 60 °C for 30 min Subsequently the manufactured chips were stored at -20 °C.

Standard hybridization protocol – The hybridization solution was composed of 1x hybridization buffer (1 M NaCl, 10 mM Tris, pH 8, 0.005 % Triton X-100, 1 mg/mL BSA, 0.1 µg/µL HS-DNA), the biotin-labeled PCR-fragments in a final concentration of 11.25 ng DNA/µL, and the 250 bp PCR-fragment TBP from *S. cerevisiae* with biotin-labeled primers in a final concentration of 4.7 ng DNA/µL as a positive control. The background of the chips was blocked with 1x hybridization buffer without HS-DNA at 58 °C for 1 hour in a slide box. Afterwards, the slides were centrifuged, and the DNA was denatured by heating at 94 °C for 5 min. For hybridization on the glass slide, we used a special kind of cover slip, the Lifter Slip (Implen, München, Germany). It has two printed bars at the edges to prevent slippage on the slide and to provide a defined volume on top of the slide. 30 µL of the denatured hybridization solution were pipetted under the pre-positioned cover slip, and an even dispersal of hybridization solution between chip and cover slip was achieved through capillary action. The slide was placed in a humid chamber, which was constructed from a 50 mL Sarstedt tube filled with tissues moistened with hybridization solution. The hybridization reaction lasted at 58 °C in a Shake 'n' Stack hybridization oven (Thermo Hybaid, Ulm, Germany) for one hour. The slide was washed 15 min with 2x saline sodium citrate (SSC) buffer (10 mM EDTA, 0.05 % SDS), then with 1x SSC (10 mM EDTA) for 15 min and thereafter for 5 min with 0.2x SSC (10 mM EDTA) to remove unspecific binding. Hybridizations for the specificity tests were repeated twice. Field samples were initially prepared in triplicate, and hybridization analyses were conducted in duplicates for each of the three samples.

Staining – Detection of the hybridized PCR-fragments was accomplished via indirect staining of the incorporated biotin molecules in the PCR-fragments. A Lifter Slip was placed onto the slide and Streptavidin-CY5 (Amersham Biosciences, Freiburg, Germany) at a final concentration of 100 ng/mL in hybridization buffer was pippetted under the cover slip. The slide was placed in the humid chamber again. The staining took place at room temperature for 30 min. For removal of excessive staining molecules, the washing protocol was performed twice with 2x SSC for 5 min and once for 5 min with 1x SSC.

Scanning and quantification of microarrays – The chip was scanned with a GenePix 4000B scanner (Molecular Devices Cooperation, Sunnyvale USA) to obtain fluorescent signals, which were processed with the GenePix 6.0 software (Molecular Devices Coperation, Sunnyvale USA). The signal-to-noise-ratios were calculated using a formula from Loy et al. (2002). Afterwards, the target signal was normalized to the signal of TBP positive control, and finally the mean value of all replicates from one culture or sample was calculated. A signal of 2 was defined as the threshold for a true signal.

Probe set and experimental set-up – In this study, we evaluated previously published probes that showed positive results in other hybridization methods. Their performance in a microarray hybridization approach should be tested. For *Alexandrium minutum,* one new probe was developed by M. Nölte and Hannes Weber (ZeTeM / University of Bremen) with a self-coded program, which performed a heuristic alignment with thermodynamic parameters. The secondary and homodimere structure was determined based on the Vienna RNA Package or on a program from the Vienna RNA Package (Hofacker et al. 1994). Tm was calculated with the Nearest-Neighbor-Interactions from the "Unified Model" (Santa Lucia 1998) or optional as a set of thermodynamic parameters. The probe ALEXMIN1 targets four out of five strains, which are placed in GenBank (see Table 2 for accession numbers). The identity of the fifth entry is questionable and did not match the others.

Results

Specificity of 18S rDNA probes – Two probes for the division Dinophyta, probe DinoB and DinoE-12, were evaluated and all *Alexandrium* 18S PCR-fragments used in this study were target organisms for these probes. Both probes showed specific signals for all species examined (Fig. 1 and 2). The DinoE-12 signals were significantly higher than those obtained with probe DinoB. The specificity of the three AOST probes for *Alexandrium ostenfeldii,* were tested with two different *A. ostenfeldii* strains (Fig. 1 and 2). We observed reduced signals with DNA from strain *A. ostenfeldii* CCMP 1773 as compared to strain *A. ostenfeldii* K0324. But DNA from strain CCMP 1773 produced entirely different signals when used in the spiked field samples (Fig. 2). No cross-hybridization with PCR-fragments from any other closely related species was detected for these three probes. The probe ALEXMIN1 for *Alexandrium minutum* was tested with two target strains, *A. minutum* BAHME91 and AL3T. Strain AL3T also showed reduced signals as compared to signals from BAHME91. An enhancement of signals was also observed in results from the field samples spiked with *A.*

minutum AL3T (Fig. 2). No unspecific binding was observed with other species for this probe. Furthermore, the same enhancement of probe signals was shown for both Dino probes hybridized with PCR-fragments of the pure cultures and the spiked field samples of *A. ostenfeldii* CCMP 1773 and *A. minutum* AL3T (Fig. 2).

Specificity of 28S rDNA probes – Probes for the *A. tamarense* "species complex" originated from the 28S rDNA and showed overall strong specific hybridization results. No probe gave unspecific cross-reactions, either with the closely related *A. ostenfeldii* PCR-fragments or with PCR-fragments from the other *A. tamarense* clades that were examined in this study. The probes ATNA01 and ATNA02 (North American Clade) were specific with high signal-to-noise-ratios for all three target strains (Fig. 3). The first probe published for the North American Clade, ATNA01 (Miller and Scholin 1998) can produce a hairpin fold that could prevent correct binding under certain conditions (John et al. 2005). Therefore, the probe was redesigned as ATNA02 by shifting the initial probe sequence five bases upstream. The shifted probe, ATNA02, showed slightly better signals in the microarray hybridization, nevertheless, both performed satisfactorily. The culture *A. tamarense* 31/9 showed hybridization signals for the ATNA01, ATNA02 and the ATWE03 probe. Cells of both clades must be present in the culture, i.e., the original culture was not clonal. Hybridization with three strains of the Western European Clade resulted in excellent signals for the ATWE03 probe, showing great discriminative potential and sensitivity with signal-to-noise-ratios ranging from 124-252. The same can be reported for the signals for probe ATME04 evaluated with four strains of the Mediterranean Clade, which performed with values from 80-190 (Fig. 3).

Spiked field samples and probe specificity – We spiked field samples with cells from pure *Alexandrium* cultures and then extracted DNA. PCR-fragments were amplified from these DNA extracts and applied to the microarray. In Figure 2, the hybridization results of two different pure cultures of *Alexandrium* spp. (*A. ostenfeldii* CCMP 1773, *A. minutum* AL3T) and of spiked field samples are shown. Whereas PCR-fragments from pure laboratory cultures showed low signals when hybridized with the target probe, the same cells produced greatly enhanced signals when they were added to the field material, amplified and hybridized.

The results of the spiked field samples for the 28S probes supported the specificity tests with pure cultures of target species. As expected, strains *A. fundyense* CA28 gave only

signals for the two probes ATNA01 and ATNA02 and the same was found for the *A. tamarense* SZNB01 strain and probe ATME04. There was a cross-hybridization detected with the natural phytoplankton background, but signals of the target species were clearly distinguishable from non-specific bindings. As shown in the specificity tests, the culture *A. tamarense* 31/9 was not clonal and contained cells from the North American Clade and the Western European Clade. Members of both clades naturally occur in the strain origin (Ireland). This result was repeated with the spiked field samples as well and represents an example of detection of two ribotypes from the *A. tamarense* "species complex", co-occurring in one bloom. However, in the field samples the Western European Clade seems to dominate the culture, whereas in the specificity tests, the probes for the North American Clade gave higher signals. This is likely resulted from DNA extractions at two different dates, and in the meantime one strain might have outgrown the other.

All probes were hybridized with all PCR-fragments (data not shown) and the 18S probes were negative with the 28S PCR-fragments of the pure cultures and the field samples and vice versa. The published SHA and FISH probes AMINC (18S) for *A. minutum* (Diercks et al. 2007), AOST01 (28S) for *A. ostenfeldii* (John et al. 2003a) and ATMA01 (18S) for the whole *A. tamarense* "species complex" (John et al. 2005) did not work at all in a microarray format (Fig. 5). All hybridization signals were clearly below the threshold of 2. Therefore we would not recommend these three probes for a DNA chip to detect species from the genus *Alexandrium*.

Discussion

In this study, we tested and evaluated the applicability of *Alexandrium* species or clade specific probes from other methods in a microarray hybridization format. Specificity and sensitivity of the probes were tested under comparable conditions (i.e., same hybridization and washing conditions for all probes and microarrays). Amplicons of 18S and 28S rDNA genes from pure cultures of target and closely related species were tested. *In silico* prediction of probe properties is not always transferable to real experimental conditions, because many factors influence the stability of probe-target hybridization (Graves 1999; Loy et al. 2005). Specificity and sensitivity of probes from other methods e.g., FISH or SHA can not be transferred to the chip format because target nucleic acids may form secondary structures in the microarray hybridization. It has been observed that not all probes working in dot-blot, SHA, and FISH formats will work in a microarray format (Medlin et al. 2006b).

One of the major drawbacks for the examination of environmental samples with this approach is a possible cross-reaction or unspecific binding. This could originate from unknown organisms in environmental samples, because the probes are only designed from known sequences. Only species with a probe on the chip can be detected and those without a probe will be overlooked (Gentry et al. 2006). The development of a functional chip is time-consuming, expensive, and all probes on one chip should work specifically under the same hybridization conditions (Boireau et al. 2005; Feriotto et al. 2002; Metfies et al. 2006).

The stringent conditions of the microarray support the analysis of hybridizations to many different probes in one single experiment. Unfortunately, they also support the occurrence of false-positives, because the stability of mismatched probe-target hybrids is influenced by many factors, such as the number or the position of mismatches (Fotin et al. 1998; Loy et al. 2002; Urakawa et al. 2003). We observed weak cross-hybridizations of the AOST probes with the two *A. minutum* strains; this was previously shown by Metfies et al. (2005). Nevertheless, in this case a signal for either one or the other species in a sample would lead to the same precautions, because they are both toxic. Although all 18S probes showed weak signals in hybridization with PCR-fragments of one or two pure cultures, the real proof of their specificity was shown in their consistent results in the spiked field samples. In this experiment, the signals for DNA from spiked field samples were higher than the signals achieved with pure cultures. One possible explanation might be that substances in the sample (e.g., other DNA) could have prevented secondary structure formation of the PCR-fragments. Under these circumstances, more PCR-fragments might have been allowed to hybridize to their target probe.

For the utilization of the microarray to reliable identify several toxic and non-toxic species of the genus *Alexandrium*, we recommend the following probes: DinoE-12 over DinoB because of improved signals in the specificity tests and proven potential for all strains in the field samples. Of the three probes available for *A. ostenfeldii,* we recommend the probe AOST1, which showed better signals in the specificity tests than in the field samples. The second probe, AOST2, has only one mismatch to the recently new described non-toxic species *A. tamutum* (Montresor et al. 2004) and is therefore unusable. The third probe AOST02 worked reliably; however the ratios were significantly lower than for probe AOST1. For differentiation of clades within the *A. tamarense* "species complex" both probes for the North American Clade performed with high discrimination power, but the ATNA02 gave slightly better signals. With this probe, the potential hairpin formation that may hamper correct binding in probe ATNA01 is avoided (John et al. 2005).

We are well aware that the utilization of PCR introduces a bias to field samples with regard to species abundance (Hansen et al. 1998; Kanagawa 2003; Polz and Cavanaugh 1998; Reysenbach et al. 1992). The results may show a distorted view of true community composition (Medlin et al. 2006a). The PCR step can be avoided if total RNA is directly labeled. But RNA extractions from field samples often show low values (Peplies et al. 2006) and the content per cell may vary under different environmental conditions. However, experiments with eukaryotic cells show that they do not always loose their rRNA when they become senescent (Scholin et al. 1999) as is regularly seen in prokaryotes. Nevertheless, for a quantitative detection of HAB species, it would be a great advantage to link hybridization signals to cell densities, which can only be achieved with a calibration curve. This procedure is tedious and time-consuming, because every probe on the chip needs an individual curve to convert the signal to cell counts. For a quantitative detection and a reliable analysis of field samples, the correlation of cell counts and RNA concentration per cell with signal intensity will be a prerequisite.

The utilization of the ALEX CHIP could improve the detection of members of this important group in a many ways. First of all, it could be used for regulatory monitoring on close spatial and temporal scales to track the occurrence and abundance of species and strains. A bloom is often not composed of a single species and therefore toxic and non-toxic microalgae species co-occur in field samples. Separation can be difficult, and by using the chip, more than one species can be detected with a single experiment. Another benefit is the possible tracing of species introduction from other regions, which might happen through resting cysts in ballast water (Bolch and De Salas 2007; Lilly et al. 2002), especially in the geographical clades of the *A. tamarense* "species complex".

This small chip with six probes from the genus *Alexandrium* represents a prototype, which can be improved in many ways. The application of probes at different taxonomic levels will allow the validation of one probe signal at different hierarchical levels (Metfies et al. 2006). The first step with a probe at a higher taxonomic level was done with Dino probes on the ALEX CHIP. The chip could be expanded by evaluation of published probes for *Alexandrium* (Hosoi-Tanabe and Sako 2005; Ki and Han 2006; Kim and Sako 2005; Sako et al. 2004) or other HAB species (Scholin et al. 1997; Töbe et al. 2006; Tyrrell et al. 2002).

Conclusions

It is important to detect and monitor HAB species, especially with promising molecular methods. The specificity, sensitively, and reliability of the ALEX CHIP showed that

this hybridization approach can significantly contribute to the detection and classification of *Alexandrium* species. This chip contains probes for three clades of the *A. tamarense* "species complex" and two additional species, *A. minutum* and *A. ostenfeldii*. Probes from other methods have been adapted successfully. Sensitive monitoring of field populations of toxic and non-toxic *Alexandrium* is possible without taxonomic expertise in a high throughput format. The application of further probes is desired to improve the scope of the ALEX CHIP.

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References

ADACHI, M., Y. SAKO, and Y. ISHIDA. 1996. Analysis of *Alexandrium* (Dinophyceae) species using sequences of the 5.8S ribsomal DNA and internal transcribed spacer regions. J. Phycol. **32:** 424-432.

ALTSCHUL, S. F., W. GISH, W. MILLER, E. W. MYERS, and D. J. LIPMAN. 1990. Basic local alingment search tool. J. Mol. Biol. **215:** 403–410.

ANDERSON, D. M., D. M. KULIS, B. A. KEAFER, K. E. GRIBBLE, R. MARIN, and C. A. SCHOLIN. 2005. Identification and enumeration of *Alexandrium* spp. from the Gulf of Maine using molecular probes. Deep Sea Res. II **52:** 2467-2490.

BALECH, E. 1995. The genus *Alexandrium* Halim (Dinoflagellata). Sherkin Island Marine Station, Ireland.

BOIREAU, W., J. C. ZEEHA, P. E. PUIGA, and D. POMPON. 2005. Unique supramolecular assembly of a redox protein with nucleic acids onto hybrid bilayer: towards a dynamic DNA chip. Biosens. Bioelectron. **20:** 1631-1637.

BOLCH, C. J. S., and M. F. DE SALAS. 2007. A review of the molecular evidence for ballast water introduction of the toxic dinoflagellates *Gymnodinium catenatum* and the *Alexandrium* "*tamarensis* complex" to Australasia. Harmful Algae **6:** 465-485.

CEMBELLA, A. D. 1998. Ecophysiology and metabolism of paralytic shellfish toxins in marine microalgae, p. 381-404. *In* D. M. Anderson, A. D. Cembella and G. M. Hallegraeff [eds.], Physiological Ecology of Harmful Algal Blooms. Springer Verlag, Berlin, Heidelberg, Germany.

CEMBELLA, A. D., A. G. BAUDER, N. I. LEWIS, and M. A. QUILLIAM. 2001. Association of the gonyaulacoid dinoflagellate *Alexandrium ostenfeldii* with spirolide toxins in size-fractionated plankton. J. Plankton Res. **23:** 413 -419.

CEMBELLA, A. D., N. I. LEWIS, and M. A. QUILLIAM. 2000. The marine dinoflagellate *Alexandrium ostenfeldii* (Dinophyceae) as the causative organism of spirolide shellfish toxins. Phycologia **39:** 67-74.

CEMBELLA, A. D., J. J. SULLIVAN, G. L. BOYER, F. J. R. TAYLOR, and R. J. ANDERSON. 1987. Variation in paralytic shellfish toxin composition within the *Protogonyaulax tamarensis/catenella* species complex, red tide Dinoflagellates. Biochem. Syst. Ecol. **15:** 171-186.

CEMBELLA, A. D., and F. J. R. TAYLOR. 1985. Biochemical variability within the *Protogonyaulax tamarensis catenella* species complex, p. 55-60. *In* D. M. Anderson,

A. W. White and D. G. Baden [eds.], Toxic Dinoflagellates. Elsevier, New York, USA.

CEMBELLA, A. D., J.-C. THERRIAULT, and P. BERLAND. 1988. Toxicity of cultured isolates and natural populations of *Protogonyaulax tamarensis* from the St. Lawrence estuary. J. Shellfish Res. **7:** 611-621.

CHEN, C. Y., and H. N. CHOU. 2002. A modified high-performance liquid chromatography method for analysis of PSP toxins in dinoflagellate, *Alexandrium minutum*, and shellfish from Taiwan. Food Res. lnt. **35:** 715-720.

DERISI, J. L., V. R. IYER, and P. O. BROWN. 1997. Exploring the metabolic and genetic control of gene expression on a genomic scale. Science **278:** 680-686.

DIERCKS, S., L. K. MEDLIN, and K. METFIES. 2007. Colorimetric detection of the toxic dinoflagellate *Alexandrium minutum* using sandwich hybridization in a microtiter plate assay. Harmful Algae, accepted.

EPPLEY, R. W., R. W. HOLMES, and J. D. H. STRICKLAND. 1967. Sinking rates of marine phytoplankton measured with a fluorometer. J. Exp. Mar. Biol. Ecol. **1:** 191-208.

FAUST, M. A., and R. A. GULLEDGE. 2002. Identifying harmful marine dinoflagellates. Smithsonian Institution, Washington, USA.

FERIOTTO, G., M. BORGATTI, C. MISCHIATI, N. BIANCHI, and R. GAMBARI. 2002. Biosensor technology and surface plasmon resonance for real-time detection of genetically modified roundup ready soybean gene sequences. J. Agric. Food Chem. **50:** 955-962.

FOTIN, A. V., A. L. DROBYSHEV, D. Y. PROUDNIKOV, A. N. PEROV, and A. D. MIRZABEKOV. 1998. Parallel thermodynamic analysis of duplexes on oligodeoxyribonucleotide microchips. Nucleic Acids Res. **26:** 1515–1521.

GENTRY, T. J., G. S. WICKHAM, C. W. SCHADT, Z. HE, and J. ZHOU. 2006. Microarray applications in microbial ecology research. Microbial Ecol. **52:** 159-175.

GODHE, A. 2002. Benthic and pelagic Dinoflagellate stages: environmental settings, cyst vaibility, and molecular idetification, Department of Marine Ecology, Marine Botany. Phd Thesis, Göteborg University, Sweden.

GRAVES, D. J. 1999. Powerful tools for genetic analysis come of age. Trends Biotechnol. **17:** 127-134.

GUILLARD, R. R. L., and J. H. RYTHER. 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* Cleve. Can. J. Microbiol. **8:** 229-239.

HALLEGRAEFF, G. M. 1993. A review of harmful algal blooms and their apparent global increase. Phycologia **32:** 79–99.

---. 2002. Aquaculturists' guide to harmful Australian microalgae. School of Plant Science, Hobart, Australia.

---. 2003. Harmful algal blooms: a global overview, p. 25-49. *In* G. M. Hallegraeff, D. M. Anderson and A. D. Cembella [eds.], Manual on Harmful Marine Microalgae. United Nations Educational, Scientific and Cultural Organization, Paris, France.

HANSEN, M. C., T. TOLKER-NIELSEN, M. GIVSKOV, and S. MOLIN. 1998. Biased 16S rDNA PCR amplification caused by interference from DNA flanking the template region. Microbiol. Ecol **26:** 141-149.

HANSEN, P. J., A. D. CEMBELLA, and Ø. MOESTRUP. 1992. The marine dinoflagellate *Alexandrium ostenfeldii*: paralytic shellfish toxin concentration, composition, and toxicity to a tintinnid ciliate. J. Phycol. **28:** 597-603.

HIGMAN, W. A., D. M. STONE, and J. R. LEWIS. 2001. Sequence comparisons of toxic and non-toxic *Alexandrium tamarense* isolates from UK waters. Phycologia **40:** 256-262.

HOAGLAND, P., D. M. ANDERSON, Y. KAORU, and A. W. WHITE. 2002. The economic effects of harmful algal blooms in the United States: estimates, assessment issues, and information needs. Estuaries **25:** 819–837.

HOFACKER, I. L., W. FONTANA, P. F. STADLER, S. BONHOEFFER, M. TACKER, and P. SCHUSTER. 1994. Fast folding and comparison of RNA secondary structures. Monatsh. Chem. **125:** 167-188.

HOSOI-TANABE, S., and Y. SAKO. 2005. Rapid detection of natural cells of *Alexandrium tamarense* and *A. catenella* (Dinophyceae) by fluorescence-*in-situ*-hybridization. Harmful Algae **4:** 319-328.

JANSON, S., and P. K. HAYES. 2006. Molecular taxonomy of Harmful Algae, p. 9-21. *In* E. Granéli and J. T. Turner [eds.], Ecology of Harmful Algae. Ecological Studies. Springer Verlag, Berlin, Heidelberg, Germany.

JOHN, U., A. CEMBELLA, C. HUMMERT, M. ELBRÄCHTER, R. GROBEN, and L. K. MEDLIN. 2003a. Discrimination of the toxigenic dinoflagellates *Alexandrium tamarense* and *A. ostenfeldii* in co-occurring natural populations from Scottish coastal waters. Eur. J. Phycol. **38:** 25-40.

JOHN, U., R. A. FENSOME, and L. K. MEDLIN. 2003b. The application of a molecular clock based on molecular sequences and the fossil record to explain biogeographic

distributions within the *Alexandrium tamarense* 'species complex' (Dinophyceae). Mol. Biol. Evol. **20:** 1015-1027.

JOHN, U., L. K. MEDLIN, and R. GROBEN. 2005. Development of specific rRNA probes to distinguish between geographic clades of the *Alexandrium tamarense* species complex. J. Plankton Res. **27:** 199–204.

KANAGAWA, T. 2003. Bias and artifacts in multitemplate polymerase chain reactions (PCR). J. Biosci. Bioeng. **96:** 317-323.

KELLER, M. D., R. C. SELVIN, W. CLAUS, and R. R. L. GUILLARD. 1987. Media for the culture of oceanic ultraphytoplankton. J. Phycol. **23:** 633-338.

KI, J.-S., and M.-S. HAN. 2006. A low-density oligonucleotide array study for parallel detection of harmful algal species using hybridization of consensus PCR products of LSU rDNA D2 domain. Biosens. Bioelectron. **21:** 1812-1821.

KIM, C.-J., and Y. SAKO. 2005. Molecular identification of toxic *Alexandrium tamiyavanichii* (Dinophyceae) using two DNA probes. Harmful Algae **4:** 984-991.

LILLY, E. L., D. M. KULIS, P. GENTIEN, and D. M. ANDERSON. 2002. Paralytic shellfish poisoning toxins in France linked to a human-introduced strain of *Alexandrium catenella* from the Western Pacific: evidence from DNA and toxin analysis. J. Plankton Res. **24:** 443-452.

LOCKHART, D. J. and others 1996. Expression monitoring by hybridization to high-density oligonucleotide arrays. Nat. Biotech. **14:** 1675-1680.

LOCKHART, D. J., and E. A. WINZELER. 2000. Genomics, gene expression and DNA arrays. Nature **405:** 827-836.

LOY, A. and others 2002. Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Appl. Environ. Microbiol. **68:** 5064-5081.

---. 2005. 16S rRNA gene-based oligonucletide microarray for environmental monitoring of the betaproteobacterial order "Rhodocyclales". Appl. Environ. Microbiol. **71:** 1373-1386.

LUNDHOLM, N., and Ø. MOESTRUP. 2006. The biogeography of Harmful Algae, p. 23-35. *In* E. Granéli and J. T. Turner [eds.], Ecology of Harmful Algae. Springer Verlag, Berlin, Heidelberg, Germany.

MACKENZIE, L., D. WHITE, Y. OSHIMA, and J. KAPA. 1996. The resting cyst and toxicity of *Alexandrium ostenfeldii* (Dinophyceae) in New Zealand. Phycologia **35:** 148-155.

MEDLIN, L. K., H. J. ELWOOD, S. STICKEL, and M. L. SOGIN. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene **71:** 491-499.

MEDLIN, L. K., M. LANGE, and U. WELLBROCK. 1998. Sequences comparison links to Europena isolates of *Alexandrium tamarense* from the Orkney Islandsto toxic North American stocks. Eur. J. Protistol. **34:** 329-335.

MEDLIN, L. K., K. METFIES, H. MEHL, K. H. WILTSHIRE, and K. VALENTIN. 2006a. Picoeukaryotic plankton diversity at the Helgoland Time Series Site as assessed by three molecular methods. Microbial Ecol. **52:** 53-71.

MEDLIN, L. K., K. VALENTIN, K. METFIES, K. TÖBE, and R. GROBEN. 2006b. Molecular tools in the study of marine microbial diversity. Encyclopedia of Life Support Systems (EOLSS), Second edition, http://www.eolss.net., Oxford, UK.

METFIES, K., S. HULJIC, M. LANGE, and L. K. MEDLIN. 2005. Electrochemical detection of the toxic dinoflagellate *Alexandrium ostenfeldii* with a DNA-biosensor. Biosens. Bioelectron. **20:** 1349-1357.

METFIES, K., and L. K. MEDLIN. 2004. DNA microchips for phytoplankton: The fluorescent wave of the future. Nova Hedwigia **79:** 321-327.

METFIES, K., K. TÖBE, C. A. SCHOLIN, and L. K. MEDLIN. 2006. Laboratory and field applications of ribosomal RNA probes to aid the detection and monitoring of Harmful Algae, p. 311-325. *In* E. Granéli and J. T. Turner [eds.], Ecology of Harmful Algae. Springer Verlag, Berlin, Heidelberg, Germany.

MILLER, P., and C. A. SCHOLIN. 1998. Identification and enumeration of cultured and wild *Pseudo-nitzschia* (Bacillariophyceae) using species-specific LSU rRNA-targeted fluorescent probes and filter-based whole cell hybridization. J. Phycol. **34:** 371-382.

MONTRESOR, M., U. JOHN, A. BERAN, and L. K. MEDLIN. 2004. *Alexandrium tamutum* sp. nov. (Dinophyceae): A new nontoxic species in the genus *Alexandrium*. J. Phycol. **40:** 398-411.

NASCIMENTO, S. M., E. L. LILLY, J. LARSEN, and S. MORRIS. 2005. Toxin profile, pigment composition, and large subunit rDNA phylogenetic analysis of an *Alexandrium minutum* (Dinophyceae) strain isolated from the Fleet Lagoon, United Kingdom. J. Phycol. **41:** 343–353.

PEPLIES, J., C. LACHMUND, F. O. GLÖCKNER, and W. MANZ. 2006. A DNA microarray platform based on direct detection of rRNA for characterization of freshwater sediment-related prokaryotic communities. Appl. Environ. Microbiol. **72:** 4829-4838.

PERSICH, G. R., D. M. KULIS, E. L. LILLY, D. M. ANDERSON, and V. M. T. GARCIA. 2006. Probable origin and toxin profile of *Alexandrium tamarense* (Lebour) Balech from southern Brazil. Harmful Algae **5:** 36-44.

POLZ, M. F., and C. M. CAVANAUGH. 1998. Bias in template-to-product ratios in multitemplate PCR. Appl. Environ. Microbiol. **64:** 3724-3730.

REYSENBACH, A.-L., L. J. GIVER, G. S. WICKHAM, and N. R. PACE. 1992. Differential amplification of rRNA genes by polymerase chain reaction. Appl. Environ. Microbiol. **58:** 3417-3418.

SAKO, Y., S. HOSOI-TANABE, and A. UCHIDA. 2004. Fluorescence-*in-situ*-hybridization using rRNA-targeted probes for simple and rapid identification of the toxic dinoflagellates *Alexandrium tamarense* and *Alexandrium catenella*. J. Phycol. **40:** 598–605.

SANTA LUCIA, J. 1998. A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics. Proc. Natl. Acad. Sci. USA **95:** 1460-1465.

SCHENA, M., D. SHALON, R. W. DAVIS, and P. O. BROWN. 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science **270:** 467-470.

SCHENA, M., D. SHALON, R. HELLER, A. CHAI, P. O. BROWN, and R. W. DAVIS. 1996. Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. Proc. Natl. Acad. Sci. USA **93:** 10614-10619.

SCHOLIN, C. A., G. M. HALLEGRAEFF, and D. M. ANDERSON. 1995. Molecular evolution of the *Alexandrium tamarense* 'species complex' (Dinophyceae): Dispersal in the North American and West Pacific regions. Phycologia **34:** 472-485.

SCHOLIN, C. A., M. HERZOG, M. SOGIN, and D. M. ANDERSON. 1994. Identification of group- and strain-specific genetic markers for the globally distributed *Alexandrium* (Dinophyceae). II. Sequence analysis of a fragment of the LSU rRNA gene. J. Phycol. **30:** 999-1011.

SCHOLIN, C. A. and others 1999. DNA probes and a receptor-binding assay for the detection of *Pseudo-nitzschia* (Bacillariophyceae) species and domoic acid activity in cultured and natural samples. J. Phycol. **35:** 1356-1367.

---. 1997. Detection and quantification of *Pseudo-nitzschia australis* in cultured and natural populations using LNA rRNA-targeted probes. Limnol. Oceanogr. **42:** 1265-1272.

TAYLOR, F. J. R., and Y. FUKUYO. 1998. The neurotoxigenic dinoflagellate genus *Alexandrium* Halim: General Introduction, p. 381-404. *In* D. M. Anderson, A. D.

Cembella and G. M. Hallegraeff [eds.], Physiological Ecology of Harmful Algae Blooms. Springer Verlag, Berlin, Heidelberg, Germany.

TAYLOR, F. J. R., Y. FUKUYO, and J. LARSEN. 1995. Taxonomy of harmful dinoflagellates, p. 283-317. *In* G. M. Hallegraeff, D. M. Anderson and A. D. Cembella [eds.], Manual on Harmful Marine Microalgae. UNESCO, Paris, France.

TÖBE, K., G. ELLER, and L. K. MEDLIN. 2006. Automated detection and enumeration for toxic algae by solid-phase cytometry and the introduction of a new probe for *Prymnesium parvum* (Haptophyta: Prymnesiophyceae). J. Plankton Res. **28:** 643-657.

TYRRELL, J. V., L. B. CONNELL, and C. A. SCHOLIN. 2002. Monitoring for *Heterosigma akashiwo* using a sandwich hybridization assay. Harmful Algae **1:** 205-214.

URAKAWA, H. and others 2003. Optimization of single-base-pair mismatch discrimination in oligonucleotide microarrays. Appl. Environ. Microbiol. **69:** 2848–2856.

VAN DOLAH, F. M. 2000. Marine algal toxins: origins, health effects, and their increased occurrence. Environ. Health Perspect. **108:** 133–141.

VILA, M. and others 2005. A comparative study on recurrent blooms of *Alexandrium minutum* in two Mediterranean coastal areas. Harmful Algae **4:** 673-695.

YE, R. W., T. WANG, L. BEDZYK, and K. M. CROKER. 2001. Applications of DNA microarrays in microbial systems. J. Microbiol. Meth. **47:** 257-272.

Table 1. Algal cultures

Species	Strain (Georaphical Clade)	Origin
A. ostenfeldii	CCMP1773	Limfjordan, Denmark, Hansen
A. ostenfeldii	K0324	Scandinavian Culture Centre for Algae and Protozoa, Denmark
A. minutum	AL3T	Gulf of Trieste, Italy, A. Beran
A. minutum	BAHME91	Biologische Anstalt Helgoland, Germany
A. fundyense	CA28 (NA)	Woods Hole, Oceanogr. Institution, D.M. Anderson
A. tamarense	BAHME225 (NA)	Biologische Anstalt Helgoland, Germany
A. tamarense	GTLI21 (NA)	Mud Creek, Moriches Bay, Long Island, USA
A. tamarense	31/9 (NA+WE)	Cork Habor, Ireland, W. Higman
A. tamarense	31/4 (WE)	Cork Habor, Ireland, W. Higman
A. tamarense	BAHME182 (WE)	Biologische Anstalt Helgoland, Germany
A. tamarense	UW42 (WE)	Belfast, UK, W. Higman
A. tamarense	SZNB01 (ME)	Gulf of Neaples, Italy, M. Montresor
A. tamarense	SZNB08 (ME)	Gulf of Neaples, Italy, M. Montresor
A. tamarense	SZNB19 (ME)	Gulf of Neaples, Italy, M. Montresor
A. tamarense	SZNB21 (ME)	Gulf of Neaples, Italy, M. Montresor

Table 2. Probe sequences and sources of this study

Probe name	Target gene	Sequence (5'-3')	Specific for	Reference
DINOB	18S	CCT CAA ACT TCC TTG CIT TA	Dinophyceae (incl. Apicomplexa)	John et al. 2003a
DINOE-12	18S	CGG AAG CTG ATA GGT CAG AA	Dinophyceae (incl. Apicomplexa)	Medlin et al. 2006a
AOST1	18S	CAA CCC TTC CCA ATA GTC AGG T	*A. ostenfeldii*	Metfies et al. 2005
AOST2	18S	GAA TCA CCA AGG TTC CAA GCA G	*A. ostenfeldii*	Metfies et al. 2005
AOST02	18S	CAC CAA GGT TCC AAG CAG	*A. ostenfeldii*	John et al. 2003a
ALEXMIN1	18S	CCC AGA AGT CAG GTT TGG AT	*A. minutum* (AY831408, AY883006, AJ535380, AJ535388)	Nölte, unpublished
ATNA01	28S	AGT GCA ACA CTC CCA CCA	*A. tamarense* (North American Clade)	Miller & Scholin, 1998
ATNA02	28S	AAC ACT CCC ACC AA GCAA	*A. tamarense* (North American Clade)	John et al. 2005
ATWE03	28S	GCA ACC TCA AAC ACA TGG	*A. tamarense* (Western European Clade)	John et al. 2005
ATME04	28S	CCC CCC CAC AAG AAA CTT	*A. tamarense* (Mediterranean Clade)	John et al. 2005
Positive control	TATA-Box	ATG GCC GAT GAG GAA CGT	*S. cerevisiae*	Metfies &Medlin 2004
Negative control		TCC CCC GGG TAT GGC CGC		Metfies &Medlin 2004

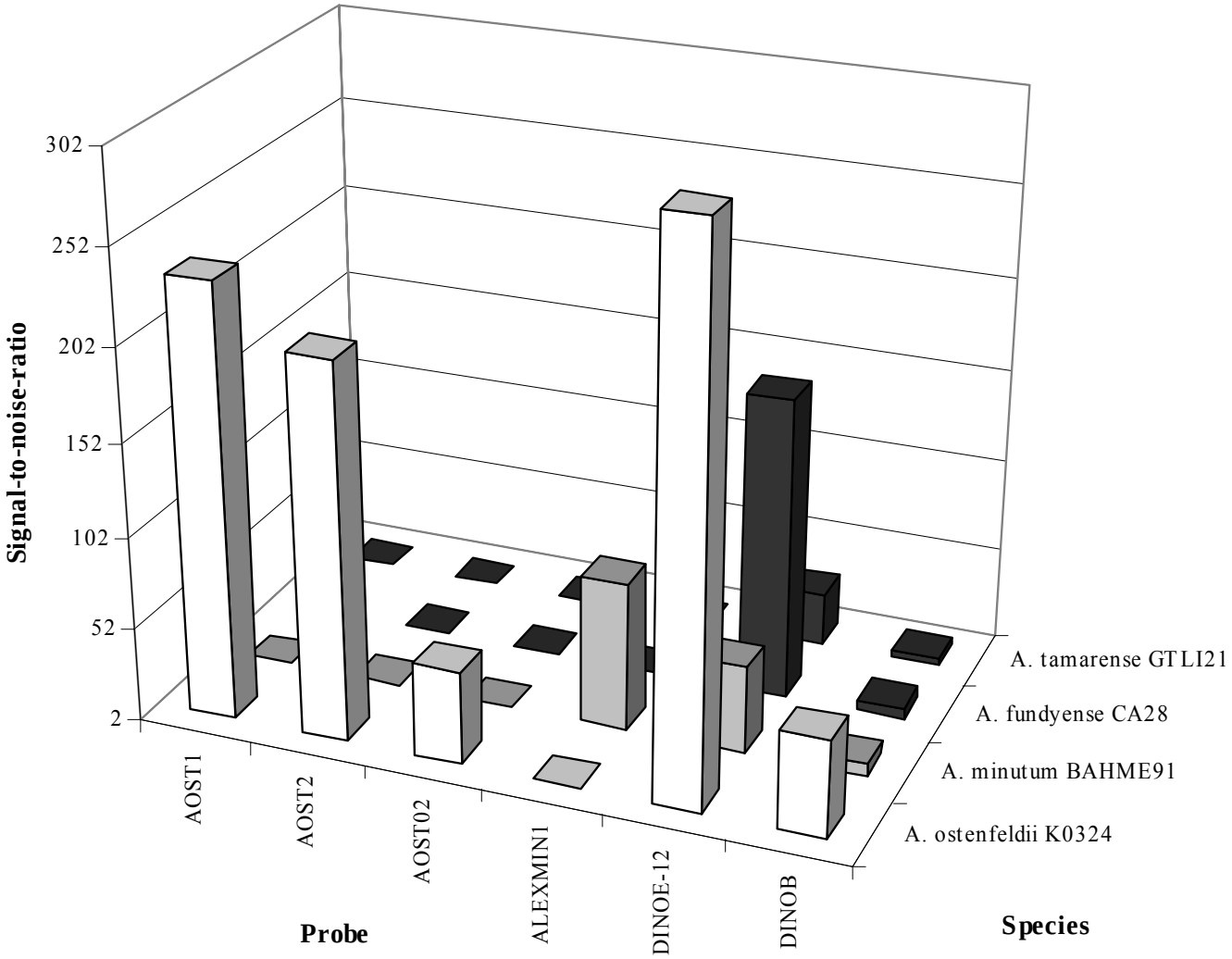

Figure 1. Specificity tests of 18S Dinophyta and *Alexandrium* probes

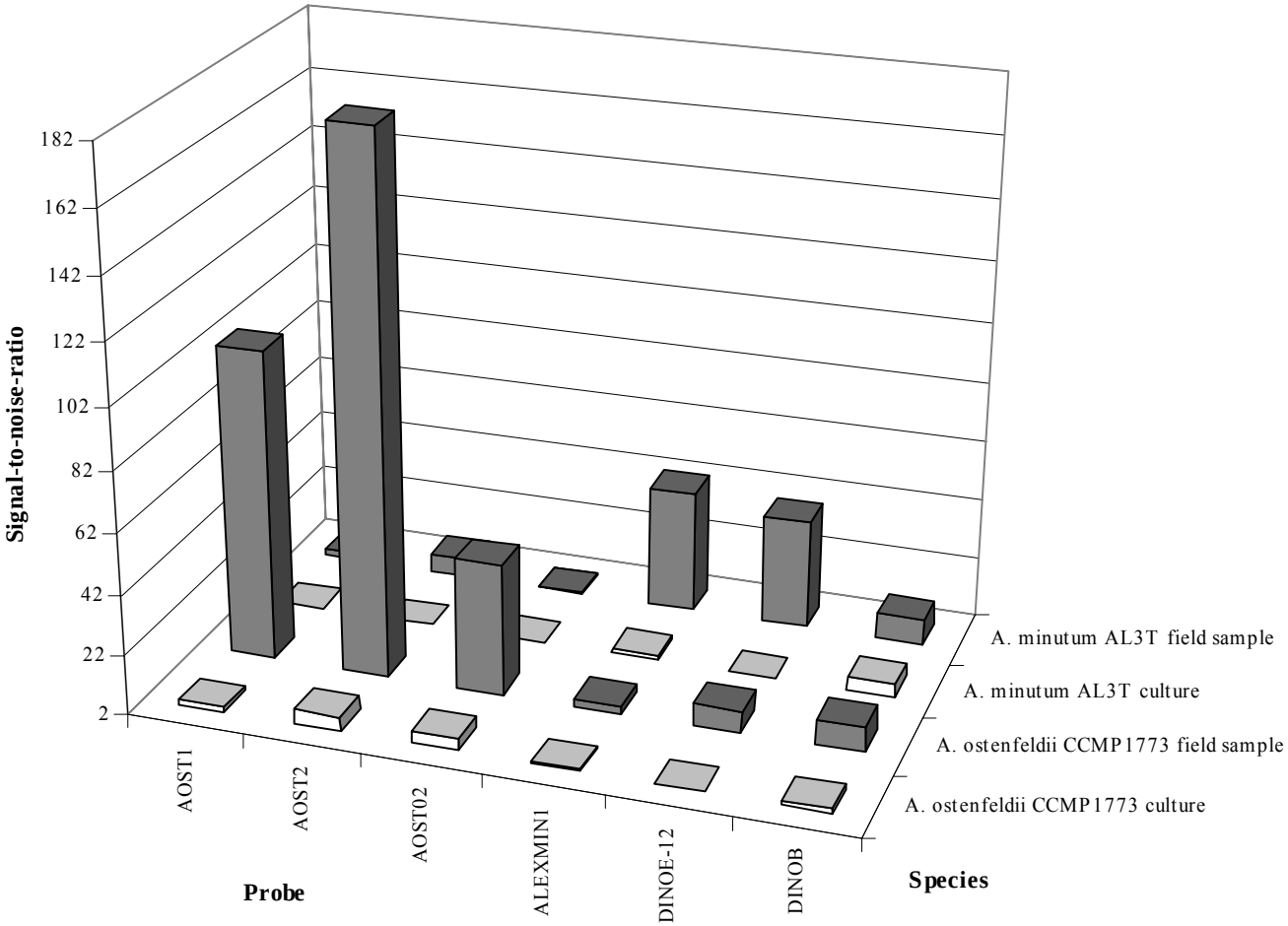

Figure 2. Results of further specificity tests of 18S Dinophyta and *Alexandrium* probes in comparison with hybridization signals of spiked field samples

