Characterization of phytoplankton in the North Sea with optical and molecular methods

by

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“Not everything that can be counted counts;
and not everything that counts can be counted.”

Albert Einstein
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Summary

Phytoplankton is the basis of the marine food web and an important element in global carbon cycling. To understand processes, interactions and also shifts within its community, a thorough observation is required, especially in the light of a changing climate and increased utilization of marine ecosystems by humanity.

A major hindrance in observation is the associated effort: Traditionally, phytoplankton biomass is mostly determined via measurement of chlorophyll-a (chl-a), while community composition is primarily investigated by microscopy. This is time consuming, which limits the number of samples that can be analyzed and prevents thereby a high spatial and temporal resolution of the investigations. Furthermore, only insufficiently detailed information is gained about species which are not readily visible under the microscope and which have no morphological features for reliable identification. To overcome these limitations and to make phytoplankton investigation more convenient, a variety of methods have evolved in the last decades, mainly based on the measurement of optical proxy values or molecular biology. Some of them provide the opportunity for continuous measurement of parameters like chl-a, other facilitate a rapid analysis of discrete samples, even on a taxonomical level.

In the present study, a selection of these convenient methods (fluorescence and absorption measurements, flow cytometry, DNA/RNA-microarrays, and ARISA fingerprinting) was used in combination with the traditional approaches for characterizing the phytoplankton community in the whole German Bight over two years and different seasons. Furthermore, a newly designed device for the continuous measurement of water constituent absorption (ft-PSICAM) was also applied in the course of this investigation. Focus of the study was an evaluation of the different methods, especially with respect to a potential usage in routine monitoring. Where possible, this was carried out by a direct comparison of the results obtained by the different methods.

Absorption coefficients measurements were found to be a more stable alternative for the determination of chl-a and total suspended matter (TSM) in the water than traditional approaches based on chl-a fluorescence and turbidity, respectively. Furthermore, with the ft-PSICAM, it was possible to measure these absorption coefficients continuously.

The various methods for the assessment of the community composition turned out to be widely complementary to each other. While microscopy provided detailed quantitative and qualitative information, but was mainly restricted to microphytoplankton observation, primarily qualitative data were obtained by the molecular methods. This made them valuable to detect the presence or absence of taxa in the nano- and picophytoplankton, where reliable taxonomical information was otherwise not acquirable, as well as general changes in the community. Quantitative information about the size distribution within the small phytoplankton and also limited information about taxonomical composition was provided by flow cytometry.
By analyzing the phytoplankton community in the German Bight with this complementary set of information, it could be found that the microphytoplankton community shows seasonal variation, with a dinoflagellate-dominated community in summer and a diatom-dominated one during spring and autumn. Moreover, also parameters like e.g. species number and biodiversity index exhibited seasonality. In contrast, such variations were not found for the nano- and picophytoplankton. Instead, an abrupt shift in this community was found from the end of 2010 to the beginning of 2011. It was characterized by an increasing contribution of cryptophytes, accompanied with a reduction in cryptophyte and prasinophyte diversity. The reason for this shift was not explainable in the course of this study.

In summary, the convenient methods were found to be a valuable addition to traditional methods by expanding the measurement spectrum. Furthermore, depending on the desired level of detail, they can also be considered suitable to reduce measurement effort in routinely observation. Nevertheless, in order to develop the full potential of these methods, further improvement is required especially with respect to obtaining quantitative taxonomical information.
1. General introduction

1.1 The North Sea

The North Sea is a shelf sea on the margin of the North Atlantic. Its average depth is about 90 m, with the deepest area (700 m) being the Norwegian Trench (Ducrotoy et al. 2000). Generally, depth increases from the Southeast to the Northwest. The North Sea covers the area between the English Channel in the southwest, the Skagerrak in the east and the Shetland Islands in the Northwest. It is bordered by the coastlines of Great Britain, Belgium, The Netherlands, Germany, Denmark, Sweden, and Norway.

![North Sea map](http://commons.wikimedia.org/wiki/File:North_Sea_location_map.svg)

**Fig. 1:** Picture of the North Sea with its surrounding countries and the borders of their exclusive economic zones (grey dotted lines). Picture modified after NordNordWest/Wikipedia, already modified by Uwe Dederig;
http://commons.wikimedia.org/wiki/File:North_Sea_location_map.svg
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It also includes the Wadden Sea, an intertidal mudflat area which is located between the Frisian Islands and the Dutch, German, and Danish coast (reaching from Den Helder to Skallingen). It is the largest area of this type in the world and exhibits a high biodiversity which was the reason for it being included in the UNESCO’s World Heritage List in 2009 (Common Wadden Sea Secretariat 2010).
The direction of the large scale current system in the North Sea is anti-clockwise (cyclonic), since water entering from the North Atlantic is guided by the Coriolis force along the British coast, while water entering via the English Channel flows eastwards along the Dutch coast (Ducrotoy et al. 2000). Due to its geographical position, the North Sea exhibits a variety of environmental conditions, which result from the interaction of oceanic and coastal influences. In its northern and north-western parts, where the water exchange with the North Atlantic is greatest, oceanic influences dominate. These regions are characterized by high salinity (about 35 PSU), relatively constant water temperatures during the year (Becker 1990), and lower nutrient concentrations (Topcu et al. 2011). In the coastal regions, which cover a relatively large proportion of the total area, temperatures are much more variable (Becker 1990). Moreover, especially in the southern and eastern parts, the coasts are influenced by riverine input and outflow from the Wadden Sea, resulting in lower salinity, increased influx of nutrients and organic matter, and often high turbidity. The combination of these factors fosters the North Sea to be, like most shelf seas, a highly productive ecosystem.

