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Analysis of phytoplankton distribution and community structure in the German Bight with respect to the different size classes

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Abstract

Investigation of phytoplankton biodiversity, ecology, and biogeography is crucial for understanding marine ecosystems. Research is often carried out on the basis of microscopic observations, but due to the limitations of this approach regarding detection and identification of picophytoplankton (0.2-2 µm) and nanophytoplankton (2-20 µm), these investigations are mainly focused on the microphytoplankton (20-200 µm). In the last decades, various methods based on optical and molecular biological approaches have evolved which enable a more rapid and convenient analysis of phytoplankton samples and a more detailed assessment of small phytoplankton. In this study, a selection of these methods (*in situ* fluorescence, flow cytometry, genetic fingerprinting, and DNA microarray) was placed in complement to light microscopy and HPLC-based pigment analysis to investigate both biomass

distribution and community structure of phytoplankton. As far as possible, the size classes were analyzed separately. Investigations were carried out on six cruises in the German Bight in 2010 and 2011 to analyze both spatial and seasonal variability. Microphytoplankton was identified as the major contributor to biomass in all seasons, followed by the nanophytoplankton. Generally, biomass distribution was patchy, but the overall contribution of small phytoplankton was higher in offshore areas and also in areas exhibiting higher turbidity. Regarding temporal development of the community, differences between the small phytoplankton community and the microphytoplankton were found. The latter exhibited a seasonal pattern regarding number of taxa present, alpha- and beta- diversity, and community structure, while for the nano- and especially the picophytoplankton, a general shift in the community between both years was observable without seasonality. Although the reason for this shift remains unclear, the results imply a different response of large and small phytoplankton to environmental influences.

Keywords: phytoplankton, biodiversity, North Sea, flow cytometry, molecular sensing, microscopy

1. Introduction

Microalgae are the main primary producers in marine ecosystems and constitute the basis of the marine food web. Although representing less than 1 % of global biomass, they are responsible for roughly 50 % of global carbon fixation and are therefore a crucial factor in the carbon cycle (Field et al. 1998). Coasts and shelf seas in particular are highly productive areas fostered by their comparably low water depth and higher nutrient input by upwelling or adjacent rivers. Due to this productivity and other benefits, 40 % of the world´s population lives within 100 km distance from the

coast (IOC/UNESCO 2011), putting pressure on the ecosystem because of e.g. increased pollution and eutrophication. These stressors as well as climate change effects will probably have an influence on the phytoplankton and changes within its community are likely to propagate also to higher trophic levels. In order to get track of potential changes and to relate them eventually to observations made on other parts of the marine ecosystem, it is important to investigate phytoplankton development comprehensively both in terms of spatiotemporal distribution and biodiversity. Information on biodiversity is of special importance, since its loss can potentially further reduce the ability of the ecosystem to cope with environmental changes or human induced stress (Yachi and Loreau 1999).

The North Sea is an example for a highly utilized coastal area (Ducrotoy et al. 2000), and a lot of knowledge has been collected about the structure and variability of its phytoplankton community, either due to long lasting time series or due to occasional research cruises (Reid et al. 1990; Tillmann and Rick 2003; Wiltshire et al. 2010). Information is available regarding seasonal succession patterns (Hagmeier and Bauerfeind 1990; Reid et al. 1990), response to environmental factors (Gillbricht 1988; Hickel 1998; Freund et al. 2012; Schlüter et al. 2012), and biodiversity (Hoppenrath 2004; Wiltshire and Dürselen 2004; Hoppenrath et al. 2007). However, since most of this information is based on microscopic observation, it covers mainly the microphytoplankton (20-200 µm). The taxonomical resolution of the information available for smaller phytoplankton is rare (Knefelkamp 2009), because it is more difficult to count microscopically and often lacks morphological features for a reliable identification. Approaches based on electron microscopy (e.g. Novarino et al. 1997) require too much effort to be used on a larger scale and have often problems similar to light microscopy regarding species identification. However, for a thorough understanding of phytoplankton ecology, information on nanophytoplankton (2-20

µm) and picophytoplankton (0.2-2 µm) of comparable quality to the information available for microphytoplankton would be advantageous.

In the present study, the spatial and temporal variability of phytoplankton community structure and biomass was assessed including all three phytoplankton size classes as far as possible. A set of various complementary methods was used, since the whole community is hardly accessible by one method alone (Peperzak 2010, Stehouwer 2013). This included light microscopy, HPLC-based phytoplankton pigment determination, *in situ* chlorophyll-*a* fluorescence measurements, flow cytometry, molecular fingerprinting, and DNA microarray analyses (Table 1).

With this suite of methods, in the North Sea almost the whole German Bight was extensively sampled over several seasons for two years (2010 and 2011). This allowed the investigation of the phytoplankton community with respect to seasonal, but also spatial differences. Thus, the provided data might be a valuable addition to the existing datasets which are mostly obtained in smaller areas or even on single spots.

2. Materials and methods

2.1. Study area and sampling

Data were obtained on six cruises conducted with the research vessel “Heincke” during 2010 (May, July, September) and 2011 (April, June, September) in the German Bight (North Sea). Both transect as well as station measurements were performed. Due to weather conditions, the order of stations was not always the same and in September, the most offshore stations could not be sampled. At each cruise, “extra” stations were integrated along the transect lines between the regular stations, but their frequency and position varied between the cruises. Continuous

measurements were carried out during the whole cruise duration at a depth of approx. 4 m. On stations, water samples for laboratory analyses were taken from a comparable depth using a sampling rosette (SBE 32, Sea-Bird Electronic, Inc.) equipped with seven 9 L “Niskin” bottles. The samples were carefully mixed and aliquots were processed for the methods described below.

2.2 Discrete measurements of phytoplankton pigments and total suspended matter

Phytoplankton pigment concentration was measured by High Performance Liquid Chromatography (HPLC) after the method of Zapata et al. (2000). Water samples (1-5 L) were filtered through pre-combusted GF/F filters (Whatman, USA, Ø 47 mm). Afterwards, the filters were shock-frozen in liquid nitrogen and stored at -80 °C. In the laboratory, pigments were extracted from the filters by incubation with 100 % acetone for 24 h at -30 °C. The extracts were transferred into 2 mL glass vials and simultaneously cleaned from particles by passing them through 0.2 µm syringe filters (regenerated cellulose, Spartan, A13). Separation and analysis of chlorophyll-*a* (chl-*a*) and group-specific marker pigments was carried out by a HPLC system from JASCO (Japan). Contribution of diatoms, dinophytes, cryptophytes, prymnesiophytes, and prasinophytes to total chl-*a* was estimated using the CHEMTAX software (Mackey et al. 1996) with initial pigment ratios derived from Schlüter et al. (2000). See SUP. 1 for details.

Concentration of total suspended matter (TSM) was determined by filtration of 1-8 L of the water sample through pre-combusted, pre-washed and pre-weighted GF/F filters (Whatman, USA, Ø 47 mm). Previous to usage, the filters were wet with purified water to avoid saturation with sea water and to reduce the amount of salt that cannot be washed out of the filter after filtration. To correct for still remaining salt at

each cruise filtered seawater was also applied to empty filters. Their average salt induced weight increase was then subtracted from all samples of the particular cruise before calculating total suspended matter concentration; see Stavn et al. (2009) for details. Additionally, manual water turbidity measurements were conducted at the stations using a Hach 2100P ISO turbidimeter (Hach, USA).

2.3 Continuous measurements of abiotic parameters and chl-*a*

Continuous measurements (at 1 min intervals) of temperature, salinity, chromophoric dissolved organic matter (CDOM), turbidity, and chlorophyll-*a* fluorescence were performed using a FerryBox system as described in Petersen et al. (2011) installed aboard the ship. The sensors mounted in the FerryBox are listed in SUP. 2. TSM and chl-*a* concentrations were calculated on the basis of continuous turbidity and chl-*a* fluorescence measurements using the coefficients given in table 2. They are the result of linear regressions between the discrete TSM and chl-*a* measurements obtained at the stations and values for the corresponding optical proxy extracted from the respective continuous data set.

Concentrations of nitrate and phosphate were measured approx. every 50 min using a Systea µMac nutrient analyzer (Systea, Italy) attached to a bypass of the FerryBox. Sample water for the nutrient analyzer was filtered by a cross-flow filter (MiniKros, pore size 0.2 µm, Spectrum Laboratories, USA) previously to analysis. In between the measurements, values for the whole time of the respective cruise were interpolated. For a correction of the field measurements, discrete water samples were taken behind the cross-flow filter, stored at -20 °C, and analyzed in the laboratory for nitrate and phosphate using an AutoAnalyzer 3 (Bran+Luebbe, Germany) and the methods from Grasshoff et al. (1983).

2.4 Microscopic cell counts

At each station, 100 mL of seawater were filled into brown glass bottles, fixed with 0.5 mL Lugol´s solution, and stored at 4-8 °C until analysis (Utermöhl 1958). Normally, 50 mL of sample were allowed to settle for 24h, but in case of increased amounts of particulate matter, only 25 mL of sample were analyzed. Microphytoplankton cells (>20 µm in largest dimension) were counted and identified to species or genus, but at least to class level using an inverted microscope (Olympus IX 51, Olympus, Japan), phase contrast and 100x or 200x magnification. No regular replicate counting of samples was performed, since in most cases a single count can be considered sufficient (Lund et al. 1958). Random re-counts of single species in different samples showed that the counting error was in average 11 % (with the highest value 22 %) in the present study. Biovolume of autotrophic cells was calculated using the mean values of cell dimensions recorded from –if possible– at least 25 individuals per taxon and the equations given in Hillebrandt et al. (1999). After correction for the effect of fixation (Montagnes et al. 1994), it was converted into carbon using the appropriate equations given in Menden-Deuer & Lessard (2000). In cases where the dimensions of a certain taxon could not be measured for a particular cruise, the average cell dimensions of this taxon from the other cruises were used instead for calculations purposes. The error introduced thereby was considered to be smaller than the error introduced by a complete omission of the particular taxon from the cruise. Diversity of the samples was estimated by species accumulation curves and by calculation of the ‘Simpson Index’ (Magurran 2004).