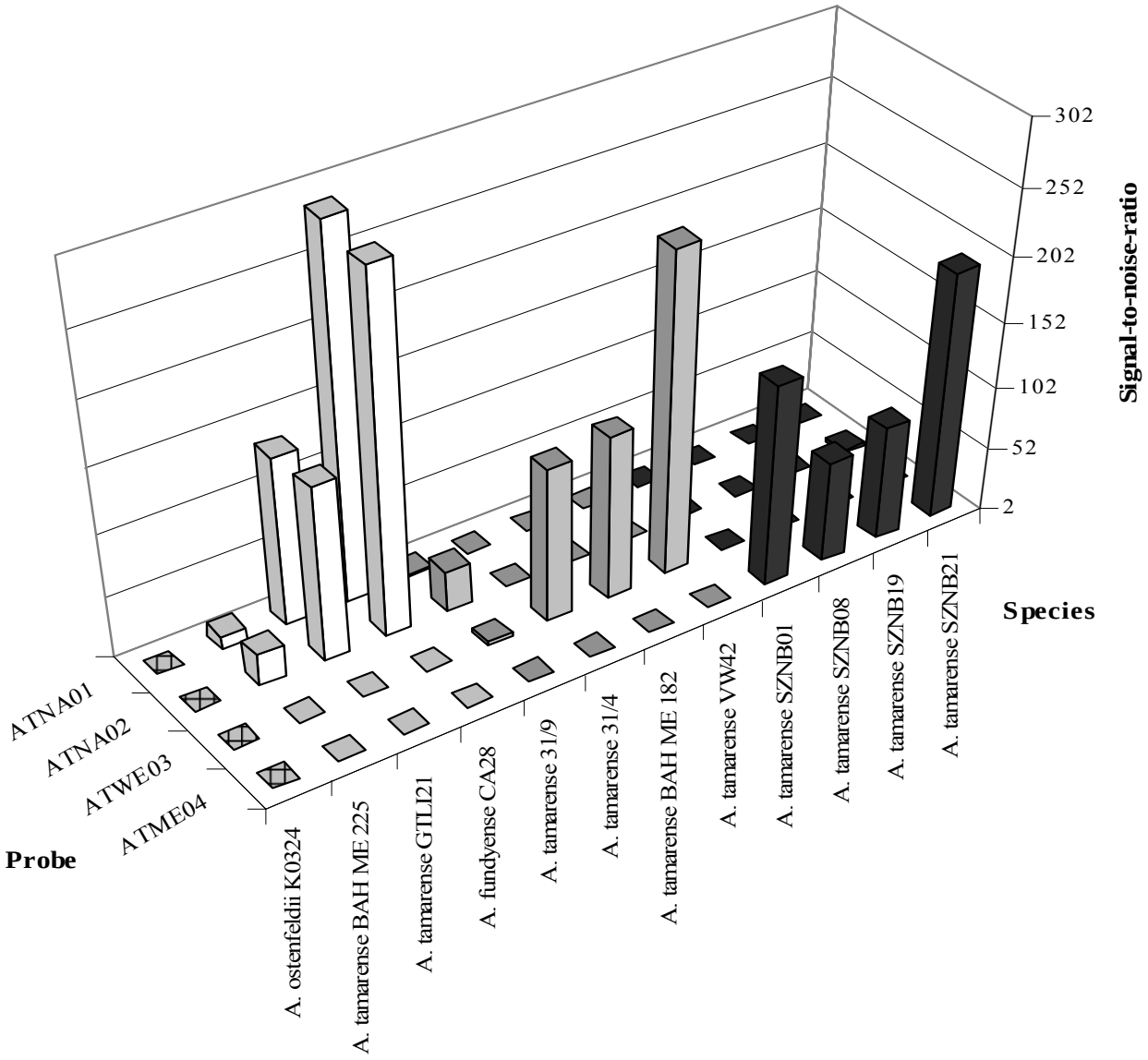

Figure 3. Specificity tests of the 28S probes for the *A. tamarense* "species complex"

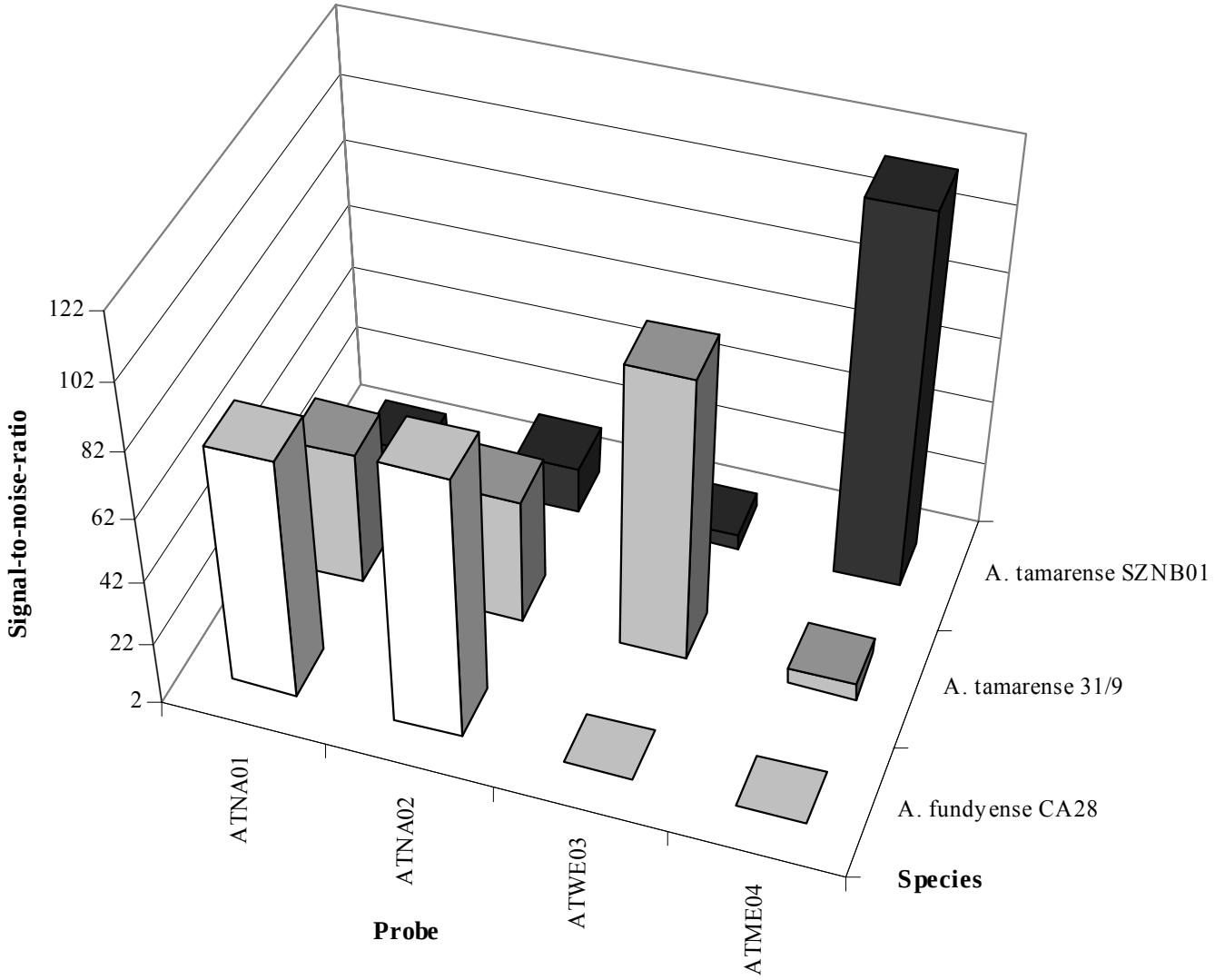

Figure 4. Hybridization results of spiked field samples for the 28S probes

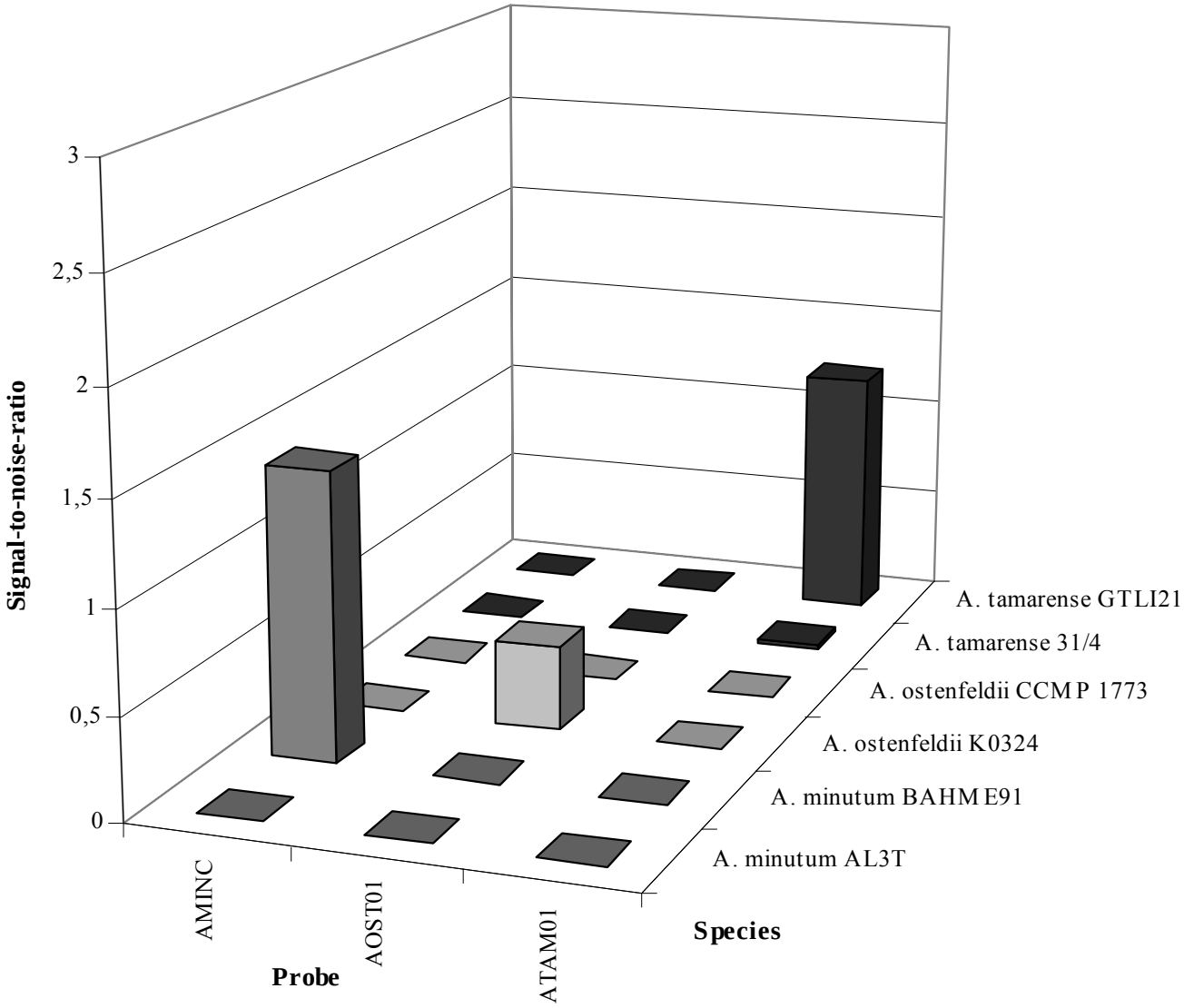

Figure 5. Hybridization results of inappropriate probes for the microarray

2.7 Publication V

DEVELOPMENT AND ASSESSMENT OF A DNA MICROARRAY FOR IDENTIFICATION OF PRASINOPHYTES

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Abstract

The reliable detection and classification of picoeukaryotes in the ocean is mainly hampered by their small size and few morphological markers. We developed an 18S rRNA gene-targeted oligonucleotide microarray consisting of 21 probes targeting prasinophytes for the characterization of microbial picoeukaryotic communities in marine environments. Probes from other hybridization methods were adapted and evaluated. New probes were designed for novel prasinophyte groups, where no probes have yet been published. The evaluation of the probe set was done under stringent conditions with 18S PCR-fragments from 20 unialgal reference cultures used as positive targets. Ambiguous hybridization results were clarified by cloning and sequencing of the concerned species. Subsequently, the chip was successfully used to analyze an environmental sample from the North Sea, and sequence analyses supported the results of the microarray. The study here demonstrated the suitability of the microarray as a reliable tool for fast and efficient monitoring of this important picoplanktonic algal group.

Introduction

Phytoplanktonic cells between 0.2 and 2 µm are termed picoplankton, and, in spite of their small size, they can contribute greatly to the global carbon cycle, biomass and, productivity in the sea (Campbell et al. 1994; Courties et al. 1994; Derelle et al. 2006; Joint et al. 1986; Li 1994). Besides the two prokaryotic cyanobacterial genera, *Synechococcus* (Johnson and Sieburth 1979; Waterbury et al. 1979) and *Prochlorococcus*, (Chisholm et al. 1988), the eukaryotic part of the picoplanktonic community is less well known. Recently, several new classes have been described (Andersen et al. 2002; Andersen et al. 1993; Chrétiennot-Dinet et al. 1995; Guillou et al. 1999a; Kawachi et al. 2002; Not et al. 2007).

In the last decade, molecular methods have facilitated the investigation of physiology, ecology, and distribution of this important part of the marine food web (Countway and Caron 2006). Methods that were previously used to study prokaryotes have been applied to eukaryotes for investigation of picoeukaryotic phytoplankton biodiversity and abundance in the ocean, where unexpected diversity has been documented (Biegala et al. 2003; Díez et al. 2001; Lovejoy et al. 2007; Marie et al. 2006; Moon-Van Der Staay et al. 2001; Not et al. 2004; Not et al. 2002; Not et al. 2007).

As one of the key taxa in the marine eukaryotic picoplankton the Prasinophyta are an exceptionally interesting group (Not et al. 2004). Since their first description in 1952 (Butcher 1952; Manton and Parke 1960), their taxonomic history is confusing, and under steady revision with still many unanswered questions (Bhattacharya and Medlin 1998; Moestrup 1991; Moestrup and Throndsen 1988; Turmel et al. 1999). One possible explanation is a missing feature unifying the group (Moestrup and Throndsen 1988). To date, 20 genera with 180 species are known within the Prasinophyta; a few were recently described (Van Den Hoek et al. 1995; Zingone et al. 2002). They are distributed worldwide and attain high abundances in several environments (Sieburth et al. 1999; Volkman et al. 1994; Zingone et al. 1999). However, their differentiation and detection is mainly hampered by the absence of reliable methods to identify and monitor small cells with few morphological features (Thomsen and Buck 1998; Zingone et al. 2006). With classical methods like light, epifluorescence, and electron microscopy it is not possible to identify picoplanktonic groups down to species level. Morphological indistinguishable species with hidden genetic diversity make the right classification impossible with this methods (Andersen et al. 1996; Janson and Hayes 2006; Massana et al. 2002; Scholin 1998b; Zingone et al. 1999). High performance liquid chromatography (HPLC) can be used to identify the major classes of the phytoplankton community (Guillou et al. 1999b), but taxonomic resolution below class level is limited.

Several groups do not possess specific diagnostic pigments or may share overlapping ones (Massana et al. 2002).

Molecular probes have especially been shown to be very useful in terms of detection and monitoring of microbial diversity. Target genes for molecular probes are normally the small and large subunit ribosomal RNA genes. They feature more or less conserved regions that allow development of probes at different taxonomic levels and have high target numbers in the cell (Groben et al. 2004). The probes can be used in many different methods and hybridization to the target can be detected by various labels attached to the end of the probe (Groben et al. 2004; Groben and Medlin 2005; Medlin and Simon 1998; Scholin 1998a). Furthermore, the probes can be tailored as a phylogenetic marker for a variety of taxonomic levels in phytoplankton from classes down to species or strains using whole-cell and cell-free formats (John et al. 2003; John et al. 2005; Metfies and Medlin 2004; Metfies et al. 2006; Scholin et al. 2003). The fluorescence-*in-situ*-hybridization (FISH) has shown great potential for identification of prasinophytes (Biegala et al. 2003; Not et al. 2004), but processing and quantitative analysis of samples by microscopy can be tedious, slow, demanding, and not even statistically adequate. Application of molecular probes and FISH technique in combination with flow cytometry has greatly increased the detection, and accuracy of monitoring of picoplanktonic communities (Biegala et al. 2003; Mackey et al. 2002; Metfies et al. 2006; Rice et al. 1997).

The application of new methods, viz. microarrays, for detection of picoplanktonic eukaryotes with potential for high throughput analysis on close temporal and spatial scales could greatly contribute in the knowledge of their species abundances, ecology, and physiology. DNA microarrays with species-specific probes have the unparallel opportunity to detect thousands of targets in one experiment. Originally developed for gene expression applications in the mid 1990s (Schena et al. 1995; Schena et al. 1996), this innovative technique as it is being transferred to species identification, has offered a promising experimental platform for microbial ecology. The oligonulceotide probes are applied onto the surface of a glass slide with special surface properties (Gentry et al. 2006; Ye et al. 2001). The most demanding challenge is the high number of unknown environmental sequences, which may result in unspecific signals or an oversight of species without a probe on the chip (Gentry et al. 2006). The development of a functional chip is time-consuming, and expensive, and all probes on the chip should work specifically under the same hybridization conditions (Boireau et al. 2005; Feriotto et al. 2002; Metfies et al. 2006). Recently, DNA microarrays have been used to detect and monitor species, their abundances and, dynamics for a

comprehensive understanding of complex microbial ecosystems. The accuracy of the so-called “phylochips” can further be enhanced by application of hierarchical probes at different taxonomic levels, because the detection of species is assessed by more than one positive probe signal (Metfies et al. 2006). Phylochips have been used mostly for prokaryotes (Gentry et al. 2006; Lehner et al. 2005; Loy et al. 2002; Loy et al. 2005; Peplies et al. 2004a; Peplies et al. 2006; Peplies et al. 2004b), but a DNA microarray was developed for detection of photosynthetic eukaryotic picoplankton using plastid RNA genes (Fuller et al. 2006a; Fuller et al. 2006b). Furthermore, there are a few publications on the successful detection and monitoring of harmful algae with microarrays (Gescher et al. 2007; Ki and Han 2006), marine microalgae on class level (Metfies and Medlin 2004), and one using prasinophyte probes (Medlin et al. 2006a).

In this study, a phylochip for the detection and monitoring of the picoplanktonic prasinophytes was developed. Some of the probes were initially designed for FISH and showed specific hybridization signals, where other probes were designed for new groups of prasinophytes. The specificity, sensitivity, and discriminative potential of the probes were tested extensively under stringent conditions. The microarray contains 21 probes at different hierarchical levels. One environmental field sample was subsequently analyzed by cloning and sequencing to prove the reliability of the microarray. These results supported the data obtained by the microarray.

Materials and Methods

Culture conditions – All algal strains were cultured under sterile conditions in seawater-based F2- and K-media (Guillard and Ryther 1962; Keller et al. 1987) at 150 µEinstein – 200 µEinstein with a light:dark cycle of 14:10 hours and at 15 or 20 °C (Table 1).

DNA extraction – The template DNA was extracted from pure cultures with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol.

PCR Amplification of 18S rRNA gene – For probe specificity tests, the entire 18S gene was amplified from the target DNA with the universal PCR primers 1F (5'-AAC CTG GTT GAT CCT GCC AGT-3') and 1528R (5'-TGA TCC TTC TGC AGG TTC ACC TAC-3') without the polylinkers (Medlin et al. 1988). The PCR protocol was 5 min 94 °C, 2 min 94 °C, 4 min 54 °C, 2 min 72 °C for 29 cycles and an extension for 7 min at 72 °C. All PCR experiments were carried out in a Mastercycler (Eppendorf, Hamburg, Germany). For the

positive control in the microarray hybridization experiments the yeast, *Saccharomyces cerevisiae* was used. A 250 bp fragment of the TATA-box binding protein-gene (TBP) of *S. cerevisiae* was amplified with the primers TBP-F (5'-ATG GCC GAT GAG GAA CGT TTA A-3') and TBP-R-Biotin (5'-TTT TCA GAT CTA ACC TGC ACC C-3'). The TBP-PCR amplification protocol was 5 min 94 °C, 1 min 94 °C, 1 min 52 °C, 1 min 72 °C for 35 cycles, and an extension for 10 min at 72 °C. All PCR-fragments were purified with the QIAquick PCR purification Kit (Qiagen, Hilden, Germany) with modifications of the protocol to enhance the yield of PCR-fragments. Step 8 (elution with elution buffer EB) was repeated with the same eluate. DNA concentration was measured with a Nanodrop Spectrophotometer (Peqlab, Erlangen, Germany).

Biotin-Labeling of the purified PCR- fragments – In the hybridization experiments, the Biotin DecaLabel DNA Labeling Kit (Fementas, St. Leon-Rot, Germany) was utilized. One labeling reaction contained 200 ng of PCR-fragments and was incubated at 37 °C, overnight (17 to 20 hours) to achieve the best biotin incorporation. The labeled PCR-fragments were purified with the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) with modifications of the manufacturer's protocol as above to enhance the yield. DNA concentration was measured as above.

Probe set and probe design – One part of the probes evaluated in this study was already published (Table 2) and tested in dot-blot and fluorescence-*in-situ*-hybridization. The other part (Table 2) was designed with the probe design and probe match tool of the ARB software (Ludwig et al. 2004) to cover all prasinophyte clades shown by Gulliou et al. (2004). Probe specificity was tested with the BLAST tool (Altschul et al. 1990).

Probe synthesis – The probes, including positive and negative controls, were synthesized from Thermo Electron Corporation (Ulm, Germany) with a C6/MMT aminolink at the 5'-end.

Microarray production – Probes were spotted onto epoxy-coated "Nexterion Slide E" slides (Peqlab Biotechnologie GMBH, Erlangen, Germany) in a final concentration of 1 µM in 3x saline sodium citrate buffer (3x SSC). We utilized the pin printer VersArray ChipWriter Pro (Bio-Rad Laboratories GmbH, München, Germany) and split pins (Point Technologies, Inc., Colorado, USA). Subsequently, the slides were baked at 60 °C for 30 min in a Shake 'n'

Stack hybridization oven (Thermo Hybaid, Ulm, Germany) for immobilization of probes. They were stored at -20 °C. The chip contained four replicates of each probe in four independent blocks.

Standard hybridization protocol – The hybridization solution was prepared with 1x hybridization buffer (1 M NaCl, 10 mM Tris, pH 8, 0.005 % Triton X-100, 1 mg/mL BSA, 0.1 µg/µL HS-DNA) and the biotin-labeled PCR-fragment at a final concentration of 11.25 ng DNA/µL. The TBP-fragment from *S. cerevisiae* with biotin-labeled primers was added as the positive control at a final concentration of 4.7 ng DNA/µL. Blocking of the background noise was conducted by pre-hybridization of the slides at 58 °C for 1 hour in a slide box with 50 mL 1x STT buffer (1 M NaCl, 10 mM Tris, pH 8, 0.005 % Triton X-100, 1 mg/mL BSA). Secondly, the slides were centrifuged, and the hybridization solution was at 94 °C for 5 min. A special cover slip, the Lifter Slip (Implen, München, Germany), was used for the hybridization. It has two printed bars at the edges to prevent slippage on the slide and to provide a definite volume on top of the slide. 30 µL were pippeted under the cover slip, and capillary action ensured even dispersal of hybridization solution between chip and cover slip. The slide was placed in a humid chamber, which was constructed from a 50 mL Sarstedt tube filled with tissues moistened with hybridization solution. The hybridization was conducted at 58 °C for 1 hour; afterwards it was washed with 2x, then 1x saline sodium citrate (2x SSC, 10 mM EDTA, 0.05 % SDS, 1x SSC, 10 mM EDTA) for 15 min and dried by centrifugation.

Staining – The hybridized PCR-fragments on the chip were stained with Streptavidin-CY5 (Amersham Biosciences, Freiburg, Germany) in 1x hybridization buffer at a final concentration of 100 ng /mL. The chip was placed at room temperature for 30 min in a humid chamber, and was washed afterwards twice with 2x SSC buffer for 5 min, and once with 1x SSC buffer for 5 min to remove excess staining moieties.

Scanning and quantification of microarrays – The chip was scanned with a GenePix 4000B scanner (Molecular Devices Cooperation, Sunnyvale, USA), and analysis of the obtained fluorescent signal intensities was done using the GenePix 6.0 software (Molecular Devices Cooperation, Sunnyvale, USA). A grid of circles was superimposed onto the scanned image to calculate the fluorescent signals and the surrounding background intensity. The signal-to-noise-ratios were determined using a formula from Loy et al. (2002) and normalized to the signal of the TBP positive control of the same microarray with the PhylochipAnalyzer

software (Metfies et al. 2007). Afterwards, the mean value for all hybridizations of one culture was calculated. A value of two was defined as the threshold for a true signal.

Sequencing – To clarify ambiguous hybridization results, the 18S gene of three cultures (*Prasinococcus* cf. *capsulatus* CCMP 1202, *Ostreococcus* spec. RCC 344, and unidentified Coccoid RCC287), and one environmental sample was cloned as described by Medlin et al. (2006) and sequenced. Purified 18S PCR-fragments were used for cycle sequencing in a Mastercycler (Eppendorf, Hamburg, Germany) with universal unlabeled PCR primers (Table 3) in a final concentration of 1 µM (MWG Biotech, Ebersberg, Germany). The following program was used: 1 min 96 °C, (10 sec 96 °C, 5 sec 50 °C, 4 min 60 °C) for 25 cycles. Afterwards the reaction mixture was purified with the DyeEx 2.0 Spin Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and electrophoresed with the capillary sequencer ABI Prism 3100, the BigDye Terminator v3.1 Cycle Sequencing Kit and Big Dye Terminator v3.1 Sequencing Buffer with approximately 50 - 100 ng of template according to the manufacturer's protocol (all Applied Biosystems, Darmstadt, Germany). The sequences were assembled and aligned with the Seqman program (DNASTAR, Madison, USA) and compared against GenBank using a BLAST search (Altschul et al. 1990).

Results

Probe development – Probes evaluated in this study were partly already published (Not et al. 2004; Simon et al. 2000; Simon et al. 1995) and represent probes for the Chlorophyta, Prasinophyta clades II, V, VI, VIIa, VIIb and the species *Bathycoccus prasinos*, *Micromonas pusilla* and *Ostreococcus tauri* (Fig. 1). They have been tested in dot-blot and FISH (Table 2). Seven new probes for clade I, III and VIIC were designed to cover the remaining prasinophyte diversity described by Guillou et al. (2004) as shown in the tree (Fig. 1). The group is paraphyletic (Nakayama et al. 1998; Steinkötter et al. 1994), thus , probe design was difficult. It was impossible to design a probe for the entire group, and even at a clade level, multiple probes were sometimes necessary to cover all members of a clade.

Probe evaluation with reference strains – Probe specificity was tested under stringent conditions, viz. the same hybridization temperature and washing conditions for all probes and chips. In general, hybridization results of this chip showed high specificity and sensitivity. Signal-to-noise-ratios of perfectly matched targets hybridized to their specific probes showed different signals, demonstrating that the intensities of individual probes varied strongly in

their sensitivity. A comparison of the signal-to-noise-ratios for two probes for the Chlorophyta was conducted (Fig. 2). All strains showed signals for probe Chlo01 and 02 probes, but they varied over a broad range. The signals for probe Chlo02 were slightly higher.

Hybridization results of 20 reference cultures to six Prasinophyta probes for clade II, V, VI, VIIA and, VIIB are shown in Figure 3. Probe Pras03 for clade VI showed strong specific signals to three out of five hybridized target strains, one target strain showed a reduced signal and the last one showed no signal. Only weak cross-hybridizations with three strains from other clades (I, II, and V) were detected. All seven strains of clade II were recognized by probe Pras04. No unspecific binding to all other closely related 14 algal templates was detected, except for strain Coccoid RCC287. Probe Pras05 (clade VIIA) also showed no cross-hybridization with non-target strains and gave strong signals for one target strain, unidentified sp. Chlorophyceae CCMP1205. The other target strain, unidentified Coccoid RCC287, did not give a signal. As mentioned above, it showed a positive signal with probe Pras04 (clade II). We sequenced the culture, and and found a misidentification, the culture is a target of probe Pras04. Probe Pras06 for clade VIIB was tested only with non-target species, because no target strains were available from this clade. Here we detected only weak cross-hybridizations. Pras01 and Pras07 target the same group, clade V according to Guillou et al. (2004). Pras07 represents an improvement over probe Pras01 and detects more species in this clade. As shown in Fig. 2 they both showed specific signals for the target strains, and Pras07, with a broader detection range, performed better that Pras01.

The remaining probes for clade I, III, and VIIC were hybridized with specific PCR-fragments, and the results are shown in Figure 4. Probe Pras08 (clade VIIC) showed a high and clear signal for the target species *Picocystis salinarium* CCMP1897. Unfortunately, several strains in other clades also cross-hybridized with this probe. Clade I was detected by three specific probes, probe Pras09A1, Pras09A2 and, Pras09D. Good results were observed for Pras09A1 and Pras09A2 when hybridized with target species, and they showed no non-target hybridization. For the third probe of this clade, probe Pras09D, and two probes of clade III, Pras10F and Pras10H, no target strains were available in culture collections, and therefore, they were only tested for cross-hybridization. All three showed no unspecific binding with all closely related species. Pras10B (clade III) showed good results in hybridization with its target strain *Nephroselmis pyriformis* CCMP717 and only weak cross-hybridizations with some other strains.

Figure 5 shows results of the hybridization of algal cultures to the two chlorophyte class level probes, Chlo01 and Chlo02, the Pras04 probe for clade II (*Mamelliales*), three

species and three sub-clade level probes. The probe for *Bathycoccus prasinos*, Bathy01, showed signals for the specific cultures *B. prasinos* RCC378 and CCMP1898 and for unidentified Coccoid RCC287, whose taxonomic classification was questionable. Sequencing of this culture revealed a mixture of *Bathycoccus prasinos* and *Micromonas pusilla*. The two *Ostreococcus* cultures, *O. tauri* RCC116 and RCC344, showed low signal intensities above the threshold for probe Ostreo01. The culture, unidentified Coccoid RCC287, gave also a signal for this probe. The probe Micro01 is specific for the species *Micromonas pusilla* and MicroA, B and C for different clades within this species complex (Guillou et al. 2004). At least five morphologically indistinguishable different groups can be detected in this complex (Slapeta et al. 2006). The cultures *M. pusilla* CCMP490 and CCMP1195 are target species and showed a signal using probe Micro01, but the signal for *M. pusilla* CCMP1195 was very low. The culture *M. pusilla* CCMP490, which belongs to the *Micromonas* clade A (Guillou et al. 2004), gave a signal for MicroA and also for MicroC. The other culture, *M. pusilla* CCMP1195, which was not in the study of Guillou et al. (2004), only showed signals for probe MicroA. One possible explanation for the mixed signals for *M. pusilla* CCMP490 could be that it the culture was not clonal. Representatives of the different *M. pusilla* clades occur worldwide (Slapeta et al. 2006), and members of two clades could easily be present in one culture. No signals were observed for MicroB.

Analysis of environmental samples – One field samples was taken on the 18.02.2001 in the PICODIV project at Helgoland Roads (54°11.3’ N, 07°54.0’E). The PCR-fragments of this sample were hybridized to the microarray to evaluate its potential after specificity of probes was proven (see Fig. 6). Sample He010218 showed high positive signals for both Chlo probes, a signal for Pras04, Micro01, Micro A and Bathy01. Furthermore, weak signals were observed for all other prasinophytes probes, except Pras09A1 and 09A2. The sample was sequenced afterwards to verify the results of by the microarray hybridization.

Sequencing results – Three cultures were sequenced to confirm their identity, because they showed confusing, weak or even no hybridization success in the microarray experiments. It was shown from the sequencing results that two cultures, *Prasinococcus* cf. *capsulatus* CCMP 1202 and *Ostreococcus* spec. RCC 344, were accurately identified. The third culture, unidentified Coccoid RCC287, was sequenced by Gulliou et al. (2004) and found to be a target for probe Pras05. We observed no signals for this probe in our microarray hybridization and resequenced the culture. The results of the sequencing analysis showed that this culture

was composed of the species *Bathycoccus prasinos* and *Micromonas pusilla*. An overview of the best BLAST hits for the obtained sequences of the cultures and field samples is shown in Table 4. The sequencing results confirmed the identity of *Prasinococcus* cf. *capsulatus* CCMP 1202 and *Ostreococcus* spec. RCC 344, which showed low or even no signal for their specific probe. Therefore, the successful probe-target duplex formation may have been hampered by other factors, e. g., secondary structure in the 18S gene (Metfies and Medlin 2004). This could be confirmed by amplification of smaller PCR-fragments of the gene with internal primer-pairs and adjacent hybridization. The hybridization results of the third culture, unidentified Coccoid RCC287, were confirmed by sequencing analysis. Hybridization signals were positive for probe Chlo01, Chlo02, and Pras04. Micro01 and Ostreo01 gave a weak signal and but highest signal-to-noise-ratio was detected for Bathy01. Low cross-hybridizations were observed for Pras08 and Pras10B. The results of the hybridization corroborated the sequencing of this culture, except for the low signal for Ostreo01. We could not find *Ostreococcus* sequences in this culture with the sequence analyses. This result can be explained by a low cross-hybridization of probe Ostreo01 or the existence of only a few *Osterococcus* cells in the culture. To detect a contaminant in a culture, many sequences from a clone library need to be examined.

Discussion

In this study, 21 phytoplankton probes at different hierarchical levels were tested and evaluated with 20 algal cultures in a microarray hybridization format. Part of the probe set was previously published, and others were newly designed. Furthermore, a smaller set of these probes was already tested on the microarray (Medlin et al. 2006a; Metfies and Medlin 2004), and showed promising perspectives, which were confirmed by this study. Specific probes were always significantly above signals for non-target species. Probes without a target strain showed no cross-hybridization with closely related strains. More distantly related species should not give a signal, because species with few mismatches would have the highest potential of cross-hybridization. This indicates that a highly specific detection of target groups is feasible with a standardized hybridization protocol. Therefore, the microarray presented here has great potential for monitoring of picoplanktonic prasinophytes, because a high number target RNAs can be hybridized in a single experiment.

Probe hybridization results for this group in FISH and dot-blot applications have been published, but it is always necessary to reevaluate the probes on the microarray. Sometimes probes that work specific in these methods perform quite differently or, even not at all in a

microarray format (Medlin et al. 2006b). The major drawback of FISH is the limited throughput, only allowing the identification of one or a few organisms at a time with a restricted number of fluorochromes (DeLong et al. 1989; Peplies et al. 2003). A comprehensive view of microbial communities is challenging, time-consuming, and difficult to achieve with this method. Furthermore, Not and co-workers (2004) reported heterogeneous signals for probes Pras01, 03, and 05 in FISH. One possible explanation may be that thick cell walls inhibit penetration of probes (Hasegawa et al. 1996). These problems do not occur in a microarray application, because only isolated nucleic acids are used, and there is no need to enter intact cells. The probes Pras04, Micro01, Ostero01, Bathy01 are specific for their target strains and delivered a bright fluorescence in FISH (Not et al. 2004) and also in a microarray hybridization (Medlin et al. 2006a).

All strains showed signals for the Chlo01 and Chlo02 probes, but they varied over a broad range. This has been observed frequently for probes that cover broad taxonomic groups (Not et al. 2004). Chlo01 has one mismatch with several 18S rRNA sequences from chlorophyte species and Chlo02 one mismatch with several green macroalgal species and three freshwater microalgal species (Simon et al. 2000). Thus, we recommend probe Chlo02 for monitoring of marine phytoplankton.

Five probes (Pras01, 04, 05, 09A1, and 09A2) were specific for their target clades (V, II, VIIA, and I, respectively) and did not cross-hybridize with closely related species. The same results were observed for the species and subclade probes of clade II (Bathy01, Ostero01, Micro01, A, B, and C). The four probes (Pras06, 09D, 10F, and 10H for clade VIIB, II, and III, respectively) where no target strain was available showed no or very weak unspecific binding with all other prasinophyte cultures. High-signal-to-noise-ratios for the target and low cross-hybridization with closely related species were observed for probe Pras03, 07, 08, and 10B (clade VI, V, III, and VIIC, respectively). Three cultures were sequenced, because for two of them, no signal for the target probe or any other probe was detected and the third one did not gave a signals for the potential target probe (Pras05), but for another one (Pras04). The two undetected cultures were identified as target strains, so the hybridization must have been hampered by other factors. The third culture was identified as target for probe Pras04 and not for Pras05, thus the hybridization results were correct.

The comparison of results from the two methods for the field sample was more challenging, because of the possibility of unknown sequences in the marine environment (Simon et al. 2000). In general, the microarray results for sample He010218 were supported by sequence data. The microarray showed the highest signals for Pras04, MicroA and

Bathy01 and the best BLAST hits were *Bathycoccus prasinos*, *Mantoniella squamata* and *Micromonas pusilla*. Some cross-hybridizations were detected for other probes on the chip. A possible explanation is unspecific binding with sequences from unknown species in the environmental sample. Probe specificity should be frequently rechecked to all newly published sequences in public databases.

Furthermore, the success of a probe-based method strongly relies on specific discrimination between perfect matched duplexes and those with mismatches (Gentry et al. 2006). The stability of these duplexes is difficult to predict and is influenced by many factors, e.g., secondary structures of target molecules and steric hindrance (Gentry et al. 2006; Loy et al. 2005). Positive *in-situ* hybridization result of a probe is not always connected to *in-silico* parameters, such as G–C content or melting temperature (Graves 1999). It is not possible to predict which probes will work under the given hybridization conditions. Cross-hybridizations are almost impossible to avoid in a microarray hybridization format with stringent conditions (Loy et al. 2005). In this study, we evaluated probes that have been designed independently, and the aim was to evaluate their performance under a given hybridization protocol. For example, the unspecific signals for probe Pras08 could not be prevented under our stringent conditions. The five strains that showed the highest cross-hybridization are closely related to the target strain *P. salinarium* CCMP1897 and have 2 mismatches with the probe sequence. However, the signal for the perfect match target is five times greater than the highest of the non-target signals. This high difference can be used to discriminate target from non-target signals.

Hierarchical probes can prevent misinterpretation of false positive signals and will further improve the reliability of the microarray. Ribosomal genes with low, moderate, and highly variable regions offer the potential to design specific probes from higher taxonomic levels down to the species level (Lange et al. 1996; Metfies and Medlin 2004; Woese 1987). For example, a signal at the species level is only considered as true if all probes in the taxonomic hierarchy also show positive signals. The PhylochipAnalyzer software offers the possibility to examine all probes in a defined hierarchy and therefore represents a major progress in data processing and interpretation of microarray experiments (Metfies et al. 2007).

Probe signals can not be directly correlated with the amount of target molecules because hybridization efficiency of probes can vary over a wide range (Loy et al. 2005; Peplies et al. 2003; Peplies et al. 2006). If quantification of cells is important, total RNA should be used. The choice of either total RNA or PCR-fragments has advantages and disadvantages. RNA hybridization theoretically offers the best possibility of quantification

and provides a less distorted view of true community composition (Peplies et al. 2006). Cell counts and signals intensities can be correlated with calibration curves, but this procedure is tedious and needs to be done individually for each probe. Possible disadvantages are low yields of RNA from environmental samples and that RNA extraction can be inhibited by complex organic molecules (Alm and Stahl 2000; Peplies et al. 2006). RNA content can vary over the cell cycle, especially for prokaryotes (Countway and Caron 2006). Picoeukaryotes are very small in size (0.2 - 2µm), and their amount of rRNA may be low as well. In contrast, the introduction of a PCR-reaction target to amplify the target gene results in a bias, and it has been shown frequently that microbial communities are not correctly reflected (Kanagawa 2003; Medlin et al. 2006a; Simon et al. 2000; Speksnijder et al. 2001; Suzuki and Giovannoni 1996; Wintzingerode et al. 1997). By using a conventional PCR-reaction, no quantification of cell densities is possible. Another possibility for quantification is offered by the Real-Time PCR or Quantitative PCR (QPCR). This modification of the conventional PCR-protocol allows the product formation in the PCR reaction to be monitored *in-situ* by fluorescence (Marie et al. 2006). Picoeukaryotes have a relatively small copy number of rRNA genes, ranging from 2 - 4 copies per cell (Zhu et al. 2005). This might cause problems in the PCR-amplification. On the other hand, benefits of the PCR-step are the amplification of low target concentrations and the easy handling and labeling of the products. Because of its advantages, we decided to perform a conventional PCR-reaction, and hybridized PCR-fragments instead of RNA. We showed the applicability of microarrays for the detection and monitoring of picophytoplankton. For quantification of cell densities, further work must be done.

The secondary structure of the applied nucleic acid may likely hamper successful probe hybridization. This steric hindrance has been frequently observed in microarray hybridizations (Metfies and Medlin 2004; Peplies et al. 2003), and application of smaller PCR-fragments sometimes resulted in signal enhancement (Metfies and Medlin 2004). Application of a helper oligonucleotide that binds to a binding site in near proximity to the real target site can increase signals in a microarray hybridization as well (Fuchs et al. 2000; Niemeyer et al. 1998). However Peplies and co-workers (2003) also found a decrease in signals for some probes.

We have no experience in reusing our microarrays for new hybridizations, but in expression analysis, it has already been tested, and microarrays have been reused up to five times (Bao et al. 2002; Dolan et al. 2001). This could possibly cut down the relatively high costs of a microarray hybridization experiment.

The current microarray could be improved with more probes to obtain a more comprehensive view of natural picoplanktonic populations, which are notoriously difficult to study (Simon et al. 2000). For example, QPCR probes for *Ostreococcus* published by Countway and Caron (2006) can be evaluated on the chip.