This ecosystem is utilized by the above mentioned countries in many ways: Besides supporting one of the world’s most active fisheries (Ducrotoy et al. 2000), it is also exploited as source for sand, gravel, oil and gas (OSPAR 2009b, 2012). Additionally, more and more of its area is used for shipping, construction of wind parks, and also for tourism and recreation, while its shores are shaped by coastal engineering. Progressing industrialization and intensified agriculture due to a growing population lead to increased pollution of the North Sea with chemicals, nutrients and radionuclides (Ducrotoy et al. 2000). Although efforts have been made to reduce these impacts, progress is achieved only slowly (OSPAR 2008, 2009a).

1.2 Phytoplankton

The term phytoplankton includes both prokaryotic and eukaryotic microalgae. Due to their photosynthetic capability, these organisms constitute the basis of the marine food web. Although phytoplankton contributes only less than 1 % to global biomass, it is responsible for almost half of the global carbon fixation, thus being also an important element of the carbon cycle (Field et al. 1998). Commonly, phytoplankton is grouped according to its size into three classes: Microphytoplankton (20-200 µm), nanophytoplankton (2-20 µm) and picophytoplankton (0.2-2 µm). In the Open Ocean, the phytoplankton communities are primarily dominated by picoplanktonic cyanobacteria, especially Prochlorococcus sp. and Synechococcus sp. (Campbell et al. 1994; Partensky et al. 1999). In contrast, coastal seas are characterized by a large diversity of micro- and nanophytoplankton. The most important classes occurring in the North Sea are the Bacillariophyceae (diatoms) and the Dinophyceae (dinoflagellates). Additionally, also members of the Cyanophyceae, Cryptophyceae,
Prymnesiophyceae, Chlorophyceae, Euglenophyceae and Prasinophyceae are commonly found (Reid et al. 1990), but in either lower abundances or with a temporally or spatially limited importance. Phytoplankton biomass and community structure in the North Sea is not constant during the year, but follows a typical succession pattern, with different contribution of diatoms, dinoflagellates and other phytoplankton. However, due to the heterogeneous environment of the North Sea, the manifestation of this pattern may be different (e.g. between onshore and offshore regions).

Generally, in spring, nutrient availability in the whole water column is high due to remineralization processes which happened during winter, riverine nutrient input, increased mixing, and low utilization by phytoplankton (Brockmann et al. 1990). When light availability becomes sufficient, these conditions allow a rapid proliferation of diatoms, resulting in large so called phytoplankton “blooms”. This development is completed at the beginning of the summer, when nutrients (especially silicate, which is required by diatoms for their cell walls) become depleted. Under high light conditions and nutrient limitation, cell division is reduced and diatoms start to accumulate carbohydrates (Richardson and Cullen 1995) which (i) are denser than water, and (ii) are also partially exudated and foster the aggregation of diatoms and the formation of “marine snow”. Both mechanisms result in higher cell sedimentation rates (Smetacek 1985; Thornton 2002). Furthermore, the beginning stratification, at least at the more offshore areas, reduces the mixing depth of the water. The combination of an enhanced sinking rate and the reduced mixing depth results in the removal of diatoms from the water column. In contrast, at onshore regions, where the water column is continuously mixed and enriched with nutrients (e.g. by riverine input), also additional diatom blooms can occur, as well as blooms of small flagellates (mainly Phaeocystis sp.; Cadée 1986).

In addition to the aforementioned mechanisms, zooplankton grazers (e.g. heterotrophic flagellates and copepods) also foster the termination of diatom spring blooms and generally control phytoplankton population. Development of the grazers is time-shifted to the growth of their food source (Sommer 2005). Thus, at the onset of spring, zooplankton abundance is low because of limited food availability and low temperatures during winter time, and therefore, grazing pressure on phytoplankton population is usually low. But until the beginning of summer, the grazer population has increased due to the high availability of food, and the grazing pressure becomes high enough to contribute significantly to loss in diatoms and total phytoplankton. When their food source is depleted, also the grazer population declines, allowing the growth of a new phytoplankton community.

In contrast to the spring bloom community, summer phytoplankton is dominated by dinoflagellates (Reid et al. 1990; Hickel 1998). They do not have the requirement of silicate for cell wall formation and have also advantages in less mixed, more oligotrophic waters (Fogg 1991). Dinoflagellates can undergo relatively large diurnal migrations (Cullen 1985;
MacIntyre et al. 1997), which allows them to take up nutrients near the thermocline, and then move to well-lit, but nutrient poor waters. Furthermore, several taxa are mixotrophic, so they can also prey on other phytoplankton cells. During the autumn the cooling of the surface layer and increased wind speed breaks down the thermocline and the mixing depth of the water column increases again, supplying new nutrients to the water column. This often results in a second bloom, which is commonly less intense than the spring bloom (Reid et al. 1990). At the end of autumn, light availability is no longer sufficient supporting intense growth of phytoplankton. Generally, besides nutrient availability, grazing pressure, and light availability, also interspecific competition plays a role in controlling growth and community composition of phytoplankton. Furthermore, cell lysis caused by viral infection has been shown to be a factor in controlling phytoplankton populations (Suttle et al. 1990; Brussaard 2004; Rhodes et al. 2008), but the impact of and the regulatory mechanisms behind these biological interactions is currently less well understood.

1.3 Phytoplankton observation in the North Sea

Due to its aforementioned importance and in order to detect or keep track of changes, the North Sea was and still is subject of many scientific investigations. With regard to its ecology, not only human activities, but also natural variability can have a great impact. At least twice, in 1979 and 1988, regime shifts were detected for this region on the basis of sudden changes in abiotic and biotic parameters (Weijerman et al. 2005). Although the last details of the exact mechanisms are not understood, changes in the relationship between climate and ocean led to alterations on all trophic levels, from phytoplankton abundance, biodiversity and spring bloom formation to zooplankton abundance and fish stock size (e.g. Reid et al. 2001; Edwards et al. 2002; Beaugrand 2004; Wiltshire and Manly 2004). Because of its pivotal role as basis of the marine food web, at least some of the observed effects on higher trophic levels are probably a result of alterations in phytoplankton spatiotemporal distribution patterns and biodiversity. This emphasizes that a detailed understanding of this community is crucial for the understanding of the complete marine ecosystem. Furthermore, regular assessment of phytoplankton biodiversity is also important for conservation purposes. Biodiversity reflects the ability of an ecosystem or a community within this system to cope with environmental changes and the aforementioned human induced stress factors (Yachi and Loreau 1999). Loss in phytoplankton diversity might lead to a general reduction in biodiversity. In turn, this could result in limitations of the ecosystem services provided by the North Sea, apart from the fact that an intact North Sea has an inherent value independently from its benefits to humanity.