2.5 Flow cytometry

For flow cytometry analyses, 3 mL samples of seawater were fixed with glutaraldehyde (0.4 % final concentration), incubated for 15 min, shock-frozen in liquid nitrogen, and stored at -20 °C. Sample analysis was carried out using a FACSCalibur (BD Biosciences, USA) or an Accuri C6 Flow Cytometer (BD, Biosciences, USA). Autofluorescence of phytoplankton was excited by blue light (488 nm) emitted by a 20 mW-laser. Isolation of eukaryotic nano- and picophytoplankton was performed manually by visual inspection of 2D-density plots (orange vs. red emission and green emission vs. sidescatter, respectively). For intercalibration between samples, yellow-green fluorescent latex beads (0.94 µm diameter, Polysciences, USA) were used and served also as reference for the normalization of cellular optical properties. In case of the FACSCalibur, TruCount beads (Becton Dickinson, USA) were used for absolute sample volume calibration. Parameters obtained for both phytoplankton fractions were cell counts, average cell size (based on side scatter) as well as red and orange fluorescence intensity. Estimations of biovolume values the members of the nano- and picophytoplankton size classes were made using the mean diameter of the respective size class for the particular station under the assumption of a spherical shape of the cells. Carbon calculation was performed as described for microphytoplankton (see 2.4) using the equation for the non-diatom phytoplankton.

2.6 Molecular biological analyses

Samples for genetic analyses of the phytoplankton community were obtained by filtration of 400-1500 mL seawater onto 0.2 µm Isopore GTTP membrane filters (Millipore, Germany). Subsequently, filters were shock-frozen in liquid nitrogen and stored at -20 °C. Genomic DNA was isolated from the filters using an E.Z.N.A Plant DNA Mini Kit (Omega Bio-Tek, USA) according to the instructions of the

manufacturer. Concentration of DNA in the obtained extracts was determined with a NanoDrop spectrophotometer (Thermo Scientific, USA). Afterwards, the 18S rDNA region of the eukaryotic ribosomal operon was used in a DNA-microarray and for automated ribosomal intergenic spacer analysis (ARISA).

DNA microarray. The protocol for microarray analyses was identical to the one described in Wollschläger et al. (2014), however, other molecular probes were used. The cells targeted in the present investigation were different clades of cryptophytes and prasinophytes. An overview of the members of these clades and the corresponding probes is given in table 3.

ARISA. For ARISA, a fragment of the internal transcribed spacer (ITS) region of the 18S rRNA gene was amplified via PCR using the forward primer 1528-6FAM (5'-ACTAGGAAGACGTCCAAGTGGATG-3') and the reverse primer ITS2 (5'-GCTGCGTTCTTCATCGATGC-3'). Per 25 µL PCR reaction, approx. 20 ng DNA were used, and the whole analysis was carried out in triplicate. The PCR-protocol started with 94 °C for three min, followed by 34 cycles of 94 °C for 45 sec, 55 °C for 1 min and 72 °C for three min. The reaction was kept at 72 °C for ten minutes and cooled down to 4 °C at the end. Subsequently, in preparation of the analysis, 1 µL of PCR solution was mixed with 15 µl Hi-Di (Applied Biosystems, USA) and 0.3 µL size-standard (GeneScan-500 ROX, Applied Biosystems, USA). The analysis of the PCR-products was carried out using an ABI 3130XL capillary sequencer (Applied Biosystems, USA), and data were evaluated using the GeneMapper 4.0 software (Applied Biosystems, USA). Fragment size patterns obtained were analyzed by non-metric multidimensional scaling.

2.7 Data analysis

Statistical, ecological and multivariate data analysis was performed using the freeware software package PAST (version 2.16, Hammer et al. 2001). Map plots were generated using the Ocean Data View Software (Schlitzer 2011). For illustration purposes, data were interpolated between the measurement points (black dots) using the DIVA gridding feature of the software.

3. Results

3.1 Abiotic parameters and chl-*a* distribution

Generally, the eastern and –to a lesser degree– the southern regions of the German Bight were found to be influenced by freshwater input from the rivers Elbe, Weser, and Ems as well as by the coastal waters of the Wadden Sea. This was indicated by lower salinity, coupled with higher concentrations of CDOM and nutrients in these areas (SUP.2). Nitrate levels were highest in the earlier periods of the year (April, May, and June). For phosphate, very low values were detected in May 2010, while seasonal differences in the other cruises were much smaller. TSM concentrations were highest in September in the shallow areas near the coast, probably because of strong wind-induced mixing resulting in increased re-suspension of mineral particles in the water column. Chl-*a* distribution (Fig. 1) was patchy and could not be directly linked to the measured nutrients (data not shown). However, chl-*a* tended to be higher near the coast, although linear correlations between chl-*a* and general coastal characteristics (using salinity as proxy) were weak (Table 4).

3.2 Contribution of phytoplankton size classes to biomass

Microscopy and flow cytometry. Carbon biomass was calculated for each of the three size classes separately on the basis of cell dimension measurements made by either microscopic observation (microphytoplankton) or flow cytometry (nano- and

picophytoplankton).The values for the different size classes were summarized to estimate total biomass of the community for the respective stations (those of May 2010 were omitted due to the lack of microphytoplankton data), and correlated linearly with HPLC-derived chl-*a* concentrations (R^2= 0.6, $p<0.001$; Fig. 2). Averaged over all cruises, microphytoplankton constituted the major part of bulk autotrophic carbon biomass (61 %), with diatoms made up for 40 and dinophytes for 21 %, respectively (other classes were negligible). Nanophytoplankton contributed over all cruises with 38 %, while picophytoplankton contribution was almost negligible (below 2 %). For this reason, nano- and picophytoplankton were summarized in the following as ultraplankton (<20 µm, Fogg 1991).

Considering the cruises separately, an often high average contribution of ultraphytoplankton to total carbon biomass was visible (Fig. 3A). In some cases, it equaled or even exceeded that of microphytoplankton (September 2010, April and June 2011). High contributions of small phytoplankton in spring and early summer are in accordance with observations made by Knefelkamp (2009) in the waters around Helgoland. On a spatial scale, contribution of ultraphytoplankton was patchy, but not completely randomly distributed (Fig. 4, left panels). As a tendency, higher proportions could be observed at the most offshore areas but occasionally also close to the coast, especially near the southwestern and eastern part of the German Bight.

Pigment data. To estimate also the contribution of the different phytoplankton- respectively size classes to total chl-*a*, based on the measured accessory pigment concentrations, the CHEMTAX-approach (Mackey et al. 1996) was used. According to these results, again groups were dominating which are commonly considered to be of microphytoplankton size (diatoms and dinoflagellates, contribution averaged over all cruises approx. 75 %). However, the majority of the microphytoplankton was assumed to be diatoms (Fig. 3B). The groups which can be associated with the

ultraphytoplankton (prasinophytes, cryptophytes, and prymnesiophytes) showed higher proportions in summer. Similar to the microscopy/flow cytometry dataset, the contribution of the smaller size classes was especially high in the more offshore areas (Fig. 4, right panels). Likewise high contributions of ultraphytoplankton near the coast as visible for carbon estimates were not observed in the pigment-based data.

3.3 General community patterns

The phytoplankton communities at the cruise stations were investigated as a whole by obtaining their genetic “fingerprints” via automated ribosomal intergenic spacer analysis (ARISA). The resulting data were analyzed by non-metric multidimensional scaling, and three distinct groups could be distinguished (Fig. 5A): The first group included all stations from 2010, with no clear differences between the seasons, while the second group was a tight cluster consisting of the stations of April and June 2011. Isolated from both groups was the September cruise 2011. In order to test whether environmental factors had an influence on the observed distribution, non-metric multidimensional scaling was also performed on environmental data available for the stations. In contrast to the ARISA data, the resulting pattern showed some seasonality (Fig. 5B): Spring cruises of both years formed one group, while the other cruises formed a second. Apparently, there was no relation between the distribution of the stations according to the ARISA data and the distribution according to the environmental parameters. This was also confirmed by a Mantel test between both similarity matrices ($R = -0.11$, $p = 0.974$).

3.4 Taxonomical composition

Microscopic observations. Microscopic analysis targeted the microphytoplankton fraction and its biodiversity, since members of this size class are readily countable

and identifiable by this method. Species accumulation curves (SUP. 4) proved that the number of samples investigated for the different cruises (n= 15-25) was sufficient to cover the majority of taxa present in the study area, since they almost reached saturation. Furthermore, they suggested the highest numbers of taxa in autumn, the smallest numbers in summer, and an intermediate value in spring. These seasonal differences in microphytoplankton biodiversity were confirmed by the average values of the Simpson index '1-D' calculated for the different cruises. It is a robust measurement of biodiversity (Magurran 2004) and more meaningful than the simple number of taxa, because it also takes the abundance per taxon into account. The Simpson index ranges from 0 to 1, with increasing values to more 'even' or diverse communities with several equally contributing taxa. Its averaged value over all respective cruise stations was 0.52 in July 2010, 0.63 in June 2011, and 0.67 in April 2011. In both years, the September cruises showed statistically significant higher values with 0.8 in 2010 and 0.85 in 2011 ($p<0.05$ according to an ANOVA), indicating a more diverse community than in the other seasons. Seasonal differences were also found with respect to the spatial variability of community composition, which can be expressed as beta-diversity (Whittaker 1960). Calculated from taxa presence/absence, beta diversity (and therefore spatial heterogeneity of the communities) was higher in summer (3.29 in July 2010 and 3.52 in June 2011), than in spring (2.87 in April 2011), while it was lowest in September of both years (1.13 in 2010 and 1.4 in 2011).