We have not tried to make longer probes for those that showed weak cross-hybridization. In another study (Metfies and Medlin 2007), longer probes for the pigmented cryptophytes were more specific.

In addition, he current chip could serve as a fast and reliable tool for quality control of culture collections. It is not necessary to have a broad taxonomic knowledge, and even a lab-trained layman can verify if the culture contains the expected cells (Metfies and Medlin 2004).

Conclusion

We demonstrated here the suitability of the microarray for detection of prasinophytes. The probes evaluated and tested in this study offer the potential to analyze a large number of picoplanktonic taxa from the Prasinophyta in one experiment. Despite its limitations, this reliable and robust method can assess the biodiversity in picoeukaryotic communities and report their occurrences in close spatial and temporal scales. It represents one of the first applications of a low-density phylochip for the simultaneous detection and identification of different picoplanktonic species. The identity of algal cultures was tested and confirmed with the microarray. In addition, one environmental sample was hybridized, and the signals of the microarray were confirmed with the subsequent sequencing.

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References

ALM, E. W., and D. A. STAHL. 2000. Critical factors influencing the recovery and integrity of rRNA extracted from environmental samples: use of an optimized protocol to measure depth-related biomass distribution in freshwater sediments. J. Microbiol. Meth. **40:** 153–162.

ALTSCHUL, S. F., W. GISH, W. MILLER, E. W. MYERS, and D. J. LIPMAN. 1990. Basic local alingment search tool. J. Mol. Biol. **215:** 403–410.

ANDERSEN, R. A., R. R. BIDIGARE, M. D. KELLER, and M. LATASA. 1996. A comparison of HPLC pigment signatures and electron microscopic observations for oligotrophic waters of the North Atlantic and Pacific Oceans. Deep Sea Res. II **43:** 517-537.

ANDERSEN, R. A., D. POTTERT, and J. C. BAILEY. 2002. *Pinguiococcus pyrenoidosus* gen. et sp. nov. (Pinguiophyceae), a new marine coccoid alga. Phycol. Res. **50:** 57-65.

ANDERSEN, R. A., G. W. SAUNDERS, M. P. PASKIND, and J. P. SEXTON. 1993. Ultrastructure and 18S rRNA gene sequence for *Pelagomonas calceolata* gen. et sp. nov. and the description of a new algal class, the Pelagophyceae classis nov. J. Phycol. **29:** 701-715.

BAO, Z., M. WENLI, S. RONG, L. LING, G. QUIYE, and Z. WENLING. 2002. Re-use of a stripped cDNA microarray. Brit J. Biomed. Sci. **59:** 118-119.

BHATTACHARYA, D., and L. K. MEDLIN. 1998. Algal phylogeny and the origin of land plants. Plant Physiol. **116:** 9–15.

BIEGALA, I. C., F. NOT, D. VAULOT, and N. SIMON. 2003. Quantitative assessment of picoeukaryotes in the natural environment by using taxon-specific oligonucleotide probes in association with tyramide signal amplification, fluorescence-*in-situ-h*ybridization and flow cytometry. Appl. Environ. Microbiol. **69:** 5519-5529.

BOIREAU, W., J. C. ZEEHA, P. E. PUIGA, and D. POMPON. 2005. Unique supramolecular assembly of a redox protein with nucleic acids onto hybrid bilayer: towards a dynamic DNA chip. Biosens. Bioelectron. **20:** 1631-1637.

BUTCHER, R. 1952. Contributions to our knowledge of the smaller marine algae. J. Mar. Biol. Assoc. UK **31:** 175-191.

CAMPBELL, L., H. A. NOLLA, and D. VAULOT. 1994. The importance of *Prochlorococcus* to community structure in the central North Pacific Ocean. Limnol. Oceanogr. **39:** 954-961.

CHISHOLM, S. W., R. J. OLSON, E. R. ZETTLER, R. GOERICKE, J. B. WATERBURY, and N. A. WELSCHMEYER. 1988. A novel free-living prochlorophyte abundant in the oceanic euphotic zone. Nature **334:** 340-343.

CHRÉTIENNOT-DINET, M.-J. and others 1995. A new marine picoeucaryote: *Ostreococcus tauri* gen. et sp. nov. (Chlorophyta, Prasinophyceae). Phycologia **34:** 285-292.

COUNTWAY, P. D., and D. A. CARON. 2006. Abundance and distribution of *Ostreococcus* sp. in the San Pedro Channel, California, as revealed by Quantitative PCR. Appl. Environ. Microbiol. **72:** 2496-2506.

COURTIES, C. and others 1994. Smallest eukaryotic organism. Nature **370:** 255.

DELONG, E. F., G. S. WICKHAM, and N. R. PACE. 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science **243:** 1360-1363.

DERELLE, E. and others 2006. From the Cover: Genome analysis of the smallest free-living eukaryote *Ostreococcus tauri* unveils many unique features. Proc. Natl. Acad. Sci. USA **103:** 11647-11652.

DÍEZ, B., C. PEDRÓS-ALIÓ, T. L. MARSH, and R. MASSANA. 2001. Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques. Appl. Environ. Microbiol. **67:** 2942–2951.

DOLAN, P. L., Y. WU, L. K. ISTA, R. L. METZENBERG, M. A. NELSON, and G. P. LOPEZ. 2001. Robust and efficient synthetic method for forming DNA microarrays. Nucleic Acids Res. **29:** e107.

FERIOTTO, G., M. BORGATTI, C. MISCHIATI, N. BIANCHI, and R. GAMBARI. 2002. Biosensor technology and surface plasmon resonance for real-time detection of genetically modified roundup ready soybean gene sequences. J. Agric. Food Chem. **50:** 955-962.

FUCHS, B. M., F. O. GLÖCKNER, J. WULF, and R. I. AMANN. 2000. Unlabeled helper oligonucleotides increase the *in-situ* accessibility of 16S rRNA for fluorescently labeled oligonucleotide probes. Appl. Environ. Microbiol. **66:** 3603–3607.

FULLER, N. J. and others 2006a. Analysis of photosynthetic picoeukaryote diversity at open ocean sites in the Arabian Sea using a PCR biased towards marine algal plastids. Aquat. Microb. Ecol. **43:** 79–93.

---. 2006b. Molecular analysis of photosynthetic picoeukaryote community structure along an Arabian Sea transect. Limnol. Oceanogr. **51:** 2502-2514.

GENTRY, T. J., G. S. WICKHAM, C. W. SCHADT, Z. HE, and J. ZHOU. 2006. Microarray applications in microbial ecology research. Microbial Ecol. **52:** 159-175.

GESCHER, C., K. METFIES, and L. K. MEDLIN. 2007. The Alex Chip-Development of a DNA Chip for identification and monitoring of *Alexandrium*. Harmful Algae, to be submitted.

GRAVES, D. J. 1999. Powerful tools for genetic analysis come of age. Trends Biotechnol. **17:** 127-134.

GROBEN, R., U. JOHN, G. ELLER, M. LANGE, and L. K. MEDLIN. 2004. Using fluorescently-labelled rRNA probes for hierarchical estimation of phytoplankton diversity – a mini-review. Nova Hedwigia **79:** 313-320.

GROBEN, R., and L. K. MEDLIN. 2005. *In -situ*-hybridization of phytoplankton using fluorescently labeled rRNA probes., p. 299-310. *In* E. A. Zimmer and E. H. Roalsen [eds.], Methods in Enzymology, Molecular Evolution: Producing the Biochemical Data. Elsevier Academic Press, San Diego, USA.

GUILLARD, R. R. L., and J. H. RYTHER. 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* Cleve. Can. J. Microbiol. **8:** 229-239.

GUILLOU, L., M.-J. CHRÉTIENNOT-DINET, L. K. MEDLIN, H. CLAUSTRE, S. LOISEAUX-DE GOER, and D. VAULOT. 1999a. *Bolidomonas*: A new Genus with two species belonging to a new algal class, The Bolidophyceae (Heterokonta). J. Phycol. **35:** 368–381.

GUILLOU, L. and others 2004. Diversity of picoplanktonic Prasinophytes assessed by direct nuclear SSU rDNA sequencing of environmental samples and novel isolates retrieved from oceanic and coastal marine ecosystems. Protist **155:** 193-214.

GUILLOU, L., S.-Y. MOON-VAN DER STAAY, H. CLAUSTRE, F. PARTENSKY, and D. VAULOT. 1999b. Diversity and abundance of Bolidophyceae (Heterokonta) in two oceanic regions. Appl. Environ. Microbiol. **65:** 4528-4536.

HASEGAWA, T. and others 1996. *Prasinoderma coloniale* gen. et sp. nov., a new pelagic coccoid prasinophyte from the western Pacific ocean. Phycologia **35:** 170–176.

JANSON, S., and P. K. HAYES. 2006. Molecular taxonomy of Harmful Algae, p. 9-21. *In* E. Granéli and J. T. Turner [eds.], Ecology of Harmful Algae. Ecological Studies. Springer Verlag, Berlin, Heidelberg, Germany.

JOHN, U., A. CEMBELLA, C. HUMMERT, M. ELBRÄCHTER, R. GROBEN, and L. K. MEDLIN. 2003. Discrimination of the toxigenic dinoflagellates *Alexandrium tamarense* and *A. ostenfeldii* in co-occurring natural populations from Scottish coastal waters. Eur. J. Phycol. **38:** 25-40.

JOHN, U., L. K. MEDLIN, and R. GROBEN. 2005. Development of specific rRNA probes to distinguish between geographic clades of the *Alexandrium tamarense* species complex. J. Plankton Res. **27:** 199–204.

JOHNSON, P. W., and J. M. SIEBURTH. 1979. Chroococcoid cyanobacteria in the sea: a ubiquitous and diverse phototrophic biomass. Limnol. Oceangr. **24:** 928-935.

JOINT, I. R., N. J. P. OWEN, and A. J. POMROY. 1986. Seasonal production of photosynthetic picoplankton and nanoplankton in the Celtic Sea. Mar. Ecol. Prog. Ser. **28:** 251–258.

KANAGAWA, T. 2003. Bias and artifacts in multitemplate polymerase chain reactions (PCR). J. Biosci. Bioeng. **96:** 317-323.

KAWACHI, M. and others 2002. The Pinguiophyceae classis nova, a new class of photosynthetic stramenopiles whose members produce large amounts of omega-3 fatty acids. Phycol. Res. **50:** 31-47.

KELLER, M. D., R. C. SELVIN, W. CLAUS, and R. R. L. GUILLARD. 1987. Media for the culture of oceanic ultraphytoplankton. J. Phycol. **23:** 633-338.

KI, J.-S., and M.-S. HAN. 2006. A low-density oligonucleotide array study for parallel detection of harmful algal species using hybridization of consensus PCR products of LSU rDNA D2 domain. Biosens. Bioelectron. **21:** 1812-1821.

LANGE, M. and others 1996. Identification of the class Prymnesiophyceae and the genus *Phaeocystis* with ribosomal RNA-targeted nucleic acid probes detected by flow cytometry. J. Phycol. **32:** 858-868.

LEHNER, A. and others 2005. Oligonucleotide microarray for identification of *Enterococcus* species. FEMS Microbiol. Lett. **246:** 133-142.

LI, W. K. W. 1994. Primary production of prochlorophytes, cyanobacteria, and eucaryotic ultraphytoplankton: Measurements from flow cytometric sorting. Limnol. Oceanogr. **39:** 169-175.

LOVEJOY, C. and others 2007. Distribution, phylogeny, and growth of cold-adapted picoprasinophytes in Arctic seas. J. Phycol. **43:** 78-89.

LOY, A. and others 2002. Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Appl. Environ. Microbiol. **68:** 5064-5081.

---. 2005. 16S rRNA gene-based oligonucletide microarray for environmental monitoring of the betaproteobacterial order "Rhodocyclales". Appl. Environ. Microbiol. **71:** 1373-1386.

LUDWIG, W. and others 2004. ARB: a software environment for sequence data. Nucleic Acids Res. **32:** 1363-1371.

MACKEY, D. J., J. BLANCHOT, H. W. HIGGINS, and J. NEVEUX. 2002. Phytoplankton abundances and community structure in the equatorial Pacific. Deep Sea Res. II **49:** 2561-2582.

MANTON, I., and M. PARKE. 1960. Further observations on small green flagellates with special reference to possible relatives of *Chromulina pusilla* Butcher. J. Mar. Biol. Assoc. UK **39:** 275-298.

MARIE, D., F. ZHU, V. BALAGUE, J. RAS, and D. VAULOT. 2006. Eukaryotic picoplankton communities of the Mediterranean Sea in summer assessed by molecular approaches (DGGE, TTGE, QPCR). FEMS Microbiol. Ecol. **55:** 403-415.

MASSANA, R., L. GUILLOU, B. DÍEZ, and C. PEDRÓS-ALIÓ. 2002. Unveiling the organisms behind novel eukaryotic ribosomal DNA sequences from the ocean. Appl. Environ. Microbiol. **68:** 4554-4558.

MEDLIN, L. K., H. J. ELWOOD, S. STICKEL, and M. L. SOGIN. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene **71:** 491-499.

MEDLIN, L. K., K. METFIES, H. MEHL, K. H. WILTSHIRE, and K. VALENTIN. 2006a. Picoeukaryotic plankton diversity at the Helgoland Time Series Site as assessed by three molecular methods. Microbial Ecol. **52:** 53-71.

MEDLIN, L. K., and N. SIMON. 1998. Phylogenetic analysis of marine phytoplankton, p. 161-186. *In* K. E. Cooksey [ed.], Molecular approaches to the study of the ocean. Chapman & Hall, London, UK.

MEDLIN, L. K., K. VALENTIN, K. METFIES, K. TÖBE, and R. GROBEN. 2006b. Molecular tools in the study of marine microbial diversity. Encyclopedia of Life Support Systems (EOLSS), Second edition, http://www.eolss.net., Oxford, UK.

METFIES, K., P. BORSUTZKI, C. GESCHER, L. K. MEDLIN, and S. FRICKENHAUS. 2007. The PhylochipAnalyzer - A programm for analyzing hierarchical probe-sets. Mol. Ecol. Notes, accepted.

METFIES, K., and L. K. MEDLIN. 2004. DNA microchips for phytoplankton: The fluorescent wave of the future. Nova Hedwigia **79:** 321-327.

---. 2007. Refining cryptophyte identification with DNA-Microarrays. Mol. Ecol. Notes, submitted.

METFIES, K., K. TÖBE, C. A. SCHOLIN, and L. K. MEDLIN. 2006. Laboratory and field applications of ribosomal RNA probes to aid the detection and monitoring of Harmful

Algae, p. 311-325. *In* E. Granéli and J. T. Turner [eds.], Ecology of Harmful Algae. Springer Verlag, Berlin, Heidelberg, Germany.

MOESTRUP, Ø. 1991. Further studies of presumedly primitive green algae, including the description of Pedinophyceae class. nov. and *Resultor* gen. nov. J. Phycol. **27:** 119-133.

MOESTRUP, Ø., and J. THRONDSEN. 1988. Light and electron microscopical studies on *Pseudoscourfieldia marina*, a primitive scaly green flagellate (Prasionphyceae) with posterior flagella. Can.J. Bot. **66:** 1415-1434.

MOON-VAN DER STAAY, S. Y., R. DE WACHTER, and D. VAULOT. 2001. Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. Nature **409:** 607-610.

NAKAYAMA, T. and others 1998. The basal position of scaly green flagellates among the green algae (Chlorophyta) is revealed by analyses of nuclear-encoded SSU rRNA sequences. Protist **149:** 367–380.

NIEMEYER, C. M., W. BÜRGER, and J. PEPLIES. 1998. Covalent DNA-streptavidin conjugates as building blocks for novel biometallic nanostructures. Angew. Chem. Int. Ed. **37:** 2265–2268.

NOT, F., M. LATASA, D. MARIE, T. CARIOU, D. VAULOT, and N. SIMON. 2004. A single species, *Micromonas pusilla* (Prasinophyceae), dominates the eukaryotic picoplankton in the western English Channel. Appl. Environ. Microbiol. **70:** 4064-4072.

NOT, F., N. SIMON, I. C. BIEGALA, and D. VAULOT. 2002. Application of fluorescent-*in-situ*-hybridization coupled with tyramide signal amplification (FISH-TSA) to assess eukaryotic picoplankton composition. Aquat. Microb. Ecol. **28:** 57-166.

NOT, F. and others 2007. Picobiliphytes: A marine picoplanktonic algal group with unknown affinities to other eukaryotes. Science **315:** 253-255.

PEPLIES, J., F. O. GLÖCKNER, and R. AMANN. 2003. Optimization strategies for DNA microarray-based detection of bacteria with 16S rRNA-targeting oligonucleotide probes. Appl. Environ. Microbiol. **69:** 1397-1407.

PEPLIES, J., F. O. GLÖCKNER, R. AMANN, and W. LUDWIG. 2004a. Comparative sequence analysis and oligonucleotide probe design based on 23S rRNA genes of Alphaproteobacteria from North Sea bacterioplankton. Syst. Appl. Microbiol. **27:** 573-580.

PEPLIES, J., C. LACHMUND, F. O. GLÖCKNER, and W. MANZ. 2006. A DNA microarray platform based on direct detection of rRNA for characterization of freshwater sediment-related prokaryotic communities. Appl. Environ. Microbiol. **72:** 4829-4838.

PEPLIES, J., S. C. K. LAU, J. PERNTHALER, R. AMANN, and F. O. GLÖCKNER. 2004b. Application and validation of DNA microarrays for the 16S rRNA-based analysis of marine bacterioplankton. Environ. Microbiol. **6:** 638-645.

RICE, J., M. SLEIGH, P. BURKILL, G. TARRAN, C. O'CONNOR, and M. ZUBKOV. 1997. Flow cytometric analysis of characteristics of hybridization of species-specific fluorescent oligonucleotide probes to rRNA of marine nanoflagellates. Appl. Environ. Microbiol. **63:** 938-944.

SCHENA, M., D. SHALON, R. W. DAVIS, and P. O. BROWN. 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science **270:** 467-470.

SCHENA, M., D. SHALON, R. HELLER, A. CHAI, P. O. BROWN, and R. W. DAVIS. 1996. Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. Proc. Natl. Acad. Sci. USA **93:** 10614-10619.

SCHOLIN, C. A. 1998a. Development of nucleic acid probe-based diagnostics for identifying and enumerating Harmful Algae Bloom species. *In* D. M. Anderson, A. D. Cembella and G. M. Hallegraeff [eds.], Physiological Ecology of Harmful Algae Blooms. Springer Verlag, Berlin, Heidelberg, Germany.

---. 1998b. Morphological, genetic, and biogeographic relationships of the toxic dinoflagellates *Alexandrium tamarense*, *A. catenella* and *A. fundyense*, p. 13-27. *In* D. M. Anderson, A. D. Cembella and G. M. Hallegraeff [eds.], Physiological Ecology of Harmful Algae Blooms. Springer Verlag, Berlin, Heidelberg, Germany.

SCHOLIN, C. A., E. G. VRIELING, L. PEPERZAK, L. L. RHODES, and P. RUBLEE. 2003. Detection of HAB species using lectin, antibody and DNA probes, p. 131-163. *In* G. M. Hallegraeff, D. M. Anderson and A. D. Cembella [eds.], Manual on Harmful Marine Microalgae. United Nations Educational, Scientific and Cultural Organization, Paris, France.

SIEBURTH, J. M., M. D. KELLER, P. W. JOHNSON, and S. M. MYKLESTAD. 1999. Widespread occurrence of the oceanic ultraplankter, *Prasinococcus capsulatus* (Prasinophyceae), the diagnostic "Golgi-decapore complex" and the newly described polysaccharide "capsulan.". J. Phycol. **35:** 1032-1043.

SIMON, N. and others 2000. Oligonucleotide probes for the identification of three algal groups by dot-blot and fluorescent whole-cell hybridization. J. Eukaryot. Microbiol. **47:** 76–84.

SIMON, N., N. LEBOT, D. MARIE, F. PARTENSKY, and D. VAULOT. 1995. Fluorescent-*in-situ*-hybridization with rRNA-targeted oligonucleotide probes to identify small phytoplankton by flow cytometry. Appl. Environ. Microbiol. **61:** 2506-2513.

SLAPETA, JAN, P. LÓPEZ-GARCÍA, and D. MOREIRA. 2006. Global dispersal and ancient cryptic species in the smallest marine eukaryotes. Mol. Biol. Evol. **23:** 23-29.

SPEKSNIJDER, A. G. C. L., G. A. KOWALCHUK, S. DE JONG, E. KLINE, J. R. STEPHEN, and H. J. LAANBROEK. 2001. Microvariation artifacts introduced by PCR and cloning of closely related 16S rRNA gene sequences. Appl. Environ. Microbiol. **67:** 469-472.

STEINKÖTTER, J., D. BHATTACHARYA, I. SEMMELROTH, C. BIBEAU, and M. MELKONIAN. 1994. Prasinophytes form independent lineages within the Chlorophyta: Evidence from ribosomal RNA sequence comparisons. J. Phycol. **30:** 340-346.

SUZUKI, M. T., and S. J. GIOVANNONI. 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. **62:** 625–630.

THOMSEN, H. A., and K. R. BUCK. 1998. Nanoflagellates of the central California waters: taxonomy, biogeography and abundance of primitive, green flagellates (Pedinophyceae, Prasinophyceae). Deep Sea Res. II **45:** 1687-1707.

TURMEL, M. and others 1999. The complete mitochondrial DNA sequences of *Nephroselmis olivacea* and *Pedinomonas minor* : two radically different evolutionary patterns within green algae. Plant Cell **11:** 1717-1730.

VAN DEN HOEK, C., D. G. MANN, and H. M. JAHNS. 1995. Algae: an introduction to phycology. Cambridge University Press, New York, USA.

VOLKMAN, J. K., S. M. BARRETT, G. A. DUNSTAN, and S. W. JEFFREY. 1994. Sterol biomarkers for microalgae from the green algal class Prasinophyceae. Org.Geochem. **21:** 1211-1218.

WATERBURY, J. B., S. W. WATSON, R. R. L. GUILLARD, and L. E. BRAND. 1979. Widespread occurrence of a unicellular, marine, planktonic, cyanobacterium. Nature **277:** 293-294.

WINTZINGERODE, F. V., U. B. GOEBEL, and E. STACKEBRANDT. 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. **21:** 213–229.

WOESE, C. 1987. Bacterial evolution. Microbiol. Rev. **51:** 221-271.

YE, R. W., T. WANG, L. BEDZYK, and K. M. CROKER. 2001. Applications of DNA microarrays in microbial systems. J. Microbiol. Meth. **47:** 257-272.

ZHU, F., R. MASSANA, F. NOT, D. MARIE, and D. VAULOT. 2005. Mapping of picoeucaryotes in marine ecosystems with quantitative PCR of the 18S rRNA gene. FEMS Microbiol. Ecol. **52:** 79-92.

ZINGONE, A., M. BORRA, C. BRUNET, G. FORLANI, W. H. C. F. KOOISTRA, and G. PROCACCINI. 2002. Phylogenetic position of *Crustomastix stigmatica* sp. nov. and D*olichomastix tenuilepis* in relation to the Mamiellales (Prasinophyceae, Chlorophyta). J. Phycol. **38:** 1024-1039.

ZINGONE, A., F. NATALE, E. BIFFALI, M. BORRA, G. FORLANI, and D. SARNO. 2006. Diversity in morphology, infectivity, molecular characteristics and induced host resistance between two viruses infecting *Micromonas pusilla*. Aquat. Microb. Ecol. **45:** 1-14.

ZINGONE, A., D. SARNO, and G. FORLANI. 1999. Seasonal dynamics in the abundance of *Micromonas pusilla* (Prasinophyceae) and its viruses in the Gulf of Naples (Mediterranean Sea). J. Plankton Res. **21:** 2143-2159.

Table 1. Origin of algal cultures used in this study and their corresponding phytogenetic clades according to Guillou et al (2004)

No.	Species	Medium	Temperature	Origin	Specific target for clade
1	unidentified sp. Prasinophyceae CCMP1413	K	20 °C	North Atlantic	VI
2	*Prasinococcus capsulatus* CCMP1193	f/2-Si	20 °C	North Atlantic, Gulf Stream	VI
3	*Prasinoderma coloniale* CCMP1220	K	20 °C	North Atlantic, Gulf Stream	VI
4	*Prasinococcus* cf. *capsulatus* CCMP1194	K	20 °C	North Atlantic, Gulf Stream	VI
5	*Prasinococcus* cf. *capsulatus* CCMP1202	K	20 °C	North Atlantic, Caribbean Sea	VI
6	*Bathycoccus prasinos* RCC496	K	15 °C	Mediterranean Sea, Spanish coast	II
7	*Bathycoccus prasinos* CCMP1898	K	15 °C	Mediterranean Sea, Gulf of Naples	II
8	*Ostreococcus tauri* RCC116	K	20 °C	Mediterranean Sea, Thau lagoon	II
9	*Ostreococcus* spec. RCC344	K	20 °C	Atlantic Ocean, Maroccan upwelling	II
10	*Micromonas pusilla* CCMP490	K	20 °C	North Atlantic, Nantucket Sound	II
11	*Micromonas pusilla* CCMP1195	f/2-Si	15 °C	North Atlantic, Gulf of Maine	II
12	*Mantoniella squamata* CCMP480	K	20 °C	Norfolk, United Kingdom	II
13	unidentified Coccoid RCC287	K	20 °C	Pacific Ocean, Equatorial Pacific	VIIa
14	unidentified sp. Chlorophyceae CCMP1205	f/2-Si	20 °C	collection site unknown, Trident cruise	VIIa
15	*Pycnococcus provasolii* CCMP1203	K	20 °C	North Atlantic	V
16	*Pycnococcus provasolii* CCMP1199	K	20 °C	North Atlantic, Gulf of Mexico	V
17	*Picocystis salinarium* CCMP1897	f/2-Si	20 °C	North Pacific, San Francisco Bay	VIIc
18	*Pterosperma cristatum* NIES221	K	20 °C	Harima-Nada, Seto Inland Sea, Japan	part of I
19	*Pyramimonas parkae* CCMP724	f/2-Si	15 °C	North Pacific, Santa Catalina Island	part of I
20	*Nephroselmis pyriformis* CCMP717	K	15 °C	North Atlantic, Galveston Channel	part of III

CCMP: Provasoli-Guillard Center for Culture of Marine Phytoplankton, USA; RCC: Roscoff Culture Collection, France;

NIES: National Institute for Environmental Studies, Japan

Table 2. Probe sequences for the microarray

Probe name	Probe sequence (5'- 3')	Target	Source
Chlo01	GTG GTG GTC CGC ACC TCG	Chlorophyta	Simon et al. 1995
Chlo02	CTT CGA GCC CCC AAC TTT	Chlorophyta	Simon et al. 2000
Pras01	ACG GTC CCG AAG GGT TGG	*Pseudoscourfieldia marina, Pycnococcus provasolii* (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 genus *Dolichomastix*	Not et al. 2004
Pras05	GCC AGA ACC ACG TCC TCG	Clade VIIA (CCMP 1205+RCC287)	Not et al. 2004
Pras06	AAT CAA GAC GGA GCG CGT	Clade VIIB (environm. sequences, OLI1059, 11305, 11345)	Not et al. 2004
Pras07	CCG ACA GAA AGA CGC AGA	*Pseudoscourfieldia marina, Pycnococcus provasolii* (Clade V)	Not et al. 2004
Pras08	ATT GTG TGG GTC TTC GGG	*Picocystis salinarium* (Clade VII C)	Gescher, this study
Pras09A1	GGT TGC GTT AGT CTT GCT	*Pterosperma cristatum* (Clade I)	Gescher, this study
Pras09A2	GCC GCC TTC GGG CGT TTT	*Pyramimonas, Prasinopapilla, Cymbomonas* (Clade I)	Gescher, this study
Pras09D	AAC TGG CTC GGT ACG CGG	*Halosphaera spec.* (Clade I)	Gescher, this study
Pras10B	TAA AAG ACC GAC CGC TTC	*Nephroselmis pyriformis, Pseudoscourfieldia marina* (CladeIII)	Gescher, this study
Pras10F	CGT TTC AAC TCG ACC AGT	*Nephroselmis pyriformis* (different from 10B) (CladeIII)	Gescher, this study
Pras10H	CAC TGG CGC GCC CCA TCT	*Nephroselmis oliviaceae* (CladeIII)	Gescher, this study
Bathy01	ACT CCA TGT CTC AGC GTT	*Bathycoccus prasinos*	Not et al. 2004
Micro01	AAT GGA ACA CCG CCG GCG	*Micromonas pusilla*	Not et al. 2004
Ostero01	CCT CCT CAC CAG GAA GCT	*Ostreococcus tauri*	Not et al. 2004
MicroA	CCG TCA AGA GGC CGC GGT	*Micromonas pusilla,* Clade A, according to Guillou et al. 2004	Simon, unpublished
MicroB	CAC GAC CAA CAG ACG GTT	*Micromonas pusilla,* Clade B, according to Guillou et al. 2004	Simon, unpublished
MicroC	ACG GCG GCG AAC CGC AAT	*Micromonas pusilla,* Clade C, according to Guillou et al. 2004	Simon, unpublished
Positive control (PC)	ATG GCC GAT GAG GAA CGT	*S. cerevisiae*, TBP	Metfies and Medlin 2005
Negative control (NC)	TCC CCC GGG TAT GGC CGC		Metfies and Medlin 2005

Table 3. Sequences of the sequencing primers

Primer	Sequence (5'-3')
1F	AAC CTG GTT GAT CCT GCC AGT
82F	GAA ACT GCG AAT GAA TGG CTC
300F	AGG GTT CGA TTC CGG AG
528F	GCG GTA ATT CCA GCT CAA A
1055F	GGT GGT GCA TGG CCG TTC TT
536R	GAT TTA CCG CGG CGG CTG
1055R	GGT GGT GCA TGG CCG TTC TT
1400R	ACG GGC GGT GTG TAC
1528R	TGA TCC TTC TGC AGG TTC ACC TAC

Table 4. Cultures and one environmental sample that were sequenced and their best BLAST hits

Cuture/Sample	Blast hits
Prasinococcus cf.capsulatus CCMP 1202	*Prasinococcus sp.* CCMP1407, MBIC11010, MBIC1101, CCMP1202
Ostreococcus spec. RCC 344	*Ostreococcus sp.* RCC 344
unidentified Coccoid RCC 287	*Bathycoccus prasinos, Micromonas pusilla*
He010218	*Bathycoccus prasinos, Mantoniella squamata, Micromonas pusilla*

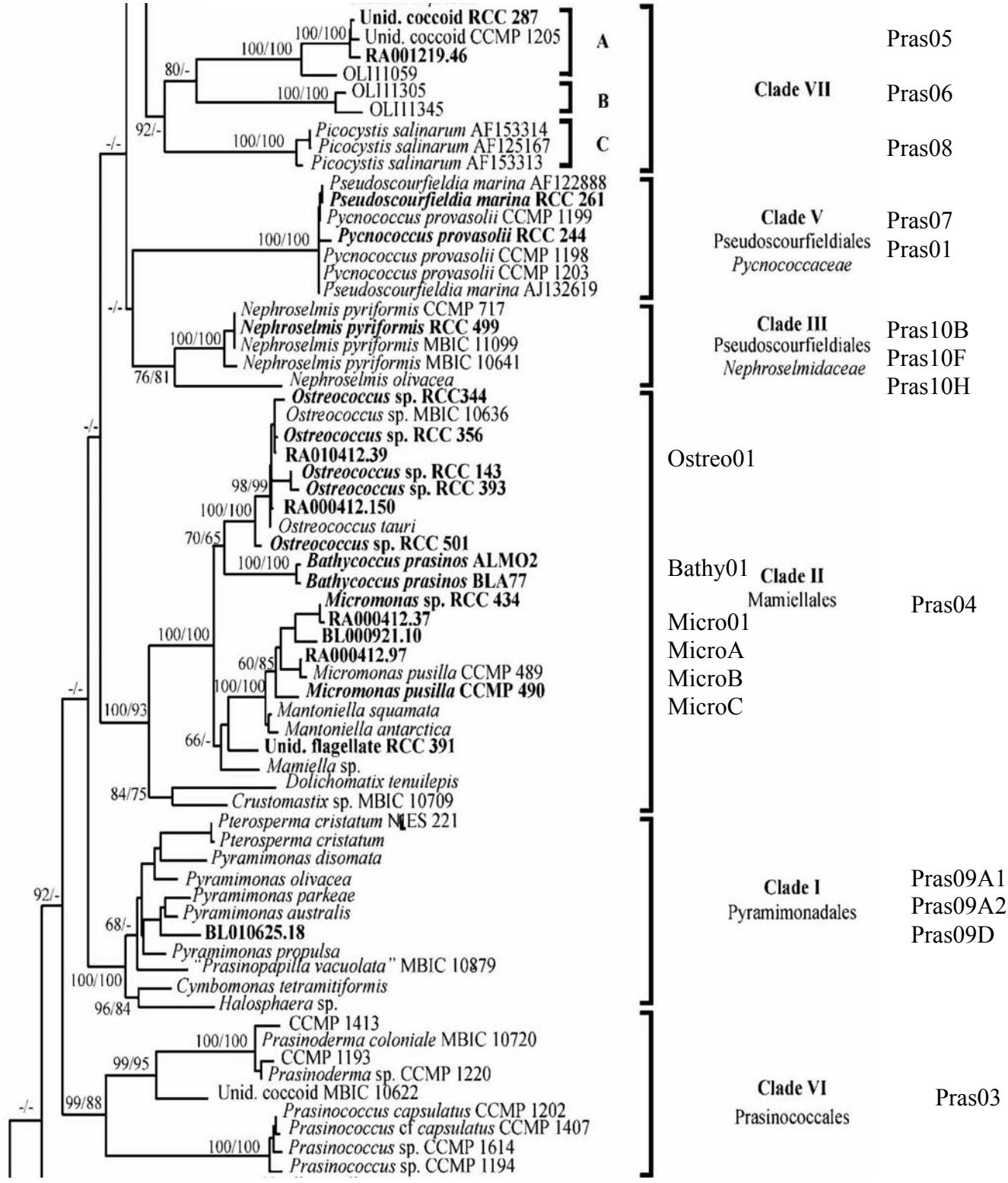

Figure 1. Tree of Prasinophyta diversity (Guillou et al. 2004). For species identification see Table1.

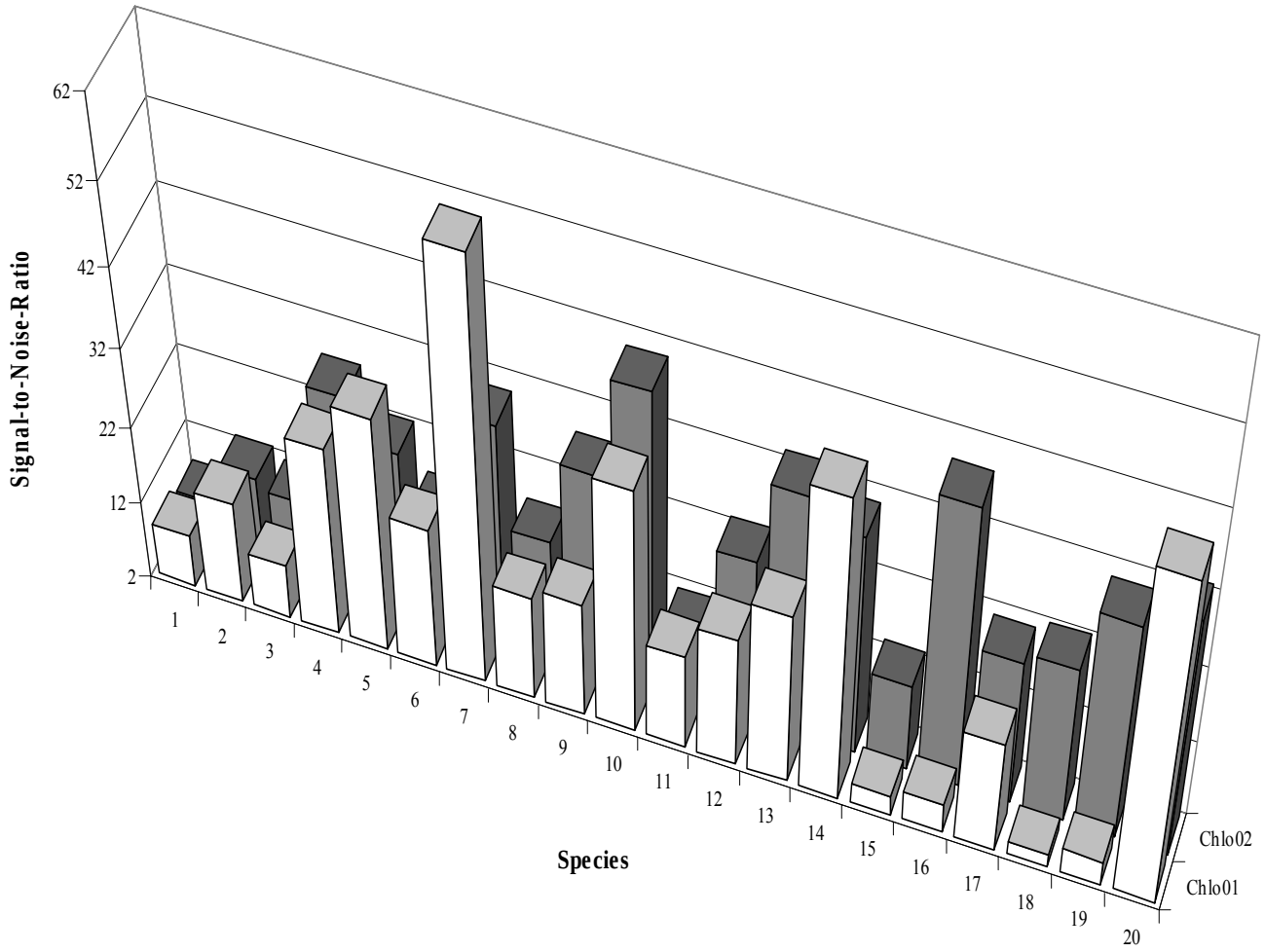

Figure 2. Signal-to-noise-ratios of all cultures hybridized to both Chlo 01 and Chlo02. Refer to Table 1 for species identification.

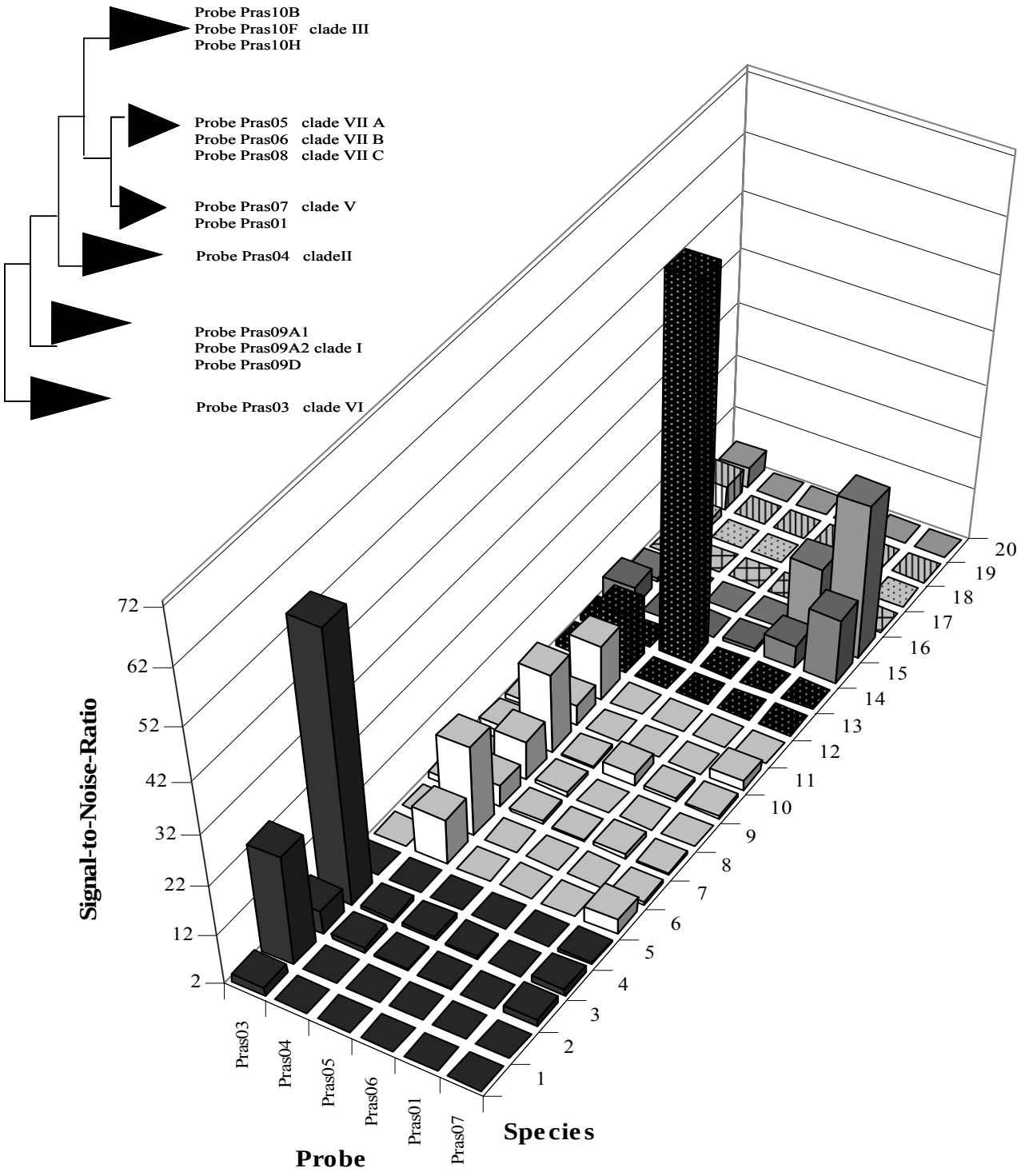

Figure 3. Signal-to-noise-ratios of all cultures hybridized to probes from clade II and clade V to VII. Refer to Table 1 for species identification.