However, since phytoplankton distribution is often patchy and changes might be both short- and long-term, observations with a high resolution in time, space, and taxonomical
complexity are the basis for an understanding of phytoplankton ecology. The “Continuous Plankton Recorder Survey” (Reid et al. 2003) is the oldest time series in the North Sea with the highest spatial coverage. For the German Bight, several other time series are also available (Tillmann and Rick 2003), with the most important being Helgoland Roads due to its high sampling frequency and long history (Wiltshire et al. 2010). They provide valuable datasets by combination of phytoplankton observation with simultaneous measurements of other oceanographically relevant parameters like temperature, salinity or nutrient concentration. However, the maintenance of these time series requires a high effort, which often results in a limited coverage (temporally, spatially, or both) or a general limitation in the establishment of such projects. Furthermore, as community structure is mainly assessed by microscopic observation, emphasize of the series is mainly the microphytoplankton, while nanophytoplankton as well as picophytoplankton is less accessible by this method due to its smaller size.

For these reasons, phytoplankton research can be significantly improved by methods which reduce measurement effort and thus allowing a higher sampling frequency, as well as by approaches which overcome limitations of microscopy. In the last decades, a variety of methods have emerged addressing this issue, mostly based on either optical or molecular biological approaches. Especially in situ methods are of convenient use and allow a high measurement frequency or even continuous operation. Moreover, they have the potential to be used in unattended systems like the FerryBox (Petersen et al. 2011), which further reduces measurement effort and greatly increases spatial coverage when mounted in ships-of-opportunity. However, in situ methods are only available for relatively few biological parameters; especially obtaining taxonomical information still requires the use of laboratory based methods. A review about currently technical and methodological trends in phytoplankton research is given by Tillmann & Rick (2003), but important laboratory and in situ approaches were also introduced in the following chapter, starting with microscopy and chl-a determination via high performance liquid chromatography (HPLC) as traditional approaches used in most time series.

1.4 Methods for phytoplankton investigation

1.4.1 Microscopy and HPLC

Basically, two aspects are of importance when investigating phytoplankton: (i) The amount of its biomass in the water and (ii) its community structure. Probably the most direct approach for investigation of phytoplankton is the analysis of water samples by microscopy. Commonly, this is done by the method of Utermöhl (1958) using samples fixed with Lugol’s solution or glutaraldehyde and an inverted microscope. Cells can be counted and identified according to morphological features often down to the species level, which directly provides
information about the community structure to the researcher. In addition, biomass can be calculated by measuring cell dimensions under the microscope (Hillebrand et al. 1999) and apply appropriate conversion factors (Menden-Deuer and Lessard 2000). A benefit of this method is that the calculation can be made for the whole sample or separately for specific taxa of interest. Thus, microscopy is a method which in principle can provide data about both biomass and taxonomical composition, at least for cells visible and identifiable microscopically. However, it suffers from being time consuming and laborious, which limits its suitability for a larger number of samples. Furthermore, its use requires a certain taxonomical experience, and although the statistical counting error can be kept relatively small (Lund et al. 1958), its accuracy varies by experience and dedication of the researcher. Finally, for many scientific questions (e.g. only mapping biomass distributions), it is by far too detailed. In this respect, other approaches are more suitable, which are explained in the following.

The concentration of chlorophyll-a (chl-a) in the water was introduced early as an indicator for phytoplankton biomass (Harvey 1933; Mineeva 2011), because it is the main pigment of photosynthesis and therefore abundant in every phytoplankton cell. Moreover, it allows the discrimination of phytoplankton biomass from detrital or heterotrophic biomass. Initially, chl-a concentration was estimated by comparison of water color with different concentration of colored solutions (Harvey 1933). Later on, pigments were extracted from the water and measured spectrophotometrically (Mackinney 1941). A derivative of this technique is the high performance liquid chromatography (HPLC), which allows not only the quantification of major pigment groups, but also an accurate separation of the single pigments. This is important, because there are a variety of different chlorophylls, chlorophyll degradation products as well as accessory or protective pigments abundant in the phytoplankton (Jeffrey et al. 1999). Since some of these pigments can (with exceptions) be attributed to certain phylogenetic groups, HPLC-based pigment data have also used for the identification and quantification of these phytoplankton groups (Mackey et al. 1996; Lewitus et al. 2005). HPLC pigment separation is based on the different polarities of the phytoplankton pigments according to their different chemical structure. Generally, the pigments feature a relatively weak polarity. In a first step, bulk pigments were extracted from a sample using an appropriate medium like acetone or methanol. After being desolved in a medium of given polarity (“liquid phase”), this mixture is applied on a column filled with alkyl-chain modified silica. This “solid phase” has a lower polarity compared to the liquid phase, thus, the solved pigments were bound to the column. By continuously decreasing the polarity of the liquid phase by changing its composition, the pigments were released from the solid phase according to their polarity; most polar pigments were released first, followed by the non-polar pigments. Currently, HPLC is considered to be the most accurate method for direct chl-a determination, and thus, this method is widely used in biological oceanography (e.g. Gibb et al. 2000; Higgins and
Mackey 2000; Irigoien et al. 2004; Yao et al. 2010; Mendes et al. 2011). However, the relation between chl-a and biomass is not constant, but varies to a certain degree with species composition and physiological conditions of the cells (Banse 1977; Hallegraeff 1977; Falkowski and Owens 1980; Geider 1987; Jiménez et al. 1987; Llewellyn and Gibb 2000). Furthermore, both microscopy and direct chl-a determination via HPLC are restricted to discrete samples. Hence, depending on sampling effort, such analyses often have a limited spatial and temporal coverage, providing merely a snapshot of phytoplankton situation.