According to taxonomical classification and cell counts, no blooms of particular species were observed during the cruises. The dominant groups were diatoms and dinoflagellates, other groups played only a marginal role. In figure 6, the percentage of dinoflagellates on total microphytoplankton population is displayed in terms of cell number, carbon and chl-*a*. It can be seen that dinoflagellates dominated the

community in the more offshore areas, especially in summer, while diatoms dominated the coastal areas. For analyzing the community compositions in more detail, a canonical correspondence analysis (CCA) was performed (Fig. 7). For clarity reasons, only those taxa were included which constituted 90 % of total dinoflagellate abundance or 80 % of non-dinoflagellate abundance of a specific cruise. Temperature, salinity, CDOM and turbidity were used as explanatory environmental variables. With respect to the arrangement of the arrows representing the environmental parameters, the ordination plot can be separated roughly into four sections: Warm/clear, warm/turbid, cold/clear, and cold/turbid environments. The positions of the different taxa in relation to the environmental variables were used to draw conclusions about their preferred occurrence. Considering the distribution of the stations, the CCA showed a separation between spring, summer and autumn communities. However, diatom taxa were present all through the year and were therefore found in all environments. Especially the genus *Chaetoceros* was an important element of the diatom community with various members. Most of them appeared to occur in clearer waters, only *Chaetoceros pseudocurvisetus* was found in more turbid regions, as well as *Eucampia zodiacus* and the majority of the pennate forms (e.g. *Navicula spp.*, *Bacillaria paxillifer*, *Pseudo-nitzschia seriata*). *Mediopyxis helysia*, a species newly recorded in the German Bight (Kraberg et al. 2012), was also found in the course of this study. In contrast to the ubiquity of the diatom taxa, most dinoflagellate taxa were located in the upper right section of the graph, indicating an association with warmer, clearer waters characteristically for the summer periods. Besides small thecate and athecate dinoflagellates, *Dinophysis acuminata* was frequently found as well as several members of the genus *Ceratium*.

Flow cytometry. Gaining taxonomical information about the ultraphytoplankton community via flow cytometry is limited. However, despite having the possibility of

differentiation between nano- and picophytoplankton, also the presence of cryptophytes can be detected by measuring the orange fluorescence originating from phycoerythrin (Li and Dickie 2001). Thus, the ratio of orange to red chl-*a* fluorescence was used in this study to estimate the proportion of cryptophytes in the ultraphytoplankton (Fig. 8). Cyanobacteria, which also show orange fluorescence, were omitted from the analysis on the basis of their smaller size (and therefore lower side-scatter). Although being a relatively crude parameter due to the variability inherent in fluorescence measurements (Falkowski and Kiefer 1985), this ratio allows the detection of differences between samples. The variation in the ratio could not be linked to variations in environmental parameters between cruise stations (data not shown). On a temporal scale, however, it indicated a relatively constant proportion of cryptophytes in all seasons of 2010. In 2011, more cryptophytes were present in April and June, while in September, the proportion of cryptophytes decreased again.

Pigment data. According to the CHEMTAX-analysis, in all seasons the majority of chl-*a* biomass was made up by diatoms (Fig. 3B). The contribution of dinoflagellates was much lower compared to the estimations made by the microscopic/flow cytometry approach (Fig. 3A), and also their dominance in the offshore regions was not visible (Fig. 6A). An apparent increase of prymnesiophytes was observed during the summer of both years, to a large degree responsible for the increased contribution of ultraphytoplankton to total chl-*a* biomass in these months (see 3.2). In contrast, prasinophyte contribution was relatively constant over all cruises. The proportion of cryptophytes in 2010 was higher in spring and autumn compared to summer of the same year, but were in general lower than in 2011. Thus, the development of this group was to a certain degree similar to the results obtained by the flow cytometric fluorescence data.

Microarray results. The DNA-microarray targeted different clades of cryptophytes and prasinophytes because both classes have been shown to be important contributors to the small phytoplankton in the German Bight (Gescher et al. 2008; Metfies et al. 2010). Since the obtained signal intensity of a DNA-microarray can be biased from several sources (Medlin et al. 2006; Wollschläger et al. 2014), the data were only interpreted with respect to the presence or absence of the different clades in this study.

The left panel of figure 9A shows the relative abundance of signals obtained for the various clades of cryptophytes on the different cruises. It can be seen that the probe specific for the whole class gave a signal at nearly all stations. The single clades showed similar presences in 2010 (only clade 4, 5 and 6 were less frequent in July) while at the beginning of 2011 the presence of all clades was considerably lower. However, to the end of the year, the values increased again, but most clades were much less present than in 2010. A similar development was also visible in the prasinophyte community (Fig. 9A, right panel): Clades were present at a high percentage of stations in 2010 cruises (with a drop of some clades in July) while in 2011, the presence of all clades was much lower but with a tendency to increase towards September.

Using the number of signal-giving probes per station as an index for cryptophyte and prasinophyte biodiversity, the results showed a high accordance of cryptophyte and prasinophyte diversity distribution (Fig. 9B). It was patchy, and direct correlations with environmental parameters, biomass distributions, or microphytoplankton diversity were not found (data not shown). However, some tendencies could be deduced from the figure: On the majority of the cruises, high diversity occurred in the southern German Bight in the region of the East Frisian Islands (approx. 53.8°N 7.4°E) as well as in the northeastern area off the coast of Sylt (54.9°N 8.3°E). In contrast, a lower

diversity was frequently observed at the Elbe estuary (53.9°N 8.7°E), and in the inner parts of the German Bight.

4. Discussion

We used an complementary approach based on light microscopy,HPLC-based phytoplankton pigment determination, *in situ* chlorophyll-*a* fluorescence measurements, flow cytometry, molecular fingerprinting and DNA microarray analyses to assess spatial and temporal variability in phytoplankton community in the German Bight, including all three phytoplankton size classes as far as possible. With exception of microscopy and HPLC, focus laid on methods which are relatively low in effort and allow therefore a high sample throughput. The fluorescence measurements give estimates of chl-a distribution in high resolution, while flow cytometry has been shown to allow a fast and accurate counting of small phytoplankton cells in a sample (Olson et al. 1985; Phinney and Cucci 1989; Vives-Rego et al. 2000). Molecular biological approaches (for an overview of common methods see de Bruin et al. 2003) are useful for obtaining taxonomic information on small and hardly identifiable cells. Fingerprinting techniques provide information on general changes in the phytoplankton community (Knefelkamp 2009; Wolf et al. 2013), although they are not suitable for an absolute assessment of biodiversity (Bent et al. 2007). The ARISA (automated ribosomal intergenic spacer analysis) approach used in this study has been often applied for prokaryotic communities (e.g. Danovaro et al. 2006; Kovacs et al. 2010), but has also been used for eukaryotes (Fechner et al. 2010; Wolf et al. 2013). In contrast, DNA-microarrays can provide taxon-specific information about the phytoplankton community in a sample (Metfies and Medlin, 2005; Kochzius et al. 2007). These data are commonly based on the detection of sequences in the 18S-

rRNA gene in the ribosomal operon by taxon-specific, complementary oligonucleotide molecular probes. This particular gene allows investigations on different taxonomical levels (Díez et al. 2001; Moon-van der Staay et al. 2001). Microarrays have frequently been used for analyzing prokaryotic communities of various origin (Nelson et al. 2011; Sessitsch et al. 2006), but also for cryptophytes and prasinophytes in the German Bight (Gescher et al. 2008; Metfies et al. 2010).

Biomass distribution and contribution of size classes. Phytoplankton biomass is certainly one of the most important and most requested parameters in biological oceanography and conveniently estimated by *in situ* chl-*a* fluorescence measurements. Such measurements are suitable for illustrating general patterns of phytoplankton distribution (patchiness) in high resolution (Fig. 1). However, their interpretation as proxy for phytoplankton biomass requires some caution due to the variability of both the fluorescence/chl-*a* and the chl-*a*/ (carbon)biomass relationship (Banse 1977; Falkowski and Kiefer 1985; Geider 1987; Hallegraeff 1977; Jiménez et al. 1987; Llewellyn and Gibb 2000). Naturally, also the estimations of carbon biomass itself by optical means can be biased by several factors, in case of microscopy by the accuracy of cell counts, the cell size measurements, or the equations used for biovolume and carbon calculation. For flow cytometry, uncertainties arise from the fact that size estimation is generally based on light scattering measurements, which depends on the orientation of the cell during measurement, and which is of course different between the spherical beads used for calibration and phytoplankton cells, which normally have other shapes and in general a different structure. Additionally, the use of average size values for the respective cruise station introduced additional uncertainties.

However, these potential biases should be small enough not to interfere with the main conclusions drawn from the data. The plausibility of the overall biomass

466 estimations is indicated by the slope between chl-*a* and estimated total carbon
467 biomass (Fig. 2), which lies with a value of approx. 38 in the range between 20 and
468 50 found for healthy cells of diatoms, dinoflagellates, and microflagellates in the
469 North Sea (Reid et al. 1990).
470 The impossibility of explaining the observed biomass distribution by the measured
471 CDOM or nutrient concentrations can be caused by different reasons: Phytoplankton
472 itself has an influence on nutrient distribution due to the uptake and release of
473 nutrients, and although higher loads of nutrients as occurring in coastal waters
474 (Radach 1992), as well as humic organic substances can promote phytoplankton
475 growth (Prakash and Rashid 1968; Carlsson and Granéli 1993), its growth exhibits
476 also a time-lag in the response to changing nutrient conditions. Internal reservoirs
477 allow cell growth also under low ambient nutrient conditions (Dortch 1982) while on
478 the other hand incorporated nutrients have to be assimilated into organic molecules
479 before they can be used for growth processes (Wheeler 1983). Also an imbalance or
480 lack of certain nutrients can limit phytoplankton growth (Tilman et al. 1982), as in the
481 case of silicon, which is required for diatom frustule formation. Thus, interpretation of
482 biomass distribution by nutrient situation can be difficult when only a snapshot of the
483 situation is available like it is the case on research cruises. This needs time series
484 data where the development of both parameters can be tracked over a longer period,
485 or modeling approaches (e.g. Baretta et al. 1995). Of course, also other parameters
486 like light availability (Loebl et al. 2009), zooplankton grazing (e.g. Calbet and Landry
487 2004), or degradation by viruses (Brussaard 2004; Rhodes et al. 2008) influence
488 phytoplankton biomass development and have to be taken into account.
489 The different results regarding the contribution of the size classes to total biomass
490 (Fig. 3 and 4) are explainable by the different methodological approaches. One is
491 based on optically derived carbon biomass estimates, while the other relies on

pigment estimates. Both parameters describe phytoplankton biomass differently and are not necessarily interchangeable (see also fig. 2). Microphytoplankton contribution is probably overestimated by the pigment-based approach at the expense of the other size classes, since its calculation is based on the assumption that diatoms and dinoflagellates respectively their marker pigments fucoxanthin and peridinin are only present in this size class. But both classes can have also smaller representatives (Moon-van der Staay et al. 2001, Gao et al. 2003) and in turn, some pigments associated with smaller phytoplankton can also occur in microphytoplankton species. Thus, the pigment based estimation of size class contribution has some uncertainties due to the impossibility to assign certain marker pigments to a certain size class. For this reason, at least regarding the ultraphytoplankton distribution the flow cytometry approach might reflect the true situation better, inasmuch as it allows also the detection of small cells not carrying marker pigments. Nevertheless, on a spatial scale, both approaches show a similar picture, and even by looking at the differences more information can be gained: The differences between both methods in detecting the ultraphytoplankton were especially high in the coastal areas (Fig. 4), indicating that in these regions this size class consisted to a considerable amount of diatoms and dinoflagellates.