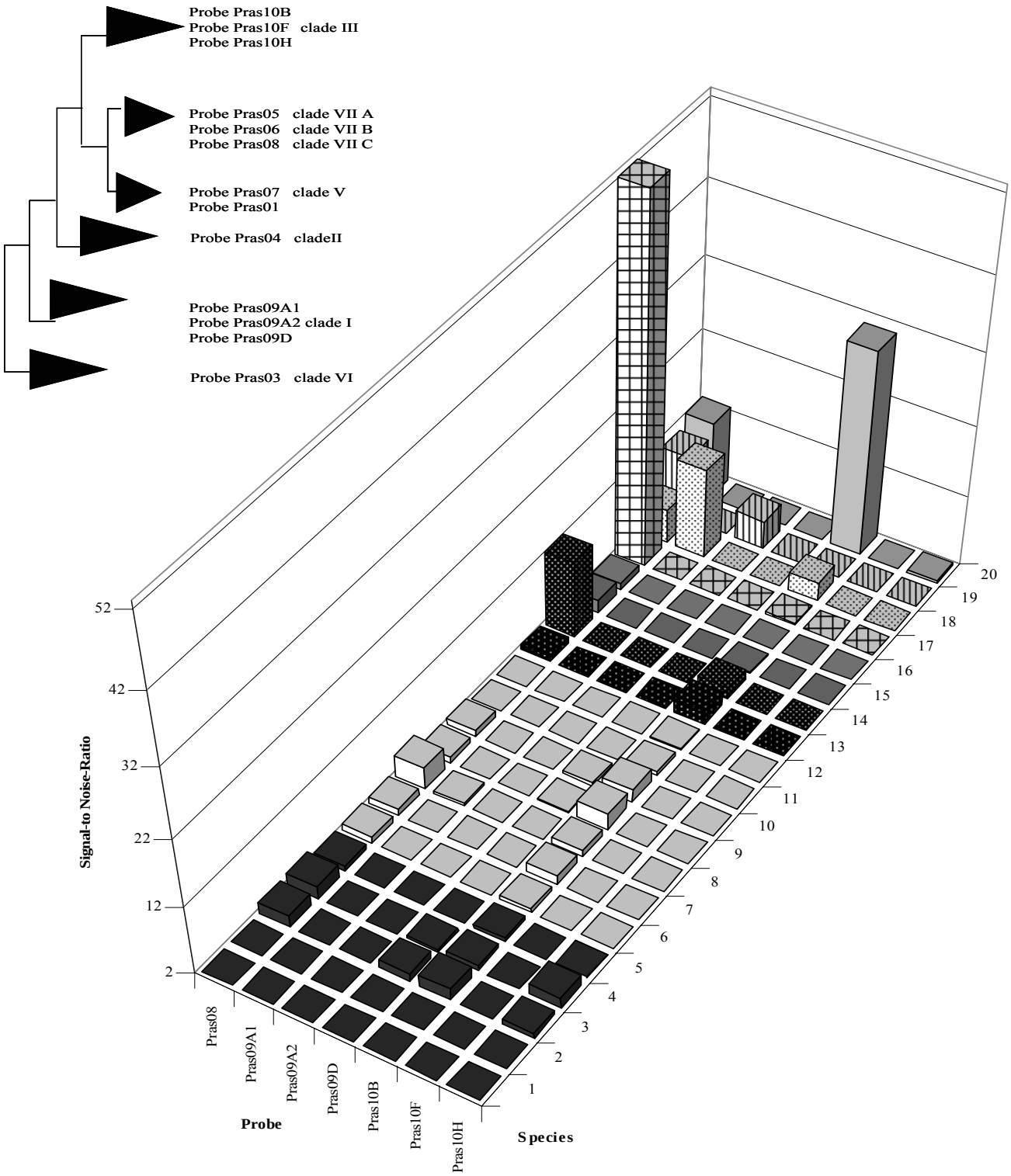

Figure 4. Signal-to-noise-ratios of all cultures hybridized to probes from clade I, clade III and clade VII. Refer to Table 1 for species identification.

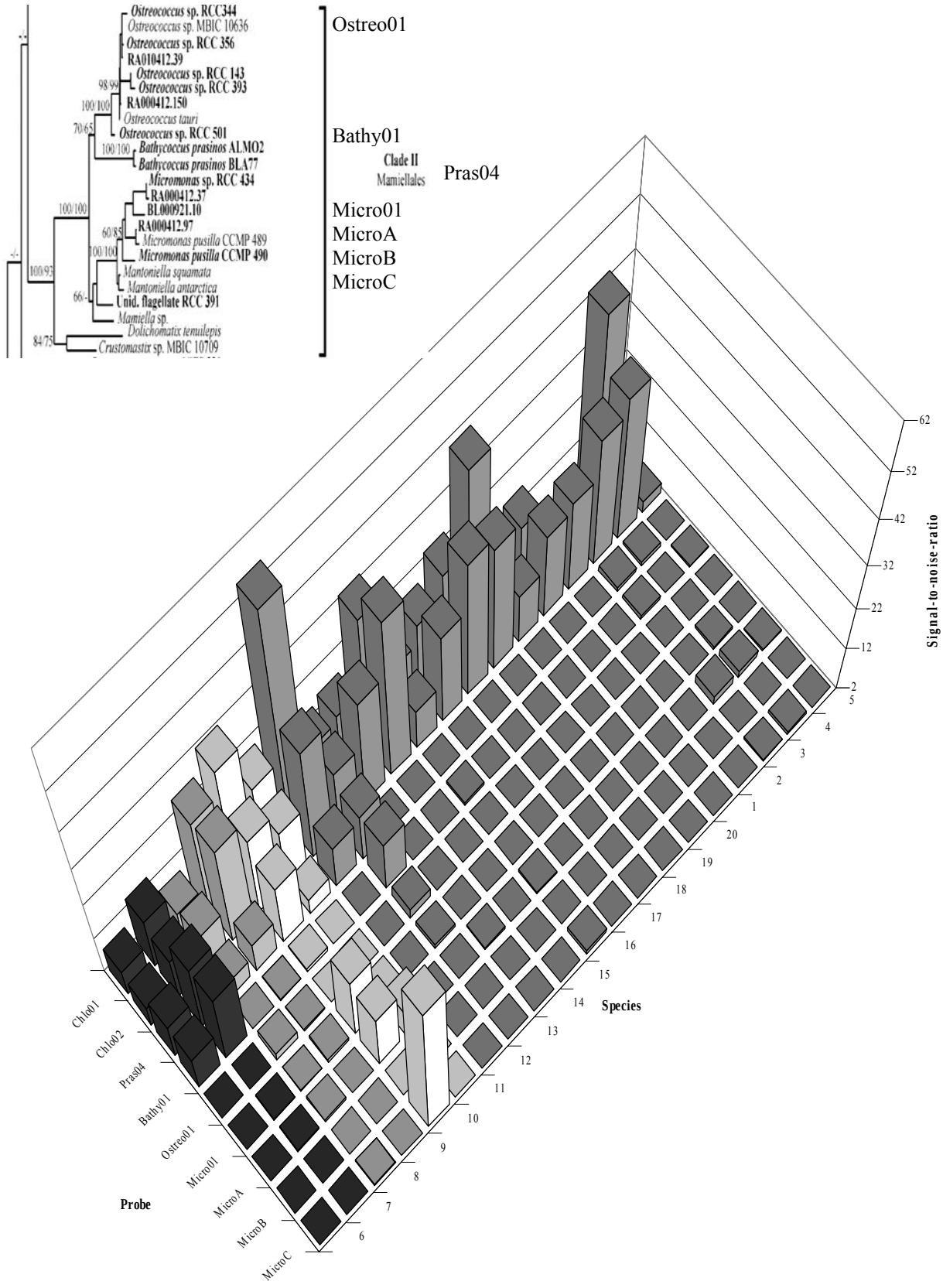

Figure 5. Signal-to-noise-ratios of all cultures hybridized to the Chlo01 and Chlo02, the Pras04, and the species level probes for clade II. Refer to Table 1 for species identification.

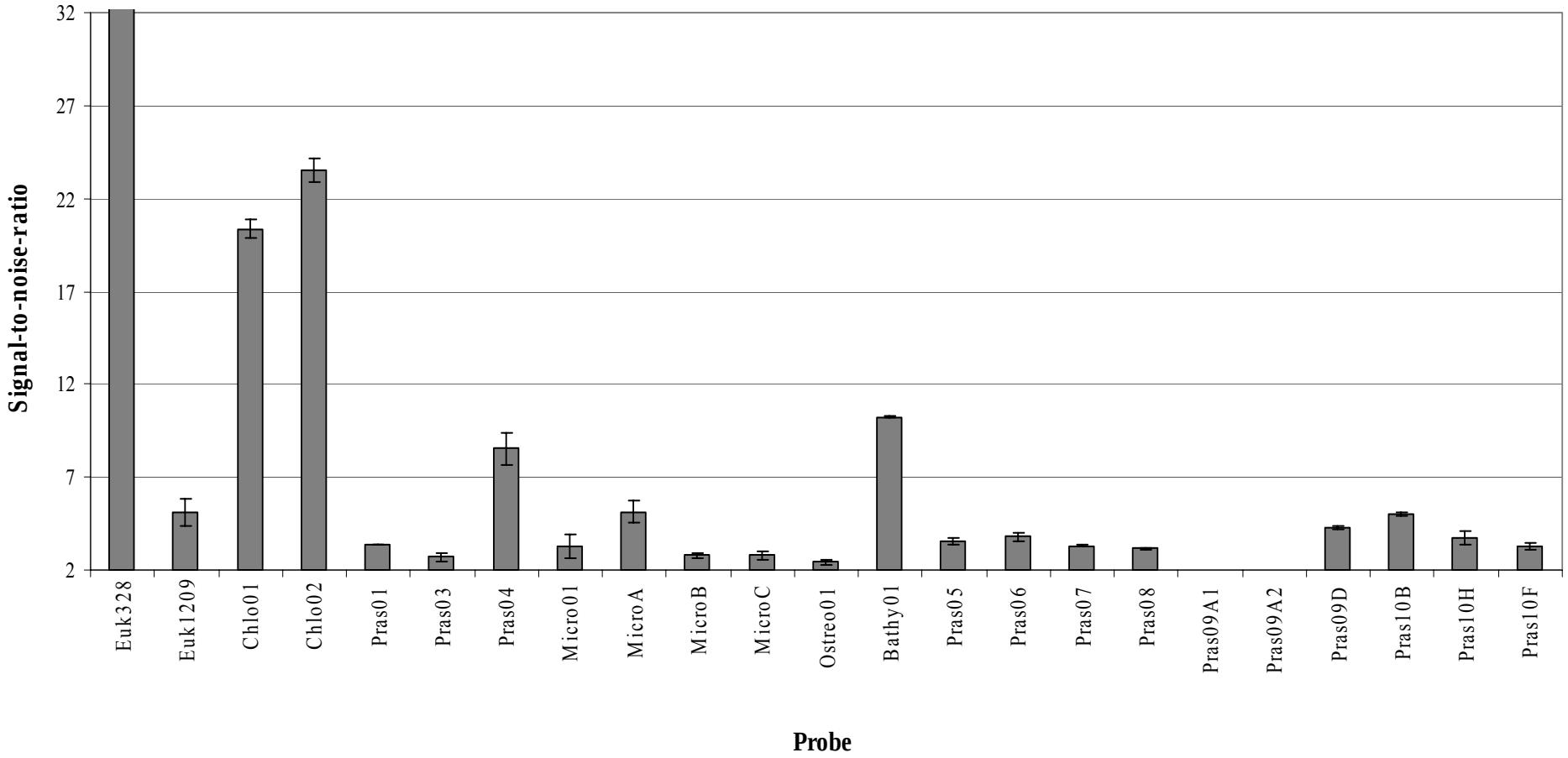

Figure 6. Signal-to- noise-ratios of the environmental sample hybridized to all prasinophyte probes on the chip

2.8 Publication VI

ASSESSMENT OF PHYTOPLANKTON DYNAMICS OVER THREE ANNUAL CYCLES AT HELGOLAND ROADS

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Abstract

The island of Helgoland has a long history in marine research and phytoplankton dynamics. In 1962, the Helgoland Roads time-series was established. For the phytoplankton community, only the > 20µm size fraction is identified on a daily basis. For picoplanktonic groups, light microscopy can not differentiate taxa or species. Molecular analyses of the picoplanktonic community have revealed frequent changes on a weekly and monthly basis, but a high congruence on a yearly basis. In this study phytoplankton field samples were taken at Helgoland in the North Sea from 2004 to 2006 in regularly intervals. The phyto- and especially picoplanktonic community compositions were successfully analyzed with the PHYTOPLANKTON CHIP for these three years. A few taxa were abundant all the time, whereas others were rare.

Introduction

It has been frequently shown in the last decade that picophytoplankton, which are composed by cells between 0.2 and 2 μm, can dominate the photosynthetic biomass in many marine ecosystems (Campbell et al. 1994; Courties et al. 1994; Joint et al. 1986; Li 1994). Thus, this community is important to the ecosystem, but it is seldom enumerated or identified because of the paucity of morphological features seen by light microscopy. Recently, molecular methods have revealed an unexpected picoplanktonic diversity (Biegala et al. 2003; Lovejoy et al. 2007; Marie et al. 2006; Moon-Van Der Staay et al. 2001; Moon-Van Der Staay et al. 2000; Not et al. 2005; Not et al. 2007; Vaulot et al. 2001; Zhu et al. 2005) and it seems that there are still many unknown species, because numerous novel sequences have been found in the marine environment (Not et al. 2004).

The island of Helgoland has a high diversity of marine life and features many different habitats (Franke et al. 2004). There has been a long history in scientific research on the island, and in 1962, a milestone in aquatic long-term monitoring series was set with the startup of the Helgoland Roads time-series (Hickel 1998; Wiltshire 2004). Today, this monitoring program is considered as one of the most important and valuable marine data sets in the world and it is especially unique in sampling length, frequency and numbers of parameters measured (Franke et al. 2004; Wiltshire 2004). The multitude of data collected in the past 40 years represents a fingerprint of history, and offer the basis for analyzing past changes, evaluating the current status of the ecosystem or predicting future changes of our aquatic system (Franke et al. 2004; Wiltshire 2004). The Helgoland Roads time-series provides one of the longest data series, especially for the monitoring of phytoplankton biodiversity. Over the entire time period, the same method, Utermöhl and manual microscopic counting has been used. Daily samples have been filtered and microalgae identified, if possible, down to the species level (Wiltshire and Dürselen 2004). This has been rarely achieved in phytoplankton monitoring, but identification of some taxa can only be very coarse. The main reasons for this are limited resources, lack of time and knowledge of personnel, and challenging morphologies of certain species. Furthermore, a frequent change in counting staff introduces a bias, which cannot be completely eliminated. Taxonomic expertise takes years to acquire (Godhe 2002) and therefore, credibility and comparability of data can be biased (Franke et al. 2004; Reid et al. 1990; Wiltshire 2004; Wiltshire and Dürselen 2004). In addition, disadvantages of light microscopy are that some species do not possess sufficient discriminative morphological markers and small cells can be especially easily overlooked in samples containing particles or aggregates. Other groups, such as cryptophytes and prasinophytes, do not preserve well,

although that they are known to be common in the North Sea (Gieskes and Kraay 1984; Reid et al. 1990). Hence, there is neither identification nor enumeration of the picoplanktonic fraction in the Helgoland Roads time-series. The reliable and exact identification of all phytoplankton species is urgently necessary for further research (Reid et al. 1990), because of their contribution to ecosystem and biomass. The requirement of continuity in counting methods should not hamper the development, evaluation, and utilization of enhanced new methods to track species and enhance the identification of community composition on finer temporal and spatial scales (Franke et al. 2004).

The first observation on picoeukaryotic diversity in phytoplankton samples from the Helgoland Roads time-series was made by Medlin et al. (2006) who compared it using three different molecular methods. High variances in species composition of the picoplanktonic community occurred on a weekly basis. In contrast, a comparison of yearly samples displayed a high consistency and suggested a seasonality in the picoplanktonic fraction (Medlin et al. 2006). In comparing the three methods, the microarray results agreed quite well with the picoeukaryotic plankton composition of clone libraries from the same samples. For a reliable examination of the picoplanktonic community dynamics, an improved microarray and sampling at closer temporal intervals are necessary.

Microarrays were introduced in the mid 1990s for the detection and monitoring of gene expression (Schena et al. 1995; Schena et al. 1996). They are based on a minimized, but high throughput form of a dot-blot supported by a glass slide with special chemical properties (Gentry et al. 2006; Ye et al. 2001). Nucleic acids with all kinds of possible scientific objectives can be spotted as targets onto the slide. The utilization of cell-free systems offer the unparalleled potential to upgrade the analysis of thousands of targets from a sample in one experiment (DeRisi et al. 1997; Gentry et al. 2006; Lockhart et al. 1996; Lockhart and Winzeler 2000; Metfies et al. 2006; Schena et al. 1995; Ye et al. 2001). This technology has fostered the development of a different kind of microarray, the so-called “phylochip”. This microarray has oligonulceotide probes derived from a conserved marker, e.g., the ribosomal RNA (rRNA). The rRNA gene features low, moderate and highly conserved regions (Woese 1987) to serve as a basis for probe design at different taxonomical levels. In addition, there is a large number of rRNA sequences available in public databases that is constantly increasing (Gentry et al. 2006). The target probes on the microarray provide the detection of organisms at different taxonomic levels, from kingdom to strain and these hierarchical probes support the precision and reliability of a phylochip, because the positive signal for a target relies on the detection by more than one probe (Metfies et al. 2006).

The phylochip has been repeatedly used for classification of prokaryotes (Gentry et al. 2006; Lehner et al. 2005; Loy et al. 2002; Loy et al. 2005; Peplies et al. 2004a; Peplies et al. 2006; Peplies et al. 2004b) and photosynthetic eukaryotic picoplankton based on their plastid 16S rRNA gene (Fuller et al. 2006a; Fuller et al. 2006b). Recently, the successful detection and monitoring of harmful algae (Gescher et al. 2007a; Ki and Han 2006) and phytoplankton community composition (Gescher et al. 2007b; Medlin et al. 2006; Metfies and Medlin 2004) has been achieved with a phylochip.

The high throughput format of the microarray offers a quick and robust tool for long-term monitoring of picoeukaryotic biodiversity at the Helgoland Roads time-series and can therefore overcome the labor intensive task of other traditional and molecular methods. The detection of species in picoplanktonic communities and their composition through time would be a great improvement in microbial ecology. The potential of the microarray (THE PHYTOPLANKTON CHIP) was tested and evaluated over three annual cycles of the Helgoland Roads time-series. The aim of this publication was to study the seasonal distribution and abundances of the North Sea phytoplankton community with emphasis on the picoplanktonic fraction. Our analysis of complex environmental samples with picoplanktonic determination and examination of their succession over three years contributes to microbial ecology.

Material and Methods

Sampling side and filtration - The Helgoland time-series site is at an anchorage area between the two islands of Helgoland, termed the Roads (54°11.3’ N, 07°54.0’E). Samples were treated in two different ways (Table 1). First, 1 - 1.5 liters of water were filtered onto a 0.2 µm Isopore GTTP membrane filter (Millipore, Schwalbach, Germany) without pre-filtration (sample treatment 1). For sample treatment 2, 1.5 - 3 liters of water were pre-filtered through 10 µm and 3 µm Isopore TCTP membrane filters and finally collected onto a 0.2 µm Sterivex-GP filter (Millipore, Schwalbach, Germany). For DNA extraction of this set of samples, the 10 µm and the 0.2 µm filter were used, thus the fraction between 10µm and 3 µm is not present. In sample treatment 1, the entire community was collected on the filter, and in sample treatment 2, the water was size fractionated. We examined two fractions, because our chip addressed primarily the picoplankton. Both sample treatments were considered equal. The picoplankton is operational defined as the < 2 µm fraction. All filters were immediately stored at -20 °C.

DNA isolation - Genomic DNA was extracted from the filters with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Concentration of the extracted DNA was determined with a Nanodrop Spectrophotometer (Peqlab, Erlangen, Germany).

PCR Amplification of 18S rRNA gene - For samples analysis with the PHYTOPLANKTON CHIP a fragment of the 18S gene was amplified with the universal specific PCR primers 82F (5'-GAA ACT GCG AAT GAA TGG CTC- 3') and 1528R (5'- TGA TCC TTC TGC AGG TTC ACC TAC- 3') from the target DNA in a Mastercycler (Eppendorf, Hamburg, Germany). The PCR protocol was 5 min 94 °C, 2 min 94 °C, 4 min 54 °C, 2 min 72 °C for 29 cycles and a 7 min extension at 72 °C. A 250 basepair (bp) PCR-fragment was amplified from the TATA-box binding protein-gene (TBP) of *Saccharomyces cerevisiae*. This fragment was used as a positive control and the same amount was added to each hybridization experiment. Primer sequences were TBP-F (5'-ATG GCC GAT GAG GAA CGT TTA A-3') and TBP-R (5'-TTT TCA GAT CTA ACC TGC ACC C- 3') and the following protocol was used 5 min 94 °C, 1 min 94 °C,1 min 52 °C, 1 min 72 °C for 35 cycles and a 10 min extension at 72 °C (Metfies and Medlin 2004). All PCR-fragments were purified with the QIAquick PCR purification Kit (Qiagen, Hilden, Germany) with one variation in the protocol. For improvement of the yield, step 8 (elution with elution buffer EB) was repeated once with the same eluate. Concentration of PCR-fragments was determined with a Nanodrop Spectrophotometer (Peqlab, Erlangen, Germany).

Labeling of PCR-fragments - The PCR-fragments were labeled with biotin. The incorporated biotin can bind to Streptavidin-CY5 and this allows the fluorescent detection of hybridized PCR-fragments. The Biotin DecaLabel DNA Labeling Kit (Fementas, St. Leon-Rot, Germany) was used according to the manufacturer's protocol. One labeling reaction contained 200 ng of PCR-fragments and was incubated overnight (17 to 20 hours) to maximize biotin incorporation. Labeled PCR-fragments were purified with the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) with the protocol modification as described above to enhance the yield of PCR-fragments. Concentration of labeled fragments was measured as described above.

Probe set and synthesis - The probes on the PHYTOPLANKTON CHIP were all tested for specificity and sensitivity before (Gescher et al. 2007a; Gescher et al. 2007b; Medlin et al.

2006; Metfies et al. 2007a; Metfies and Medlin 2004). Their origin and sequences are shown in Table 2. All probes were synthesized from Thermo Electron Corporation (Ulm, Germany) and carried a C6/MMT aminolink at the 5'-end.

Microarray production - We used epoxy-coated "Nexterion Slide E" slides (Peqlab Biotechnologie GMBH, Erlangen, Germany) to produce the PHYTOPLANKTON CHIP. All probes were diluted to a final concentration of 1 µM in 3x saline sodium citrate buffer and were printed onto the slides. A VersArray ChipWriter Pro (Bio-Rad Laboratories GmbH, München, Germany) and split pins (Point Technologies, Inc., Colorado, USA) were utilized. All microarrays contained four replicates of each probe in four independent blocks. Cross-linking of probes on the slide was achieved by incubation for 30 min at 60 °C in a Shake 'n' Stack hybridization oven (Thermo Hybaid, Ulm, Germany). Afterwards, the PHYTOPLANKTON CHIPS were stored at -20 °C.

Hybridization protocol - The hybridization solution contained 1x hybridization buffer (1 M NaCl, 10 mM Tris, pH 8, 0.005 % Triton X-100, 1 mg/mL BSA/ 0.1 µg/µL HS-DNA), the biotin-labeled PCR-fragment of the field sample in a final concentration of 25 ng DNA/µL and the biotin-labeled PCR-fragment TBP from *S. cerevisiae* in a final concentration of 1.3 ng DNA/µL as the positive control. The background of the chips was blocked with 1x hybridization buffer without HS-DNA at 58 °C for 1 hour in a slide box. The chips were centrifuged to remove the buffer and the hybridization solution was denatured at 94 °C for 5 min. For hybridization on the glass slides, a special cover slip, the Lifter Slip (Implen, München, Germany) was used. The advantages of this cover slip are two printed bars at the edges to prevent slippage and a defined volume on top of the slide. It was placed onto the chip and a volume of 30 µL was pipetted under the cover slip. Capillary action ensured even dispersal of hybridization solution between chip and cover slip. Hybridization was conducted at 58 °C for 1 hour in a humid chamber. The chamber was constructed from a 50 mL Sarstedt tube filled with tissues moistened with hybridization solution. The chips were washed afterwards 15 min with 2x saline sodium citrate (2x SSC, 10 mM EDTA, 0.05 % SDS), 15 min with 1x saline sodium citrate (1x SSC, 10 mM EDTA) and finally 5 min with 0.2 x saline sodium citrate (0.2x SSC, 10 mM EDTA). They were dried by centrifugation. Hybridization analyses of the field samples were conducted on duplicate chips and each chip contained quadruple arrays of the probes.

Staining - Hybridized microarrays were stained with Streptavidin-CY5 (Amersham Biosciences, Freiburg, Germany) in 1x hybridization buffer at a final concentration of 100 ng/mL. Staining took place at room temperature for 30 min in a humid chamber. The washing protocol was as follows twice with 2x SSC buffer for 5 min and once with 1x SSC buffer for 5 min to remove excess staining moieties.

Scanning and quantification of microarrays - The stained microarrays were scanned with a GenePix 4000B scanner (Molecular Devices Cooperation, Sunnyvale, USA) and analyzed with the GenePix 6.0 software (Molecular Devices Cooperation, Sunnyvale, USA). Signal intensities were processed by a grid of individual circles, which was superimposed onto the scanned image to measure the signals and the surrounding background intensity. Signal-to-noise-ratios were determined with the formula according to Loy et al. (2002) and normalized to the signal of the TBP positive control of the same microarray with the PhylochipAnalyzer software (Metfies et al. 2007b). The mean value was determined for all replicates of one sample. The value of 2 represents the threshold for a true signal for all probes on the PHYTOPLANKTON CHIP.

Results

Comparison of the two sample treatments - Two different treatments were used for sampling, because for particular periods no samples of treatment 1 were available. For evaluation of comparability of these both methods two hybridizations from one calendar week in 2005 when the two sampling methods overlapped, are shown in Fig. 1. The hybridization from 12.7.2005 was done with PCR-fragments of the 10 µm and the 0.2 µm filter of treatment 2, the PCR-fragments from 14.7.2005 were amplified from the DNA extract of the 0.2 µm filter of treatment 1. In general, more signals were observed from the sample of treatment 2, which shows an exclusive signal for probe Euk328, Hetero01, Prym02, DinoB, Chlo01 and Bathy01. For treatment 1, only two signals were detected for probe Euk1209 and Chlo02 that could not be found in the other sample. Both samples showed comparable hybridization success for the probes DinoE-12, CryptoB, Crypt46 and Crypt04-25.

Environmental conditions - Abiotic factors, viz., temperature, salinity and nutrients, at the sampling point Helgoland Roads are presented in Figs. 2 - 4. In general, they did not significantly differ from the values normally measured within the Helgoland Roads time-series (Wiltshire and Dürselen 2004).

Temperatures - Fig. 2 shows the temperature in the sampling period from 2004 to 2006. In 2004, there were higher temperatures in the spring (calendar week [CW] 0504 - CW1204), but then a slower increase from spring to summer (CW1204 - CW3004). 2005 and 2005 showed nearly the same curve progression, but in general, the temperatures in 2006 were higher and reached 20 °C in summer. The entire year was warmer, and from CW2806 - CW5006 water temperatures did not decrease as much as in the two other years. The winter of 2006 showed also higher water temperatures in comparison to 2004 and 2005.

Salinities - In Fig. 3, the salinities at the Helgoland Roads are shown for the years 2004-2006. In 2004, the salinity was very variable and fluctuated for a long period. This might be caused by heavy rainfalls and influx of fresh water from the rivers Eider and Elbe. By comparison, the salinity in 2005 was much more stable with only a few big changes. In spring 2005 between CW0805 and CW1805 and in fall from CW3805 until the end of the year, two long periods of constant conditions were detected. The salinity in 2006 is the most persistent of the three years sampled. In this year, only three big pulses were found, and two long stable periods between CW1006 and CW2406, and CW3106 and CW4706. The constant temperatures and salinities in comparison indicate that the water masses were quite stable in these periods.

Nutrients - In Fig. 4, the nutrients, SiO_2, PO_4, NO_3 and NH_4, are presented. In general, it seems that the whole system is limited by ammonium and phosphate and more and higher nitrate peaks were detected in 2005 and 2006 as compared to 2004. The nutrients, in 2004, were depleted fairly early (CW2104), and especially nitrate did not increase very strongly in winter. In 2005, high amounts of NO_3 and SiO_2 can be found in the spring, they decreased in summer (CW2405), and increased again in winter 2005 (CW4505). In 2006, nutrients were available longer than in the two years before. They showed a first decrease in the CW 2906 and higher peaks were detected in the fall as well. The nutrients in 2006 are carried further in the summer.

Microarray analysis - The normalized signal-to-noise-ratios of all three years are presented in four diagrams with nine different colors, each representing a range of signal intensities (Figs. 5 - 8). A value of 2 was defined as threshold for a positive signal, so values below 2 are regarded as negative. The graphs were prepared with the Scilab program (www.scilab.org). The green color demonstrated absence to low signal intensities, followed

by black in the middle and the highest values were shown in red. The probes on the PHYTOPLANKTON CHIP are listed on the y axis and the calendar weeks (CW) of the samples are shown on the x axis. The probes at the class level, the dinoflagellates, the cryptophytes and the prasinophytes are presented separately.

Class level probes - The signals of the class level probes are presented in Fig. 5. The two eukaryotic probes, Euk1209 and Euk328, showed confusing results in all years. They are designed to bind to every eukaryotic target, but in some samples they did not show a signal at all. The first probe, Euk1209 is known to produce very low signals in a microarray hybridization approach (Metfies and Medlin 2004). This is likely caused by the position of the probe in the rRNA molecule. Certain sites may be blocked for probe binding by secondary-structure formation (Metfies and Medlin 2004). This result can be confirmed here. The second probe, Euk328, is known to show better signal-to-noise-ratios in a microarray hybridization (Metfies and Medlin 2004). There are more probe pairs in the class level where the probes with the same target group lie in two different regions of the 18S rRNA gene. One of the probes may be inhibited by secondary structure. It will only react if target concentrations are extremely high. Serial dilution of target DNA will result in a disappearance of the prior probe signal at lower concentrations (Niestroj 2007). Thus, any signal by this probe can be interpreted semi-quantitatively. These probe pairs are Boli01 vs. Boli02, Prym01 vs. Prym02, DinoB vs. DinoE-12, CryptoA vs. CryptoB and Chlo02 vs. Chlo01.

In general, the dominant contribution to the picoplanktonic community came from the cryptophytes and dinoflagellates. The probes DinoE-12 and CryptoB showed high signals within all samples in the three examined years. These probes target a large group of organisms. The DinoE-12 targets the alveolate groups I + II, the dinoflagellates, and apicomplexa. Probe CryptoB detects the pigmented cryptophytes. In 2005 and 2006 more and higher signals were found in comparison to 2004. No phytoplankton blooms were detected with the microarray analysis in 2004; neither in spring nor in the fall. We found low signals for probe Pela02, Hetero01, and Chlo02 in one fall sample, and signals for probe Boli02 in two fall samples. In summary, the year 2004 had very few signals for the class level probes. In 2005, a small spring bloom was detected with signals for the probe Pela02, Hetero01, Boli02, NS04, Prym02, DinoB, and Chlo02. Furthermore, we found a fall bloom with signals for probe Hetero01, Prym02, DinoB, Chlo01, and Chlo02. The probe Prym01 targets the same taxonomic groups as probe Prym02, but here the signals should be regarded as semi-quantitative. This probe showed no signals in all examined samples. There were many

positive probe signals in 2006. No distinct blooms were detected and the signals showed high values over the whole year. After the constantly present signals for probes DinoE-12 and CryptoB, numerous signals were found for the probe Prym02, Chlo02, Boli02, Pela02, and Hetero01. Additionally, signals of probe Boli01, Prym03 and Chlo02 were sporadically present.

Dinoflagellate probes - The two dinoflagellate probes, DinoB and DinoE-12, two probes for the species *Alexandrium minutum,* and three probes for the species *Alexandrium ostenfeldii* are shown in Fig. 6. These probes are on this chip because of our on-going interest in automated detection of toxic algae. As mentioned before, the probe DinoE-12 showed high signals-to-noise-ratios in all samples, whereas signals for the probe DinoB could not be found in 2004, and only sporadically in 2005 and 2006. In 2004, no signals were detected for the five *Alexandrium* species probes, in 2004, only few signals have been found. The highest values and abundances were measured in 2006 for all species probes.

Cryptophyte probes - The cryptophytes are characterized by eleven probes targeting the six major clades in their RNA phylogeny. For clade I, II, and III two probes for each clade were used. The second probe for each clade represented an elongation of the first probe with higher specificity (Metfies et al. 2007a; Metfies and Medlin 2007). Only for clade VII in the cryptophyte tree no specific probe could be designed (Metfies and Medlin 2007). Probe Crypt46 targets clades 4 and 6 together. Fig. 7 shows the signal-to-noise-ratios for all cryptophyte probes together with class level probe CryptoA and B for all pigmented cryptophytes. In general, species detected by probe Crypt04-25 for clade IV, and Crypt46 for clade IV and VI were most frequently found in all years and all seasons. In 2004, several cryptophytes were detected, and they showed the highest contribution to the picoplanktonic community in 2004 (Fig. 5). 2005 showed two blooms in spring and fall, and a decrease in detected cryptophytes in summer. Members of all clades were detected in this year, but clade III and V were less abundant. In 2006, representatives of all clades were present through the entire year.

Prasinophyte probes - The prasinophytes are an important group within the picoplankton and belong to the chlorophytes. They are characterized by two probes for the chlorophytes, thirteen probes for the six major prasinophyte clades, three probes for species in clade II, and three probes for clades within the species concept of *Micromonas pusilla*. The

results of their microarray analysis are shown in Fig. 8. The probes, Chlo01 and 02, which target different species within the chlorophytes, showed nearly no signals in 2004, high signals in the last quarter of 2005 and targets for probe Chlo2 were nearly present all through the whole year 2006. The two class probes for the chlorophyceae, which target a higher taxonomic level in the taxonomic tree than the prasinophytes, did not show agreement with all prasinophyte signals. For some positive prasinophyte signals, no corresponding Chlo signals could be found. Representatives of the prasinophytes were detected in all years. 2004 showed the lowest abundances, whereas in 2006 the highest signals have been found. The only important contribution of prasinophytes to the picoplanktonic community was detected in spring 2006. Clade II (*Mamelliales*), detected by probe Pras04, is the dominant clade in all years. In this clade, the species *Bathycoccus prasinos* has been found frequently by probe Bathy01. *Micromonas pusilla* was found in some samples in all years. The species has been detected by probe Micro01 and its clades by probe MicroA, B, and C; which seem to be equally represented in Helgoland. The third species in clade II, *Osterococcus tauri*, was only found once in 2005 and in six samples from 2006. After clade II, the second abundant species are members of clade VIIC (*Picocystis salinarium*), detected by probe Pras08. The clades I (Pyramimonadales), V (Pseudocourfieldales and *Pycnococcaceae*) and, VIIB (clade composed of environmental sequences) were only sporadically found by probe Pras09A1, 09A2, 07, and 06. Probe Pras01, which also targets clade V, showed no signals in all years. The probes Pras03 (clade VI, Prasinoccocales) and Pras05 (clade VII, composed of unidentified cultures and environmental sequences) were only found in 2006. Pras09D (clade I) and 10B (clade III) were rarely detected in 2004 and 2005, but showed high signals in 2006. Probes Pras10F (clade III) had only one signal in 2004, and Pras10H (clade III) in 2005.

Cluster analysis - A cluster analysis was conducted for probes at the class level for the three years separately and all three years together, and for probes at clade or species level for the three years separately with the Cluster/Treeview program (Eisen and Brown 1999). This method groups the field samples by complete linkage clustering such that similar samples are combined in clusters. The results are shown in Figs. 9 - 11. In the procedure, no further normalization was applied to the data already shown in Figs. 5 - 8. The method should evaluate if samples of the spring and autumn bloom or non-bloom periods of one year show comparable probe patterns.

Clustering of class level probes in three separated years - The clustering of class level probes separated for the three years is shown in Fig. 9, and the same color scale as for Figs. 4 - 8 was used. Year 2004 had the lowest sample number and the sampling started later. In the clustering, no real groups were found and the clusters recovered were highly similar. All samples are relative similar and no big differences were revealed. Two spring samples group together, the fall samples as well except for two samples. We observed non-bloom samples from the summer scattered over the tree. In 2005, we found a small spring bloom from 0505 - CW1505 and a fall bloom from CW2805 - CW5105. All spring samples except for this one clustered together in the right clade of the tree. Here four non-bloom and one fall samples fell into the same cluster. Only two non-bloom and one fall sample were found in the left clade of the tree, where all fall samples except for one were found. The clustering of 2006 showed a different picture in comparison to the other two years. This year had the highest number of samples and many probes showed signals throughout the entire year. A small group of spring samples clustered in the left corner of the tree. In the middle of the tree all fall samples were grouped together with five non-bloom, and one spring sample. The remaining spring samples and two non-bloom samples were clustered together in the right corner.

Clustering of class level probes of all three years together - All years were clustered together to evaluate if same seasons of different years may group because of similar probe patterns (Fig. 10). Three main clades were observed with no seasonal grouping. All samples of the first clade showed signals for the probe Hetero01 and Prym02. Most of the samples have been taken in 2005 and the Hetero01 signals are likely associated with heterotrophic flagellates, because no signals for the bolidophyte (Boli01 and 02) or pelagophyte probes (Pela01 and 02) were observed. These probes target organisms, which are also detected by the Hetero01 probe, but at a lower taxonomic level. It is likely that many diatoms in the water at this time. The large cluster of samples in the centre is composed by samples with few signals except for the highly abundant probes. Within this group samples from all years and all seasons can be found together. The right clade shows only samples from 2006, where many signals were detected through the entire year. These samples are separated because of the high signal-to-noise-ratios for the Prym02 and the Chlo02 probe.

Clustering of clade and species level probes in three separated years - The clustering of probes at the clade and species level in Fig. 11 showed, in general, the same patterns as observed in the class level cluster analysis for the separated three years . In 2004, the samples

taken at different times were mixed and did not cluster distinctly. In general, fall samples were in the left corner and spring samples in the right corner, with non-bloom samples in the middle. In 2005, the spring samples are grouped together in the left branch, whereas the majority of fall samples clustered in the right area. Comparable results were observed in 2006, with most spring samples left with a few fall samples, and a big branch to the right with fall and non-bloom samples, and four spring samples scattered amongst the others.

Discussion

Comparison of the two sample treatments - The two samples in Fig. 1 were taken on two different days in one week, and within two days of one another so there could be changes in the species composition. Nevertheless, the higher signals of DinoE-12, CryptoB and Crypt04-25 agreed generally. The signals for the sample without the 3 - 10 µm fraction were higher for probe CryptoB, Crypt04-25 and Crypt46, whereas the DinoE-12 gave higher signals for the sample with the whole phytoplankton fraction. In general, the two samples showed similar hybridization patterns and for the detection of picoplankton, the 0.2 µm fraction is more important than the fraction between 3 µm and 10 µm. In conclusion, we think that the abundances in the picoplanktonic community can be monitored with these two comparable sampling treatments.

Environmental conditions - For interpretation of phytoplankton abundances and successions it is necessary to observe the abiotic factors in their habitat as well. The phytoplankton development can be described as a function of the physical environment (Gillbricht 1988), and the environmental conditions can be very variable in the waters around Helgoland (Drebes 1974). The three years provided variable abiotic conditions and several microalgal groups may have responded to these changes. The temperatures in 2004 and 2005 were relatively stable, whereas higher temperatures were detected in 2006. The warmer water temperatures in 2006 were reflected in an increase in the phytoplankton abundance and diversity as compared to the other two years. The salinity was influenced by heavy rainfalls, which caused influx of fresh water from the rivers. In 2004, many changes in the salinity were detected and 2005 showed the most stable conditions. The changing salinities in 2004 decreased growth in the microalgae community, and only in periods with stable salinities, numerous and high signals were observed for certain probes. In 2004 and 2005, the high amounts of nutrients were available in the spring, decreased very fast by the end of May and

showed no big increase in the fall. The high amount and long availability of nutrients in 2006 supported the growth of microalgae combined with the high water temperatures.

Microarray analysis - The Figs. 5 - 8 show the results of the hybridization of the field samples to the current PHYTOPLANKTON CHIP. In general, the results of the phytoplankton dynamics at the Helgoland Roads showed different species abundances and characteristics for the three examined years as expected for a variable and changing environment such as the North Sea.

Class level probes - The two eukaryotic probes showed confusing results in all years. For probe Euk1209 this was expected and explained by secondary structure formation of the target molecule. Probe Euk328 was known to shower higher signal-to-noise-ratios. We assume that every field sample contained eukaryotic PCR-fragments, because all samples showed specific hybridization signals for other algal probes on the chip. Thus, the problem can be only technical. In some experiments, the probe Euk328 showed high values as expected and in others no signal was observed. The samples have not been hybridized chronologically, so our explanation is that the probe may have been broken at some point. This could be confirmed by resynthesis of probe Euk328 and comparison of both probes in a hybridization.

2004 had the lowest sample number and sampling started later. In the beginning, seven weeks were not processed and an early spring bloom may have been missed. No fall bloom was detected in this year. This might be caused by the nutrient decrease in summer. In 2004, salinity was very unstable. This might be caused by heavy rainfalls and influx of fresh water from the rivers Eider and Elbe. The fresh water can flush the phytoplankton community out and therefore, low signals have been detected with the microarray. The absence of the spring bloom in 2005 can not be explained by the nutrient data. SiO_2 and NO_3 show high values in spring 2005. The salinity in this year was more constant and only a few big pulses of rainfalls were detected. The long stable salinity period in fall 2005 can be related to the phytoplankton bloom. In 2006, there are many positive probe signals through the entire year. This is likely caused by nutrients that were available longer than in the previous two years. High nutrients were present further into the summer. The decrease in nutrient availability in summer occurred at CW2906, whereas the decrease was at CW2104 in 2004 and at CW2304 in 2005. The nitrate increased again in fall 2006, and this corresponded to phytoplankton fall bloom,

which started in CW4106. The salinity in this year was very stable as well and therefore likely supported the growth of species through the entire year.