1.4.2 Techniques based on optical proxy data

**Fluorescence.** Instead of directly measuring chl-a concentration in the water, an often used approach is its estimation via fluorescence. Light energy which is absorbed by the light harvesting complexes of the chloroplast excites the chlorophyll molecules in the reaction centres. If it cannot be utilized in photochemistry, the energy has to be dissipated; otherwise, the photosystem might be subject to photooxidative damage. Dissipation can take place either as heat or as the emission of photons. The latter process is called chlorophyll fluorescence, by which light is emitted mainly in the red region of the spectrum around 680 nm. In part, fluorescence depends on the content of chl-a in the cell, and it has been shown that the fluorescence signal can be calibrated against HPLC measurements of this pigment (Wiltshire et al. 1998). However, the calibration is not stable over longer time periods and for different environments. The reason is that fluorescence is a parameter which is strongly linked to the physiology of the cell, and therefore there are various factors influencing the signal (Falkowski and Kiefer 1985; Cunningham 1996). Light is not only absorbed by chlorophyll, but also by accessory pigments. They channel this energy to the chlorophyll, therefore increasing the mean optical cross section of the light harvesting complexes. Due to this additional energy input, concentration of these pigments also affects chl-a fluorescence. In turn, the concentration of these pigments is determined on the one hand by species and on the other hand by acclimatization processes to long term light conditions (Falkowski and Kiefer 1985; Cunningham 1996; Chekalyuk and Hafez 2011). Additionally, light absorption efficiency of phytoplankton pigments is determined by the relationship between cell size and intracelular pigment concentration, the so called “pigment packaging effect” (Morel and Bricaud 1981). Short term acclimatization processes (e.g. non-photochemical quenching via the xanthophyll cycle; Szabo et al. 2005) can also influence the variable fluorescence yield and the detectable signal. Finally, the general status of the cell, e.g. nutrient limitation, is discussed affecting chl-a fluorescence (Kiefer 1973; Parkhill et al. 2001; Kruskopf and Flynn 2006). Despite their variability, fluorescence measurements are commonly used in biologic oceanography for mapping chl-a distributions horizontally and vertically (e.g. Uehlinger 1985; Lazzara et al. 1996; Seppälä and Balode 1998; Wiltshire et al. 1998; Petersen et al. 2008).
This is mainly the result of the convenience and rapidity of the measurements, but also of the large number of commercial instruments available on the market (Moore et al. 2009) from which many provide the opportunity to be used in situ and/or continuously. Besides being used for the mere estimation of chl-a concentration in the water, fluorescence measurements are also used for identifying phytoplankton groups (Yentsch and Phinney 1985; Beutler et al. 2002; Millie et al. 2002). With exceptions, the members of each major phytoplankton group can be characterized by a specific set of pigments. Thus, absorption-emission spectra created for these groups by measuring chl-a fluorescence at different excitation wavelengths results in some kind of spectral “fingerprints”. On their basis, proportions of different groups to a natural assemblage of phytoplankton can be calculated (Beutler et al. 2002).

Absorption. An alternative method for chl-a determination via an optical proxy is the measurement of water constituent absorption. Besides chromophoric dissolved organic matter (CDOM) and non-pigmented particular material, the main components that determine light absorption in water are –besides water itself– phytoplankton pigments (Kirk 1994). Chl-a exhibits an absorption maximum in the blue region of the visible spectrum and a second, smaller one in the red region. Other pigments, like chl-b, chl-c, carotenoids and phycobilines have their maxima mostly in the region between the both absorption maxima of chl-a, enabling the cell to use also light energy from this spectral region (“green gap”) for photosynthesis. Because absorption is a more physical parameter of the cell, it is less susceptible for physiological changes. The proportionality between absorption coefficients measured in the regions of the absorption maxima of chl-a and the concentration of this pigment in the water are basically influenced by only two factors: The pigment packaging effect and the presence of other pigments which also absorb to a certain degree at these wavelengths (Kirk 1976; Hoepffner and Sathyendranath 1992). When measuring in the red absorption maximum, the latter factor is negligible to a large extend, as only few pigments absorb there. Similar to fluorescence measurements, absorption measurements can also not only be used for chl-a determination, but also for the discrimination of phytoplankton groups in the water (Hoepffner and Sathyendranath 1991; Johnsen et al. 1994; Millie et al. 2002). Basis for such calculations are variations in the absorption spectra in these groups according to differences in the featured pigments. There are several instruments available for the determination of absorption in seawater (Pegau et al. 1995; Moore et al. 2009), many of them suitable for continuous or in situ operation. However, measurement is complicated due to two issues: First, the concentration of absorbing material in seawater is usually very low (with the exception of certain coastal regions), and high sensitive measurements are required for a detection. Second, particles like detritus, suspended minerals and phytoplankton cells do not only absorb light, but also scatter light. Because not all of these scattered photons reach the detector, this leads to an overestimation of light loss and therefore absorption. The
first problem can be solved by using relatively large devices with a long optical path length for obtaining sufficient sensitivity, or by concentrating the sample via filtration ("filter pad method"; Yentsch 1962). Concerning scattering-induced light loss, often empirical correction procedures were applied (Roesler 1998; Finkel and Irwin 2001). Recently, systems using integrating spheres like the ICAM (integrating cavity absorption meter; Fry et al. 1992; Pope et al. 2000) and the technically simpler PSICAM (point source integrating cavity absorption meter; Kirk 1997; Lerebourg et al. 2002; Röttgers et al. 2005) have shown to be powerful tools for absorption measurements. Due to their design, they provide a long optical path length although being relatively small, and scattering effects on the measurements are negligible. Furthermore, in contrast to other systems, they determine the absorption spectra in the whole range of the visible spectrum. This additionally provided information can be used for the aforementioned determination of phytoplankton groups. Effort has also been made to adapt systems based on integrating cavities for continuous operation (Musser et al. 2009), but these systems have not been applied in the field yet.