Both flow cytometry and pigment analysis agreed with a higher contribution of small cells to biomass in the more offshore, nutrient poor regions. This is in accordance with observations made by Agawin et al. (2000) and Sabetta et al. (2008), and can be explained by the competitive advantage of smaller cells under low nutrient conditions (Chisholm 1992; Fogg 1991). They have a higher surface/volume ratio which enhances the utilization of available nutrients. This ratio is lower for larger cells, which are in addition more affected by sinking and were therefore successively removed from the community when nutrient levels decline. However, the flow

cytometry approach indicated also frequently high contributions of small phytoplankton in coast-near areas of the German Bight. This was also observed by Hesse et al. (1989), which attributed it primarily to frontal zone effects due to tidal mixing or river plumes. But coastal waters are also often quite turbid what can limit the availability of light (seen SUP. 1 for distributions of TSM, but also CDOM). Since small cells are also more efficient in light absorption than larger ones due to a lower pigment packaging effect (Morel and Bricaud 1981; Kirk 1994), they might have also a certain competitive advantage under low light conditions what could be an explanation for their stronger presence in certain coastal areas. Of course, additional factors which have not been assessed in this study, like selective grazing by zooplankton can also shape the phytoplankton size class distribution (Riegman et al. 1993; Gaul and Antia 2001; Lindén and Kuosa 2004).

Taxonomic composition. When looking at the taxonomic composition of the phytoplankton, the ARISA fingerprint (Fig. 5A) suggested differences in the community between 2010, the first half of 2011, and the end of 2011. Similar differences were not observed in the microscopy-based microphytoplankton data, instead, the values for Simpson-index, for beta-diversity, and the position of stations in the CCA (same seasons of both years close to each other; Fig. 7) indicated seasonal behavior of the community. Minor variations between 2010 and 2011, as observable for the summer stations, are probably related to differences in phytoplankton seasonal succession. The higher occurrence of dinoflagellates in summer of both years is in accordance with results published by e.g. Hagmeier & Bauerfeind (1990), Peeters & Peperzak (1990) and Hickel (1998). Under stratified, oligotrophic conditions they have competitive advantages over diatoms (Fogg 1991) because they require no silicon for cell wall formation and are able to exploit nutrient rich water near or below the thermocline due to diurnal migrations (Cullen 1985;

MacIntyre et al. 1997). Especially the genus *Ceratium* was frequently abundant and contributed to biomass, what is a typical feature of the North Sea in the second half of the year (Reid et al. 1990, see also various datasets of the Helgoland Roads time series at www.pangaea.de).

Seasonality with a lower contribution of diatoms in summer was also seen in the pigment-based class estimations. However, dinoflagellate contribution in this season was underestimated compared to the microscopic data, in favor of an increased contribution of prymnesiophytes. Partially, this could be explained by the high pigment diversity within the dinoflagellates due to acquisition of different types of chloroplasts by multiple endosymbiotic events (Zapata et al. 2012). This might limit the validity of the initial pigment ratios assumed for dinoflagellates in the CHEMTAX analysis and weaken its ability to estimate this group correctly. Furthermore, the chl-*a* but also other pigment concentrations were quite low in the offshore regions (where the majority of the dinoflagellates was present according to microscopy), what could have also introduced a bias in the pigment analysis.

Regarding the results for the ultraphytoplankton, the molecular probe specific for all cryptophytes indicated a ubiquitous presence of this group in the German Bight thorough the year, what is in accordance with results from Metfies et al. (2010), and what is also supported by the flow cytometry and pigment data. Cryptophyte and prasinophyte diversity was spatially variable, but although not always harboring the highest biodiversity, turbid coastal areas regularly showed high diversities on all cruises. Since in these regions the ultraphytoplankton exhibited often also high carbon biomass proportions (compare figure 4), they appear to provide generally a suitable environment for small phytoplankton.

A response of the small phytoplankton community to seasonal changes of the environmental parameters similar to the microphytoplankton was not observed, with

exception for the prymnesiophytes in the CHEMTAX-analysis. In contrast, a rather sharp change in the community between 2010 and 2011 was indicated by an increased cryptophyte contribution to biomass as well as a lower diversity in the cryptophytes and prasinophytes (Fig. 3B, 8). In September 2011, the community appeared to change again to some extent. Such a difference in the behavior of larger and smaller phytoplankton to seasonal changes of environmental parameters has been also observed by Not et al. (2007) for picophytoplankton. However, these changes generally matched the pattern of the ARISA. Interestingly, the fingerprint reflected the changes in the small phytoplankton, but not the seasonality of the microphytoplankton. This indicates a large impact of the small phytoplankton community on this method, which can be explained by its high biodiversity (especially of picophytoplankton) in marine ecosystems (Moon-van der Staay et al. 2001; Vaulot et al. 2008; Knefelkamp 2009; Not et al. 2007). Furthermore, fingerprinting methods tend to neglect rare species (Liu et al. 1997), and in terms of cell numbers, the ultraphytoplankton was approx. 100x more abundant than the microphytoplankton (data not shown). Thus, the signal obtained from the microphytoplankton was probably masked by the one obtained from the nano- and picophytoplankton. For this reason, samples should be size-fractionated by filtering in advance for a more accurate analysis of the different size classes by fingerprinting methods like the ARISA.

4. Conclusions

Patterns found in this study like the occurrence of higher biomass near the coast, or the increased contribution of dinoflagellates (e.g. *Ceratium*) especially in summer agreed well with older observations. They appear to be stable features largely

596 untouched by climate change effects like the temperature increase of about 1.1 °C in
597 the German Bight over the last 45 years (Wiltshire and Manly 2004). Furthermore,
598 the results of this study indicated a behavior or development of the
599 ultraphytoplankton community which is different from those of the microphytoplankton
600 community. This finding emphasizes both the value but also the requirement of using
601 other methods in addition to routinely microscopic observation. While the
602 microphytoplankton followed a well-known succession according to seasonal
603 changes in environmental parameters, the factors governing the changes in
604 distribution and diversity of the small phytoplankton remain largely unknown. This
605 makes the interpretation of the observed patterns difficult, and qualitative and
606 quantitative taxonomical data for comparison are scarce. To close this knowledge
607 gap, the database regarding the ultraphytoplankton in the German Bight has to be
608 broadened. This is of particular importance with respect to the nanophytoplankton
609 which contribution in terms of biomass is most likely underestimated by pigment
610 analysis or light microscopy alone. Since variability in the phytoplankton community
611 can be high, a comprehensive analysis of phytoplankton community in the German
612 Bight requires high frequency measurements over a larger area to cover variability
613 both in time and space. In turn, this requires the use of methods relatively low in
614 effort, like optical and molecular biological approaches. The complementary use of *in*
615 *situ* measurements, microscopy, flow cytometry, DNA-microarray, and ARISA in
616 addition to microscopy and pigment analyses as used in this study can be seen as a
617 first attempt in this direction, because it has shown to provide useful taxonomical and
618 quantitative information on different levels of detail. Furthermore, the chosen
619 methods have the advantage of being well suited for routine usage, what might be
620 not yet the case for more sophisticated approaches. Thus, they can also be
621 considered as being suitable to build up long-term datasets in complement to

622 microscopic observations. This complementary use of different methods in time
623 series would also provide means to identify breaks due to changes in the responsible
624 investigator or the used equipment, which can bias in microscopic datasets. (Wilshire
625 and Dürselen 2004; Peperzak 2010).
626 For future investigations, of course not all the methods used in the present study are
627 necessary to achieve an overview about the phytoplankton community. The final
628 choice should depend on the particular scientific question. For detailed taxonomic
629 investigation, microscopy is still the method of choice, but since its usefulness
630 declines with cell size, molecular methods targeting single taxa (like microarrays) are
631 of great advance. However, the use of microarrays requires the pre-selection of taxa,
632 so it should be complemented with a fingerprint method like the ARISA applied on
633 size-fractionated samples to see changes in the general community. A disadvantage
634 of (DNA-based) microarrays is furthermore the limited quantitative information (see
635 Wollschläger et al. 2014 for a more detailed discussion of this method). But since
636 technology advances rapid, especially in the molecular field, maybe other
637 approaches which are currently relatively sophisticated and expensive (e.g.
638 pyrosequencing) might be an alternative in the future. Flow cytometry or other forms
639 of automated phytoplankton counting can in general be considered as very useful
640 due to their ability to provide a rapid overview over the size distribution within the
641 community and their potential for automation. For some applications, these methods
642 might even replace microscopy. Pigment-based approaches like CHEMTAX provide
643 comprehensive information about the community structure on class level; however,
644 the accuracy of the information depends on the availability of pigment ratios for the
645 classes present in the investigated region. A major drawback is the limited potential
646 of this method to be automated and the resulting low spatiotemporal resolution.

Maybe similar methods not relying directly on pigments, but on absorption or fluorescence spectra might be more useful for routinely usage.

However, to go the step from a proper description of phytoplankton dynamics to a real explanation, it is of course necessary to measure also its antagonists like zooplankton and viruses in comparable detail. Also here, an integrative approach of several methods, including especially molecular and automatic counting techniques, would be of great advantage. Only if these data are available, the assemblage can finally be modelled and the results validated by the observations.