In the three years examined we observed the highest contribution of cryptophytes and dinoflagellates to the phytoplanktonic community. These class level probes target very large taxonomic groups and further investigation of these two groups is urgently necessary. It seems that that the dinoflagellates are especially important for community succession, because of their heterotrophic and parasitic life cycles. It can be assumed that they regulate the picoplanktonic community, which is not regularly counted using light microscopy in the Helgoland Roads time-series. Additional probes at lower taxonomic levels can greatly contribute to our knowledge in succession and abundances in this community. The signals of probes at class levels can be divided into smaller taxonomic units with probes at clade or species level. The probe DinoE-12 detects autotrophic, heterotrophic and parasitic dinoflagellates, and alveolates I+II. The alveolates have been recently described, and oligonuclotide probes have been already developed (Groisillier et al. 2006). These probes can be evaluated and adapted for microarray analysis. Clade level probes of cryptophytes on the microarray should be used regularly to monitor the abundances of cryptophytes.

Dinoflagellate probes - The low signal-to-noise-ratios for the *Alexandrium ostenfeldii* and *Alexandrium minutum* probes may be artifacts caused by cross-hybridization with unknown sequences in the environmental samples. In specificity tests and with spiked field samples, these probes are highly specific and sensitive. Detection and reliable identification of these species is really important, because both are toxic and can cause Harmful Algal Blooms (Gescher et al. 2007a). On the other hand, representatives of both species have been found sporadically in samples at Helgoland (Malte Elbrächter, personal communication). They are not listed in the current phytoplankton species list of Helgoland (Hoppenrath 2004), but they might have been confused in the light microscope with small cells of another dinoflagellate species, *Fragilidium subglobosum*, which is often detected at Helgoland and in the North Sea (Malte Elbrächter, personal communication). Here fluorescence-*in-situ*-hybridization using the automatic machine, the ChemScan, would provide resolution of this rare event (Töbe and Medlin 2005).

Cryptophyte probes - Species of the two cryptophytes clades IV and VI seem to be relatively halo-, temperature- and nutrient tolerant and grew under all different environmental factors, whereas the other clades seem to respond to the changing environmental conditions.

Similar results have been observed in Arcachon lagoon (France) for 2006, where members of cryptophyte clade I and II were the most abundant taxa in field samples (Medlin, et al., unpublished). Cerino and Zongone (2006) found some cryptomonad species present throuout the year in the Mediteranean Sea, others occasionally and still others only at certain times of the year. In 2004, few signals on the class level were detected, but in contrast, many cryptophytes were found in the samples. This may be explained by the hydrographic conditions that have favored the growth of cryptophytes. Two phytoplankton blooms have been found in 2005 and the decrease of abundances in the summer can be explained by the unstable salinity and depletion of nutrients. In 2006, more clades were present through the entire year, which were likely caused by more stable and nutrient rich conditions. The community composition is reflected in an increased diversity of microarray signals.

Prasinophyte probes - The prasinophytes were abundant in the spring and autumn blooms of all years with the highest abundances in 2006. It seemed that the environmental conditions in 2006 may have favored the growth of prasinophytes. Members of clade II were most abundant and therefore have the greatest tolerance to changes in the abiotic factors. The two probes for the chlorophytes showed high variations in signals for different prasinophyte target strains in fluorescence-*in-situ*-hybridization (Not et al. 2004) and microarray analysis (Gescher et al. 2007b). This might explain the discrepancy between the prasinophyte and chlorophyte probes. The first showed a signal in some samples, where no chlorophyte probe was positive. The signals for the prasinophytes were low, and it could be that in these samples the amount of target DNA was too low for a chlorophyte signal. Probe Pras01 and Pras03 are both specific for clade V, but Pras07 has a broader detection range (Gescher et al. 2007b). We found a few signals for probe Pras07 and no signal for Pras01. This might be explained by the broader detection range, and the signals may have there origin in DNA from species, which are not targeted by probe Pras01.

Cluster analysis - The clustering results showed very different results for the three examined years. If samples grouped together, the spring and autumn bloom samples clustered rather separately or with non-bloom samples than both together in one branch. This may be explained by changing species abundances in the two blooms of one year. All three examined years showed very different abiotic conditions and variable phytoplankton abundances. Therefore, long-term monitoring of phytoplankton dynamics is very valuable and urgently necessary to track these different patterns. In summary, all cluster analyses showed the same

general trends, and clustering of microarray data is possible to compare the different probe patterns in one year and between years.

In general, the data obtained by the PHYTOPLANKTON CHIP showed highly abundant taxa through all three years and some taxa that were rare. The first part seemed to be very stable and unaffected by environmental changes. The latter part is sensitive and appeared to grow only under certain environmental conditions. A comparable observation has been reported for prokaryotes from the southern California coast (Fuhrman et al. 2006). For some bacterial taxa, the patterns in distribution and abundance were highly predictable and significantly influenced by a broad range of biotic and abiotic factors (Fuhrman et al. 2006). Fuhrman and co-workers also found less predictable subsets of taxa and some occurred only a few times over the 4.5 years of sampling. This is similar to our observations. For prokaryotes at this sampling side, it seemed that the repeating predictable patterns are less functionally interchangeable and that their distribution and abundance is regulated by biotic and abiotic factors, and nutrients. A further investigation of more annual cycles of phytoplankton samples could resolve if there are also repeating cyclical patterns that can predict environmental conditions

We are well aware of the fact, that the utilization of PCR-fragments introduces a bias to the analyses and it has been shown frequently that microbial communities may not be reflected correctly (Kanagawa 2003; Medlin et al. 2006; Simon et al. 2000; Speksnijder et al. 2001; Suzuki and Giovannoni 1996; Wintzingerode et al. 1997). But the benefits of the PCR-step are the amplification of low target concentrations and the easy handling and labeling. The hybridization of RNA theoretically offers the possibility of quantification and delivers a less distorted view of true community composition (Peplies et al. 2006). Possible disadvantages are surely low yields of RNA from environmental samples and that extraction can be inhibited by complex organic molecules (Alm and Stahl 2000; Peplies et al. 2006). Furthermore, the RNA content can vary over the cell cycle (Countway and Caron 2006) and especially for picoeukaryotes their rRNA content can be low. In contrast, an alternative approach of quantifying bacteria by hybridization of RNA to a set of non specific probes was presented (Pozhitkov et al. 2007). The signal intensities of one species are viewed as a whole and considered to be a “fingerprint”. The method is quantitative, and does not rely on PCR amplification, or probe design and probe validation (Pozhitkov et al. 2007). We decided to analyze the field samples with PCR-fragments, because of its advantages. We showed the applicability of microarrays for the detection and monitoring of picophytoplankton. The next step in improving the PHYTOPLANKTON CHIP would be a quantification of cell densities.

The number of unknown sequences in the marine environment is expected to be high (Simon et al. 2000) and can result in unspecific probe binding. In the three years analyzed, we found several probes without signals in one ore more of the 70 samples, and it can be suggested that most of the probes worked specifically and did not bind to non-target sequences.

The microarray offers several benefits for detection and monitoring of picoeukaryotes in comparison to other methods like fluorescence-*in-situ*-hybridization. The primary advantages are the high throughput of samples and the unlimited number of probes for one experiment. An adequate chip offers the possibility to reach a comprehensive view of microbial communities in relatively short time with few experiments. There are no difficulties with autofluorescence of organisms and thick cell walls, that inhibit penetration of probes (Hasegawa et al. 1996; Not et al. 2004). In addition, compared to the manual counts of phytoplankton samples, which are routinely done within the Helgoland Roads time-series, there are even more benefits. The utilization of the chip does not require taxonomic expertise, which is difficult to acquire (Godhe 2002). The frequent changes of personnel responsible for counting with variable taxonomic knowledge has introduced a bias in the data of the time-series (Wiltshire and Dürselen 2004). The handling and hybridization of the PHYTOPLANKTON CHIP can be learned by an experienced laboratory technician within a week. Wiltshire and Dürselen (2004) suggested storing the samples for 10 years for backtracking and the storage of extracted nucleic acids is easy and space-saving. There is also the possibility to try extraction of DNA from old samples preserved in Lugol's solution. It has been shown that the PCR-reaction may be inhibited by Lugol's solution (Godhe et al. 2002; Marín et al. 2001), but DNA from Lugol's fixed samples has been amplified successfully by PCR (Bertozzini et al. 2005; Bowers et al. 2000; Penna et al. 2007; Tengs et al. 2001). The results obtained with the microarray could be compared to the manual counts from the corresponding days.

In general, the utilization of the PHYTOPLANKTON CHIP for assessment of phytoplankton dynamics in the North Sea can greatly improve our knowledge in microbial ecology. It facilitates the reliable and fast detection and monitoring of phytoplankton and especially of picoplanktonic taxa that are not counted in the microscopic investigation. We observed high stability and changes in species composition of the phytoplanktonic community at the same time. Therefore, it is important to monitor these abundances because phytoplankton is at the root of the marine food web. Their progressions have a great impact on the marine habitat and affect many other species and organisms. Sampling for detection of changes and special events e.g., bloom formation, will require massive, continuous data-

collecting or monitoring (Banse 1995). Only sampling on close temporal and spatial scales will offer the possibility to investigate the changes in the community and causal relationships of the phytoplankton composition and anthropogenic events, e.g., eutrophication and climate change. The resulting conclusions are indispensable for understanding of the long-term effects (Reid et al. 1990). The connection of man-made input of nutrients to the North Sea and the phytoplankton stocks have been investigated thoroughly, but are still not clearly understood, as natural variability is large and hydrographical factors possibly dominate (Hickel et al. 1993). Even mild climatic changes do measurably affect the plankton community (Banse 1995; Edwards et al. 2002) and have caused an obvious warming of 1.1 °C in the North Sea since 1962 (Wiltshire and Manly 2004). It has been shown that in warm winters, the algal spring bloom is delayed and shifted to the end of the first quarter of the year (Wiltshire and Manly 2004). This phenomenon is likely explained by delayed mixing of water layers in spring (Wiltshire and Manly 2004). This caused a restriction of nutrients, which delayed phytoplankton growth (Wiltshire and Manly 2004). This data showed the first indication of a warming related shift in phytoplankton succession, which will affect other members of the food web who are dependant on the microalgae as a food resource (Wiltshire and Dürselen 2004). The long-term consequences will probably include regime shifts in the North Sea system.

The chip can further facilitate the detection of species disappearance caused by environmental changes. The identification of non-indigenous species in the North Sea (Reise et al. 2006) is also possible with the corresponding probe on the chip. In addition, the chip can be improved by extension with more probes for diatoms and other key species. A further extension to the PhylochipAnalyzer program by multiple array feature analysis, e.g., clustering methods, can greatly facilitate the analysis of huge amounts of data that need to be processed. For a long-term comparison of reliability of analysis a comparison of microarray data with other methods, especially molecular approaches, is desired.

In summary, the utilization of the newly developed PHYTOPLANKTON CHIP for the Helgoland Roads time-series phytoplankton sampling can improve and enhance the data obtained by this historically important and long time established sampling series to an extremely high degree. For example the time intervals can be shortened, data from more phytoplankton taxa can be obtained, all data possess a greater reliability and the taxonomic resolution is deeper and more precise for more different groups. The data that will be collected offer the possibility of examination of phytoplankton against the background of climate change (Wiltshire and Dürselen 2004).

Conclusions

The correct classification and enumeration of phytoplankton and especially picoplanktonic taxa is nearly impossible with traditional methods, such as light and epifluorescence microscopy. Molecular methods offer new possibilities to investigate phytoplankton dynamics and have revealed unexpected diversity in the picoplanktonic fraction. The utilization of DNA microarrays with species-specific probes is a relatively new application for the assessment of species composition in environmental samples. In this study we used the PHYTOPLANKTON CHIP to analyze field samples from three years from Helgoland. Our results demonstrate the applicability and reliability of the chip. It has the potential to detect phytoplankton abundances with a deeper taxonomical resolution and a high through put format. The data obtained by this reliable, robust and efficient method greatly contribute to our knowledge in microbial ecology.

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References

ALM, E. W., and D. A. STAHL. 2000. Critical factors influencing the recovery and integrity of rRNA extracted from environmental samples: use of an optimized protocol to measure depth-related biomass distribution in freshwater sediments. J. Microbiol. Meth. **40:** 153–162.

BANSE, K. 1995. Science and organization in open-sea research: The plankton. Helgol. Mar. Res. **49:** 3-18.

BERTOZZINI, E., A. PENNA, E. PIERBONI, I. BRUCE, and M. MAGNANI. 2005. Development of new procedures for the isolation of phytoplankton DNA from fixed samples. J. Appl. Phycol. **17:** 223-229.

BIEGALA, I. C., F. NOT, D. VAULOT, and N. SIMON. 2003. Quantitative assessment of picoeukaryotes in the natural environment by using taxon-specific oligonucleotide probes in association with tyramide signal amplification, fluorescence-*in-situ-h*ybridization and flow cytometry. Appl. Environ. Microbiol. **69:** 5519-5529.

BOWERS, H. A., T. TENGS, H. B. GLASGOW, J. M. BURKHOLDER, P. A. RUBLEE, and D. W. OLDACH. 2000. Development of Real-Time PCR assays for rapid detection of *Pfiesteria piscicida* and related dinoflagellates. Appl. Environ. Microbiol. **66:** 4641-4648.

CAMPBELL, L., H. A. NOLLA, and D. VAULOT. 1994. The importance of *Prochlorococcus* to community structure in the central North Pacific Ocean. Limnol. Oceanogr. **39:** 954-961.

CERINO, F., and A. ZINGONE. 2006. A survey of cryptomonad diversity and seasonality at a coastal Mediterranean site. Eur. J. Phycol. **41:** 363-378.

COUNTWAY, P. D., and D. A. CARON. 2006. Abundance and distribution of *Ostreococcus* sp. in the San Pedro Channel, California, as revealed by Quantitative PCR. Appl. Environ. Microbiol. **72:** 2496-2506.

COURTIES, C. and others 1994. Smallest eukaryotic organism. Nature **370:** 255.

DERISI, J. L., V. R. IYER, and P. O. BROWN. 1997. Exploring the metabolic and genetic control of gene expression on a genomic scale. Science **278:** 680-686.

DREBES, G. 1974. Marines Phytoplankton. Eine Auswahl der Helgoländer Planktonalgen (Diatomeen, Peridineen). Georg Thieme Verlag Stuttgart, Germany.

EDWARDS, M., G. BEAUGRAND, P. C. REID, A. A. ROWDEN, and M. B. JONES. 2002. Ocean climate anomalies and the ecology of the North Sea. Mar. Ecol. Prog. Ser. **239:** 1–10.

EISEN, M. B., and P. O. BROWN. 1999. DNA arrays for analysis of gene expression, p. 179-205. *In* S. M. Weissman [ed.], cDNA Preparation and Characterization. Academic Press.

ELLER, G., K. TÖBE, and L. K. MEDLIN. 2007. Hierarchical probes at various taxonomic levels in the Haptophyta and a new Division level probe for the Heterokonta. J. Plankton Res., in press.

FRANKE, H.-D., F. BUCHHOLZ, and K. H. WILTSHIRE. 2004. Ecological long-term research at Helgoland (German Bight, North Sea): retrospect and prospect-an introduction. Helgol. Mar. Res. **58:** 223-229.

FUHRMAN, J. A., I. HEWSON, M. S. SCHWALBACH, J. A. STEELE, M. V. BROWN, and S. NAEEM. 2006. Annually reoccurring bacterial communities are predictable from ocean conditions. PNAS **103:** 13104-13109.

FULLER, N. J. and others 2006a. Analysis of photosynthetic picoeukaryote diversity at open ocean sites in the Arabian Sea using a PCR biased towards marine algal plastids. Aquat. Microb. Ecol. **43:** 79–93.

---. 2006b. Molecular analysis of photosynthetic picoeukaryote community structure along an Arabian Sea transect. Limnol. Oceanogr. **51:** 2502-2514.

GENTRY, T. J., G. S. WICKHAM, C. W. SCHADT, Z. HE, and J. ZHOU. 2006. Microarray applications in microbial ecology research. Microbial Ecol. **52:** 159-175.

GESCHER, C., K. METFIES, and L. K. MEDLIN. 2007a. The Alex Chip-Development of a DNA Chip for identification and monitoring of *Alexandrium*. Harmful Algae, to be submitted.

---. 2007b. Development and assessment of a DNA microarray for identification of Prasinophyta. Appl. Environ. Microbiol., to be submitted.

GIESKES, W. W. C., and G. W. KRAAY. 1984. Phytoplankton, its pigments, and primary production at a central North Sea station in May, July and September 1981. Neth. J. Sea. Res. **18:** 51-70.

GILLBRICHT, M. 1988. Phytoplankton and nutrients in the Helgoland region. Helgol. Mar. Res. **42:** 435-467.

GODHE, A. 2002. Benthic and pelagic dinoflagellate stages: environmental settings, cyst vaibility, and molecular idetification, Department of Marine Ecology, Marine Botany. Phd Thesis, Göteborg University, Sweden.

GODHE, A., D. M. ANDERSON, and A.-S. REHNSTAM-HOLM. 2002. PCR amplification of microalgal DNA for sequencing and species identification: studies on fixatives and algal growth stages. Harmful Algae **1:** 375-382.

GROISILLIER, A., R. MASSANA, K. VALENTIN, D. VAULOT, and L. GUILLOU. 2006. Genetic diversity and habitats of two enigmatic marine alveolate lineages. Aquat. Microb. Ecol. **42:** 277-291.

GUILLOU, L., S.-Y. MOON-VAN DER STAAY, H. CLAUSTRE, F. PARTENSKY, and D. VAULOT. 1999. Diversity and abundance of Bolidophyceae (Heterokonta) in two oceanic regions. Appl. Environ. Microbiol. **65:** 4528-4536.

HASEGAWA, T. and others 1996. *Prasinoderma coloniale* gen. et sp. nov., a new pelagic coccoid prasinophyte from the western Pacific ocean. Phycologia **35:** 170–176.

HICKEL, W. 1998. Temporal variability of micro- and nanoplankton in the German Bight in relation to hydrographic structure and nutrient changes. ICES J. Mar. Sci. **55:** 600-609.

HICKEL, W., P. MANGELSDORF, and J. BERG. 1993. The human impact in the German Bight: Eutrophication during three decades (1962-1991). Helgol. Mar. Res. **47:** 243-263.

HOPPENRATH, M. 2004. A revised checklist of planktonic diatoms and dinoflagellates from Helgoland (North Sea, German Bight). Helgol. Mar. Res. **58:** 243-251.

JOHN, U., A. CEMBELLA, C. HUMMERT, M. ELBRÄCHTER, R. GROBEN, and L. K. MEDLIN. 2003. Discrimination of the toxigenic dinoflagellates *Alexandrium tamarense* and *A. ostenfeldii* in co-occurring natural populations from Scottish coastal waters. Eur. J. Phycol. **38:** 25-40.

JOINT, I. R., N. J. P. OWEN, and A. J. POMROY. 1986. Seasonal production of photosynthetic picoplankton and nanoplankton in the Celtic Sea. Mar. Ecol. Prog. Ser. **28:** 251–258.

KANAGAWA, T. 2003. Bias and artifacts in multitemplate polymerase chain reactions (PCR). J. Biosci. Bioeng. **96:** 317-323.

KI, J.-S., and M.-S. HAN. 2006. A low-density oligonucleotide array study for parallel detection of harmful algal species using hybridization of consensus PCR products of LSU rDNA D2 domain. Biosens. Bioelectron. **21:** 1812-1821.

LANGE, M. and others 1996. Identification of the class Prymnesiophyceae and the genus *Phaeocystis* with ribosomal RNA-targeted nucleic acid probes detected by flow cytometry. J. Phycol. **32:** 858-868.

LEHNER, A. and others 2005. Oligonucleotide microarray for identification of *Enterococcus* species. FEMS Microbiol. Lett. **246:** 133-142.

LI, W. K. W. 1994. Primary production of prochlorophytes, cyanobacteria, and eucaryotic ultraphytoplankton: Measurements from flow cytometric sorting. Limnol. Oceanogr. **39:** 169-175.

LIM, E. L., L. A. AMARAL, D. A. CARON, and E. F. DELONG. 1993. Application of rRNA-based probes for observing marine nanoplanktonic protists. Appl. Environ. Microbiol. **59:** 1647-1655.

LOCKHART, D. J. and others 1996. Expression monitoring by hybridization to high-density oligonucleotide arrays. Nat. Biotechnol. **14:** 1675-1680.

LOCKHART, D. J., and E. A. WINZELER. 2000. Genomics, gene expression and DNA arrays. Nature **405:** 827-836.

LOVEJOY, C. and others 2007. Distribution, phylogeny, and growth of cold-adapted picoprasinophytes in Arctic seas. J. Phycol. **43:** 78-89.

LOY, A. and others 2002. Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Appl. Environ. Microbiol. **68:** 5064-5081.

---. 2005. 16S rRNA gene-based oligonucletide microarray for environmental monitoring of the betaproteobacterial order "Rhodocyclales". Appl. Environ. Microbiol. **71:** 1373-1386.

MARIE, D., F. ZHU, V. BALAGUE, J. RAS, and D. VAULOT. 2006. Eukaryotic picoplankton communities of the Mediterranean Sea in summer assessed by molecular approaches (DGGE, TTGE, QPCR). FEMS Microbiol. Ecol. **55:** 403-415.

MARÍN, I., A. AGUILERA, B. REGUERA, and J. P. ABAD. 2001. Preparation of DNA suitable for PCR amplification from fresh or fixed single dinoflagellate cells. Biotechniques **30:** 88–93.

MASSANA, R., L. GUILLOU, B. DÍEZ, and C. PEDRÓS-ALIÓ. 2002. Unveiling the organisms behind novel eukaryotic ribosomal DNA sequences from the ocean. Appl. Environ. Microbiol. **68:** 4554-4558.

MEDLIN, L. K., K. METFIES, H. MEHL, K. H. WILTSHIRE, and K. VALENTIN. 2006. Picoeukaryotic plankton diversity at the Helgoland Time Series Site as assessed by three molecular methods. Microbial Ecol. **52:** 53-71.

METFIES, K. and others 2007a. An optimised protocol for the identification of diatoms, flagellated algae and pathogenic protozoa with phylochips. Mol. Ecol. Notes, in press.

METFIES, K., P. BORSUTZKI, C. GESCHER, L. K. MEDLIN, and S. FRICKENHAUS. 2007b. The PhylochipAnalyzer - A programm for analyzing hierarchical probe-sets. Mol. Ecol. Notes, accepted.

METFIES, K., and L. K. MEDLIN. 2007. Refining cryptophyte identification with DNA-Microarrays. Mol. Ecol. Notes, submitted.

METFIES, K., S. HULJIC, M. LANGE, and L. K. MEDLIN. 2005. Electrochemical detection of the toxic dinoflagellate *Alexandrium ostenfeldii* with a DNA-biosensor. Biosens. Bioelectron. **20:** 1349-1357.

METFIES, K., and L. K. MEDLIN. 2004. DNA microchips for phytoplankton: The fluorescent wave of the future. Nova Hedwigia **79:** 321-327.

---. 2007. Refining cryptophyte identification with DNA-Microarrays. Mol. Ecol. Notes, submitted.

METFIES, K., K. TÖBE, C. A. SCHOLIN, and L. K. MEDLIN. 2006. Laboratory and field applications of ribosomal RNA probes to aid the detection and monitoring of Harmful Algae, p. 311-325. *In* E. Granéli and J. T. Turner [eds.], Ecology of Harmful Algae. Springer Verlag, Berlin, Heidelberg, Germany.

MOON-VAN DER STAAY, S. Y., R. DE WACHTER, and D. VAULOT. 2001. Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. Nature **409:** 607-610.

MOON-VAN DER STAAY, S.-Y., G. W. M. VAN DER STAAY, L. GUILLOU, and D. VAULOT. 2000. Abundance and diversity of prymnesiophytes in the picoplankton community from the equatorial Pacific Ocean inferred from 18S rDNA sequences. Limnol. Oceanogr. **45:** 98-109.

NIESTROJ, R. 2007. Untersuchung der Biodiversität des eukaryontischen Picoplanktons in Wasserproben aus der Nordsee. Diploma thesis. Technischen Fachhochschule Berlin, Germany.

NOT, F., M. LATASA, D. MARIE, T. CARIOU, D. VAULOT, and N. SIMON. 2004. A single species, *Micromonas pusilla* (Prasinophyceae), dominates the eukaryotic picoplankton in the western English Channel. Appl. Environ. Microbiol. **70:** 4064-4072.

NOT, F. and others 2005. Late summer community composition and abundance of photosynthetic picoeukaryotes in Norwegian and Barents Seas. Limnol. Oceanogr. **50:** 1677-1686.

---. 2007. Picobiliphytes: A marine picoplanktonic algal group with unknown affinities to other eukaryotes. Science **315:** 253-255.

PENNA, A. and others 2007. Monitoring of HAB species in the Mediterranean Sea through molecular methods. J. Plankton Res. **29:** 19-38.

PEPLIES, J., F. O. GLÖCKNER, R. AMANN, and W. LUDWIG. 2004a. Comparative sequence analysis and oligonucleotide probe design based on 23S rRNA genes of Alphaproteobacteria from North Sea bacterioplankton. Syst. Appl. Microbiol. **27:** 573-580.

PEPLIES, J., C. LACHMUND, F. O. GLÖCKNER, and W. MANZ. 2006. A DNA microarray platform based on direct detection of rRNA for characterization of freshwater sediment-related prokaryotic communities. Appl. Environ. Microbiol. **72:** 4829-4838.

PEPLIES, J., S. C. K. LAU, J. PERNTHALER, R. AMANN, and F. O. GLÖCKNER. 2004b. Application and validation of DNA microarrays for the 16S rRNA-based analysis of marine bacterioplankton. Environ. Microbiol. **6:** 638-645.

POZHITKOV, A. E., K. D. BAILEY, and P. A. NOBLE. 2007. Development of a statistically robust quantification method for microorganisms in mixtures using oligonucleotide microarrays. J. Microbiol. Meth., accepted.

REID, P. C., C. LANCELOT, W. W. C. GIESKES, E. HAGMEIER, and G. WEICHART. 1990. Phytoplankton of the North Sea and its dynamics: A review. Neth. J. Sea Res. **26:** 295-331.

REISE, K., S. OLENIN, and D. THIELTGES. 2006. Are aliens threatening European aquatic coastal ecosystems? Helgol. Mar. Res. **60:** 77-83.

SCHENA, M., D. SHALON, R. W. DAVIS, and P. O. BROWN. 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science **270:** 467-470.

SCHENA, M., D. SHALON, R. HELLER, A. CHAI, P. O. BROWN, and R. W. DAVIS. 1996. Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. Proc. Natl. Acad. Sci. USA **93:** 10614-10619.

SIMON, N., N. LEBOT, D. MARIE, F. PARTENSKY, and D. VAULOT. 1995. Fluorescent-*in-situ*-hybridization with rRNA-targeted oligonucleotide probes to identify small phytoplankton by flow cytometry. Appl. Environ. Microbiol. **61:** 2506-2513.

SIMON, N. and others 2000. Oligonucleotide probes for the identification of three algal groups by dot-blot and fluorescent whole-cell hybridization. J. Eukaryot. Microbiol. **47:** 76–84.

SPEKSNIJDER, A. G. C. L., G. A. KOWALCHUK, S. DE JONG, E. KLINE, J. R. STEPHEN, and H. J. LAANBROEK. 2001. Microvariation artifacts introduced by PCR and cloning of closely related 16S rRNA gene sequences. Appl. Environ. Microbiol. **67:** 469-472.

SUZUKI, M. T., and S. J. GIOVANNONI. 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. **62:** 625–630.

TENGS, T., H. A. BOWERS, A. P. ZIMAN, D. K. STOECKER, and D. W. OLDACH. 2001. Genetic polymorphism in *Gymnodinium galatheanum* chloroplast DNA sequences and development of a molecular detection assay. Mol. Ecol. **10:** 515-523.

TÖBE, K., and L. K. MEDLIN. 2005. Cytometry for the automated detection of microalgae. Phycologia **44:** 101.

VAULOT, D. and others 2001. Diversity of eukaryotic picoplankton in costal waters. J. Phycol. **37:** 50-50.

WILTSHIRE, K. H. 2004. Editorial. Helgol. Mar. Res. **58:** 221-222.

WILTSHIRE, K. H., and C.-D. DÜRSELEN. 2004. Revision and quality analyses of the Helgoland Reede long-term phytoplankton data archive. Helgol. Mar. Res. **58:** 252-268.

WILTSHIRE, K. H., and B. F. J. MANLY. 2004. The warming trend at Helgoland Roads, North Sea: phytoplankton response. Helgol. Mar. Res. **58:** 269-273.

WINTZINGERODE, F. V., U. B. GOEBEL, and E. STACKEBRANDT. 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. **21:** 213–229.

WOESE, C. 1987. Bacterial evolution. Microbiol. Rev. **51:** 221-271.

YE, R. W., T. WANG, L. BEDZYK, and K. M. CROKER. 2001. Applications of DNA microarrays in microbial systems. J. Microbiol. Meth. **47:** 257-272.

ZHU, F., R. MASSANA, F. NOT, D. MARIE, and D. VAULOT. 2005. Mapping of picoeucaryotes in marine ecosystems with quantitative PCR of the 18S rRNA gene. FEMS Microbiol. Ecol. **52:** 79-92.

Table 1. Overview of field samples and treatment from 2004-2006

Calendar Week	Date	Sample Treatment
0804	19.02.2004	1
1004	04.03.2004	1
1204	18.03.2004	1
1404	01.04.2004	1
1604	15.04.2004	1
2004	13.05.2004	1
2404	10.06.2004	1
2604	24.06.2004	1
2804	08.07.2004	1
3004	22.07.2004	1
3604	04.09.2004	1
3804	16.09.2004	1
4004	30.09.2004	1
4104	09.10.2004	1
4604	11.11.2004	1
4904	02.12.2004	1
5104	16.12.2004	1

Calendar Week	Date	Sample Treatment
0105	06.01.2005	1
0305	20.01.2005	1
0505	03.02.2005	1
0705	17.02.2005	1
0905	03.03.2005	1
1105	17.03.2005	1
1305	31.03.2005	1
1505	14.04.2005	1
2105	23.05.2005	1
2205	02.06.2005	1
2605	28.06.2005	2
2605	30.06.2005	1
2805	12.07.2005	2
2805	14.07.2005	1
3005	26.07.2005	2
3005	28.07.2005	1
3205	09.08.2005	2
3705	13.09.2005	2
3905	27.09.2005	2
4105	11.10.2005	2
4305	25.10.2005	2
4505	08.11.2005	2
4705	22.11.2005	2
4905	06.12.2005	2
5105	20.12.2005	2

Calendar Week	Date	Sample Treatment
0206	12.01.2006	1
0406	26.01.2006	1
0506	04.02.2006	1
0806	23.02.2006	1
1006	09.03.2006	1
1206	23.03.2006	1
1406	06.04.2006	1
1606	20.04.2006	1
1806	02.05.2006	2
2006	16.05.2006	2
2306	07.06.2006	2
2506	20.06.2006	2
2706	04.07.2006	2
2906	18.07.2006	2
3406	22.08.2006	1
3506	29.08.2006	1
3606	07.09.2006	1
3706	12.09.2006	1
3806	19.09.2006	1
3906	26.09.2006	1
4006	05.10.2006	1
4106	10.10.2006	1
4206	17.10.2006	1
4306	24.10.2006	1
4406	03.11.2006	1
4506	09.11.2006	1
4606	14.11.2006	1
4706	21.11.2006	1
4906	05.12.2006	1
5006	12.12.2006	1

Table 2. Probes on the PHYTOPLANKTON CHIP

Probe	Specific for	Sequence (5'-3')	Reference
TBP	*Saccharomyces cerevisiae* (Positive Control)	ATG GCC GAT GAG GAA CGT	Metfies and Medlin 2004
NC	Negative Control	TCC CCC GGG TAT GGC CGC	Metfies and Medlin 2004
Euk328	Kingdom Eukaryotes	ACC TGG TTG ATC CTG CCA G	Moon-Van der Staay et al. 2000
Euk1209	Kingdom Eukaryotes	GGG CAT CAC AGA CCT G	Lim et al., 1993
Boli01	Class Bolidophyceae	CAG TCT GAT GAA CTG CGT	Guillou et al., 1999
Boli02	Class Bolidophyceae	TAC CTA GGT ACG CAA ACC	Guillou et al., 1999
Prym01	Class Prymnesiophyceae	ACA TCC CTG GCA AAT GCT	Lange et al., 1996
Prym02	Class Prymnesiophyceae	GGA ATA CGA GTG CCC CTG AC	Simon et al., 2000
Prym03	Phylum Prymnesiophyta	GTC AGG ATT CGG GCA ATT	Eller et al. 2007
Hetero01	Phylum Heterokonta	ACG ACT TCA CCT TCC TCT	Sinon et al. 2000
Pela01	Class Pelagophyceae	ACG TCC TTG TTC GAC GCT	Simon et al., 2000
Pela02	Pelagophyceae clade	GCA ACA ATC AAT CCC AATC	Simon et al., 2000
NS04	New Stramenopiles clade	TAC TTC GGT CTG CAA ACC	Massana et al. 2002
Chlo01	Phylum Chlorophyta	GCT CCA CGC CTG GTG GTG	Simon et al., 1995
Chlo02	Phylum Chlorophyta	CTT CGA GCC CCC AAC TTT	Simon et al., 2000
Pras01	Prasinophyceae (clade V), *P.marina, P.provasolii*	ACG GTC CCG AAG GGT TGG	Not et al. 2004
Pras03	Prasinococcales (clade VI)	GCC ACC AGT GCA CAC CGG	Not et al. 2004
Pras04	Prasinophyceae, Mamiellales (clade II)	CGT AAG CCC GCT TTG AAC	Not et al. 2004
Osrteo01	*Ostreococcus tauri*	CCT CCT CAC CAG GAA GCT	Not et al. 2004
Bathy01	*Bathycoccus prasinos*	ACT CCA TGT CTC AGC GTT	Not et al. 2004
Micro01	*Micromonas pusilla*	AAT GGA ACA CCG CCG GCG	Not et al. 2004
Pras05	Prasinophyceae (clade VIIA), CCMP 1205+RCC287	GCC AGA ACC ACG TCC TCG	Not et al. 2004
Pras06	Prasinophyceae (clade VIIB), env sequences, OLI1059, 11305, 11345	AAT CAA GAC GGA GCG CGT	Not et al. 2004

Pras07	Prasinophyceae (clade V), *P.marina, P.provasolii*	CCG ACA GAA AGA CGC AGA	Not et al. 2004
Pras08	Prasinophyceae (clade VII C), *P.salinarium*	ATT GTG TGG GTC TTC GGG	Gescher et al. 2007b
Pras09A1	Prasinophyceae (clade I), *P.cristatum*	GGT TGC GTT AGT CTT GCT	Gescher et al. 2007b
Pras09A2	Prasinophyceae (clade I), *Pyramimonas, Prasinopapilla, Cymbomonas*	GCC GCC TTC GGG CGT TTT	Gescher et al. 2007b
Pras09D	Prasinophyceae (clade I), *Halosphaera* spec.	AAC TGG CTC GGT ACG CGG	Gescher et al. 2007b
Pras10B	Prasinophyceae (cladeIII), *N.pyriformis, P.marina*	TAA AAG ACC GAC CGC TTC	Gescher et al. 2007b
PRAS10H	Prasinophyceae (cladeIII), *N.oliviaceae*	CAC TGG CGC GCC CCA TCT	Gescher et al. 2007b
Pras10F	Prasinophyceae (cladeIII), *N.oliviaceae*	CGT TTC AAC TCG ACC AGT	Gescher et al. 2007b
CryptoA	Pigmented Cryptophyceae	CAC TAA GAC ATG CAT GGC	Metfies et al. 2007a
CryptoB	Pigmented Cryptophyceae	ACG GCC CCA ACT GTC CCT	Metfies et al. 2007a
Crypt01	Cryptophyta clade I	CATT ACC CCA GTC CCA T	Metfies et al. 2007a
Crypt01-25	Cryptophyta clade I	CAT TAC CCC AGT CCC ATA ACC AAC G	Metfies et al. 2007a
Crypt02	Cryptophyta clade II	GTC CCA CTA CCC TAC AGT	Metfies et al. 2007a
Crypt02-25	Cryptophyta clade II	GCG TCC CAC TAC CCT ACA GTT AAG T	Metfies et al. 2007a
Crypt03	Cryptophyta clade III	TTC CCG CGC ACC ACG GTT	Metfies et al. 2007a
Crypt03-25	Cryptophyta clade III	GTG TTC CCG CGC ACC ACG GTT AAA T	Metfies et al. 2007a
Crypt04-25	Cryptophyta clade IV	CAC CTC CAC CAT AA AGGC ATG AGG T	Metfies and Medlin 2007
Crypt05	Cryptophyta clade V	CTC GCA ATC AAG CTC CAT	Metfies et al. 2007a
Crypt053	Cryptophyta clade V	GTC CCA ACG CCC CTC AGT	Metfies et al. 2007a
Crypt053-25	Cryptophyta clade V	TGC GTC CCA ACG CCC CAC AGT GAA G	Metfies et al. 2007a
Crypt46	Cryptophyta clades IV & VI	CAA GGT CGG CTT TGA ATC	Metfies et al. 2007a
DinoB	Phylum Dinophyta and Apicomplexa	CCT CAA ACT TCC TTG CIT TA	John et al., 2003
DinoE-12	Phylum Dinophyta and Apicomplexa	CGG AAG CTG ATA GGT CAG AA	Medlin et al. 2006
Aost1	*Alexandrium ostenfeldii*	CAA CCC TTC CCA ATA GTC AGG T	Metfies et al. 2005
Aost2	*Alexandrium ostenfeldii*	GAA TCA CCA AGG TTC CAA GCA G	Metfies et al. 2005

Aost02	*Alexandrium ostenfeldii*	CAC CAA GGT TCC AAG CAG	John et al. 2003
Alexmin1	*Alexandrium minutum* (AY831408, AY883006, AJ535380, AJ535388)	CCC AGA AGT CAG GTT TGG AT	Gescher et al. 2007a
Alexmin2	*Alexandrium minutum* (U27499)	CCC TTC CAA AAG TCA GGT TTA G	Nölte, unpublished

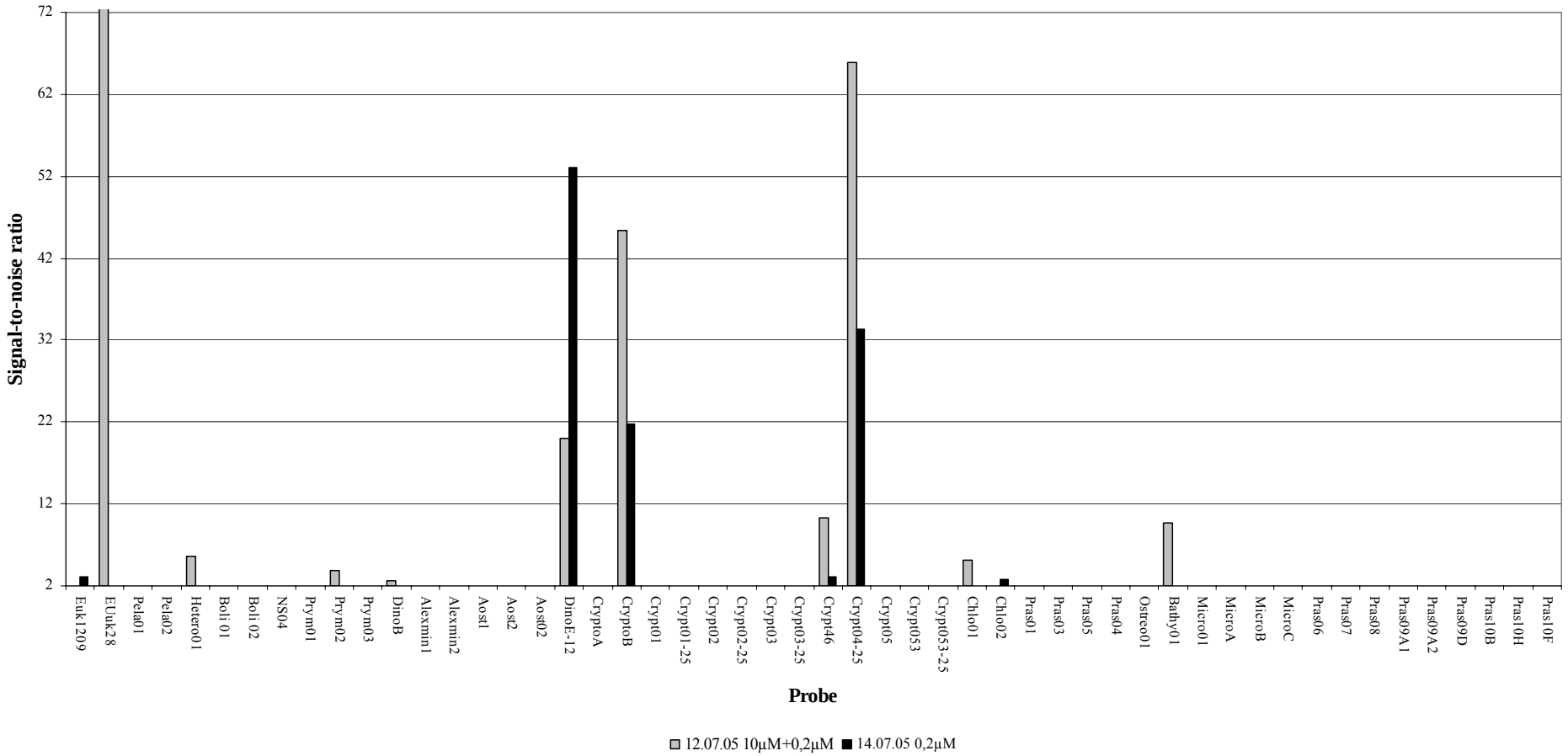

Figure 1. Analysis of two phytoplankton samples from 2005 with the two different treatments.