**Automatic counting techniques.** These methods emphasize the rapid gain of information about phytoplankton cell numbers and size distribution, while taxonomical classification is limited. One of the first devices for the automated counting of suspended particles was the Coulter Counter (Coulter 1956). It was based on the conductivity change of a solution between two electrodes when a particle passes by. This method is actually not an optical one, and is mentioned here only briefly for the sake of completeness. Furthermore, since it is not able to distinguish between types of particles, the Coulter Counter is only of limited use for the analysis of environmental samples with respect to phytoplankton. However, another automatic counting technique based on the optical properties of the particles has been proven valuable for phytoplankton research over the last decades: Flow cytometry is a method originally developed for medical research but has also been widely used in the field of marine microbiology (Phinney and Cucci 1989; Dubelaar and Jonker 2000; Veldhuis and Kraay 2000; Wang et al. 2010). Basically, it works similarly to the Coulter Counter, but relies on the alteration of a light beam by a passing particle. A schematic drawing of the instrument is provided in figure 2.
Fig. 2: Schematic setup of a standard flow cytometer. Picture modified after Dubelaar and Jonker (2000)

Samples containing live or fixated cells are confined in a thin water jet which forces the particles to align in a row. The velocity of the water stream is adjusted in such a way that the particles pass one or more lasers individually. Thus, information can be obtained and recorded for every single particle in the sample. Light beam attenuation indicates a particle in the water stream, thus allowing enumeration. Forward- and sidescatter of the particle is also determined and used e.g. to deduce its size. Furthermore, particle fluorescence is measured, which provides additional information about the particle type. Since phytoplankton cells are autofluorescent due to the presence of chl-a and other pigments, it allows the discrimination between phytoplankton and non-living particles. In addition, cells can be treated with fluorescence dyes in advance to distinguish between cell types or physiological condition, e.g. between living and dead cells (Caron et al. 1998). Of course, this requires the selection of proper excitation and detection wavelengths. When analyzing unstained cells, fluorescence is measured mostly in the red and the orange region of the spectrum. As mentioned before, red fluorescence results from chl-a and can therefore be used as biomass-proxy, while orange fluorescence is caused by the accessory pigment phycoerythrin. Being a specific pigment for these groups, it can be used as proxy for the presence of cyanobacteria (Olson et al. 1985) and cryptophytes (Li and Dickie 2001). Another attractive feature of flow cytometry is the possibility of cell sorting according to their optical features. This offers the opportunity to conduct physiological or biochemical experiments on a certain group or type of cells. Commonly available commercial flow cytometry devices allow the analysis of a large fraction of the phytoplankton, especially the...
important and less investigated pico- and nanophytoplankton. Nevertheless, they are limited to a cell size below approximately 150 µm due to restrictions like e.g. the size of the water jet orifice, disturbances of the water jet by large particles, fragmentation of larger chains or settling of large cell in the tubes of the system (Dubelaar and Jonker 2000). Thus, in order to extend the usage of flow cytometry for phytoplankton research by increasng the detectable size spectrum, effort has been made to adapt existing systems or design new devices (Dubelaar et al. 1989; Cavender-Bares et al. 1998; Olson et al. 2003). However, as particle number is commonly inversely correlated with size (Chisholm 1992), it takes considerably longer and requires more sample water to count a statistically meaningful number of larger cells. Another modification was the adaptation of flow cytometry for in situ operation (Dubelaar and Gerritzen 2000). Furthermore, in order to increase its value for taxonomical investigations, Sieracki et al. (1998) combined flow cytometry with in situ plankton video imaging, providing additionally images of individual cells in the size range of microphytoplankton. These images can be used for an identification of the cells as well as for calculation of biovolume. However, especially chain forming cells are a large error source in these applications (overestimations in biomass ranging from 20-100 %; Álvarez et al. 2012). Thus, such methods might be of limited use in the North Sea, where diatoms are the dominant group which includes many chain forming species.

### 1.4.3 Molecular sensing

In recent years, a variety of molecular methods have emerged for the investigation of phytoplankton, as it is summarized by de Bruin et al. (2003). They have several advantages compared to traditional techniques like microscopy: The analyses are relatively rapid and require no morphologic and less taxonomic expertise from the researcher. Since identification relies on nucleic acid sequences and no longer on morphological characteristics, even organisms which lack such features can be taxonomically classified (Ebenezer et al. 2012). This also enables the differentiation of organisms below the species level (strains, ecotypes). Moreover, hardly visible organisms (e.g. from the picophytoplankton) can be investigated in the same way as larger ones. Thus, the taxonomical resolution of these techniques is potentially high.
Valuable phylogenetic markers for molecular taxonomical investigations are ribosomal RNAs (rRNA) or their respective genes (Amann and Ludwig 2000; Moon-van der Staay et al. 2000; Díez et al. 2001; Moon-van der Staay et al. 2001). Because rRNAs are integrative parts of the ribosomes and therefore required for protein biosynthesis, they are ubiquitous in eukaryotic cells. Their genes are organized in an operon the structure of which (for eukaryotes) is shown in figure 3. It codes for the 18S rRNA required for the small subunit of the ribosome, as well as for the 5.8S and the 25S, which are used in the large subunit. The 5S rRNA is also a component of the large subunit, but it is transcribed separately, thus is in a strict sense no component of the actual operon. The regions between the coding genes (the rDNA) are the internally transcribed spacers (ITS). Taxonomical analyses often target the 18S rRNA or its respective gene, the 18S rDNA, because its sequence is more conserved than that of the other ribosomal genes, but has still also regions of certain variability. Hence, it provides the possibility for investigations on different taxonomical levels. An additional advantage of using ribosomal genes for phylogenetic investigations is the availability of huge databases for sequence comparison (Amann and Ludwig 2000). Although the number of molecular techniques is high and will probably increase in the future, their majority can be basically sorted into three groups: (i) Fingerprinting techniques, (ii) hybridization-based approaches, and (iii) sequencing techniques. In the following, a short overview about the measurement principles and the kind of provided information of these groups is given.