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950

951 **Tables**

952

953 Tab.1: Overview of methods used

Method	Target	Parameter derived
Microscopy	Microphytoplankton (>20 µm)	Cell counts and sizes of single taxa
Pigment analysis (HPLC)	Community as a whole	Contribution of phytoplankton classes
in situ fluorescence	Community as a whole	Bulk biomass distribution (as chl-*a*)
Flow Cytometry	Nanophytoplankton (2-20 µm) Picophytoplankton (0.2-2 µm)	Cell counts and biomass proxies
Molecular fingerprinting (ARISA)	Community as a whole	Community structure
Microarray	Selected clades of nanophytoplankton (2-20 µm) and picophytoplankton (0.2-2 µm)	Presence of investigated clades

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955

Tab.2: Coefficients obtained from linear regression used for the conversion of chl-*a* fluorescence into chl-*a* concentration and turbidity into TSM concentration, respectively. The equation used for conversion was *parameter = (optical proxy value – offset) / slope*.

	Cruise	Slope	Slope error	Offset	Offset Error	R^2
Chl-a	May 2010	0.35	0.03	0.88	0.34	0.94
	July 2010*	149.42	20.29	136.19	77	0.77
	September 2010	0.5	0.08	0.23	0.34	0.68
	April 2011	0.49	0.05	1.13	0.16	0.76
	June 2011	0.44	0.04	1.17	0.14	0.81
	September 2011	0.36	0.05	1.25	0.15	0.7
TSM	May 2010	0.43	0.03	-0.62	0.17	0.97
	July 2010*	0.36	0.02	0.28	0.04	0.93
	September 2010	0.34	0.03	0.97	0.35	0.87
	April 2011	0.45	0.01	0.68	0.04	0.98
	June 2011	0.28	0.03	0.81	0.1	0.77
	September 2011	0.38	0.01	1.16	0.06	0.99

***In this cruise, the continuous measurements were conducted by the ECO FLNTU sensor instead of the SCUFA-II.**

963

964 Tab.3: Molecular probes used in the course of this study with their respective target

965 taxa.

target taxon	probe sequence (5'…3')	probe name	reference
All Cryptophytes	ACGGCCCCAACTGTCCCT	Crypto B	Metfies & Medlin 2007
Cryptophytes clade 1 *Cryptomonas*	CATTACCCCAGTCCCATAACCAAGG	Crypt01-25	Metfies & Medlin 2007
Cryptophytes clade 2 *Rhinomonas* *Rhodomonas*	GCGTCCCACTACCCTACAGTTAAGT	Crypt02-25	Metfies & Medlin 2007
Cryptophytes clade 3 *Hanusia* *Guillardia*	GTGTTCCCGCGCACCACGGTTAAAT	Crypt03-25	Metfies & Medlin 2007
Cryptophytes clade 4 and 6 *Plagioselmis* *Teleaulax* *Geminigera* *Komma* *Chroomonas* *Hemiselmis* *Plagiomonas*	CAAGGTCGGCTTTGCCTC	Crypt46	Metfies & Medlin 2007
Cryptophytes clade 5 *Proteomonas*	TGCGTCCCAACGCCCCACAGTGAAG	Crypt053-25	Metfies & Medlin 2007
Prasinophytes clade 1 *Pterosperma cristatum*	GGTTGCGTTAGTCTTGCT	Pras09A1	Gescher et al. 2008
Prasinophytes clade 1 *Pyramimonas spp.* *Prasinopapilla spp.* *Cymbomonas spp*.	GCCGCCTTCGGGCGTTTT	Pras09A2	Gescher et al. 2008
Prasinophytes clade 1 *Halosphaera spp.*	AACTGGCTCGGTACGCGG	Pras09D	Gescher et al. 2008
Prasinophytes clade 2 *Mamiellales* (except *Dolichomastix*)	CGTAAGCCCGCTTTGAAC	Pras04	Not et al. 2004
Prasinophytes clade 3 *Nephroselmis pyriformis* *Pseudoscoufieldia marina*	TAAAAGACCGACCGCTTC	Pras10B	Gescher et al. 2008
Prasinophytes clade 3 *Nephroselmis pyriformis*	CGTTTCAACTCGACCAGT	Pras10F	Gescher et al. 2008
Prasinophytes clade 3 *Nephroselmis olivacea*	CACTGGCGCGCCCCATCT	Pras10H	Gescher et al. 2008
Prasinophytes clade 5 *Pseudoscoufieldia marina* *Pycnococcus provasolii*	ACGGTCCCGAAGGGTTGG	Pras01	Not et al. 2004
Prasinophytes clade 5 *Pseudoscoufieldia marina* *Pycnococcus provasolii*	CCGACAGAAAGACGCAGA	Pras07	Not et al. 2004
Prasinophytes clade 6 *Prasinococcales*	GCCACCAGTGCACACCGG	Pras03	Not et al. 2004
Prasinophytes clade 7A Unclassified sequences	GCCAGAACCACGTCCTCG	Pras05	Not et al. 2004
Prasinophytes clade 7B	AATCAAGACGGAGCGCGT	Pras06	Not et al. 2004

Unclassified sequences			
Prasinophytes clade 7C *Picocystis salinarum*	ATTGTGTGGGTCTTCGGG	Pras08	Gescher et al. 2008

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969 Tab.4: Linear regressions between chl-*a* concentration and salinity. With exception of

970 June 2011, $p<0.05$ in all cases.

Cruise	Slope	Slope error	Offset	Offset error	R^2	n
May 2010	-3.38	0.09	120.99	2.96	0.42	1897
July 2010	-1.3	0.02	44.98	0.68	0.36	6899
September 2010	-0.57	0.02	21.92	0.76	0.15	3362
April 2011	-0.42	0.02	16.41	0.5	0.08	9049
June 2011	-0.06	0.03	5.49	1.05	0	5941
September 2011	-0.4	0.01	16.39	0.21	0.19	16202

971

Figures

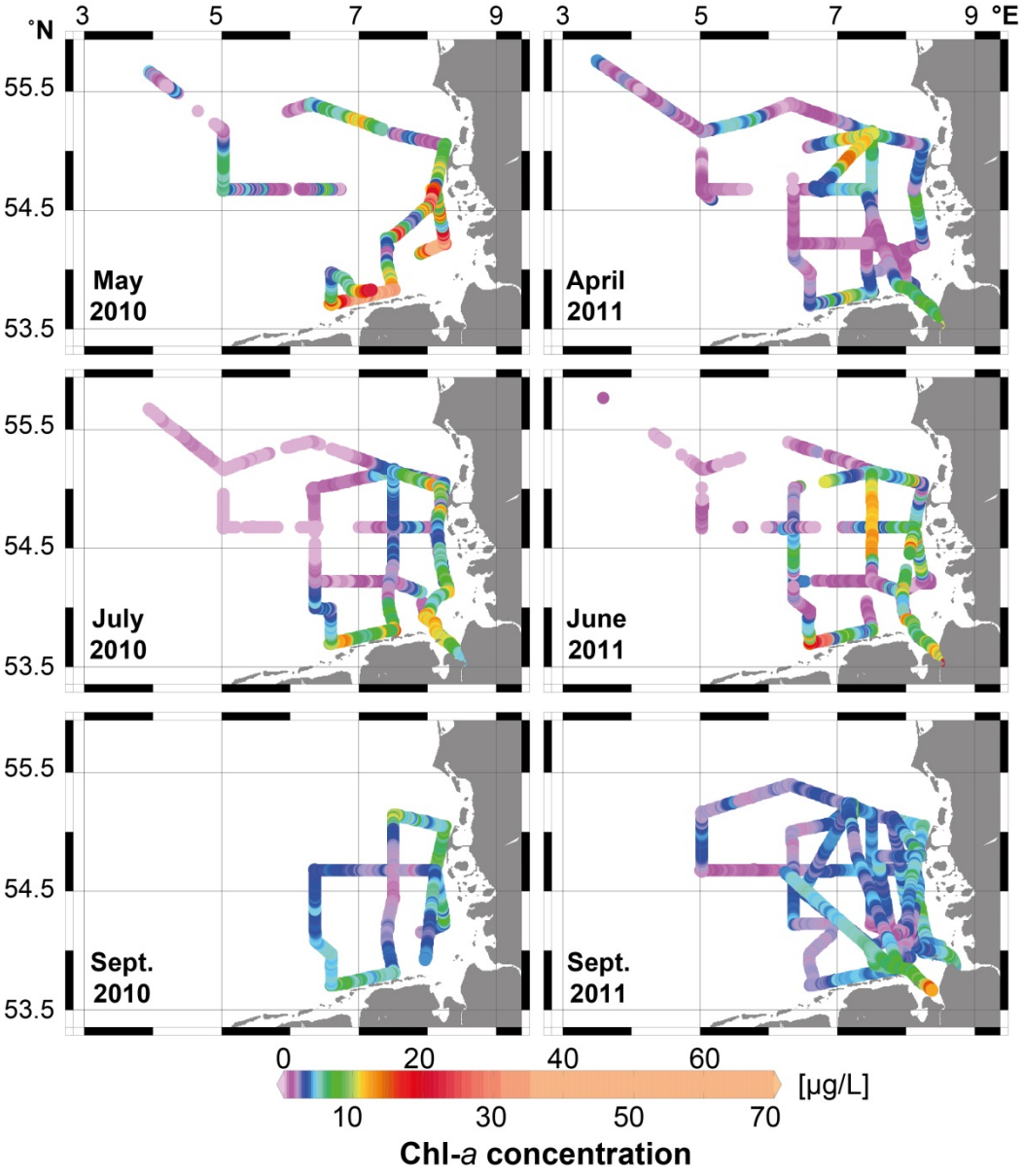

Fig.1: Map plots of continuously measured fluorescence-based chl-*a* concentrations. The color scaling of the figure is non-linear to present differences more clearly.