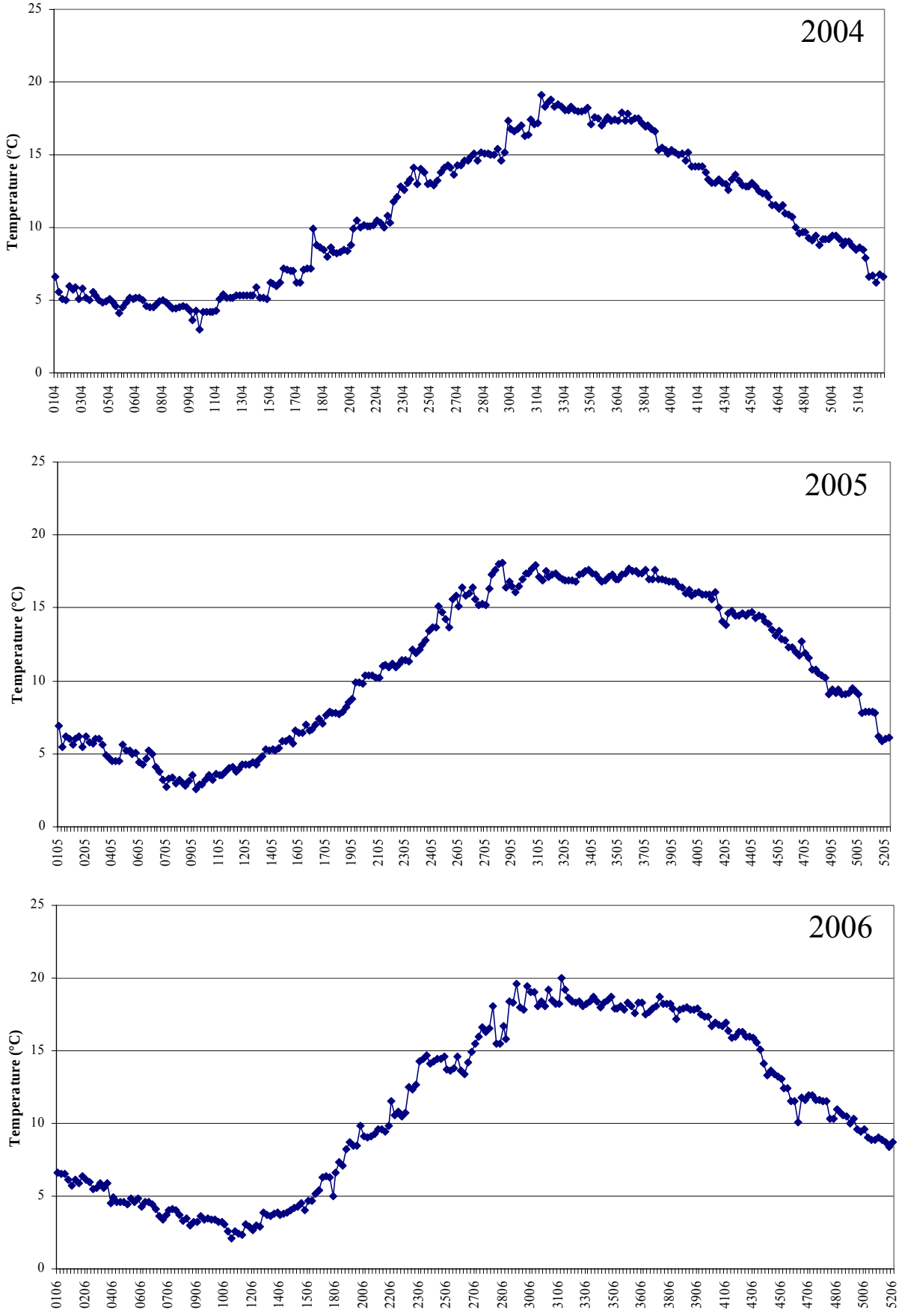

Figure 2. Temperatures at the Helgoland Roads from 2004 - 2006

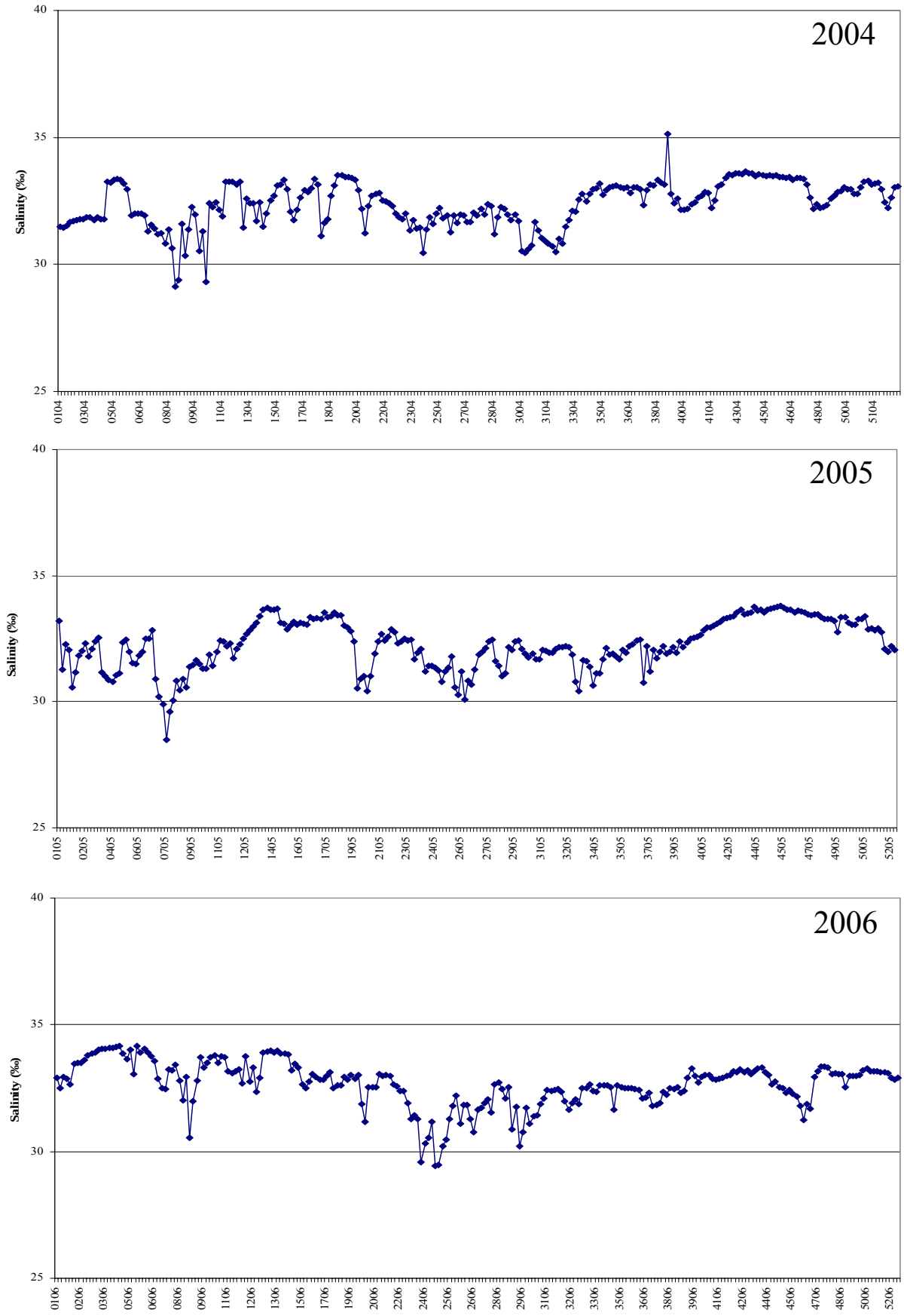

Figure 3. Salinity at the Helgoland Roads from 2004 - 2006

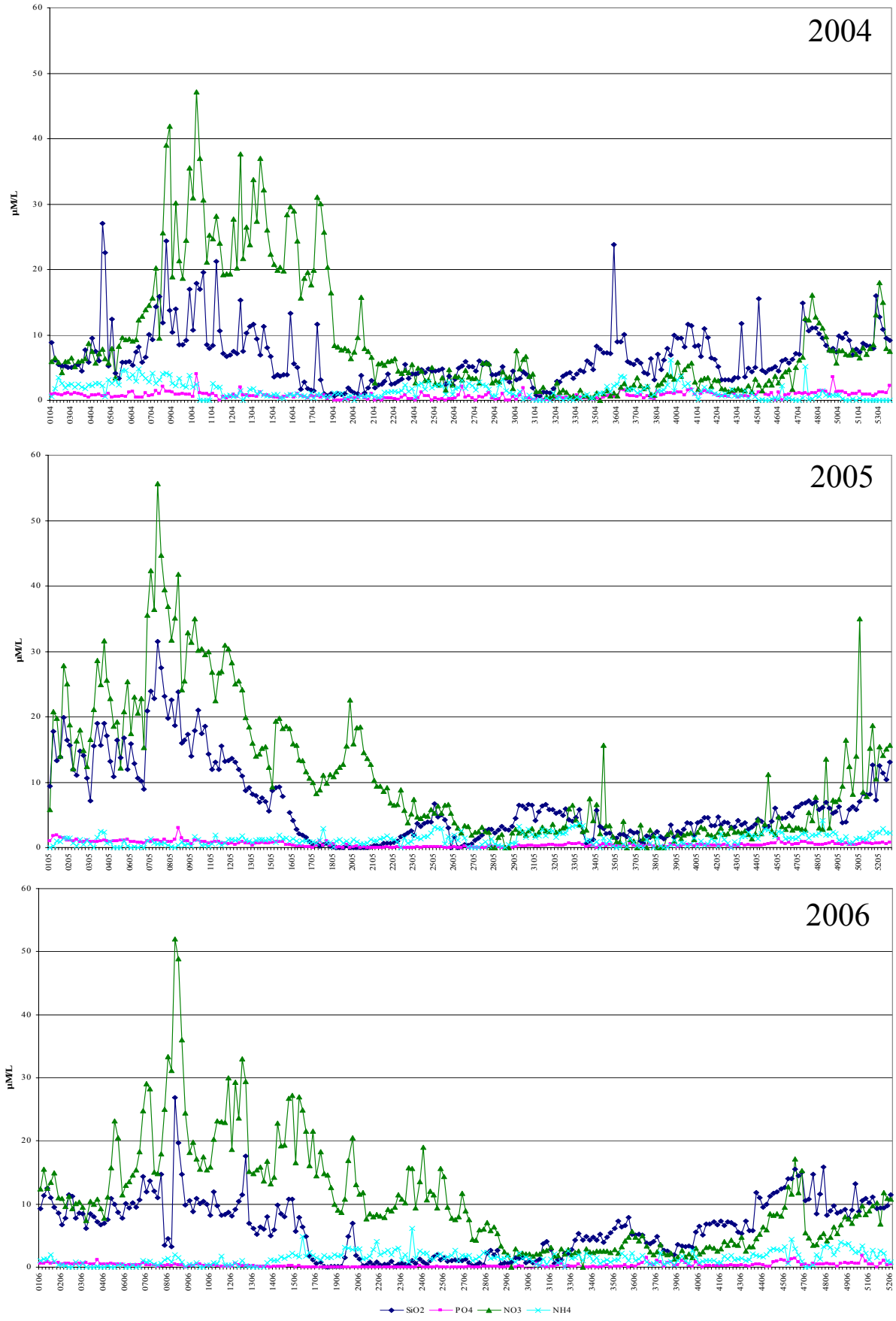

Figure 4. Nutrients at the Helgoland Roads from 2004 - 2006

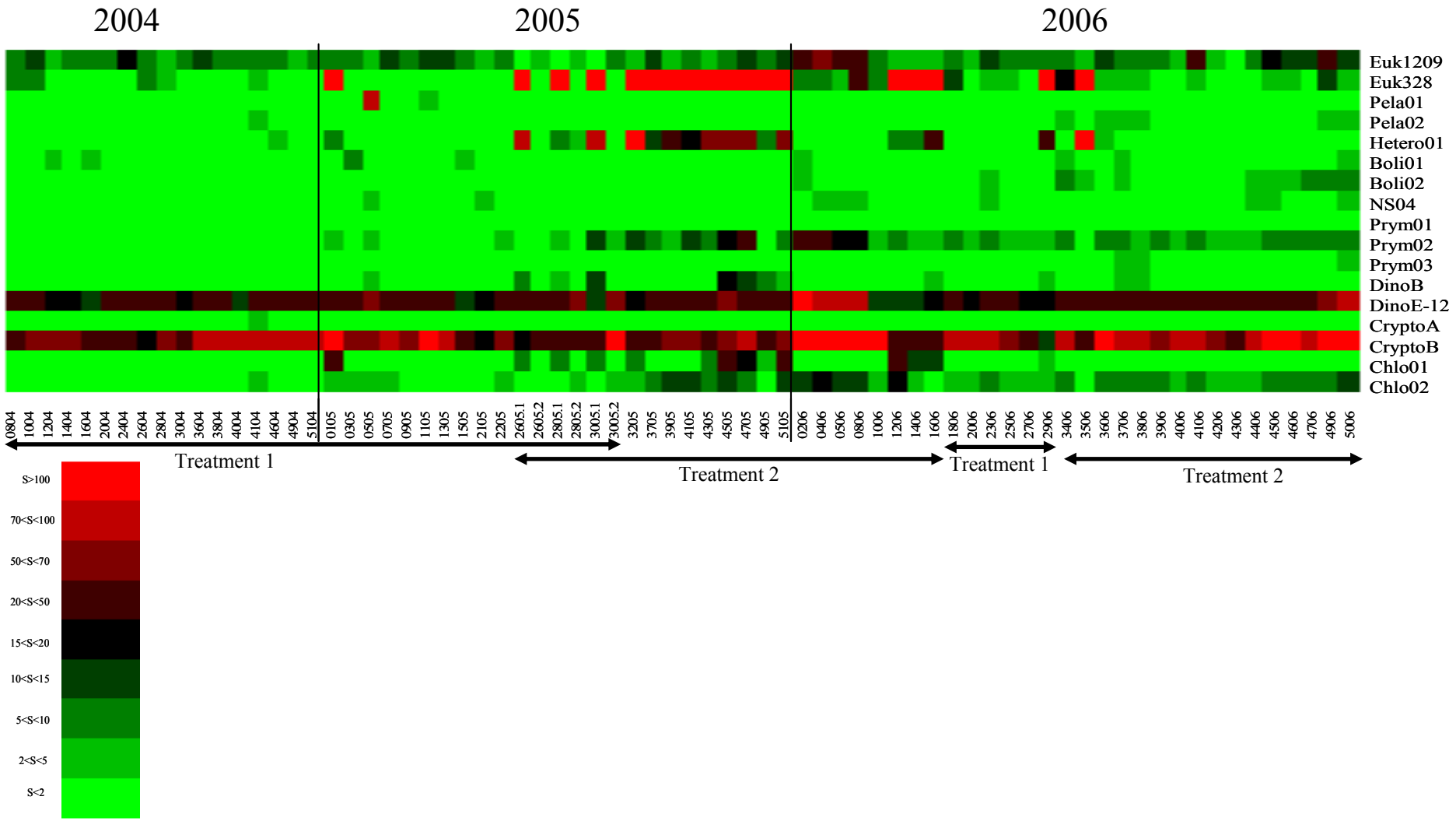

Figure 5. Results of the microarray analysis of environmental samples with the class level probes. The signal-to-noise-ratios of the probes are presented in nine different groups. Refer to scale for color coding.

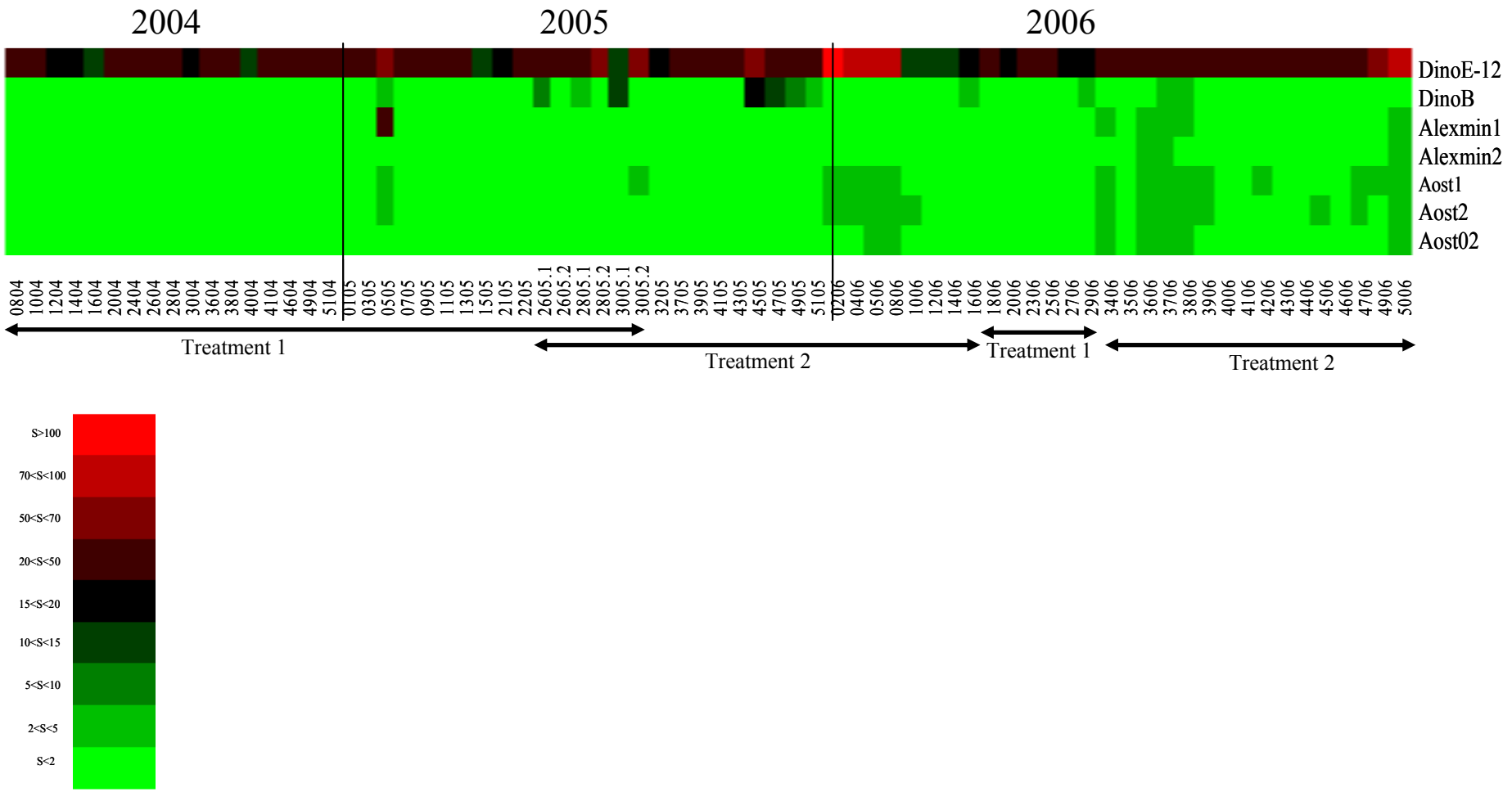

Figure 6. Results of the microarray analysis of environmental samples with the dinoflagellate probes. The signal-to-noise-ratios of the probes are presented in nine different groups. Refer to scale for color coding.

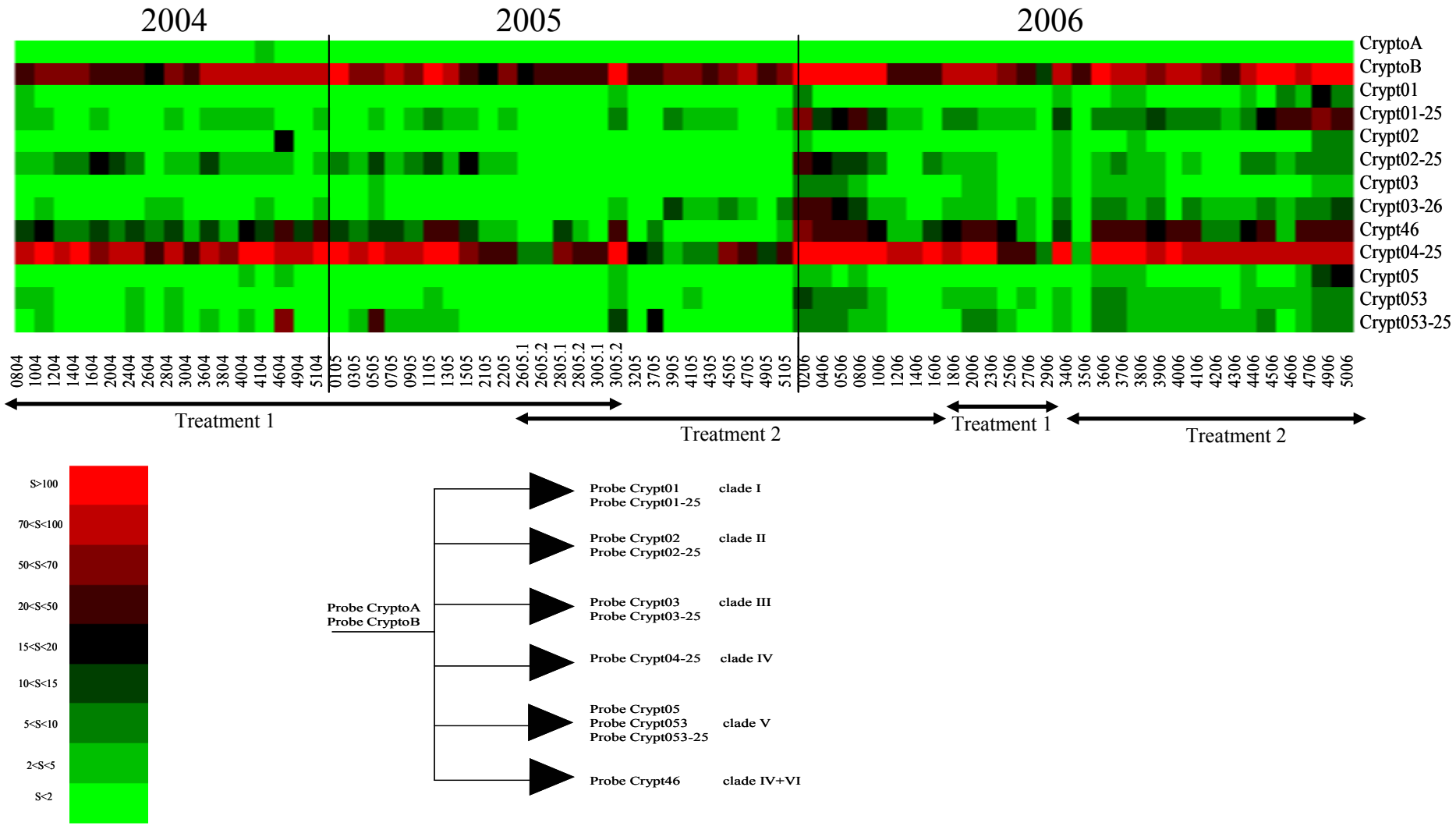

Figure 7. Results of the microarray analysis of environmental samples with the cryptophyte probes. The signal-to-noise-ratios of the probes are presented in nine different groups. Refer to scale for color coding. The tree shows an overview of probe levels.

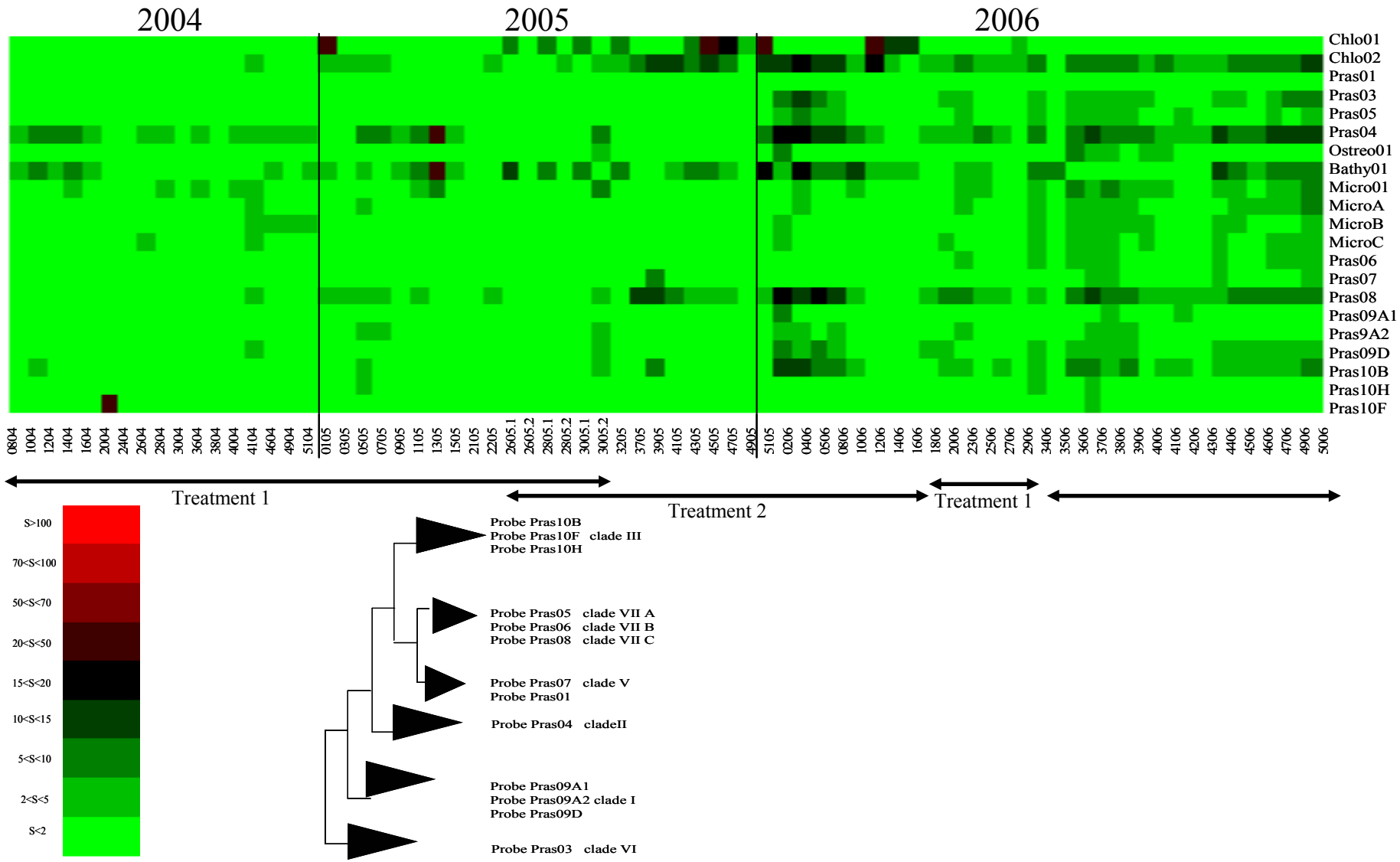

Figure 8. Results of the microarray analysis of environmental samples with the prasinophyte probes. The signal-to-noise-ratios of the probes are presented in nine different groups. Refer to scale for color coding. The tree shows an overview of probe levels.

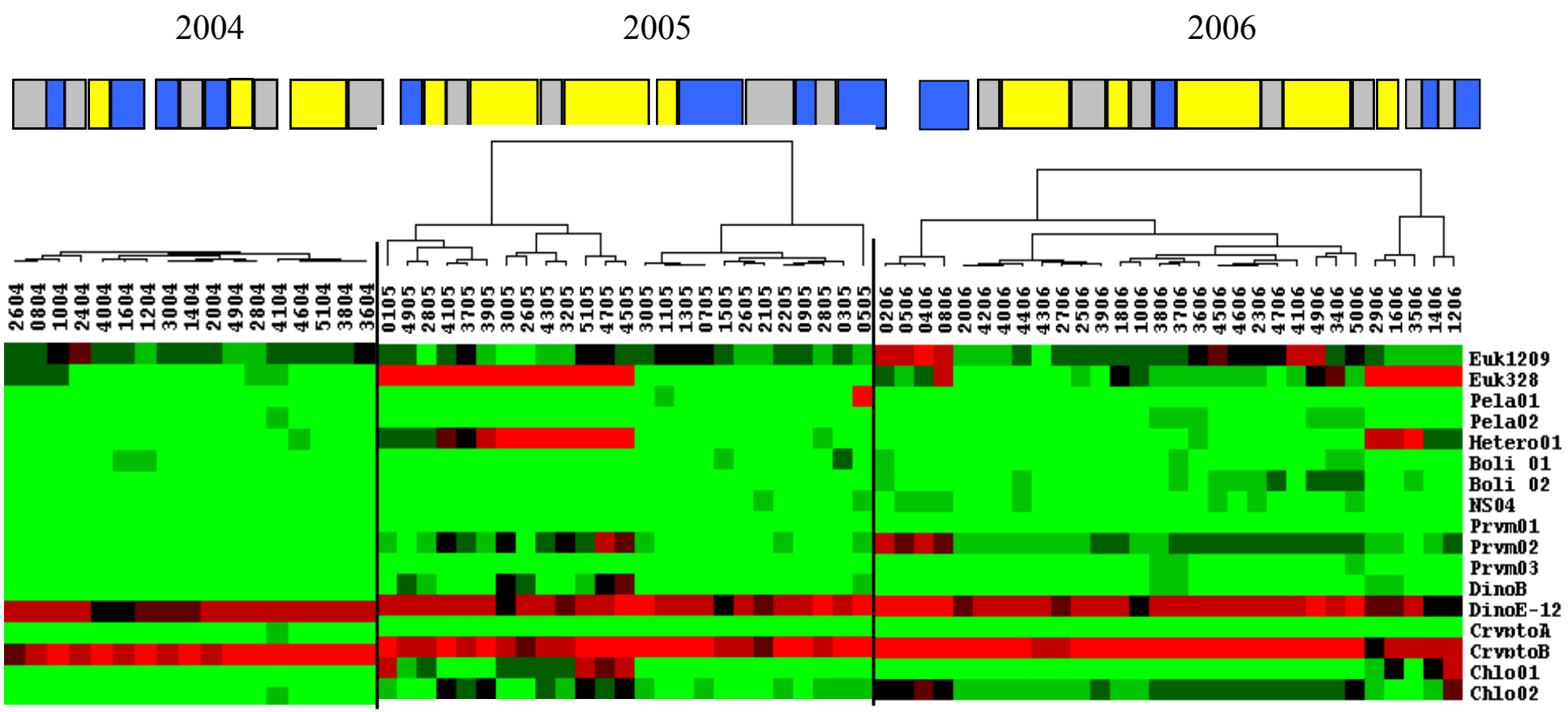

Figure 9. Clustering of microarray results from class level probes for the three years separately. The signal-to-noise-ratios of the probes are presented in nine different groups. Refer to scale in Figs. 5 - 8 for color coding. The bars show an overview of clustered samples in one clade. The different periods are presented as follows: **grey** = no bloom, **blue** = spring bloom, **yellow** = fall bloom.

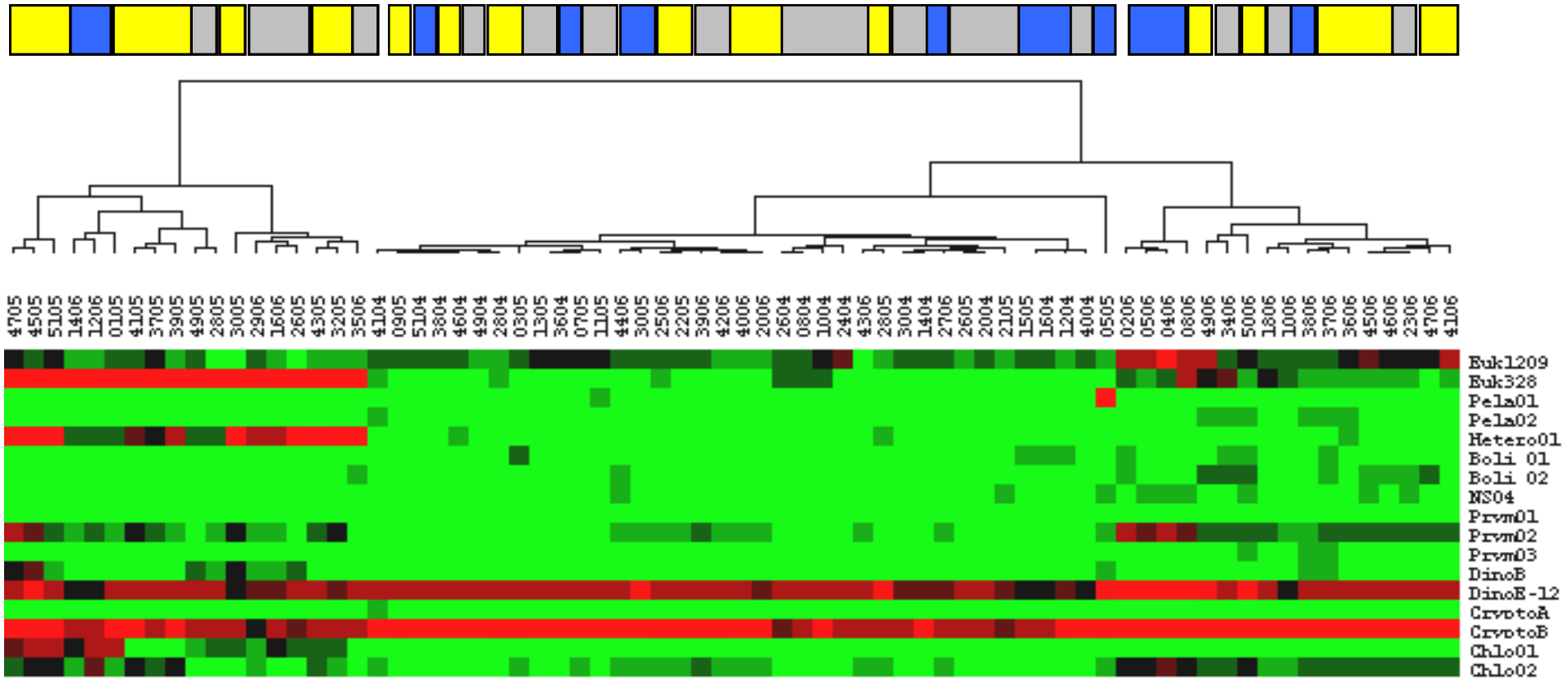

Figure 10. Clustering of microarray results from class level probes for all three years together. The signal-to-noise-ratios of the probes are presented in nine different groups. Refer to scale in Figs. 5 - 8 for color coding. The bars show an overview of clustered samples in one clade. The different periods are presented as follows: **grey** = no bloom, **blue** = spring bloom, **yellow** = fall bloom.

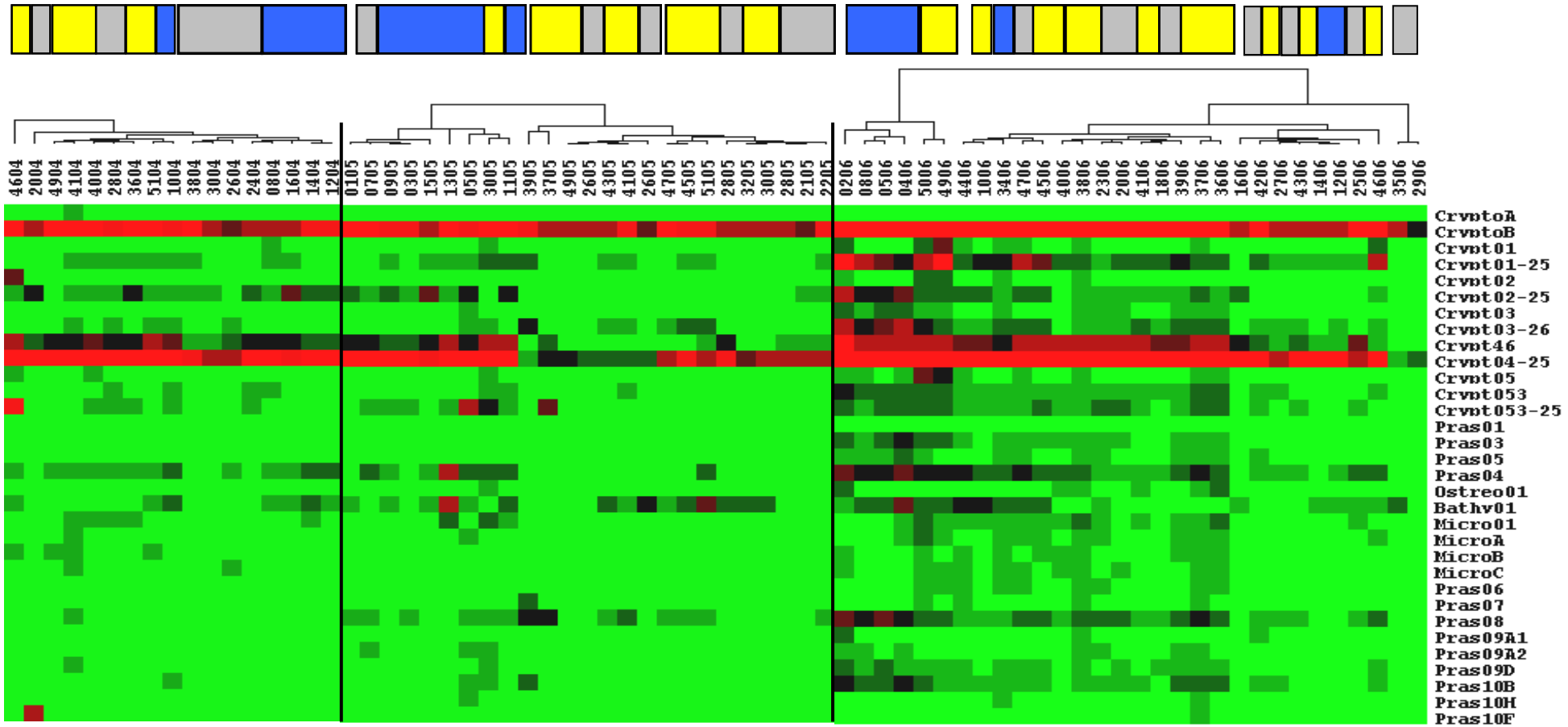

Figure 11. Clustering of microarray results from clade and species level probes for the three years separately. The signal-to-noise-ratios of the probes are presented in nine different groups. Refer to scale in Figs. 5 - 8 for color coding. The bars show an overview of clustered samples in one clade. The different periods are presented as follows: **grey** = no bloom, **blue** = spring bloom, **yellow** = fall bloom.

3. Synthesis

3.1 Evaluation of different approaches for improvement of microarray analysis

The application of phylochips to analyze complex phytoplankton communities offers the possibility of monitoring on long-time scales and at close temporal resolution, and therefore greatly contributes our knowledge of phytoplankton biodiversity (Medlin et al. 2006). With this method it is possible to hybridize a sample to several thousands of potential targets in a single experiment (Ye et al. 2001). However, the development and evaluation of a reliable phylochip is demanding and several challenges must be considered such as the unpredictability of *in-situ* probe performance, the stringent conditions in the hybridization experiment, cross-hybridization of non-specific targets, and the high number of unknown organisms in the environment.

3.1.1 Microarrays for quantification of microalgae

Another difficulty is the ambition to relate cell numbers to signal intensities for quantification. For each probe used, it is necessary to develop a calibration curve and to convert the signal into cell counts. This relation is required for a reliable analysis of field samples, especially for toxic algae. Only hybridization of total RNA offers the possibility for this calculation. In contrast, it has been shown that the utilization of PCR-fragments introduces a bias to the community structure in a sample with uneven target amplification (Hansen et al. 1998; Kanagawa 2003; Medlin et al. 2006; Polz and Cavanaugh 1998; Reysenbach et al. 1992). But RNA is difficult to extract and to use, and the content in field samples is rather low (Peplies et al. 2006). RNA content per cell may vary under different environmental conditions, and a high sampling volume up to several liters is necessary to harvest enough cells. Thus, quantification with a microarray can only be considered as semi-quantitative.

In **Publication I** the protocol described by Metfies and Medlin (2004) was evaluated and optimized for the application of total RNA. All steps that are necessary for microarray hybridization are described in detail for the possible reproduction by lab experienced laymen. Furthermore, the advantages and drawbacks of the microarray application were presented in this chapter of a method handbook for phytoplankton analysis. The RNA extraction protocol from Qiagen (Hilden, Germany) was improved, and a commercially available kit from Fermentas (Valencia, USA) was tested for direct RNA labeling. Within the scope of

Publication I, it was evaluated if a quantification of cell densities could be achieved with this method (data not shown). The RNA content per cell was determined and a calibration curve was constructed for two probes. The relation of cell numbers to signal intensities is a prerequisite for an accurate cell density determination. This is advantageous for monitoring of phytoplankton biodiversity, and even indispensable for the detection of toxic algae. The latter was tested for quantification of *Alexandrium fundyense* in spiked field samples in an intercalibration workshop. The results are shown in the publication by (Godhe et al. 2007).

3.1.2 Analysis of hierarchical probe-sets

The hierarchical probe approach uses the positive signal of several probes on different hierarchical levels as a confirmation for a correct signal. Therefore, this approach offers the possibility of signal validation. Rechecking the signals for each probe manually can be tedious and demanding if a high sampling volume and numerous probes have to be examined. Furthermore, it is necessary to introduce standards for the representation of chip description and data-processing details. Automation of analysis and data processing represents a further improvement of the microarray as a fast and robust tool for monitoring of phytoplankton biodiversity.

The design and test-run of a program for the automated analysis of hierarchical probe sets was shown in **Publication II**. The "PhylochipAnalyzer" allows the assessment of biodiversity with different resolutions and was developed under Borland-Delphi as GUI-based Windows-program. The two important functions, hierarchy editing and data analysis, were interconnected. First, the individual hierarchical tree determination is conducted using a spot description file in GenePix Array List format (GAL) generated by the GenePix software (Axon Instruments Inc., USA). This tree is saved as an Extensible Markup Language (XML)-file for later data analysis. Spot-intensity data are read from files with externally defined format, such as tab-delimited tables. The program validates automatically all signals in a hierarchical tree branch and deletes false positive detections. Subsequently, the data are transferred to an Excel file. The applicability and the proof of the program were evaluated by hybridization of an 18S PCR-fragment of *Micromonas pusilla* (Prasinophyta) to the current chip. This experiment was analyzed with the PhylochipAnalyzer.