**Fingerprinting techniques.** These techniques include e.g. denaturing gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP) or automated ribosomal intergenic spacer analysis (ARISA). They are based on differences in the behavior of DNA sequences during electrophoretic separation, depending on their length or base sequence. Since both can be variable between organisms, the result of sample analysis is a band pattern which is related to the organisms the sample contains. It can be used to compare their diversity under the reasonable assumption that similar patterns indicate similar communities. However, these approaches are not suitable for directly assessing biodiversity.
in a given sample because of various reasons: The fragments visible represent not necessarily species, but, depending of the degree of difference in the nucleic acid sequence, taxa from various levels. For this reason, commonly the term “operational taxonomic unit” (OTU) is used when referring to taxa obtained with these methods. A second factor limiting the use of fingerprint methods for direct diversity determination is that their resolution is limited by their ability in discriminating fragments. Thus, often more than one taxon contributes to a fragment visible in the analysis (Bent et al. 2007; Gillevet et al. 2009). Finally, rare species are prone to be missed due to the low contribution of their DNA to total DNA in a sample (Liu et al. 1997). Nevertheless, although not yet automatable, fingerprinting approaches are valuable tools for providing a fast qualitative overview about communities and enable the detection of differences or possible changes therein. In this study, ARISA has been chosen as fingerprint method. It is based on length differences in the sequence of PCR-amplified ITS1 regions of the eukaryotic ribosomal operon between taxonomical groups. Although frequently used to study prokaryotic marine diversity (Graham et al. 2004; Danovaro et al. 2006; Kovacs et al. 2010), it is less established for eukaryotic communities (Fechner et al. 2010; Wolf et al. 2013).

**Hybridization based approaches.** These methods take advantage of oligonucleotide probes for the detection of specific sequences. The probes have to be designed in advance with a sequence complementary to their respective target. Molecular probes can be used for various purposes, from dot blots to the analysis of environmental samples, as it is summarized by Amann et al. (1995). For the investigation of marine microbial diversity two methods using molecular probes are especially important: Fluorescence in situ hybridization (FISH) and microarrays. FISH is based on the linkage of fluorescently labeled oligonucleotide probes to cells according to the abundance of specific gene sequences. This allows the visualization and differentiation of otherwise indistinguishable cells, for example in a microscopic sample (Simon et al. 2000) or during flow cytometry analysis (Simon et al. 1995). However, the use of this method is limited by the number of distinguishable fluorescence dyes. Furthermore, if microscopy is involved, the effort in analyzing the samples remains quite high. In contrast, microarrays have the potential to provide a more rapid overview over a phytoplankton community (Kochzius et al. 2007). Originally invented for gene expression studies (de Bruin et al. 2003), this method is designed for detecting a high number of sequences in one given sample at once. Target of the molecular probes can be either RNA or DNA; as mentioned before, often 18S rRNA or rDNA is used. The oligonucleotide probes are fixed on a solid surface, the microarray chip (often also called “phylochip” when used for taxonomical investigations). Restrictions in the number of simultaneously detectable species are given only by the size of the chip and the effort emerging from the design of the molecular probes. When applied on rDNA, the principle of
the method as applied in marine microbiology is as described in the following: After genomic DNA is extracted from a water sample, rDNA is amplified and the PCR-products are given on the chip. The complementary sequences hybridize to the molecular probes, while others were removed. Bound DNA is commonly detected by fluorescence staining or the use of sandwich hybridization techniques, which includes the hybridization of an additional probe carrying the detectable signal to the target sequence (Ranki et al. 1983; Rautio et al. 2003; Metfies et al. 2005; Diercks-Horn et al. 2011). For the application of the microarray on rRNA, the method is similar, but the amplification step can be omitted. Instead, precautions have to be taken with respect to the higher fragility of the rRNA. The presence of a certain taxon can be determined from the presence of a signal from the corresponding position on the chip. The intensity of the fluorescence signal is proportional to the amount of bound nucleic acid, thus, in principle, it can be used to estimate the abundance of the respective species in the sample. Microarrays have been used in marine microbiology for the analysis of both prokaryotic (Lau et al. 2013) and eukaryotic phytoplankton communities (Gescher et al. 2008; Metfies et al. 2010). Recently, attempts have also been made to adapt microarrays for the use in semi-automated devices (Diercks et al. 2008; Diercks-Horn et al. 2011).

**Sequencing.** The highest degree of information about a phytoplankton community is obtained by directly assessing gene sequences of its members and comparing them with already available genetic data (de Bruin et al. 2003). Due to this level of detail, sequencing approaches require a high effort. Because emphasis of the present study was the evaluation of relatively rapid methods for phytoplankton community investigation, a sequencing approach was not included. However, for the sake of completeness, the principles are described briefly. Traditional, sequencing involves extraction of DNA from a sample and amplification of the gene of interest, followed by cloning the obtained fragments in vectors to create a DNA-library (Amann et al. 1995). This allows separation of the sequences from different species present in the sample. Afterwards, the incorporated DNA has to be extracted again from the single clones, purified and amplified via PCR. The last step is the sequencing itself, for which different methods are available, but most of them are derivatives of the chain-termination method of Sanger (Sanger et al. 1977). Currently, newly emerging next generation sequencing techniques like 454-pyrosequencing (Margulies et al. 2005) increase rapidity this approach and may also facilitate a routinely use of sequencing in phytoplankton ecology in the future.
2. Aims and outline of the thesis