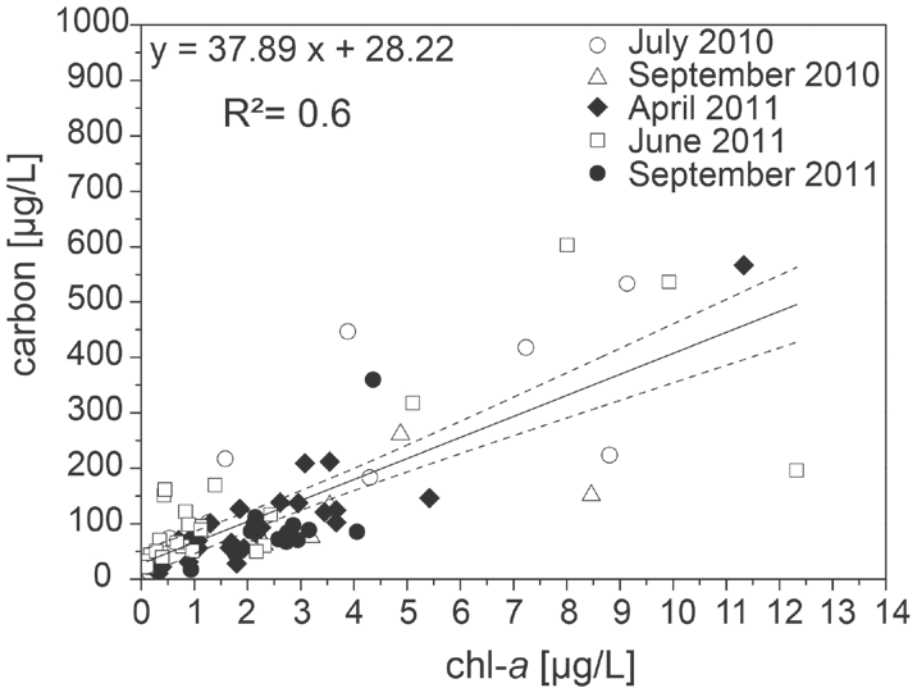

Fig.2: Correlation between HPLC measured chl-*a* concentrations and corresponding calculated total carbon based on cell size measurements by microscopy and flow cytometry. The dotted lines represent the 95 % confidence interval of the linear fit. Both slope and offset are different from zero at p=0.05.

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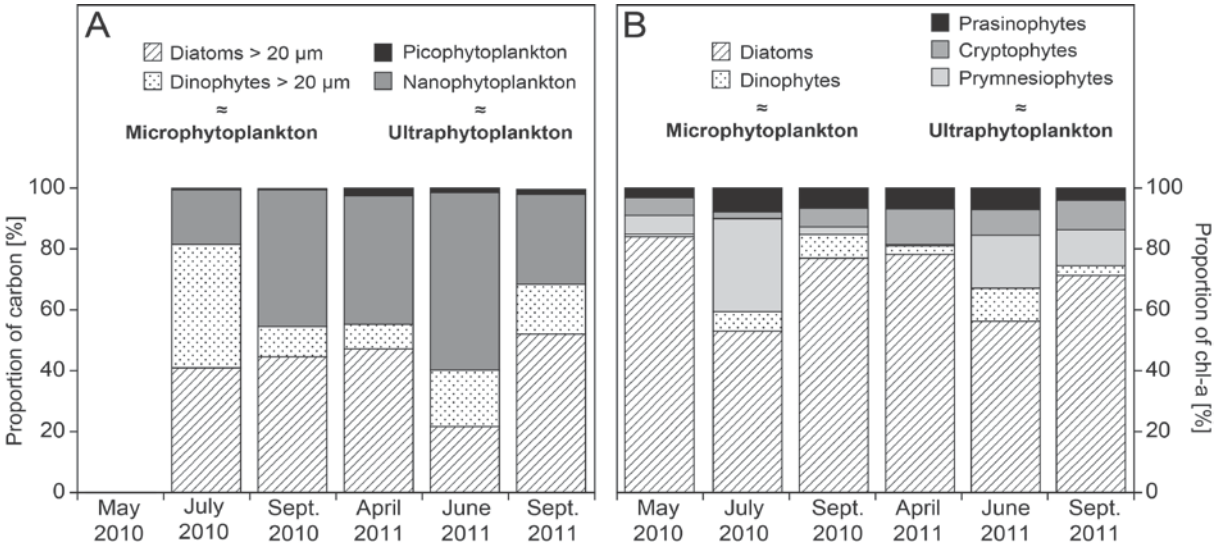

987

988 Fig.3: (A) Mean contribution of phytoplankton (size) classes to total carbon biomass

989 estimated by a combination of microscopy and flow cytometry for the different

990 cruises. Data were not available for May 2010. (B) Mean contribution of

991 phytoplankton classes to total chl-*a* biomass based on CHEMTAX pigment data

992 analysis.

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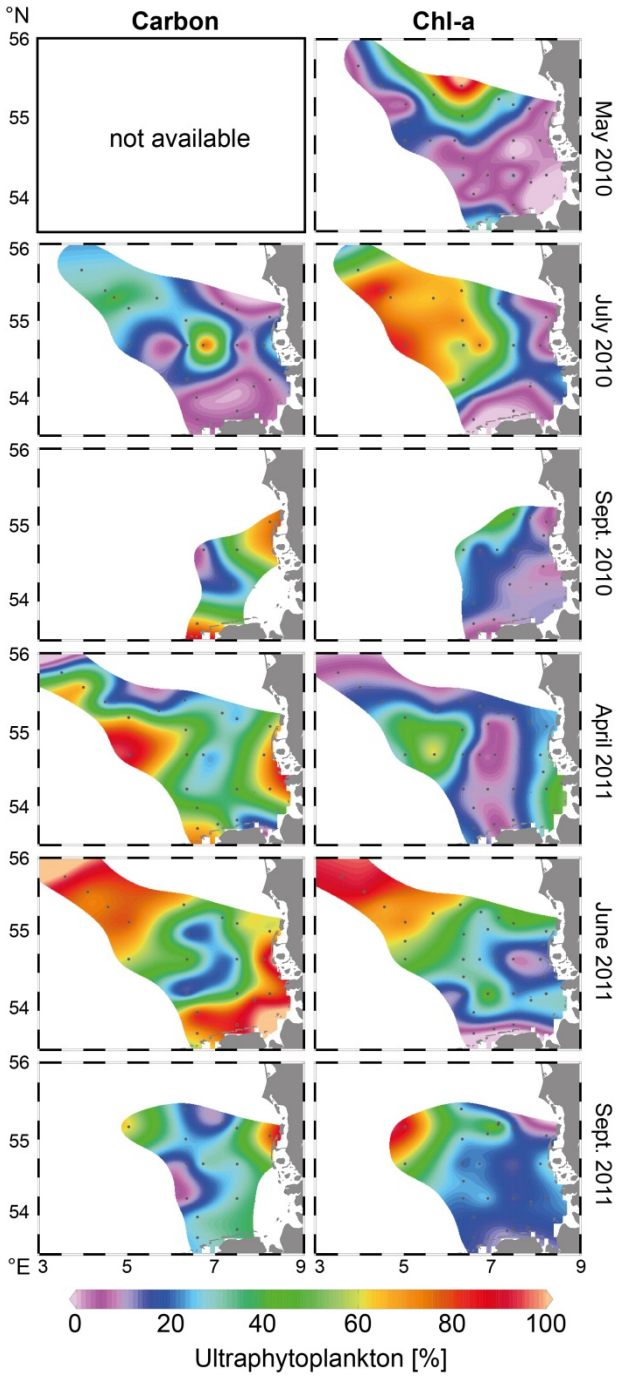


Fig.4: Spatial distribution of ultraphytoplankton contribution to biomass on the different cruises. Left panel shows the values estimated from microscopic and flow cytometry observation, the right panel those based on pigment data.