3.1.3 Evaluation of Locked Nucleic Acids for signal enhancement

Microarrays are powerful tools for species detection and monitoring of phytoplankton. Reliable identification of species with molecular probe-based methods demands high

specificity and sensitivity of the utilized probes. Problems emerge with the limited possibilities of probe design from relatively conserved markers, e.g., the rRNA genes (Woese 1987). In some cases, it is nearly impossible to find more than one unique probe sequence for a species or a great group of organisms. It has been shown recently, that probes covering higher taxonomic groups vary in hybridization signals over a broad range (Not et al. 2004). Because of that, enhancement and improvement of moderately working probes is highly desirable for microarray analysis. The presentation of Locked Nucleic Acids (LNAs) for modification of probes offers the possibility to enhance their specificity and sensitivity. LNAs are bicyclic RNA analogs with exceptionally high affinities and specificities towards their complementary DNA and RNA target molecules (Koshkin et al. 1998; Singh et al. 1998). It was stated that they are usable in any hybridization assay as a modified probe or primer to increase specificity and reproducibility (Kongsbak 2002).

In the microarray part of **Publication III** the evaluation of LNAs for enhancement of five moderately working microarray probes was shown. Because of the promising results of LNAs in many applications, e.g., real-time PCR, gene expression profiling, genotyping assays and fluorescence *in-situ* hybridization (Jacobsen et al. 2002a; Jacobsen et al. 2002b; Nielsen and Kauppinen 2002; Silahtaroglu et al. 2004; Silahtaroglu et al. 2003; Ugozzoli et al. 2004) it was assessed if they could possibly overcome problems of low hybridization efficiency and cross hybridization of probes to closely related non-target species. In this study, previously published and microarray tested probes were used. The five conventional probes were compared to two different LNA-modified probes, respectively. Each conventional probe sequence was synthesized with two different locked nucleic acid modifications, LNA2 or LNA3 varying in the number of LNAs/probe, the length, and in the methylation of cytosine. We tested the potential of the probes by hybridization with target and non-target species. These results showed an enhancement of signals for the LNA-modified probes, but also decreased specificity. All LNA2 probes except for probe CryptoB performed better than the LNA3 probes. The LNA-modified probes cross-hybridized with four widely related algal targets of the genus *Alexandrium*. The improvement of the microarray probes with LNA substitution showed promising results for signal enhancement, but the specificity was simultaneously reduced. Because of that, the use of LNA-modified probes in our microarray hybridization protocol is not recommended.

In summary, a RNA hybridization protocol was evaluated and successfully adapted to the microarray with a commercially available RNA labeling kit from Fermentas. All steps were described and pictured in detail as a method description for a manual for phytoplankton analysis. Furthermore, a computer program, the PhylochipAnalyzer, was developed for the analysis of hierarchical probe sets. It enhances the robustness of the results by consistency checks at different taxonomic levels, and decreases the time for data processing of hierarchical phylochips. In addition, Locked Nucleic Acids (LNAs) were evaluated for the improvement of conventional molecular probes on the mircoarray. The results revealed that the LNAs may have great potential for signal enhancement, but they decreased the specificity as well.

3.2 Specificity tests of probes for the PHYTOPLANKTON CHIP

3.2.1 Probes for the detection of toxic microalgae

The increase of harmful algal blooms (HABs) and their resulting impacts on humans, ecosystems, fishery, tourism, and aquaculture demand a reliable and efficient detection of the causative microalgae. The traditional methods e.g., culturing, epifluorescence and electron microscopy, and high-performance liquid chromatography (HPLC) do not feature the possibilities to differentiate closely related and often co-occurring toxic and non-toxic species in a bloom. The genus *Alexandrium* contains probably the most intensive studied HAB species, although not all of them are known to be toxic (Janson and Hayes 2006). The toxic species produce toxins related to the Paralytic Shellfish Poisoning complex (PSP) and are difficult to distinguish morphologically. They differ only in minute details and these patterns may also vary with environmental factors (Hosoi-Tanabe and Sako 2006).

The **Publication VI** describes the evaluation and specificity test of molecular probes for several species belonging to the genus *Alexandrium*. The microarray contained nine probes from other methods (fluorescence-*in-situ*-hybridization, sandwich hybridization) for *Alexandrium ostenfeldii* and three clades of the *A. tamarense* "species complex" (North American, Western European and Mediterranean). Furthermore, one new probe for the species *A. minutum* was developed. The 18S and 28S genes of pure cultures of target and closely related species and spiked field samples from the Weser estuary (German Bight) were amplified by PCR. Subsequently, the specificity of probes was tested by hybridization of PCR-fragments to the microarray. Furthermore, we compared three probes specific for *A. ostenfeldii* and two specific for the North American clade of the *A. tamarense* "species complex". A hybridization protocol and a recommendation of the best performing probe to

detect a particular species or clade was provided. For the 18S probes, it was observed that the signal-to-noise-ratios for pure culture PCR-fragments were significantly lower than the signals for field samples spiked with the same species. The only difference was the natural phytoplankton background with unknown sequences or substances in the sample. This background could have influenced the formation of secondary structure of the PCR-fragments. Because of that, more DNA might have hybridized to the particular probe and caused the higher signals. The specificity tests of the 28S probes showed overall high specificity and revealed a co-occurrence of species from the North American and the Western European clade in one culture from Cork (Ireland). Both clades might co-occur at this sampling side. The result was confirmed with the hybridization of spiked field samples and offered a good example for discrimination of a toxic (North American) from a non-toxic clade (Western European) in one bloom.

3.2.2 Probes for detection of picoplankton

It has been shown frequently in the last decades that the contribution of picoplankton to global carbon cycle, biomass and productivity in the marine environment can be tremendous (Campbell et al. 1994; Courties et al. 1994; Joint et al. 1986; Li 1994). The picoplanktonic part of the phytoplankton is composed of cells between 0.2 and 2 μm and can occasionally achieve high abundances (Countway and Caron 2006; Li 1994). The three parts of the photosynthetic picoplankton are: the two prokaryotic cyanobacterial genera *Synechococcus* and *Prochlorococcus*, and the picoplanktonic eukaryotes. The latter are less well investigated and approximately 40 picoplanktonic species are known today (Not et al. 2004). The prasinophytes represent one particular interesting groups, because they have been recently shown as one of the key picoplankton group in marine waters (Not et al. 2004). About 20 genera with 180 species are known nowadays, a few have been recently described (Van Den Hoek et al. 1995; Zingone et al. 2002). Detection of their diversity and distribution is mainly hampered by small size, few morphological markers, and the absence of methods for reliable identification and monitoring (Thomsen and Buck 1998; Zingone et al. 2006).

In **Publication V,** molecular probes for the detection of members of the prasinophytes were tested. The first part of the probe-set was already published and evaluated in dot-blot and fluorescence-*in-situ*-hybridization. The other part was newly designed with the probe design and probe match tool of the ARB software (Ludwig et al. 2004) to cover the known prasinophyte diversity (Guillou et al. 2004). The probe specificity was tested with the BLAST tool (Altschul et al. 1990). 21 probes on the chip were evaluated under stringent conditions

with 18S PCR-fragments from 20 unialgal reference cultures. The results of the target hybridization were always significantly above signals for non-target species or if no target strain was available for a probe test, at least no cross-hybridization was observed with closely related strains. If hybridizations showed indistinct results, the 18S gene of the culture was sequenced. Afterwards, the microarray was used to analyze one environmental sample. This sample was cloned and sequenced as well. The results of the sequences analyses supported the microarray hybridization results.

In summary, the microarray results presented for the genus *Alexandrium* showed a specific, sensitive, and reliable detection and differentiation of species and clades. It was certified that this hybridization approach can significantly contribute to the classification and monitoring of toxic species. No taxonomic expertise is needed and sample processing can be conducted in a high throughput format. This chip contained probes for three clades of the *A. tamarense* "species complex", and two additional species, *A. minutum* and *A. ostenfeldii*. Secondly, the suitability of the microarray for detection of prasinophytes was presented. The probes evaluated and tested in this study offer the potential to analyze a large number of picoplanktonic taxa from the prasinophytes in one experiment. This indicates that a highly specific detection is feasible with a standardized hybridization protocol. Therefore, the microarray has great potential for monitoring of picoplanktonic prasinophytes.

3.3 Analysis of field samples with the PHYTOPLANKTON CHIP

The Helgoland Roads time-series was started in 1962 and provides one of the longest data series for monitoring of phytoplankton biodiversity (Hickel 1998; Wiltshire 2004). The sampling is conducted every workday, and the >20µm phytoplankton community is identified by Utermöhl method and light microscopy (Wiltshire and Dürselen 2004). The disadvantages of this method are the difficult identification of species with few morphological markers and frequent change in counting staff. Furthermore, there is no identification of the picoplanktonic fraction in the Helgoland Roads time-series. There has been a first investigation on the picoeukaryotic diversity in samples from Helgoland with comparison and assessment of three different molecular methods by Medlin et al. (2006). The microarray results of this study agreed with the data obtained by the other two methods used and therefore, the microarray offers a quick and robust tool for long-time monitoring of picoeukaryotic biodiversity in the Helgoland Roads time-series. Furthermore, the data suggested a seasonality in the phytoplankton species composition. The extension of the microarray with more probes and

the analysis of three annual cycles of the Helgoland Roads time-series to gain more information about the seasonal distribution and abundances of the North Sea phytoplankton community were the major aims of this study.

In **Publication VI** the PHYTOPLANKTON CHIP was utilized for the comprehensive analysis of three years of samples from Helgoland. The DNA was extracted and the 18S gene was amplified. The samples were hybridized to the chip and the results demonstrate the applicability and reliability of the chip. The data of the phytoplankton dynamics at the Helgoland Roads showed different species abundances and characteristics for the three years, because the North Sea is a variable and changing environment. Abiotic conditions varied over the three years and several microalgal groups may have responded to these changes In the three years, we observed the highest contribution of cryptophytes and dinoflagellates to the phytoplanktonic community. The cryptophytes represent an important picoplanktonic group and were characterized by eleven probes targeting the six major clades in their RNA phylogeny. In general, species in clade IVand VI were most frequently found. They seem to be relatively halo-, temperature- and nutrient tolerant and could grow under all different environmental factors, whereas other clades grew only under certain conditions. It seems that that the abundant groups are very important for community succession. The PHYTOPLANKTON CHIP has the potential to detect phytoplankton abundances with deep taxonomical resolution and a high through put format. The data obtained by this reliable, robust and efficient method greatly contribute to our knowledge in microbial ecology.

4. Future Research

A protocol for microarray hybridization of RNA was presented in **Publication I**. For the quantification of cell densities it is urgently necessary to relate cell counts and RNA content to the microarray signals. This was also important for the subset of probes evaluated in **Publication IV** and **Publication V**. Each probe on a chip demands its own calibration curve and this requires various hybridizations of target organisms to achieve a reliable correlation. In this context, further investigations of RNA content per cell under different environmental conditions and over the growth cycle of the cell are required. The RNA isolation is a crucial step in this procedure. First of all, the commercialized kit used to extract the RNA has limitations with low or high cell numbers. The columns were likely not sensitive enough or became saturated. A further investigation of different methods or kits would be highly desirable. Secondly, the obtained RNA yield is influenced by different persons. This bias could be eliminated with an automated RNA isolation, e.g., with a 96 well plate format and a pipetting robot.

The PhylochipAnalyzer program developed in **Publication II** could be improved by automated comparison of a set of hybridized chips. The upload of all raw data, the automatic normalization against positive control, and the output of analysis as a 3-D-plot would bring a great benefit for the reliable and fast interpretation of larger data sets. The integration of output formats from other scanner programs could broaden the application of the program. Another refinement can be achieved by the storage of hybridization data like, e.g., in the EMMA program for expression analysis (Dondrup et al. 2003).

The LNAs evaluated in **Publication III** did not show their promising properties for enhancement of probe signals in the microarray hybridization format. If there is no possibility for modification of the hybridization protocol or the design of a new probe for a particular target group, other methods for signal enhancement of a microarray hybridization can be evaluated. The weakness of fluorescence signal collected through the scanner might be solved by using optical thin films as support for probes or sequences instead of glass slides. It was shown that a theoretical enhancement of twenty-fold (compared to glass substrate) was achievable (Barritault et al. 2004). Further promising is the utilization of rotating, circular microchambers for the hybridization solution. They cover exactly the spotted area of the microarray and rotate while the microarray is stationary. The microchamber bottom wall drags the solution past the microarray spots with velocity. A 5-fold increase of the

hybridization intensity was found in comparison to a conventional microscope slide with a coverslip (Vanderhoeven et al. 2005).

The sequences of the probes on the chip in **Publication IV-VI** must be regularly checked for specificity in public databases, because the number of sequences is growing daily. Furthermore, the chip can be extended by integration of more probes for e.g., species of the genus *Alexandrium*, other harmful algal species, and members of the prasinophytes or other picoplanktonic taxa. If the PHYTOPLANKTON CHIP should be used in other geographical regions, a preliminary overview of abundant species is needed and afterwards the chip can be adapted.

Especially the current PHYTOPLANKTON CHIP in **Publication VI** for monitoring of phytoplankton dynamics in the North Sea can be improved by extension with probes for diatoms. A further improvement of the PhylochipAnalyzer program can greatly facilitate the analysis of huge amounts of data that need to be processed. For a long-term comparison of reliability of analysis a comparison of microarray data with other methods, especially molecular approaches, is desired.

5. Summary

The aim of this doctorial thesis was the evaluation and development of a DNA chip as a robust and rapid tool for the detection and monitoring of phytoplankton dynamics. The reliable and comprehensive data census of microalgal biodiversity and tracing of their abundances is urgently necessary. They represent the base of the aquatic food web and contribute enormously to the global carbon cycle, biomass and productivity in the marine environment. Furthermore, the punctual and reliable detection of Harmful Algal Blooms is required because they threaten humans, ecosystems, fishery, tourism, and aquaculture. The determination and enumeration of microalgae is demanding and for many traditional methods, highly trained taxonomists are needed. This is especially challenging if cells are small and lack morphological markers. The introduction of molecular approaches has facilitated the microalgal research in many ways and they offer a multitude of new possibilities.

First of all, the microarray hybridization protocol introduced by Metfies and Medlin (2004) was improved for isolation, labeling and microarray hybridization of RNA. The approach presented here offers the possibility to extract whole RNA and to relate the obtained amount of nucleic acid to cell numbers. A commercial RNA isolation kit was tested and the protocol was optimized. The labeling kit used is commercially available as well and was evaluated for application in the microarray approach. The advantages, drawbacks, and potential pitfalls are discussed. The entire method is described in detail with possible source or supplier for chemicals and materials, and displayed by pictures as a manual for a book on phytoplankton analysis.

The introduction of phylochips with hierarchical probes-sets has facilitated species identification in environmental samples enormously. The application of probes at several taxonomical levels allows the assessment of biodiversity with different resolutions. However, the processing of obtained data at different hierarchical levels can be challenging, especially if high samples numbers and several probes have to be analyzed. The PhylochipAnalyzer program was developed to facilitate the analysis of phylochips. The program contains two features; first the upload and editing of the phylogenetic trees, and secondly the analysis of scanner data files with the determined tree. A signal is accounted as positive only if all probes in the hierarchy show a hybridization result. The applicability of the program was proven by a hybridization of an 18S PCR-fragment of *Micromonas pusilla*, a prasinophyte. This species was detected at four taxonomical levels on the current microarray, the Euk328 and 1209 probes for eukaryotes, the Chlo01 and 02 probes for chlorophytes, the Pras04 probe for the

prasinophyte clade II (*Marmelliales*) and finally the Micro01 probe for the species. The utilization of the PhylochipAnalyzer significantly increases the robustness of the results retrieved from phylochip experiments by hierarchy editing and evaluation of data.

Locked Nucleic Acids (LNAs) were presented in 1998 as a class of bicyclic RNA analogs, and they show high affinities and specificities towards their complementary nucleic acid target molecules. An enhancement of specificity and sensitivity is suggested by the insertion of LNAs in molecular probes. A comparison of five conventional probes and ten LNA-modified probes were tested on the microarray. The hybridization of target and widely related species 18S PCR-fragments were analyzed and showed enhancement of signals for the LNA probes. But unfortunately, the discriminative potential of the LNA-modified probes was low and they showed also positive signals for non-target organisms with several mismatches in the probe sequence. Thus, the LNA technology may have great potential in methods that use probes in suspension and in gene expressions studies, but under the accurately defined conditions of this microarray hybridization approach they did not improve the method.

Furthermore, the potential of a microarray (ALEX CHIP) for detection and discrimination of *Alexandrium* species was shown. This genus features toxic and non-toxic species that are difficult to distinguish by morphology alone. The genus *Alexandrium* is probably the best investigated group triggering Harmful Algal Blooms. Nine probes were taken from other methods and one new probe was developed. The probes targeted the 18S and 28S rRNA gene and were specific for *Alexandrium ostfeldii*, *A. minutum* and three different clades in the *A. tamarense* "species complex". Probe specificity was evaluated by hybridization of PCR-fragments of target and closely related species. In addition, a comparison of three probes specific for *A. ostenfeldii* and two probes specific for the North American Clade of the *A. tamarense* "species complex" was conducted. Field samples with a natural phytoplankton background were spiked with cells of pure target *Alexandrium* cultures and the extracted DNA was amplified by PCR. Subsequently, they were hybridized to the ALEX CHIP to prove the applicability and reliability. The results presented showed that the chip is an effective and fast application to detect and differentiate toxic and non-toxic *Alexandrium* members even in mixed species assemblages.

The reliable detection and monitoring of picoplanktonic taxa is mainly hampered by their small size and few morphological markers. The utilization of molecular probes and the microarray technology offers the potential to identify picoeukaryotes from environmental samples. A microarray was developed that facilitates the detection of members of the Prasinophyta. Probes for members of the prasinophytes that were initially developed for other

hybridization methods were adapted to the microarray protocol. Furthermore, new probes were designed for the prasinophyte groups that were not yet covered. This chip contained, altogether, 21 probes for the characterization of microbial picoeukaryotic communities. The probe set was assessed and specificity and discriminative potential was tested under stringent conditions with 18S PCR-fragments from 20 unialgal reference cultures. Afterwards, indistinct hybridization results were clarified with sequencing of the concerned species. The chip was proven with the application of one environmental sample; a sequence analysis of the environmental sample supported the results of the microarray. Therefore, it was shown that the microarrays can serve as a reliable tool for fast and efficient monitoring of this important picoplanktonic algal group.

All probes with positive specificity results in this thesis were applied together to one microarray, the PHYTOPLANKTON CHIP. Phytoplankton field samples were taken at the island of Helgoland in the North Sea from 2004 to 2006 at regular intervals. The island of Helgoland has a long history in marine research and phytoplankton dynamics. In 1962, the Helgoland Roads time-series was established. For the phytoplankton community, only the > 20 µm size fraction is identified on a daily basis. For picoplanktonic groups, light microscopy can not differentiate taxa or species. The phyto- and especially picoplanktonic dynamics were successfully analyzed with the PHYTOPLANKTON CHIP in these three annual cycles. In general, the data showed highly abundant taxa present through all three years and some taxa that were rare. The first group of taxa seemed to be very stable and unaffected by environmental changes. The latter group is sensitive and could only grow under certain environmental conditions. In summary, the PHYTOPLANKTON CHIP has the potential to analyze phytoplankton abundances with deep taxonomical resolution in a high through put format. The results obtained by this reliable, robust and efficient method can greatly contribute to our knowledge in microbial ecology.

6. Zusammenfassung

Ziel dieser Doktorarbeit war die Entwicklung und Beurteilung eines DNA-Chips als sicheres und schnelles Instrument für den Nachweis und das Monitoring von Phytoplanktondynamiken. Die zuverlässige und umfassende Datenerhebung der Biodiversität von Mikroalgen und die Überwachung ihrer Abundanzen ist unbedingt erforderlich, da sie die Basis des marinen Nahrungsnetzes darstellen und enorm zu Kohlenstoffkreislauf und Biomasseproduktion im marinen Lebensraum beitragen. Des Weiteren ist eine rechtzeitige und zuverlässige Bestimmung von toxischen Algenblüten erforderlich, da sie Menschen, Ökosysteme, Fischerei, Tourismus und Aquakultur bedrohen. Die Bestimmung und Auszählung von einzelligen Algen ist anspruchsvoll, und für die Verwendung vieler klassischer Methoden sind hochqualifizierte Experten nötig. Eine spezielle Herausforderung stellen hierbei besonders kleine Zellen mit wenigen morphologischen Kennzeichen dar. Die Einführung von molekularen Methoden hat die Erforschung von Mikroalgen und ihrer Biodiversität erleichtert und viele neue Möglichkeiten eröffnet.

Zunächst wurde das von Metfies und Medlin (2004) veröffentlichte Mikroarray-Hybridisierungsprotokoll für die Isolation, Markierung und Hybridisierung von RNA verbessert. Die präsentierte Methode bietet die Möglichkeit, die gesamte RNA zu extrahieren und die erhaltene Menge an Nukleinsäuren mit den Zellzahlen in Verbindung zu setzten. Ein kommerziell erhältliches RNA-Isolationskit wurde getestet und das Isolierungsprotokoll optimiert. Auch das verwendete Markierungskit ist käuflich zu erwerben; es wurde für die Verwendung in der Mikroarrayhybridisierung bewertet; Vorzüge, Nachteile und möglichen Problemstellungen des Weiteren diskutiert. Das gesamte Prozedere ist mit möglichen Bezugsquellen oder Firmen für die benötigten Chemikalien und Materialen im Detail beschrieben. Da diese Anleitung Teil eines Buches für Phytoplanktonanalysen ist, sind Abbildungen der einzelnen Geräte und Schritte zur Verdeutlichung dargestellt.

Mit der Einführung von Phylochips, die hierarchische Sondensets verwenden, wurde die Artenbestimmung von Feldproben erheblich erleichtert. Die Anwendung von Sonden auf mehreren taxonomischen Ebenen erlaubt die Einschätzung der Biodiversität mit verschiedenerer Auflösung. Dennoch kann die Bearbeitung der erhobenen Daten schwierig sein, besonders wenn ein großes Probenvolumen und viele Sonden analysiert werden müssen. Das PhylochipAnalyzer Computerprogramm wurde entwickelt, um die Analyse von Phylochips zu unterstützen; es enthält zwei Funktionen, zum einen das Hochladen und Editieren des phylogenetischen Baumes und zum anderen die Auswertung der Scannerdateien

mit dem vorher festgelegten Baum. Ein Signal wird nur dann als korrekt definiert, wenn alle Sonden im betreffenden Teil des Baumes ein Hybridisierungsergebnis zeigen. Die Anwendbarkeit des Programms wurde mit der Hybridisierung eines 18S PCR-Fragments von *Micromonas pusilla*, einer Prasinophyceae, gestestet. Diese Art wird mit dem aktuellen Mikroarray auf vier taxonomischen Ebenen detektiert, mit den Euk328 und 1209 Sonden für Eukaryota, den Chlo01 und 02 Sonden für Chlorophyta, der Pras04 Sonde für den Prasinophytceae clade II (*Marmelliales*) und letztendlich der Micro01 Sonde für die Spezies. Die Verwendung des PhylochipAnalyzers erhöht die Verlässlichkeit der von einem Phylochip Experiment erhaltenen Daten mit Hilfe der Baumbearbeitung und anschließender Bewertung der Daten.

Locked Nucleic Acids (LNAs) wurden 1998 als eine Klasse von bicyclischen RNA Analoga präsentiert, und sie zeigen hohe Affinitäten und Spezifitäten zu ihren komplementären Nukleinsäurezielmolekülen. Die Eigenschaften versprechen eine Verbesserung der Spezifität und Sensitivität bei Einbau von LNAs in molekularen Sonden. Im Mikroarrayteil der Publikation wurden fünf herkömmliche Sonden mit zehn LNA-modifizierten Sonden verglichen; die Hybridisierungen mit 18S PCR-Fragmenten von Zielarten und weiter verwandten Arten analysiert. Diese zeigten eine Verbesserung der Signalstärke für die LNA-modifizierten Sonden. Leider war das Unterscheidungspotential der LNA-modifizierten Sonden allerdings sehr gering, so dass sie auch für Nicht-Zielarten mit vielen Basenunterschieden in der Sondensequenz positive Hybridisierungssignale zeigten. Daher ist abschließend zu sagen, dass die LNA-Technologie großes Potential für die Anwendung in Lösungen und bei der Untersuchung von Genexpressionen haben mag, aber unter den definierten Bedingungen für diesen Mikroarray Hybridisierungsansatz keine Verbesserung der Methodik gezeigt hat.

Zudem wurde das Potential eines Mikroarrays (ALEX CHIP) für die Bestimmung und Unterscheidung von *Alexandrium* Arten gezeigt. In dieser Gattung sind giftige und ungiftige Arten bekannt, die anhand ihrer morphologischen Merkmale nur schwer zu unterscheiden sind. Sie stellen wahrscheinlich eine der am besten untersuchten Gruppen dar, die toxischen Algenblüten auslösen können. Neun Sonden von anderen Sonden-basierten Methoden wurden ausgewählt und eine neue Sonde entwickelt; die Sonden hatten entweder 18S oder 28S rRNA Gene als Zielmoleküle und waren für *Alexandrium ostfeldii*, *A. minutum* und drei verschiedene Clades im *A. tamarense* "Spezieskomplex" spezifisch. Die Spezifitäten der Sonden wurden mit Hybridisierungen von PCR-Fragmenten der Zielarten und dicht verwandten Arten getestet; zusätzlich verglichen wir drei Sonden für *A. ostenfeldii* und zwei

für den North American Clade des *A. tamarense* "Spezieskomplexes". Feldproben mit natürlichem Phytoplankton-hintergrund wurden mit Zellen von Reinkulturen der Zielarten versetzt und die extrahierte DNA per PCR amplifiziert. Um die Anwendbarkeit und die Zuverlässigkeit des ALEX CHIP zu prüfen, wurden die PCR-Fragmente anschließend hybridisiert. Die gezeigten Ergebnisse verdeutlichen, dass der Mikroarray eine effektive und schnelle Anwendung zur Detektierung und Unterscheidung von giftigen und ungiftigen *Alexandrium* Arten darstellt.

Die verlässliche Bestimmung und Überwachung von pikoplanktonischen Taxa ist hauptsächlich durch ihre geringe Größe und die wenigen morphologischen Merkmale behindert. Die Verwendung von molekularen Sonden und der Mikroarray-Technologie ermöglicht es, die Pikoeukaryoten aus Umweltproben zu identifizieren. Deshalb wurde ein Mikroarray entwickelt, der die Detektion von Prasinophyceen Arten unterstützen soll. Ein Teil des Sets bestand aus Sonden, die ursprünglich für andere Hybridisierungsmethoden entwickelt wurden, der zweite Teil bestand neu konzipierte Sonden für die bis dahin nicht abgedeckten Gruppen. Der Chip umfasste insgesamt 21 Sonden für die Charakterisierung von pikoeukaryotischen Gemeinschaften; diese wurden bewertet und ihre Spezifitäten und Unterscheidungsfähigkeit wurden unter gleichen Bedingungen mit 18S PCR-Fragments von 24 reinen Referenzkulturen getestet. Anschließend erfolgte eine Klärung von nicht eindeutigen Hybridisierungsergebnissen mit Hilfe der DNA Sequenzierung der betroffenen Arten. Der Chip wurde mit einer Feldprobe getestet, und Sequenzergebnisse bestätigten die Ergebnisse des Mikroarrays. Demnach erwies sich, dass der Mikroarray als verlässliches Werkzeug für schnelle und effiziente Überwachung dieser wichtigen pikoplanktonischen Gruppe dienen kann.

Alle Sonden, die in dieser Doktorarbeit ein positives Resultat erzielten, konnten anschließend auf dem PHYTOPLANKTON CHIP verwendet werden. Von 2004 - 2006 wurden in regelmäßigen Abständen Phytoplankton Feldproben vor der Insel Helgoland in der Nordsee genommen. Helgoland hat eine lange Geschichte in der Meeresforschung. 1962 wurde die Helgoland Reede Langzeitreihe ins Leben gerufen. Seit dem werden Phytoplanktonabundanzen für Arten < 20 µm mit Hilfe des Lichtmikroskops aufgenommen und untersucht. Die Identifizierung von Mikroalgen ist schwierig, und besonders pikoplanktonische Gruppen oder Arten lassen sich mit dem Lichtmikroskop nicht unterscheiden. Um weitere Erkenntnisse über die phyto- und im speziellen pikoplanktonischen Artenzusammensetzungen in der Nordsee und die sich wiederholende Gemeinschaftskomposition zu erhalten, wurden drei Jahresgänge der Phytoplankton

Feldproben erfolgreich mit diesem Chip untersucht. Die Ergebnisse dieser Studie zeigten in allen drei Jahren sehr häufige vorkommende taxonomische Gruppen und andere, die nur sehr selten vorkamen. Erstere zeigten sehr stabiles Wachstum und schienen von den veränderten Unweltbedingungen nicht beeinflusst zu sein. Der andere Teil scheint sehr sensibel zu sein und konnte nur unter bestimmten Umweltbedingungen wachsen. Zusammenfassend ist zu sagen, dass der PHYTOPLANKTON CHIP das Potential besitzt, Phytoplanktonabundanzen mit großer taxonomischer Auflösung in hohem Probendurchsatz zu detektieren und zu überwachen. Die mit dieser zuverlässigen, robusten und effizienten Methode erhaltenen Ergebnisse können unser Wissen über mikrobiologische Ökologie in großem Umfang erweitern.

7. References

ADACHI, M., Y. SAKO, and Y. ISHIDA. 1994. Restriction fragment length polymorphism of ribosomal DNA internal transcribed spacer and 5.8S regions in Japanese *Alexandrium* species (Dinophyceae). J. Phycol. **30:** 857-863.

---. 1996a. Analysis of *Alexandrium* (Dinophyceae) species using sequences of the 5.8S ribsomal DNA and internal transcribed spacer regions. J. Phycol. **32:** 424-432.

---. 1996b. Identification of the toxic dinoflagellates *Alexandrium catenella* and *A. tamarense* (Dinophyceae) using DNA probes and whole-cell hybridization. J. Phycol. **32:** 1049-1052.

ALTSCHUL, S. F., W. GISH, W. MILLER, E. W. MYERS, and D. J. LIPMAN. 1990. Basic local alingment search tool. J. Mol. Biol. **215:** 403–410.

AMANN, R. I., B. J. BINDER, R. J. OLSON, S. W. CHISHOLM, R. DEVEREUX, and D. A. STAHL. 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analysing mixed microbial populations. Appl. Environ. Microbiol. **56:** 1919-1925.

ANDERSEN, R. A., R. R. BIDIGARE, M. D. KELLER, and M. LATASA. 1996. A comparison of HPLC pigment signatures and electron microscopic observations for oligotrophic waters of the North Atlantic and Pacific Oceans. Deep Sea Res. II **43:** 517-537.

ANDERSEN, R. A., D. POTTERT, and J. C. BAILEY. 2002. *Pinguiococcus pyrenoidosus* gen. et sp. nov. (Pinguiophyceae), a new marine coccoid alga. Phycol. Res. **50:** 57-65.

ANDERSEN, R. A., G. W. SAUNDERS, M. P. PASKIND, and J. P. SEXTON. 1993. Ultrastructure and 18S rRNA gene sequence for *Pelagomonas calceolata* gen. et sp. nov. and the description of a new algal class, the Pelagophyceae classis nov. J. Phycol. **29:** 701-715.

ANDERSON, D. M. 1998. Preface, p. 1-2. *In* D. M. Anderson, A. D. Cembella and G. M. Hallegraeff [eds.], Physiological Ecology of Harmful Algal Blooms. Springer Verlag, Berlin, Heidelberg, Germany.

ANDERSON, D. M., D. M. KULIS, B. A. KEAFER, and E. BERDALET. 1999. Detection of the toxic dinoflagellate *Alexandrium fundyense* (Dinophyceae) with oligonucleotide and antibody probes: Variability in labeling intensity with physiological condition. J. Phycol. **35:** 870-883.

BALECH, E. 1995. The genus *Alexandrium* Halim (Dinoflagellata). Sherkin Island Marine Station, Ireland.

BARRITAULT, P., S. GETIN, P. CHATON, B. ANDRE, F. VINET, and B. FOUQUE. 2004. Optical thin films serving biotechnology: fluorescence enhancement of DNA-chip. Proc. SPIE **5250:** 12-20.

BHATTACHARYA, D., and L. K. MEDLIN. 1998. Algal phylogeny and the origin of land plants. Plant Physiol. **116:** 9–15.

BIEGALA, I. C., F. NOT, D. VAULOT, and N. SIMON. 2003. Quantitative assessment of picoeukaryotes in the natural environment by using taxon-specific oligonucleotide probes in association with tyramide signal amplification, fluorescence-*in-situ-h*ybridization and flow cytometry. Appl. Environ. Microbiol. **69:** 5519-5529.

BOIREAU, W., J. C. ZEEHA, P. E. PUIGA, and D. POMPON. 2005. Unique supramolecular assembly of a redox protein with nucleic acids onto hybrid bilayer: towards a dynamic DNA chip. Biosens. Bioelectron. **20:** 1631-1637.

BOLD, H. C., and M. J. WYNNE. 1978. Introduction to the algae: Structure and Reproduction. Prentice Hall, Englewood Cliffs, USA.

BORNET, B., E. ANTOINE, S. FRANCOISE, and C. MARCAILLOU-LE BAUT. 2005. Development of sequence characterized amplified region markers from intersimple sequence repeat fingerprints for the molecular detection of toxic phytoplankton *Alexandrium catenella* (Dinophyceae) and *Pseudo-nitzschia pseudodelicatissima* (Bacillariophyceae) from french coastal waters. J. Phycol. **41:** 704–711.

BOWERS, H. A., T. TENGS, H. B. GLASGOW, J. M. BURKHOLDER, P. A. RUBLEE, and D. W. OLDACH. 2000. Development of Real-Time PCR assays for rapid detection of *Pfiesteria piscicida* and related dinoflagellates. Appl. Environ. Microbiol. **66:** 4641-4648.

BRAASCH, D. A., and D. R. COREY. 2001. Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA. Chem. Biol. **8:** 1-7.

BRATBAK, G., W. WILSON, and M. HELDAL. 1996. Viral control of *Emiliania huxleyi* blooms? J. Marine Syst. **9:** 75-81.

BRETON, E., C. BRUNET, B. SAUTOUR, and J.-M. BRYLINSKI. 2000. Annual variations of phytoplankton biomass in the Eastern English Channel: comparison by pigment signatures and microscopic counts. J. Plankton Res. **22:** 1423-1440.

BROWN, M. R., and S. W. JEFFREY. 1992. Biochemical composition of microalgae from the green algal classes Chlorophyceae and Prasinophyceae. 1. Amino acids, sugars and pigments. Journal of Experimental Marine Biology and Ecology **161:** 91-113.

BRUSSAARD, C. P. D., B. KUIPERS, and M. J. W. VELDHUIS. 2005. A mesocosm study of *Phaeocystis globosa* population dynamics: I. Regulatory role of viruses in bloom control. Harmful Algae **4:** 859-874.

BRUSSAARD, C. P. D., R. THYRHAUG, D. MARIE, and G. BRATBAK. 1999. Flow cytometric analyses of virus infection in two marine phytoplankton species, *Micromonas pusilla* (Prasinophyceae) and *Phaeocystis pouchetii* (Prymnesiophyceae). J. Phycol. **35:** 941-948.

BUTCHER, R. 1952. Contributions to our knowledge of the smaller marine algae. J. Mar. Biol. Assoc. UK **31:** 175-191.

CAMPBELL, L., H. LIU, H. A. NOLLA, and D. VAULOT. 1997. Annual variability of phytoplankton and bacteria in the subtropical North Pacific Ocean at Station ALOHA during the 1991–1994 ENSO event. Deep Sea Res. I **44:** 167–192.

CAMPBELL, L., H. A. NOLLA, and D. VAULOT. 1994. The importance of *Prochlorococcus* to community structure in the central North Pacific Ocean. Limnol. Oceanogr. **39:** 954-961.

CARON, D. A., P. D. COUNTWAY, and M. V. BROWN. 2004. The growing contributions of molecular biology and immunology to Protistan ecology: molecular signatures as ecological tools. J. Euk. Microbiol. **51:** 38-48.

CARON, D. A., E. R. PEELE, E. L. LIM, and M. R. DENNETT. 1999. Picoplankton and nanoplankton and their trophic coupling in surface waters of the Sargasso Sea south of Bermuda. Limnol. Oceanogr. **44:** 259-272.

CEMBELLA, A. D., J. J. SULLIVAN, G. L. BOYER, F. J. R. TAYLOR, and R. J. ANDERSON. 1987. Variation in paralytic shellfish toxin composition within the *Protogonyaulax tamarensis/catenella* species complex, red tide dinoflagellates. Biochem. Syst. Ecol. **15:** 171-186.

CEMBELLA, A. D., and F. J. R. TAYLOR. 1986. Electrophoretic variability within the *Protogonyaulax tamarensis/catenella* species complex: Pyridine linked dehydrogenases. Biochem. Syst. Ecol. **14:** 311-323.

CEMBELLA, A. D., J.-C. THERRIAULT, and P. BERLAND. 1988. Toxicity of cultured isolates and natural populations of *Protogonyaulax tamarensis* from the St. Lawrence estuary. J. Shellfish Res. **7:** 611-621.

CHEUNG, V. G., M. MORLEY, F. AGUILAR, A. MASSIMI, R. KUCHERLAPATI, and G. CHILDS. 1999. Making and reading microarrays. Nat. Genet. **21:** (1 Suppl),15-19.

CHISHOLM, S. W., R. J. OLSON, E. R. ZETTLER, R. GOERICKE, J. B. WATERBURY, and N. A. WELSCHMEYER. 1988. A novel free-living prochlorophyte abundant in the oceanic euphotic zone. Nature **334:** 340-343.

CHRÉTIENNOT-DINET, M.-J. and others 1995. A new marine picoeucaryote: *Ostreococcus tauri* gen. et sp. nov. (Chlorophyta, Prasinophyceae). Phycologia **34:** 285-292.

COCHLAN, W. P., and P. J. HARRISON. 1991. Kinetics of nitrogen (nitrate, ammonium and urea) uptake by the picoflagellate *Micromonas pusilla* (Prasinophyceae). J. Exp. Mar. Biol. Ecol. **153:** 129-141.

COCHLAN, W. P., P. J. HARRISON, P. J. CLIFFORD, and K. YIN. 1990. Observations on double chlorophyll maxima in the vicinity of the Fraser River plume, Strait of Georgia, British Columbia. J. Exp. Mar. Biol. Ecol. **143:** 139-146.

COTTREL, M. T., and C. A. SUTTLE. 1995. Dynamics of a lytic virus infecting the photosynthetic marine picoflagellate *Micromonas pusilla*. Limnol. Oceanogr. **40:** 730-739.

COTTRELL, M. T., and C. A. SUTTLE. 1991. Wide-spread occurrence and clonal variation in viruses which cause lysis of a cosmopolitan, eukaryotic marine phytoplankter *Micromonas pusilla*. Mar. Ecol. Prog. Ser. **78:** 1-9.

COUNTWAY, P. D., and D. A. CARON. 2006. Abundance and distribution of *Ostreococcus sp.* in the San Pedro Channel, California, as revealed by Quantitative PCR. Appl. Environ. Microbiol. **72:** 2496-2506.

COURTIES, C., R. PERASSO, M.-J. CHRETIENNOT-DINET, M. GOUY, L. GUILLOU, and M. TROUSSELLIER. 1998. Phylogenic analysis and genome size of *Osterococcus tauri* (Chlorophyta, Prasinophyceae). J. Phycol. **34:** 844-849.

COURTIES, C. and others 1994. Smallest eukaryotic organism. Nature **370:** 255.

COYNE, K. J., D. A. HUTCHINS, C. E. HARE, and S. C. CARY. 2001. Assessing temporal and spatial variability in *Pfiesteria piscicida* distributions using molecular probing techniques. Aquat. Microb. Ecol. **24:** 275-285.

DAUGBJERG, N., Ø. MOESTRUP, and P. ARCTANDER. 1995. Phylogeny of genera of Prasinophyceae and Pedinophyceae (Chlorophyta) deduced from molecular analysis of the rbcL gene. Phycol. Res. **43:** 203-213.

DELONG, E. F. 1998. Molecular Phylogenetics: New perspective on the ecology, evolution and biodiversity of marine organisms, p. 1-. *In* K. E. Cooksey [ed.], Molecular approaches to the study of the ocean. Chapman & Hall, London, UK.

DELONG, E. F., G. S. WICKHAM, and N. R. PACE. 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science **243:** 1360-1363.

DERELLE, E. and others 2002. DNA libraries for sequencing the genome of *Osterococcus tauri* (Chlorophyta, Prasinophyceae): The smallest free-living eukaryotic cell. J. Phycol. **38:** 1150-1156.

---. 2006. From the Cover: Genome analysis of the smallest free-living eukaryote *Ostreococcus tauri* unveils many unique features. Proc. Natl. Acad. Sci. USA **103:** 11647-11652.

DERISI, J. L., V. R. IYER, and P. O. BROWN. 1997. Exploring the metabolic and genetic control of gene expression on a genomic scale. Science **278:** 680-686.

DEVEREUX, R., M. D. KANE, J. WINFREY, and D. A. STAHL. 1992. Genus- and group- specific hybridisation probes for determination and environmental studies of sulphate reducing bacteria. Syst. Appl. Microbiol. **15:** 601-609.

DÍEZ, B., C. PEDRÓS-ALIÓ, T. L. MARSH, and R. MASSANA. 2001a. Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques. Appl. Environ. Microbiol. **67:** 2942–2951.

DÍEZ, B., C. PEDRÓS-ALIÓ, and R. MASSANA. 2001b. Study of genetic diversity of eukaryotic picoplankton in different oceanic regions by small-subunit rRNA gene cloning and sequencing. Appl. Environ. Microbiol. **67:** 2932-2941.

DODGE, J. D. 1985. Atlas of dinoflagellates : a scanning electron microscope survey. Farrand Press, London, UK.

DONDRUP, M. and others 2003. EMMA: a platform for consistent storage and efficient analysis of microarray data. J. Biotechnol. **106:** 135-146.

DUPUY, C. and others 2000. Feeding rate of the oyster *Crassostrea gigas* in a natural planktonic community of the Mediterranean Thau Lagoon. Mar. Ecol. Prog. Ser. **205:** 171-184.