2.1 Aims of the thesis

Investigation of phytoplankton ecology is difficult compared to analyses of terrestrial plant communities, because the marine environment lies outside of our direct experience and the target organisms are of microscopically size. Furthermore, acquiring samples is relatively costly, often limiting the spatial and temporal resolution of conducted research. However, knowledge about the phytoplankton community is important with respect to an understanding of the functioning of the marine ecosystem, since microalgae are the primary producers in world’s oceans. Against this background, different methods have evolved targeting various aspects of this community, from bulk biomass and size class distribution to the presence of certain phylogenetic groups and information about specific taxa. All approaches have certain advantages and disadvantages and differ in effort. In the present study, a selection of these methods is used for the investigation of North Sea phytoplankton during several cruises in the German Bight. The chosen methods are chl-a determination via HPLC, chl-fluorescence and water constituent absorption measurements, as well as microscopy, flow cytometry, ARISA and DNA/RNA-microarrays. Thus, this selection includes direct approaches for phytoplankton biomass determination and community structure analysis (HPLC and microscopy), as well as indirect approaches based on the measurement of optical proxies and molecular sensing.

Aim of the work is the evaluation of the above mentioned methods with respect to the routine assessment of phytoplankton biomass and community structure in a heterogenous, coastal environment. Emphasis is laid on the comparison of relatively new, rapid techniques (like chl-a determination by absorption measurements and community analyses using microarrays) with well-established methods (HPLC, chl-a fluorescence measurements, and microscopy). In this context, also a promising new method for absorption determination is adapted for continuous operation and included in the evaluation. Furthermore, phytoplankton biomass distribution and community structure in the German Bight is characterized using these methods, and the results are interpreted in the light of supplementary oceanographic data.

Basically, two questions are going to be answered:

1. What kind of additional information about the phytoplankton community is provided by the new approaches, and how is their performance compared to the traditional ones?

2. Which combination of methods can be recommended for an efficient, routine observation of North Sea phytoplankton with a simultaneous reduction in effort?

Finally, suggestions for further work are going to be given.
2.2 Outline of the thesis

In **Manuscript I** of this thesis, effort has been made to develop a method for the continuous measurement of spectral absorption coefficients. For this purpose, the point-source integrating cavity absorption meter (PSICAM), a laboratory-based system for absorption measurement using an integrating sphere, was modified for continuous operation and tested in the field for the first time. The manuscript discusses the performance of this flow through-PSICAM (ft-PSICAM) and also potential error sources. Furthermore, the determination of chl-a and total suspended matter (TSM) concentration by means of total water constituent absorption coefficients is tested.

A general evaluation of absorption measurements as an alternative approach to the traditional determination of these bulk parameters by fluorescence and turbidity is given in **Manuscript II**. It was done by the comparison of linear regressions between both parameters and their respective optical proxies. This has been done for both manual and continuous measurements. Furthermore, the progression of chl-a and TSM concentrations calculated on the basis of continuously obtained optical proxy data were compared.

**Manuscript III** evaluated the results of a DNA-microarray (the so-called “phylochip”) with respect to the results of non-molecular methods, namely cell counts, pigment data and fluorescence measurements obtained by flow cytometry. The comparison was carried out both in qualitative and quantitative terms. Furthermore, data obtained from a RNA-microarray carried out by an automated biosensor were correlated with microscopic cell counts.

In **Manuscript IV**, the results obtained from the various methods were used for a characterization of phytoplankton in terms of biomass distribution and community composition in the whole German Bight. The analysis was carried out on the basis of data obtained on several cruises in different seasons. Focus was the spatial and seasonal variation in the different parts of the community.
3. Manuscripts

3.1 List of manuscripts

I. Jochen Wollschläger, Maik Grunwald, Rüdiger Röttgers, and Wilhelm Petersen
Flow-through-PSICAM: A new approach for determining water constituents absorption continuously
Published in: Ocean Dynamics 63 (7), 761-775. The final publication is available at http://link.springer.com/article/10.1007/s10236-013-0629-x

II. Jochen Wollschläger, Rüdiger Röttgers, Wilhelm Petersen, and Karen H. Wiltshire
Evaluation of different optical methods for the determination of chlorophyll-a and total suspended matter
Submitted to: Journal of Experimental Marine Biology and Ecology

III. Jochen Wollschläger, Anja Nicolaus, Karen H. Wiltshire, and Katja Metfies
Assessment of North Sea phytoplankton via molecular sensing – A method evaluation
Submitted to: Journal of Plankton Research

IV. Jochen Wollschläger, Katja Metfies, Karen H. Wiltshire, and Wilhelm Petersen
Analysis of phytoplankton distribution and community structure in the German Bight with respect to the different size classes
Prepared for submission
3.2 Statement of the author’s contribution to the manuscripts

**Manuscript I**
The experiment was planned together by all persons mentioned. Development of the ft-PSICAM has been done by Maik Grunwald while its use in the field was performed by the author. Conventional PSICAM measurements were done by Rüdiger Röttgers. Analysis of the data and writing of the manuscript was done by the author with assistance of Maik Grunwald and Rüdiger Röttgers.

**Manuscript II**
The experiments were planned together by all persons mentioned. The author did the data analyses and the ft-PSICAM measurements. Conventional PSICAM measurements were done by Rüdiger Röttgers, while Wilhelm Petersen provided fluorescence and turbidity measurements from the FerryBox. The author wrote the manuscript, which was reviewed by Karen Wiltshire.

**Manuscript III**
The experiments were planned together with Katja Metfies and Karen Wiltshire. Basically, the author performed the experimental work, but additional measurements with the microarray were also done by Anja Nicolaus. Data were analyzed by the author. Biosensor measurements were made by Katja Metfies. The manuscript was written by the author and Katja Metfies.