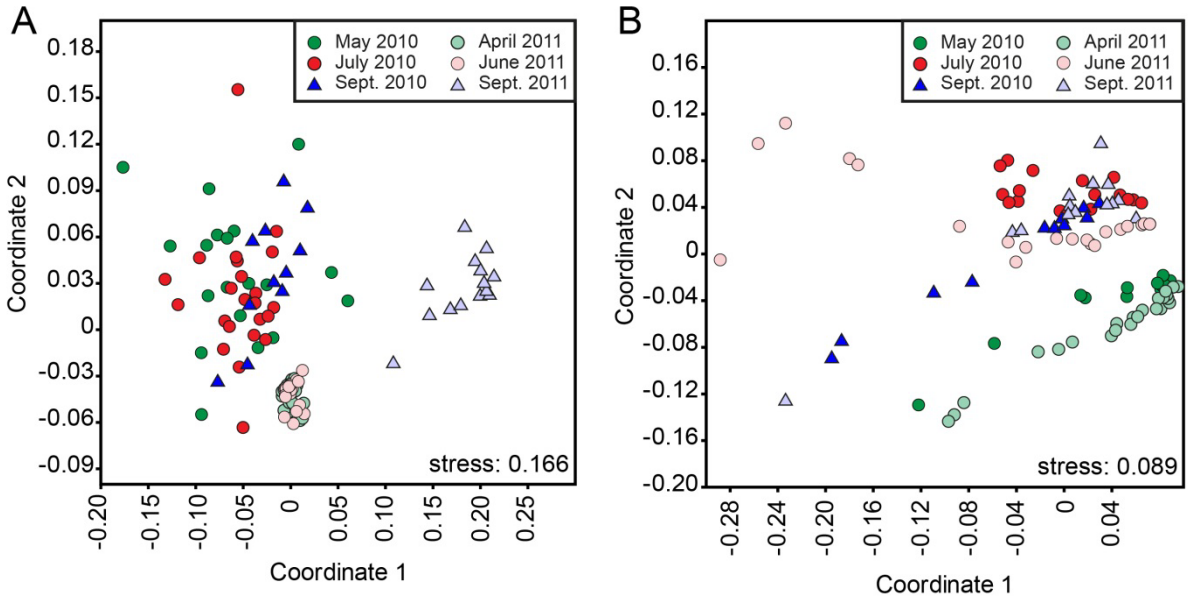
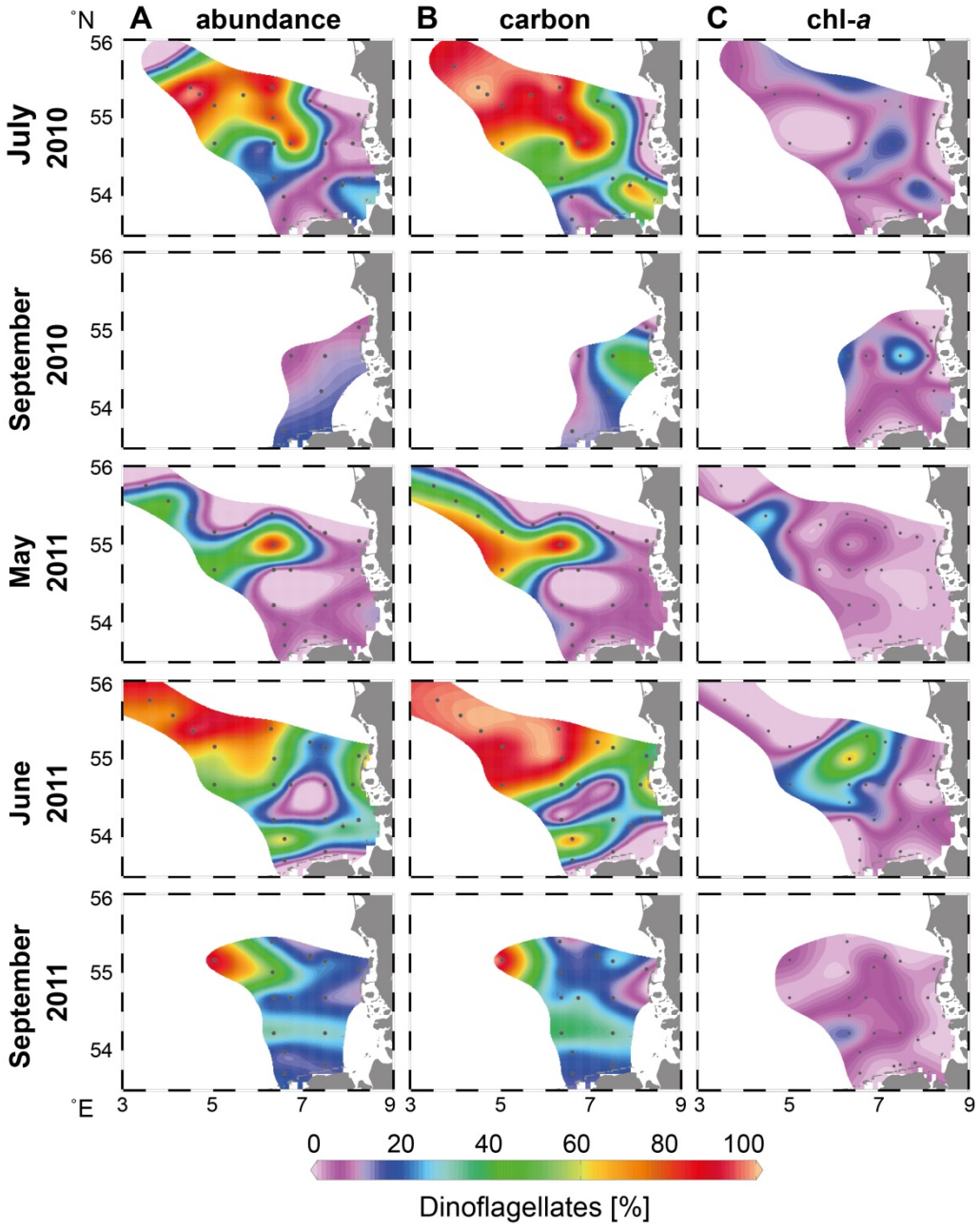


Fig.5: (A) Non-metric multidimensional scaling plots based on fragment patterns obtained by ARISA. (B) The same done for environmental data available for the stations (temperature, salinity, CDOM, and turbidity).

1007



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1009 Fig.6: Distribution of dinoflagellates in terms of (A) cell numbers, (B) carbon, and (C)

1010 chl-*a* (estimated by CHEMTAX).

1011

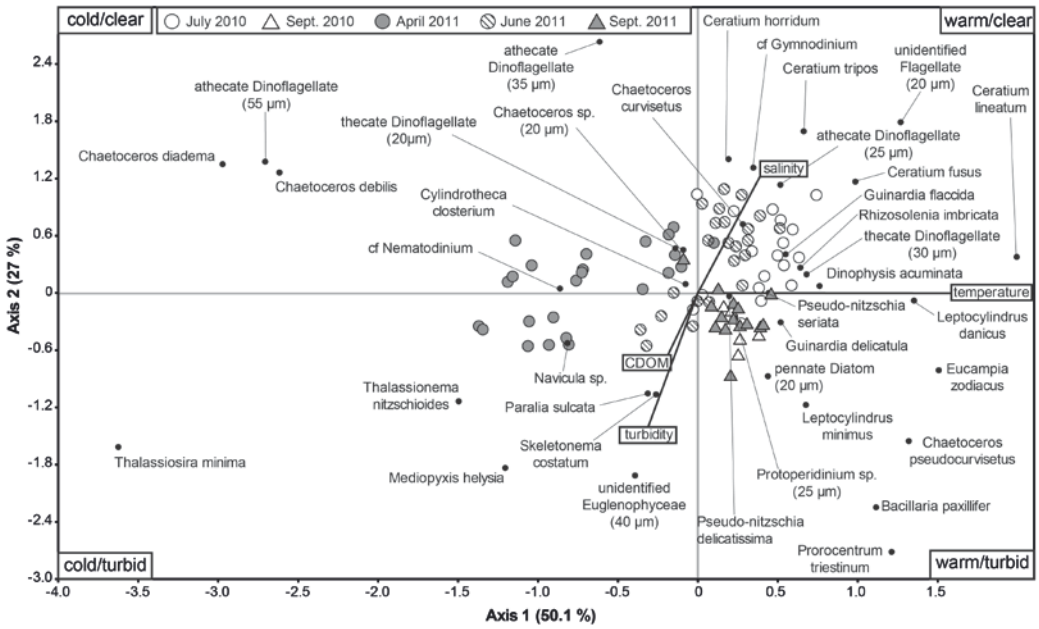

Fig.7: Canonical correspondence analysis of the cruises based on microphytoplankton abundance data. Only a limited set of taxa were used (see text), and abundance data were logarithmized previous to analysis to downweight exceptional high values at some stations. Variability explained by the ordination axes is statistically significant ($p<0.01$, 999 permutations).

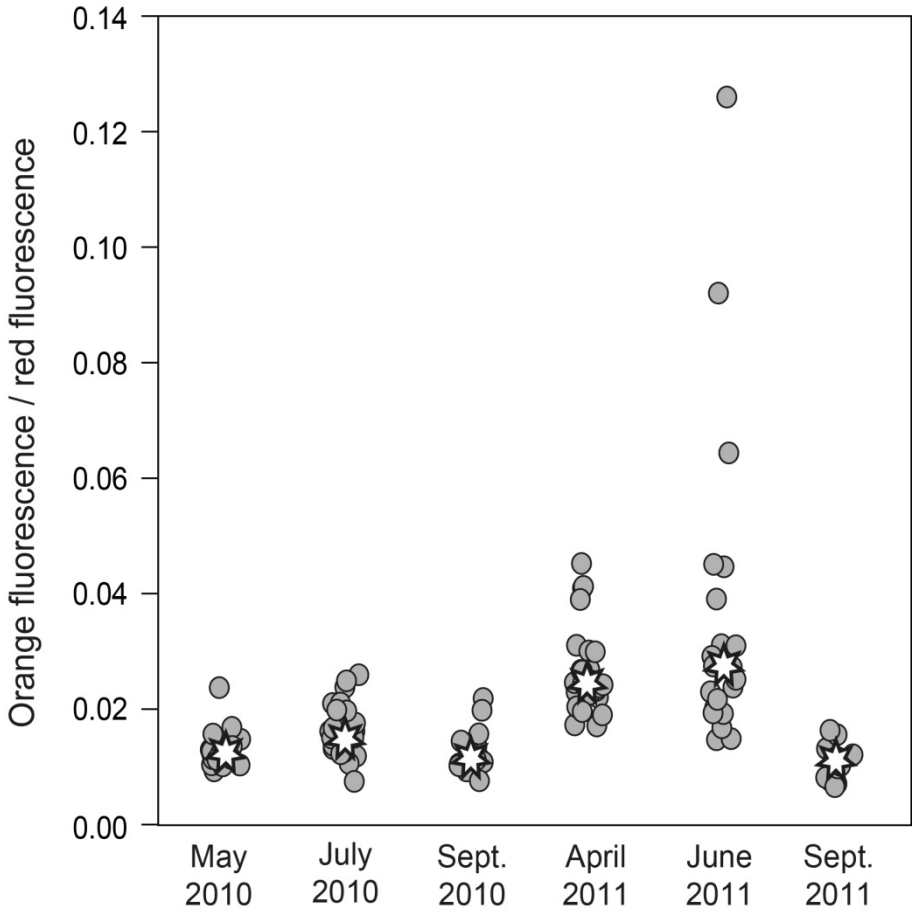

Fig.8: Ratio of orange to red fluorescence measured for the ultraphytoplankton fraction on the stations of the different cruises. The stars mark the median value of the respective cruise.