DURAND, M. D., R. J. OLSON, and S. W. CHISHOLM. 2001. Phytoplankton population dynamics at the Bermuda Atlantic Time-series Station in the Sargasso Sea. Deep Sea Res. II **48:** 1983–2003.

DYHRMAN, S. T., D. ERDNER, J. L. DU, M. GALAC, and D. M. ANDERSON. 2006. Molecular quantification of toxic *Alexandrium fundyense* in the Gulf of Maine using real-time PCR. Harmful Algae **5:** 242-250.

ETSCHEID, M., and D. RIESNER. 1998. TGGE and DGGE, p. 135-143. *In* A. Karp, P. G. Isaac and D. S. Ingram [eds.], Molecular tools for screening biodiversity. Chapman&Hall, London, UK.

FALKOWSKI, P. G. 1980. Primary productivity in the sea, Environmental Science Research. Plenum Press, New York, USA.

FAUST, M. A., and R. A. GULLEDGE. 2002. Identifying harmful marine dinoflagellates. Smithsonian Institution, Washington, USA.

FAWLEY, M. W., Y. YUN, and M. QIN. 2000. Phylogenetic analyses of 18s rDNA sequences reveal a new coccoid lineage of the prasinophyceae (Chlorophyta). J. Phycol. **36:** 387-393.

FERIOTTO, G., M. BORGATTI, C. MISCHIATI, N. BIANCHI, and R. GAMBARI. 2002. Biosensor technology and surface plasmon resonance for real-time detection of genetically modified roundup ready soybean gene sequences. J. Agric. Food Chem. **50:** 955-962.

FOUILLAND, E., C. DESCOLAS-GROS, C. COURTIES, Y. COLLOS, A. VAQUER, and A. GASC. 2004. Productivity and growth of a natural population of the smallest free-living eukaryote under nitrogen deficiency and sufficiency. Microbial Ecol. **48:** 103-110.

FRANKE, H.-D., F. BUCHHOLZ, and K. H. WILTSHIRE. 2004. Ecological long-term research at Helgoland (German Bight, North Sea): retrospect and prospect-an introduction. Helgol. Mar. Res. **58:** 223-229.

FULLER, N. J. and others 2006a. Analysis of photosynthetic picoeukaryote diversity at open ocean sites in the Arabian Sea using a PCR biased towards marine algal plastids. Aquat. Microb. Ecol. **43:** 79–93.

---. 2006b. Molecular analysis of photosynthetic picoeukaryote community structure along an Arabian Sea transect. Limnol. Oceanogr. **51:** 2502-2514.

GALLUZZI, L. and others 2005. Development of a qualitative PCR method for the *Alexandrium* spp. (Dinophyceae) detection in contaminated mussels (*Mytilus galloprovincialis*). Harmful Algae **4:** 973-983.

GALLUZZI, L., A. PENNA, E. BERTOZZINI, M. VILA, E. GARCÉS, and M. MAGNANI. 2004. Development of a Real-Time PCR Assay for rapid detection and quantification of *Alexandrium minutum* (a dinoflagellate). Appl. Environ. Microbiol. **70:** 1199-1206.

GARCÉS, E., M. DELGADO, M. MASÓ, and J. CAMP. 1998. Life history and *in situ* growth rates of *Alexandrium taylori* (Dinophyceae, Pyrrophyta). J. Phycol. **34:** 880-887.

GENTRY, T. J., G. S. WICKHAM, C. W. SCHADT, Z. HE, and J. ZHOU. 2006. Microarray applications in microbial ecology research. Microbial Ecol. **52:** 159-175.

GIESKES, W. W. C., and G. W. KRAAY. 1983. Dominance of Cryptophyceae during the phytoplankton spring bloom in the central North Sea by HPLC analysis of pigments. Mar. Biol. **75:** 179–185.

---. 1984. Phytoplankton, its pigments, and primary production at a central North Sea station in May, July and September 1981. Neth. J. Sea. Res. **18:** 51-70.

GODHE, A. 2002. Benthic and pelagic dinoflagellate stages: environmental settings, cyst vaibility, and molecular idetification, Department of Marine Ecology, Marine Botany. Phd Thesis, Göteborg University, Sweden.

GODHE, A. and others 2007. Intercalibration of classical and molecular techniques for identification of *Alexandrium fundyense* (Dinophyceae) and estimation of cell densities. Harmful Algae **6:** 56-72.

GRANÉLI, E., and J. T. TURNER. 2006a. An introduction to Harmful Algae, p. 3-7. *In* E. Granéli and J. T. Turner [eds.], Ecology of Harmful Algae. Ecological Studies. Springer Verlag, Berlin, Heidelberg, Germany.

---. 2006b. Preface, p. V-VII. *In* E. Granéli and J. T. Turner [eds.], Ecology of Harmful Algae. Ecological Studies. Springer Verlag, Berlin, Heidelberg, Germany.

GRAVES, D. J. 1999. Powerful tools for genetic analysis come of age. Trends Biotechnol. **17:** 127-134.

GROBEN, R., U. JOHN, G. ELLER, M. LANGE, and L. K. MEDLIN. 2004. Using fluorescently-labelled rRNA probes for hierarchical estimation of phytoplankton diversity – a mini-review. Nova Hedwigia **79:** 313-320.

GROBEN, R., and L. K. MEDLIN. 2005. *In -situ*-hybridization of phytoplankton using fluorescently labeled rRNA probes., p. 299-310. *In* E. A. Zimmer and E. H. Roalsen [eds.], Methods in Enzymology, Molecular Evolution: Producing the Biochemical Data. Elsevier Academic Press, San Diego, USA.

GUILLOU, L., M.-J. CHRÉTIENNOT-DINET, L. K. MEDLIN, H. CLAUSTRE, S. LOISEAUX-DE GOER, and D. VAULOT. 1999a. *Bolidomonas*: A new genus with two species belonging to a new algal class, the Bolidophyceae (Heterokonta). J. Phycol. **35:** 368–381.

GUILLOU, L. and others 2004. Diversity of picoplanktonic Prasinophytes assessed by direct nuclear SSU rDNA sequencing of environmental samples and novel isolates retrieved from oceanic and coastal marine ecosystems. Protist **155:** 193-214.

GUILLOU, L., S.-Y. MOON-VAN DER STAAY, H. CLAUSTRE, F. PARTENSKY, and D. VAULOT. 1999b. Diversity and abundance of Bolidophyceae (Heterokonta) in two oceanic regions. Appl. Environ. Microbiol. **65:** 4528-4536.

HALIM, Y. 1960. *Alexandrium minutum* n. gen., n. sp. dinoflagelle provocant des "eaux rouges". Vie et Milieu **11:** 102-105.

HALLEGRAEFF, G. M. 2002. Aquaculturists' guide to harmful Australian microalgae. School of Plant Science, Hobart, Australia.

---. 2003. Harmful algal blooms: a global overview, p. 25-49. *In* G. M. Hallegraeff, D. M. Anderson and A. D. Cembella [eds.], Manual on Harmful Marine Microalgae. United Nations Educational, Scientific and Cultural Organization, Paris, France.

HALLEGRAEFF, G. M., and S. W. JEFFREY. 1984. Tropical phytoplankton species and pigments of continental shelf waters of North and North-West Australia. Mar. Ecol. Prog. Ser. **20:** 59-74.

HANDY, S. M., D. A. HUTCHINS, S. C. CARY, and K. J. COYNE. 2006. Simultaneous enumeration of multiple raphidophyte species by quantitative real-time PCR: capabilities and limitations. Limnol. Oceanogr. Meth. **4:** 193-204.

HANSEN, M. C., T. TOLKER-NIELSEN, M. GIVSKOV, and S. MOLIN. 1998. Biased 16S rDNA PCR amplification caused by interference from DNA flanking the template region. FEMS Microbiol. Ecol **26:** 141-149.

HARDY, A. C. 1965. The open sea: its natural history. Collins, London, UK.

HICKEL, W. 1998. Temporal variability of micro- and nanoplankton in the German Bight in relation to hydrographic structure and nutrient changes. ICES J. Mar. Sci. **55:** 600-609.

HIGMAN, W. A., D. M. STONE, and J. R. LEWIS. 2001. Sequence comparisons of toxic and non-toxic *Alexandrium tamarense* isolates from UK waters. Phycologia **40:** 256-262.

HOEPFFNER, N., and L. W. HAAS. 1990. Electron microscopy of nanoplankton from the North Pacific central gyre. J. Phycol. **26:** 421-439.

HOSOI-TANABE, S., and Y. SAKO. 2006. Development and application of fluorescence-*in-situ*-hybridization (FISH) method for simple and rapid identification of the toxic dinoflagellates *Alexandrium tamarense* and *Alexandrium catenella* in cultured and natural seawater. Fish. Sci. **72:** 77–82.

ISHIZAKA, J. and others 1997. Size and taxonomic plankton community structure and carbon flow at the equator, 175 °E during 1990–1994. Deep Sea Res. II **44:** 1927–1949.

JACOBSEN, N. and others 2002a. LNA-enhanced detection of single nucleotide polymorphisms in the apolipoprotein E. Nucleic Acids Res. **30:** e100.

---. 2002b. Genotyping of the apolipoprotein B R3500Q mutation using immobilized Locked Nucleic Acid capture probes. Clin. Chem. **48:** 657-660.

JACQUET, S., J.-F. LENNON, D. MARIE, and D. VAULOT. 1998. Picoplankton population dynamics in coastal waters of the northwestern Mediterranean Sea. Limnol. Oceangr. **43:** 1916-1931.

JANSON, S., and P. K. HAYES. 2006. Molecular taxonomy of Harmful Algae, p. 9-21. *In* E. Granéli and J. T. Turner [eds.], Ecology of Harmful Algae. Ecological Studies. Springer Verlag, Berlin, Heidelberg, Germany.

JOHN, U., A. CEMBELLA, C. HUMMERT, M. ELBRÄCHTER, R. GROBEN, and L. K. MEDLIN. 2003a. Discrimination of the toxigenic dinoflagellates *Alexandrium tamarense* and *A. ostenfeldii* in co-occurring natural populations from Scottish coastal waters. Eur. J. Phycol. **38:** 25-40.

JOHN, U., R. A. FENSOME, and L. K. MEDLIN. 2003b. The application of a molecular clock based on molecular sequences and the fossil record to explain biogeographic distributions within the *Alexandrium tamarense* 'species complex' (Dinophyceae). Mol. Biol. Evol. **20:** 1015-1027.

JOHN, U., L. K. MEDLIN, and R. GROBEN. 2005. Development of specific rRNA probes to distinguish between geographic clades of the *Alexandrium tamarense* species complex. J. Plankton Res. **27:** 199–204.

JOHNSON, P. W., and J. M. SIEBURTH. 1979. Chroococcoid cyanobacteria in the sea: a ubiquitous and diverse phototrophic biomass. Limnol. Oceangr. **24:** 928-935.

---. 1982. *In-situ* morphology and occurrence of eucaryotic phototrophs of bacterial size in the picoplankton of estuarine and oceanic waters. J. Phycol. **18:** 318-327.

JOHNSON, Z. I., E. R. ZINSER, A. COE, N. P. MCNULTY, E. M. S. WOODWARD, and S. W. CHISHOLM. 2006. Niche partitioning among *Prochlorococcus* ecotypes along ocean-scale environmental gradients. Science **311:** 1737-1740.

JOINT, I. R., N. J. P. OWEN, and A. J. POMROY. 1986. Seasonal production of photosynthetic picoplankton and nanoplankton in the Celtic Sea. Mar. Ecol. Prog. Ser. **28:** 251–258.

KAAS, H., J. LARSEN, F. MØHLENBERG, and K. RICHARDSON. 1991. The *Chrysochromulina polylepis* bloom in the Kattegat (Scandinavia) May-June 1988. Distribution, primary production and nutrient dynamics in the late stage of the bloom. Mar. Ecol. Prog. Ser. **79:** 151-161.

KAMIKAWA, R. and others 2007. Application of real-time PCR assay for detection and quantification of *Alexandrium tamarense* and *Alexandrium catenella* cysts from marine sediments. Harmful Algae **6:** 413-420.

KANAGAWA, T. 2003. Bias and artifacts in multitemplate polymerase chain reactions (PCR). J. Biosci. Bioeng. **96:** 317-323.

KAWACHI, M. and others 2002. The Pinguiophyceae classis nova, a new class of photosynthetic stramenopiles whose members produce large amounts of omega-3 fatty acids. Phycol. Res. **50:** 31-47.

KI, J.-S., and M.-S. HAN. 2006. A low-density oligonucleotide array study for parallel detection of harmful algal species using hybridization of consensus PCR products of LSU rDNA D2 domain. Biosens. Bioelectron. **21:** 1812-1821.

KONGSBAK, L. 2002. LNA: Fine-tuning of primers and probes. LNA **01:** 1.

KOSHKIN, A. A. and others 1998. LNA (Locked Nucleic Acids): Synthesis of the adenine, cytosine, guanine, 5-methylcytosine, thymine and uracil bicyclonucleoside monomers, oligomerisation, and unprecedented nucleic acid recognition. Tetrahedron **54:** 3607-3630.

LEHNER, A. and others 2005. Oligonucleotide microarray for identification of *Enterococcus* species. FEMS Microbiol. Lett. **246:** 133-142.

LEMIEUX, C., C. OTIS, and M. TURMEL. 2000. Ancestral chloroplast genome in *Mesostigma viride* reveals an early branch of green plant evolution. Nature **403:** 649-652.

LETELIER, R. M., R. R. BIDIGARE, D. V. HEBEL, M. E. ONDRUSEK, C. D. WINN, and D. M. KARL. 1993. Temporal variability of phytoplankton community structure at the U.S.-JGOFS time-series Station ALOHA (22°459N, 158°009W) based on HPLC pigment analysis. Limnol. Oceanogr. **38:** 1420-1437.

LI, W. K. W. 1994. Primary production of prochlorophytes, cyanobacteria, and eucaryotic ultraphytoplankton: Measurements from flow cytometric sorting. Limnol. Oceanogr. **39:** 169-175.

LI, W. K. W. and others 1983. Autotrophic picoplankton in the Tropical Ocean. Science **219:** 292-295.

LILLY, E. L., K. M. HALANYCH, and D. M. ANDERSON. 2005. Phylogeny, biogeography, and species boundaries within the *Alexandrium minutum* group. Harmful Algae **4:** 1004-1020.

LIM, E. L., M. R. DENNETT, and D. A. CARON. 1999. The ecology of *Paraphysomonas imperforata* based on studies employing oligonucleotide probe identification in coastal water samples and enrichment cultures. Limnol. Oceanogr. **44:** 37-51.

LITAKER, R. W., and P. A. TESTER. 2006. Molecular approaches to the study of phytoplankton life cycles: implications for Harmful Algal Bloom ecology, p. 299-309. *In* E. Granéli

and J. T. Turner [eds.], Ecology of Harmful Algae. Springer Verlag, Berlin, Heidelberg, Germany.

LOCKHART, D. J. and others 1996. Expression monitoring by hybridization to high-density oligonucleotide arrays. Nat. Biotechnol. **14:** 1675-1680.

LOCKHART, D. J., and E. A. WINZELER. 2000. Genomics, gene expression and DNA arrays. Nature **405:** 827-836.

LOY, A. and others 2002. Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Appl. Environ. Microbiol. **68:** 5064-5081.

---. 2005. 16S rRNA gene-based oligonucletide microarray for environmental monitoring of the betaproteobacterial order "Rhodocyclales". Appl. Environ. Microbiol. **71:** 1373-1386.

LUDWIG, W. and others 2004. ARB: a software environment for sequence data. Nucleic Acids Res. **32:** 1363-1371.

LUNDHOLM, N., and Ø. MOESTRUP. 2006. The biogeography of Harmful Algae, p. 23-35. *In* E. Granéli and J. T. Turner [eds.], Ecology of Harmful Algae. Springer Verlag, Berlin, Heidelberg, Germany.

MACKEY, D. J., J. BLANCHOT, H. W. HIGGINS, and J. NEVEUX. 2002. Phytoplankton abundances and community structure in the equatorial Pacific. Deep Sea Res. II **49:** 2561-2582.

MANTON, I., and M. PARKE. 1960. Further observations on small green flagellates with special reference to possible relatives of *Chromulina pusilla* Butcher. J. Mar. Biol. Assoc. UK **39:** 275-298.

MARIE, D., N. SIMON, and D. VAULOT. 2005. Phytoplankton cell counting by flow cytometry., p. 253-267. *In* R. A. Andersen [ed.], Algal Culturing Techniques: A Book for All Phycologists. Elsevier Academic Press, London, UK.

MARIE, D., F. ZHU, V. BALAGUE, J. RAS, and D. VAULOT. 2006. Eukaryotic picoplankton communities of the Mediterranean Sea in summer assessed by molecular approaches (DGGE, TTGE, QPCR). FEMS Microbiol. Ecol. **55:** 403-415.

MASÓ, M., and E. GARCÉS. 2006. Harmful microalgae blooms (HAB); problematic and conditions that induce them. Mar. Pollut. Bull. **53:** 620-630.

MASSANA, R., V. BALAGUE, L. GUILLOU, and C. PEDRÓS-ALIÓ. 2004. Picoeukaryotic diversity in an oligotrophic coastal site studied by molecular and culturing approaches. FEMS Microbiol. Ecol. **50:** 231-243.

MASSANA, R., L. GUILLOU, B. DÍEZ, and C. PEDRÓS-ALIÓ. 2002. Unveiling the organisms behind novel eukaryotic ribosomal DNA sequences from the ocean. Appl. Environ. Microbiol. **68:** 4554-4558.

MEDLIN, L. K., H. J. ELWOOD, S. STICKEL, and M. L. SOGIN. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene **71:** 491-499.

MEDLIN, L. K., and I. KACZMARSKA. 2004. Evolution of the diatoms: V. Morphological and cytological support for the major clades and a taxonomic revision. Phycologia **43:** 245-270.

MEDLIN, L. K., M. LANGE, and U. WELLBROCK. 1998. Sequences comparison links to Europena isolates of *Alexandrium tamarense* from the Orkney Islandsto toxic North American stocks. Eur. J. Protistol. **34:** 329-335.

MEDLIN, L. K., K. METFIES, H. MEHL, K. H. WILTSHIRE, and K. VALENTIN. 2006. Picoeukaryotic plankton diversity at the Helgoland Time Series Site as assessed by three molecular methods. Microbial Ecol. **52:** 53-71.

MEDLIN, L. K., and N. SIMON. 1998. Phylogenetic analysis of marine phytoplankton, p. 161-186. *In* K. E. Cooksey [ed.], Molecular approaches to the study of the ocean. Chapman & Hall, London, UK.

MELKONIAN, M. 1990. Prasinophyceae., p. 600–607. *In* L. Margulis, J. O. Corliss, M. Melkonian and D. J. Chapman [eds.], Handbook of Protoctista. Jones and Bartlett Publishers, Boston, USA.

METFIES, K., S. HULJIC, M. LANGE, and L. K. MEDLIN. 2005. Electrochemical detection of the toxic dinoflagellate *Alexandrium ostenfeldii* with a DNA-biosensor. Biosens. Bioelectron. **20:** 1349-1357.

METFIES, K., and L. K. MEDLIN. 2004. DNA microchips for phytoplankton: The fluorescent wave of the future. Nova Hedwigia **79:** 321-327.

METFIES, K., K. TÖBE, C. A. SCHOLIN, and L. K. MEDLIN. 2006. Laboratory and field applications of ribosomal RNA probes to aid the detection and monitoring of Harmful Algae, p. 311-325. *In* E. Granéli and J. T. Turner [eds.], Ecology of Harmful Algae. Springer Verlag, Berlin, Heidelberg, Germany.

MOESTRUP, Ø. 1991. Further studies of presumedly primitive green algae, including the description of Pedinophyceae class. nov. and *Resultor* gen. nov. J. Phycol. **27:** 119-133.

---. 2004. IOC Taxonomic Reference list of Toxic Algae, Intergovernmental Oceanographic Commission of UNESCO.

MOESTRUP, Ø., and J. THRONDSEN. 1988. Light and electron microscopical studies on *Pseudoscourfieldia marina*, a primitive scaly green flagellate (Prasionphyceae) with posterior flagella. Can.J. Bot. **66:** 1415-1434.

MOON-VAN DER STAAY, S. Y., R. DE WACHTER, and D. VAULOT. 2001. Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. Nature **409:** 607-610.

MOON-VAN DER STAAY, S.-Y., G. W. M. VAN DER STAAY, L. GUILLOU, and D. VAULOT. 2000. Abundance and diversity of prymnesiophytes in the picoplankton community from the equatorial Pacific Ocean inferred from 18S rDNA sequences. Limnol. Oceanogr. **45:** 98-109.

MORRIS, I. 1980. The physiological ecology of phytoplankton. Blackwell Scientific Publications, Oxford, UK.

MULLIS, K. B., and F. FALOONA. 1987. Specific synthesis of DNA in vitro via polymerase-catalyzed chain reaction. Meth. Enzymol. **155:** 336.

MULLIS, K. B., F. FALOONA, S. SCHARF, R. SAIKI, G. HORN, and H. EHRLICH. 1986. Specific enzymatic amplification of DNA in vitro: The polymerase chain reaction. Cold spring Harbor symposia on quantitative biology **51:** 263.

MURPHY, L. S., and E. M. HAUGEN. 1985. The distribution and abundance of phototrophic ultraplankton in the North Atlantic. Limnol. Oceanogr. **30:** 47–58.

MUYZER, G., E. C. DE WAAL, and A. G. UITTERLINDEN. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:** 695-700.

NASCIMENTO, S. M., E. L. LILLY, J. LARSEN, and S. MORRIS. 2005. Toxin profile, pigment composition, and large subunit rDNA phylogenetic analysis of an *Alexandrium minutum* (Dinophyceae) strain isolated from the Fleet Lagoon, United Kingdom. J. Phycol. **41:** 343–353.

NIELSEN, P. S., and S. KAUPPINEN. 2002. The use of LNA oligonucleotide microarrays provides superior sensitivity and specificity in expression profiling. LNA **17:** 1-3.

NOT, F., M. LATASA, D. MARIE, T. CARIOU, D. VAULOT, and N. SIMON. 2004. A single species, *Micromonas pusilla* (Prasinophyceae), dominates the eukaryotic picoplankton in the western English Channel. Appl. Environ. Microbiol. **70:** 4064-4072.

NOT, F., N. SIMON, I. C. BIEGALA, and D. VAULOT. 2002. Application of fluorescent-*in-situ*-hybridization coupled with tyramide signal amplification (FISH-TSA) to assess eukaryotic picoplankton composition. Aquat. Microb. Ecol. **28:** 57-166.

NOT, F. and others 2007. Picobiliphytes: A marine picoplanktonic algal group with unknown affinities to other eukaryotes. Science **315:** 253-255.

O'KELLY, C. J., M. E. SIERACKI, E. C. THIER, and I. C. HOBSON. 2003. A transient bloom of *Ostreococcus* (Chlorophyta, Prasinophyceae) in West Neck Bay, Long Island, New York. J. Phycol. **39:** 850-854.

OLSON, R. J., E. R. ZETTLER, and K. O. ANDERSON. 1989. Discrimination of eukaryotic phytoplankton cell types from light scatter and autofluorescence properties measured by flow cytometry. Cytometry **10:** 636–643.

PARTENSKY, F., D. VAULOT, A. COUTÉ, and A. SOURNIA. 1988. Morphological and nuclear analysis of the bloom forming dinoflagellates *Gyrodinium* cf *aureolum* and *Gymnodinium nagasakiense* (Dinophyceae). J. Phycol. **24:** 408-415.

PAULSEN, O. 1904. Plankton-investigations in the waters round Iceland in 1903., p. 1-40, Meddelelser fra Kommission for Havundersoegelser Koebenhaven Serie Plankton.

PEPLIES, J., F. O. GLÖCKNER, R. AMANN, and W. LUDWIG. 2004a. Comparative sequence analysis and oligonucleotide probe design based on 23S rRNA genes of Alphaproteobacteria from North Sea bacterioplankton. Syst. Appl. Microbiol. **27:** 573-580.

PEPLIES, J., C. LACHMUND, F. O. GLÖCKNER, and W. MANZ. 2006. A DNA microarray platform based on direct detection of rRNA for characterization of freshwater sediment-related prokaryotic communities. Appl. Environ. Microbiol. **72:** 4829-4838.

PEPLIES, J., S. C. K. LAU, J. PERNTHALER, R. AMANN, and F. O. GLÖCKNER. 2004b. Application and validation of DNA microarrays for the 16S rRNA-based analysis of marine bacterioplankton. Environ. Microbiol. **6:** 638-645.

PIENAAR, R. N., and S. D. SYM. 2002. The genus *Pyramimonas* (Prasinophyceae) from southern African inshore waters. South African Journal of Botany **68:** 283–298.

POLZ, M. F., and C. M. CAVANAUGH. 1998. Bias in template-to-product ratios in multitemplate PCR. Appl. Environ. Microbiol. **64:** 3724-3730.

POTTER, D., T. C. LAJEUNESSE, G. W. SAUNDERS, and R. A. ANDERSON. 1997. Convergent evolution masks extensive biodiversity among marine coccoid picoplankton. Biodivers. Conserv. **6:** 99-107.

PRESCOTT, G. W. 1968. The Algae : A Review. Houghton Mifflin Company, Boston, USA.

RAVEN, J. A. 1998. Small is beautiful: the picophytoplankton. The Twelfth Tansley Lecture. Funct. Ecol. **12:** 503–513.

RAVEN, J. A., and J. E. KÜBLER. 2002. New light on the scaling of metabolic rate with the size of algae. J. Phycol. **38:** 11-16.

RAYMONT, J. E. G. 1963. Plankton and productivity in the oceans. Pergamon Press, Oxford, UK.

REID, P. C., C. LANCELOT, W. W. C. GIESKES, E. HAGMEIER, and G. WEICHART. 1990. Phytoplankton of the North Sea and its dynamics: A review. Neth. J. Sea Res. **26:** 295-331.

REYSENBACH, A.-L., L. J. GIVER, G. S. WICKHAM, and N. R. PACE. 1992. Differential amplification of rRNA genes by polymerase chain reaction. Appl. Environ. Microbiol. **58:** 3417-3418.

RICE, J., M. SLEIGH, P. BURKILL, G. TARRAN, C. O'CONNOR, and M. ZUBKOV. 1997. Flow cytometric analysis of characteristics of hybridization of species-specific fluorescent oligonucleotide probes to rRNA of marine nanoflagellates. Appl. Environ. Microbiol. **63:** 938-944.

RODRIGUEZ, F., E. DERELLE, L. GUILLOU, F. LE GALL, D. VAULOT, and H. MOREAU. 2005. Ecotype diversity in the marine picoeukaryote *Ostreococcus* (Chlorophyta, Prasinophyceae). Environ. Microbiol. **7:** 853-859.

ROMARI, K., and D. VAULOT. 2004. Composition and temporal variability of picoeukaryote communities at a coastal site of the English Channel from 18S rDNA sequences. Limnol. Oceanogr. **49:** 784-798.

ROUND, F. E. 1981. The ecology of algae. University Press, Cambridge, UK.

SÁEZ, A. G., I. PROBERT, M. GEISEN, P. QUINN, J. R. YOUNG, and L. K. MEDLIN. 2003. Pseudo-cryptic speciation in coccolithophores. Proc. Natl. Acad. Sci. USA **100:** 7163-7168.

SAHLSTEN, E., and B. KARLSON. 1998. Vertical distribution of virus-like particles (VLP) and viruses infecting *Micromonas pusilla* during late summer in the southeastern Skagerrak, North Atlantic. J. Plankton Res. **20:** 2207-2212.

SCANLAN, D. J. 2003. Physiological diversity and niche adaptation in marine *Synechococcus*. Adv. Microb. Physiol. **47:** 1–64.

SCHENA, M., R. A. HELLER, T. P. THERIAULT, K. KONRAD, E. LACHENMAIER, and R. W. DAVIS. 1998. Microarrays: biotechnology's discovery platform for functional genomics. Trends Biotechnol. **16:** 301-306.

SCHENA, M., D. SHALON, R. W. DAVIS, and P. O. BROWN. 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science **270:** 467-470.

SCHENA, M., D. SHALON, R. HELLER, A. CHAI, P. O. BROWN, and R. W. DAVIS. 1996. Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. Proc. Natl. Acad. Sci. USA **93:** 10614-10619.

SCHLÜTER, L., and F. MØHLENBERG. 2003. Detecting presence of phytoplankton groups with non-specific pigment signatures. J. Appl. Phycol. **15:** 465-476.

SCHOLIN, C. A. 1998a. Development of nucleic acid probe-based diagnostics for identifying and enumerating Harmful Algae Bloom species. *In* D. M. Anderson, A. D. Cembella and G. M. Hallegraeff [eds.], Physiological Ecology of Harmful Algae Blooms. Springer Verlag, Berlin, Heidelberg, Germany.

---. 1998b. Morphological, genetic, and biogeographic relationships of the toxic dinoflagellates *Alexandrium tamarense*, *A. catenella* and *A. fundyense*, p. 13-27. *In* D. M. Anderson, A. D. Cembella and G. M. Hallegraeff [eds.], Physiological Ecology of Harmful Algae Blooms. Springer Verlag, Berlin, Heidelberg, Germany.

SCHOLIN, C. A., G. M. HALLEGRAEFF, and D. M. ANDERSON. 1995. Molecular evolution of the *Alexandrium tamarense* 'species complex' (Dinophyceae): Dispersal in the North American and West Pacific regions. Phycologia **34:** 472-485.

SCHOLIN, C. A., M. HERZOG, M. SOGIN, and D. M. ANDERSON. 1994. Identification of group- and strain-specific genetic markers for the globally distributed *Alexandrium* (Dinophyceae). II. Sequence analysis of a fragment of the LSU rRNA gene. J. Phycol. **30:** 999-1011.

SCHOLIN, C. A., E. G. VRIELING, L. PEPERZAK, L. L. RHODES, and P. RUBLEE. 2003. Detection of HAB species using lectin, antibody and DNA probes, p. 131-163. *In* G. M. Hallegraeff, D. M. Anderson and A. D. Cembella [eds.], Manual on Harmful Marine Microalgae. United Nations Educational, Scientific and Cultural Organization, Paris, France.

SHAPIRO, L. P., and L. CAMPBELL. 1998. Immunofluorescence approaches in the study of phytoplankton, p. 247-258. *In* K. E. Cooksey [ed.], Molecular approaches to the study of the ocean. Chapman & Hall, London, UK.

SHERR, E. B., and B. F. SHERR. 1991. Planktonic microbes: Tiny cells at the base of the ocean's food webs. Trends Ecol. Evol. **6:** 50-54.

SIEBURTH, J. M., M. D. KELLER, P. W. JOHNSON, and S. M. MYKLESTAD. 1999. Widespread occurrence of the oceanic ultraplankter, *Prasinococcus capsulatus* (Prasinophyceae), the diagnostic "Golgi-decapore complex" and the newly described polysaccharide "capsulan.". J. Phycol. **35:** 1032-1043.

SILAHTAROGLU, A. N., H. M. PFUNDHELLER, A. A. KOSHKIN, N. TOMMERUP, and S. KAUPPINEN. 2004. LNA-modified oligonucleotides are highly efficient as FISH probes. Cytogen. Gen. Res. **107:** 32-37.

SILAHTAROGLU, A. N., N. TOMMERUP, and H. VISSING. 2003. FISHing with locked nucleic acids (LNA): evaluation of different LNA/DNA mixmers. Mol. Cell. Probe **17:** 165-169.

SIMON, N. and others 2000. Oligonucleotide probes for the identification of three algal groups by dot-blot and fluorescent whole-cell hybridization. J. Eukaryot. Microbiol. **47:** 76–84.

SIMON, N., N. LEBOT, D. MARIE, F. PARTENSKY, and D. VAULOT. 1995. Fluorescent-*in-situ*-hybridization with rRNA-targeted oligonucleotide probes to identify small phytoplankton by flow cytometry. Appl. Environ. Microbiol. **61:** 2506-2513.

SINGH, S. K., A. A. KOSHKIN, J. WENGEL, and P. NIELSEN. 1998. LNA (locked nucleic acids): synthesis and high-affinity nucleic acid recognition. Chem. Commun.**:** 455 - 456.

SLAPETA, JAN, P. LÓPEZ-GARCÍA, and D. MOREIRA. 2006. Global dispersal and ancient cryptic species in the smallest marine eukaryotes. Mol. Biol. Evol. **23:** 23-29.

SOMMER, H., and K. F. MEYER. 1937. Paralytic shellfish poisining. Arch. Pathol. **24:** 560-598.

SOMMER, H., W. H. WHEDON, C. A. KOFOID, and A. STOHLER. 1937. Relation of paralytic shellfish poisoning to plankton organisms of the Genus *Gonyaulax*. Arch. Pathol. **24:** 537-559.

SOURNIA, A. 1978. Phytoplankton manual. UNESCO, Paris, France.

SOURNIA, A., M.-J. CHRÉTIENNOT-DINET, and M. RICARD. 1991. Marine pyhtoplankton: how many species in the world ocean? J. Plankton Res. **13:** 1093-1099.

SOUTHERN, E., K. MIR, and M. SHCHEPINOV. 1999. Molecular interactions on microarrays. Nat. Genet. **21:** (1 Suppl):5-9.

STEELE, J. H. 1974. The structure of marine ecosystems. Harvard University Press, Cambridge, USA.

STEIDINGER, K. A., and E. GARCÉS. 2006. Importance of life cycles in the ecology of harmful microalgae, p. 37-49. *In* E. Granéli and J. T. Turner [eds.], Ecology of Harmful Algae. Ecological Studies. Springer Verlag, Berlin, Heidelberg, Germany.

STEINKÖTTER, J., D. BHATTACHARYA, I. SEMMELROTH, C. BIBEAU, and M. MELKONIAN. 1994. Prasinophytes form independent lineages within the Chlorophyta: Evidence from ribosomal RNA sequence comparisons. J. Phycol. **30:** 340-346.

SYM, S. D., and R. N. PIENAAR. 1993. The class Prasinophyceae. Prog. Phycol. Res. **9:** 281–376.

TAYLOR, F. J. R., and Y. FUKUYO. 1998. The neurotoxigenic dinoflagellate genus *Alexandrium* Halim: General Introduction, p. 381-404. *In* D. M. Anderson, A. D. Cembella and G. M. Hallegraeff [eds.], Physiological Ecology of Harmful Algae Blooms. Springer Verlag, Berlin, Heidelberg, Germany.

TAYLOR, F. J. R., Y. FUKUYO, and J. LARSEN. 1995. Taxonomy of harmful dinoflagellates, p. 283-317. *In* G. M. Hallegraeff, D. M. Anderson and A. D. Cembella [eds.], Manual on Harmful Marine Microalgae. UNESCO, Paris, France.

TENGS, T., H. A. BOWERS, A. P. ZIMAN, D. K. STOECKER, and D. W. OLDACH. 2001. Genetic polymorphism in *Gymnodinium galatheanum* chloroplast DNA sequences and development of a molecular detection assay. Mol. Ecol. **10:** 515-523.

THOMSEN, H. A., and K. R. BUCK. 1998. Nanoflagellates of the central California waters: taxonomy, biogeography and abundance of primitive, green flagellates (Pedinophyceae, Prasinophyceae). Deep Sea Res. II **45:** 1687-1707.

THRONDSEN, J. 1976. Occurrence and productivity of small marine flagellates. Nor. J. Bot. **23:** 269-293.

THRONDSEN, J., and A. ZINGONE. 1994. Micromonads of the Mediterranean Sea. G. Bot. Ital. **128:** 1031–1044.

THURMAN, H. V. 1997. Introductory Oceanography. Prentice Hall, Upper Saddle River, New Jersey, USA.

TÖBE, K., G. ELLER, and L. K. MEDLIN. 2006. Automated detection and enumeration for toxic algae by solid-phase cytometry and the introduction of a new probe for *Prymnesium parvum* (Haptophyta: Prymnesiophyceae). J. Plankton Res. **28:** 643-657.

TURMEL, M. and others 1999. The complete mitochondrial DNA sequences of *Nephroselmis olivacea* and *Pedinomonas minor* : two radically different evolutionary patterns within green algae. Plant Cell **11:** 1717-1730.

UGOZZOLI, L. A., D. LATORRA, R. PUCKET, K. ARAR, and K. HAMBY. 2004. Real-time genotyping with oligonucleotide probes containing Locked Nucleic Acids. Anal. Biochem. **324:** 143-152.

VALENTIN, K., H. MEHL, and L. K. MEDLIN. 2005. Picoplankton culture assessment using single strand conformation polymorphism and partial 18S sequencing. J. Plankton Res. **27:** 1149-1154.

VAN DEN HOEK, C., D. G. MANN, and H. M. JAHNS. 1995. Algae: an introduction to phycology. Cambridge University Press, New York, USA.

VAN HANNEN, E. J., M. P. VAN AGTERVELD, H. J. GONS, and H. J. LAANBROEK. 1998. Revealing genetic diversity of eukaryotic microorganisms in aquatic environments by denaturing gradient gel electrophoresis. J. Phycol. **34:** 206-213.

VANDERHOEVEN, J., K. PAPPAERT, B. DUTTA, P. VANHUMMELEN, and G. DESMET. 2005. DNA microarray enhancement using a continuously and discontinuously rotating microchamber. Anal. Chem. **77:** 4474-4480.

VAQUER, A., M. TROUSSELLIER, C. COURTIES, and B. BIBENT. 1996. Standing stock and dynamics of picophytoplankton in the Thau Lagoon (northwest Mediterranean coast). Limnol. Oceanogr. **41:** 1821-1828.

VAULOT, D. 2001. Phytoplankton. Encyclopedia of Life Sciences. Nature Publishing Group, London, UK.

VELDHUIS, M. J. W., and G. W. KRAAY. 1990. Vertical distribution and pigment composition of a picoplanktonic prochlorophyte in the subtropical North Atlantic: A combined study of HPLC-analysis of pigments and flow cytometry. Mar. Ecol. Prog. Ser. **68:** 121-127.

---. 2000. Application of flow cytometry in marine phytoplankton research: current applications and future perspectives. *In* M. Reckermann and F. Colijin [eds.], Aquatic flow cytometry: achievements and prospects. Sci. Mar. **64**:121-134.

VESTER, B., and L. WENGEL. 2004. LNA (Locked Nucleic Acid): high-affinity targeting of complementary RNA and DNA. Biochemistry USA **43:** 13233-13241.

VILA, M. and others 2005. A comparative study on recurrent blooms of *Alexandrium minutum* in two Mediterranean coastal areas. Harmful Algae **4:** 673-695.

VOLKMAN, J. K., S. M. BARRETT, G. A. DUNSTAN, and S. W. JEFFREY. 1994. Sterol biomarkers for microalgae from the green algal class Prasinophyceae. Org.Geochem. **21:** 1211-1218.

VRIELING, E. G., and D. M. ANDERSON. 1996. Immunofluorescence in phytoplankton research: applications and potential. J. Phycol. **32:** 1-16.

WATERBURY, J. B., S. W. WATSON, R. R. L. GUILLARD, and L. E. BRAND. 1979. Widespread occurrence of a unicellular, marine, planktonic, cyanobacterium. Nature **277:** 293-294.

WILTSHIRE, K. H. 2004. Editorial. Helgol. Mar. Res. **58:** 221-222.

WILTSHIRE, K. H., and C.-D. DÜRSELEN. 2004. Revision and quality analyses of the Helgoland Reede long-term phytoplankton data archive. Helgol. Mar. Res. **58:** 252-268.

WILTSHIRE, K. H., and B. F. J. MANLY. 2004. The warming trend at Helgoland Roads, North Sea: phytoplankton response. Helgol. Mar. Res. **58:** 269-273.

WOESE, C. 1987. Bacterial evolution. Microbiol. Rev. **51:** 221-271.

WORDEN, A. Z. 2006. Picoeukaryote diversity in coastal waters of the Pacific Ocean. Aquat. Microb. Ecol. **43:** 165-175.

WORDEN, A. Z., J. K. NOLAN, and B. PALENIK. 2004. Assessing the dynamics and ecology of marine picophytoplankton: The importance of the eukaryotic component. Limnol. Oceanogr. **49:** 168-179.

WORDEN, A. Z., and B. PALENIK. 2002. Picoeukaryote abundance, diversity, growth and grazing mortality at a California Current monitoring site, Eos Trans. AGU 83(4), Ocean Sciences Meeting Abstract, OS42A–95.

YE, R. W., T. WANG, L. BEDZYK, and K. M. CROKER. 2001. Applications of DNA microarrays in microbial systems. J. Microbiol. Meth. **47:** 257-272.

ZEHR, J. P., and W. D. HIORNS. 1998. Molecular approaches to studies of the activities of marine organisms, p. 91-111. *In* K. E. Cooksey [ed.], Molecular approaches to the study of the ocean. Chapman & Hall, London, UK.

ZHU, F., R. MASSANA, F. NOT, D. MARIE, and D. VAULOT. 2005. Mapping of picoeucaryotes in marine ecosystems with quantitative PCR of the 18S rRNA gene. FEMS Microbiol. Ecol. **52:** 79-92.

ZINGONE, A., M. BORRA, C. BRUNET, G. FORLANI, W. H. C. F. KOOISTRA, and G. PROCACCINI. 2002. Phylogenetic position of *Crustomastix stigmatica* sp. nov. and D*olichomastix tenuilepis* in relation to the Mamiellales (Prasinophyceae, Chlorophyta). J. Phycol. **38:** 1024-1039.

ZINGONE, A., and H. O. ENEVOLDSEN. 2000. The diversity of harmful algal blooms: a challenge for science and management. Ocean Coast Manag. **43:** 725-748.

ZINGONE, A., F. NATALE, E. BIFFALI, M. BORRA, G. FORLANI, and D. SARNO. 2006. Diversity in morphology, infectivity, molecular characteristics and induced host resistance between two viruses infecting *Micromonas pusilla*. Aquat. Microb. Ecol. **45:** 1-14.

ZINGONE, A., D. SARNO, and G. FORLANI. 1999. Seasonal dynamics in the abundance of *Micromonas pusilla* (Prasinophyceae) and its viruses in the Gulf of Naples (Mediterranean Sea). J. Plankton Res. **21:** 2143-2159.

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