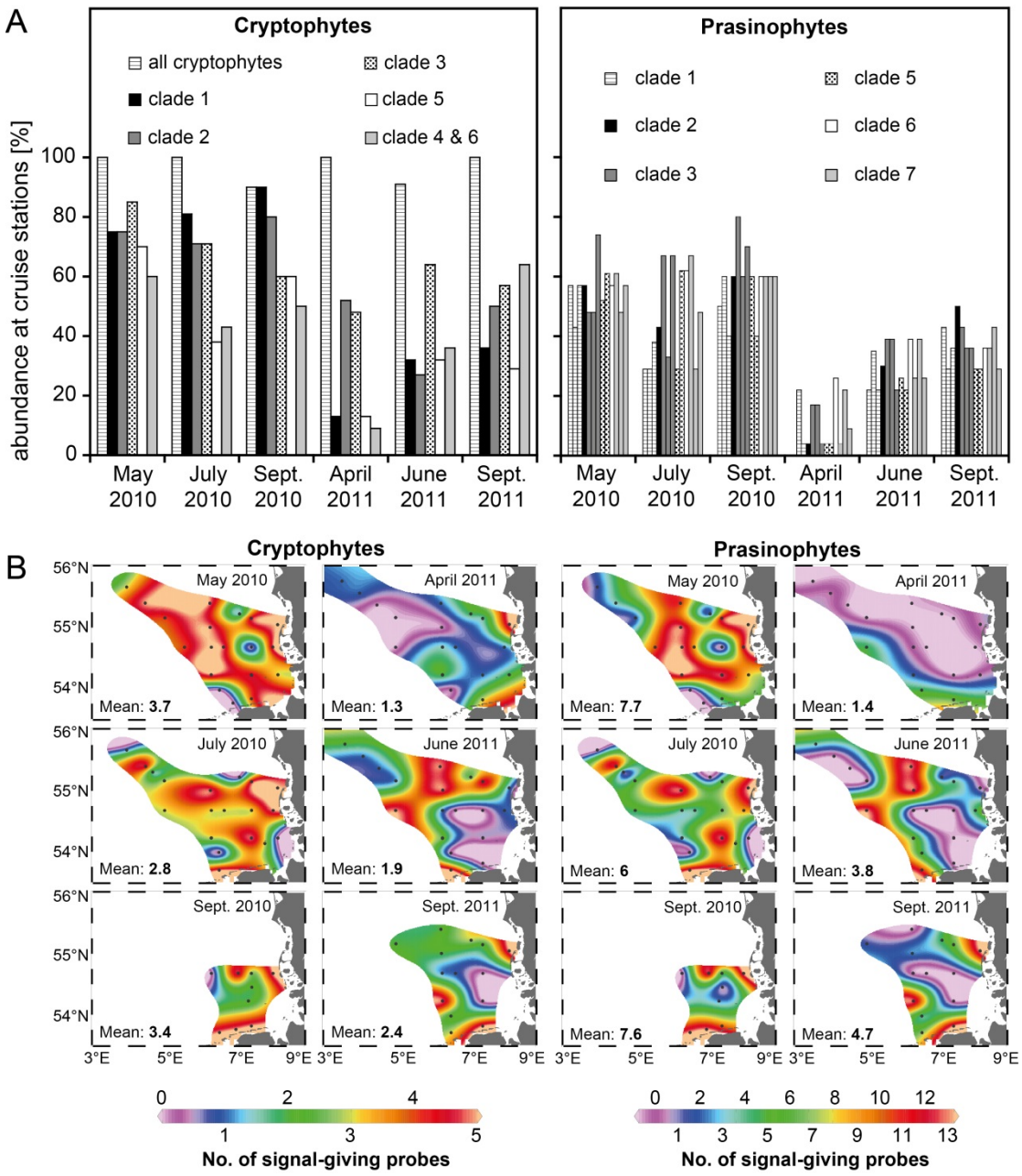

Fig.9: (A) Relative abundance of positive signals from molecular probes specific for different clades of cryptophytes (left panel) and prasinophytes (right panel). Regarding the latter, more than one probe was specific for a certain clade. The order of columns is identical to the order of the probes given in table 3.

(B) Number of probes giving a positive signal per station, shown for the different cruises.

1036 **Supplementary Material**

1037

1038 SUP.1: Initial ratios of marker pigments used in the CHEMTAX-analysis for the
1039 different groups.

	Peridinin	19 but fuco	Alloxanthin	Fuco-xanthin	19-hex-fuco	Neoxanthin	Prasino-xanthin	Viola-xanthin	Lutein	Zeaxanthin	chl-*b*
Dinophytes	0.533	0	0	0	0	0	0	0	0	0	0
Diatoms	0	0	0	0.779	0	0	0	0	0	0	0
Prymnesiophytes	0	0.023	0	0.304	0.27	0	0	0	0	0	0
Cryptophytes	0	0	0.405	0	0	0	0	0	0	0	0
Prasinophytes	0	0	0	0	0	0.096	0.458	0.138	0.034	0.061	0.606

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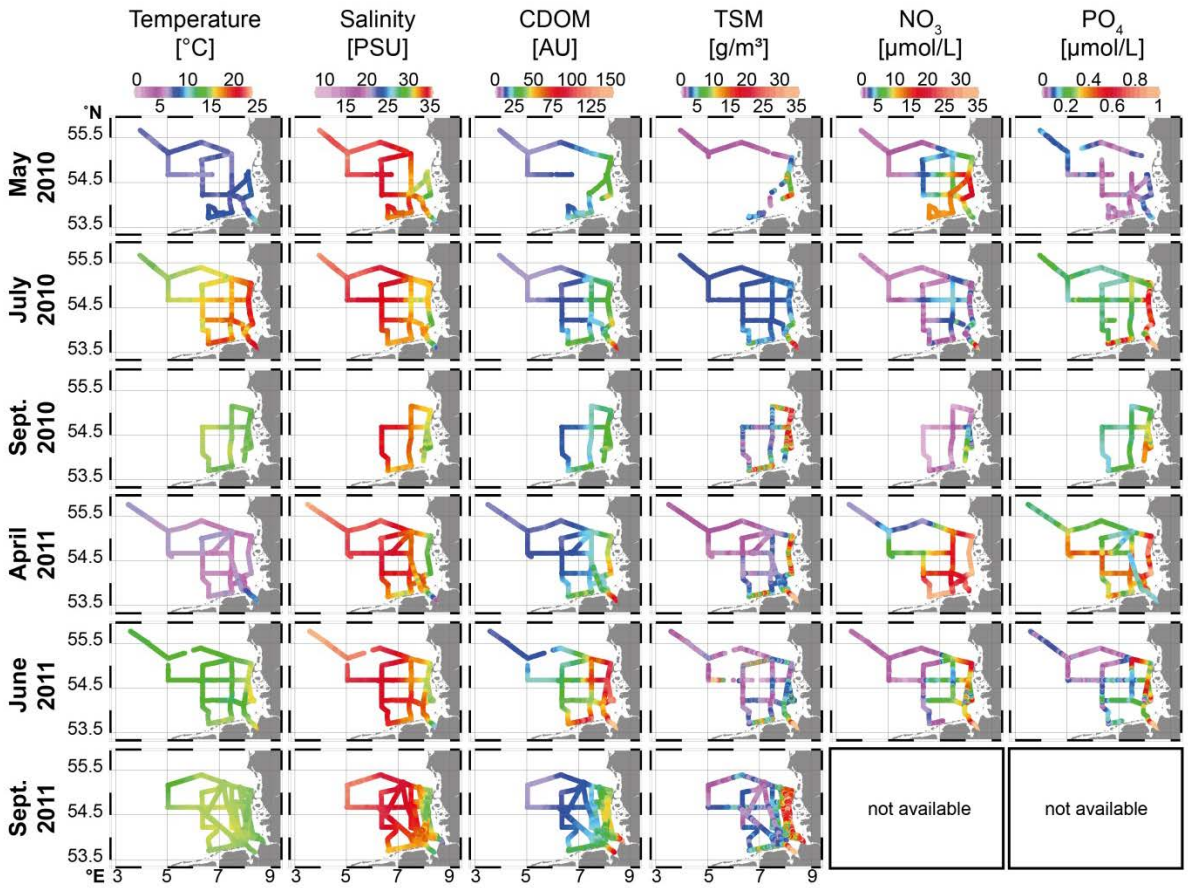
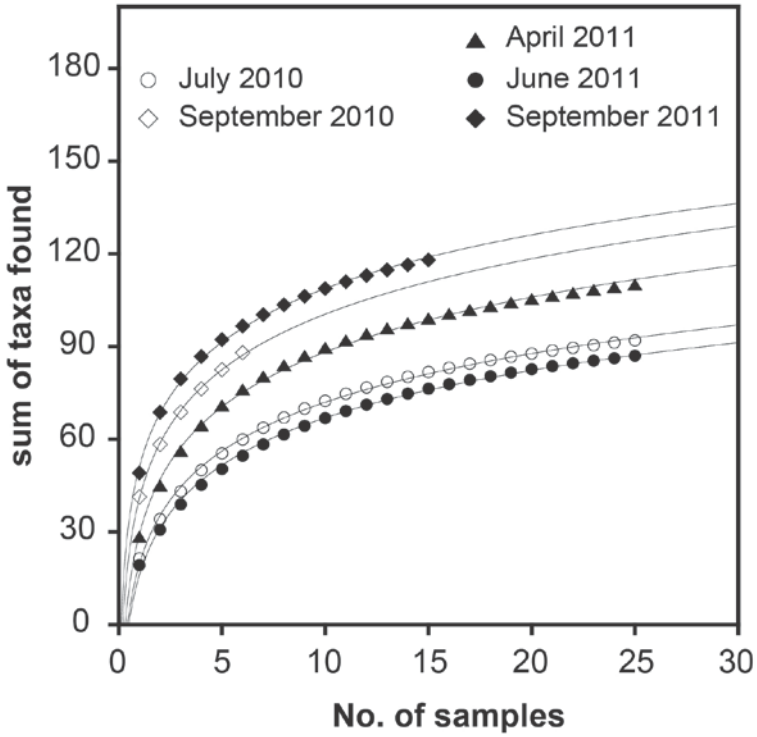
SUP.2: Parameters measured by sensors mounted in the FerryBox used in this study.

Parameter	Unit	Sensor	Manufacturer
Practical Salinity	PSU	Citadel Thermosalinograph CT	Teledyne RD Instruments, USA
Temperature	°C		
Turbidity	NTU	SCUFA-II / ECO FLNTU	Turner Designs, USA / WetLabs, USA
Fluorescence	AU		
CDOM	AU	Cyclops-7	Turner Designs, USA



SUP.3: Map plots of continuously measured environmental parameter. The color scaling of the figure is non-linear to present differences more clearly. Although nutrient values smaller than 0.05 µmol/L can be considered as below the detection limit, they were still shown to illustrate distributions.



SUP.4: Species accumulation curves for the different cruises based on microscopic observations of the microphytoplankton fraction.