**Manuscript IV**
The experiments were planned together with all mentioned persons. Wilhelm Petersen provided the FerryBox data. The author did most of the experimental work and the data analysis, and also wrote the manuscript.
3.3 Manuscript I

Flow-through-PSICAM: A new approach for determining water constituents absorption continuously

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Abstract

Determination of spectral absorption coefficients in seawater is of interest for biologic oceanographers for various reasons, but faces also several problems, especially if continuous measurements are required. We introduce the flow-through point-source integrating cavity absorption meter (ft-PSICAM) as a new tool for the continuous measurement of spectral absorption coefficients in a range of 400-710 nm. A description of the system is given and its performance in comparison with a conventional PSICAM has been evaluated on two cruises in 2011 in the southern part of the North Sea (German Bight). Furthermore, factors influencing the measurement are discussed. When comparing the data of both systems, a good linear correlation has been found for all wavelengths ($r^2 > 0.91$). Deviations between systems were different with respect to the wavelength examined with slopes of linear fits between 1.1 and 1.65 and offsets between -0.1 and 0.01, with the higher values at shorter wavelengths. They were caused mainly due to contamination of the flow-through system during operation by phytoplankton particles.

Focus was also laid on the measurement of chlorophyll-a concentrations ([chl-a]) and total suspended matter concentrations ([TSM]) on the basis of absorption coefficient determination. For this, appropriate relationships were established and [chl-a] and [TSM] values were calculated from the relevant ft-PSICAM absorption coefficients. Their progression matches well with the progression of fluorescence and turbidity measurements made in parallel. In conclusion, the ft-PSICAM is successful in measuring spectral absorption coefficients continuously and resolving relative changes in seawater optical properties.

Keywords: Point-source integrating cavity absorption meter (PSICAM), absorption, continuous measurement, chlorophyll-a, total suspended matter, North Sea
1. Introduction

Materials in seawater which absorb light in the visible part of the light spectrum are, apart from the water itself, the chromophoric dissolved organic matter (CDOM or gelbstoff) as well as living and nonliving particles, i.e. phytoplankton particles containing different kinds of pigments and nonphytoplankton particles (detritus, mineral particles and heterotrophic organisms; Bricaud et al. 1998; Babin et al. 2003). The corresponding spectral absorption coefficients are called $a_{\text{cdom}}$ (CDOM) and $a_p$ (particles), the latter subdivided into $a_{\Phi}$ for phytoplankton pigments and $a_{\text{nap}}$ for the non-phytoplankton part of particle absorption.

Thus, determination of the spectral absorption coefficients of seawater is of importance, because they contain information about all these constituents. For example, $a_{\Phi}$ can be used for the calculation of phytoplankton productivity as it allows to draw conclusions about the concentration of chlorophyll-a in a sample (Sathyendranath et al. 1987; Bricaud et al. 1998). Eventually, it can also provide information about phytoplankton taxonomy and size classes based on the different absorption properties of pigments abundant in different groups (Sathyendranath et al. 1987; Millie et al. 2002; Devred et al. 2011). Concentration and distribution of CDOM and non-phytoplankton particles (provided by $a_{\text{cdom}}$ and $a_{\text{nap}}$) are important factors governing the light regime in the water (Bowers and Binding 2006; Nelson and Siegel 2013) and therefore influencing photosynthetic activity of the phytoplankton. Furthermore, information about $a_{\text{cdom}}$ and $a_{\text{nap}}$ are of interest for validation and calibration of remote sensing data or for comparison with model calculations. This is especially important in coastal regions, where both parameters do not necessarily covary with chlorophyll-a concentration (Babin et al. 2003; Tilstone et al. 2012). Concerning the measurement of spectral absorption coefficients there are two major issues: On the one hand, the measurements should be as exact as possible. On the other hand, they should be provided in a high temporal and spatial resolution, since trends and relative changes can only be inadequately resolved by point measurements. Furthermore, data obtained during operational measurements are considered to be useful for detecting long-time changes in phytoplankton biomass and composition, respectively, as well as single events like harmful algae blooms (HABs) (Cullen et al. 1997).

The measurement of spectral absorption coefficients in seawater deals with several difficulties, and different measurement techniques have evolved to encounter these problems: Simple photometric measurements of seawater are normally not useful, because of the usually low concentration of absorbing material in the sample. Additionally, loss of photons due to scattering on particles in the sample resembles a higher absorption than there actually is (Kirk 1994). A more elaborated, commonly used, and often modified measurement technique is the filter pad method (Yentsch 1962). Particles in a sample are concentrated on a glass fiber filter and filter and filtrate are measured separately to get $a_p$.
and $a_{\text{cdom}}$, respectively. The use of an integrating sphere even allows the collection of scattered photons (Maske and Haardt 1987). Nevertheless, using this method the optical path length is increased due to light reflections in the filter, and the absorption values have to be corrected to correspond to the absorption of the same amount of phytoplankton being in solution. The used correction factor ($\beta$) is still a source of uncertainty and controversy (Roesler 1998; Finkel and Irwin 2001). Finally, the different variants of the filter pad technique are laboratory-based methods which are not suitable for continuous or in situ measurement of absorption coefficients in seawater. For this purpose, different devices have been developed in the last years: Reflective tube absorption meters (RTAMs) like the ac-9 and ac-s from WETLabs (Pegau et al. 1995; Twardowski et al. 1999; Slade et al. 2010), tethered optical profiling systems (TOPS), isotropic point sources (IPS), and compound radiometers. A comparison between these different systems is given by Pegau et al. (1995). A flaw concerning these methods is the remaining need for scattering correction. Laboratory integrating cavity measurement systems like the ICAM (Elterman 1970; Fry et al. 1992; Pope and Fry 1997) and the technically simpler PSICAM (Kirk 1997; Leathers et al. 2000; Lerebourg et al. 2002; Röttgers et al. 2005) overcome the problem of particle scattering by introducing the sample in a diffuse light field set up in an integrating tube or sphere, respectively. Scattered photons cannot go lost, but are reflected from the cavity wall until they are absorbed or reach the detector. Additionally, the optical path length is increased drastically by the reflective walls allowing the measurement of very clear waters with a relatively small device. Theoretical considerations and first experimental results concerning the construction of a flow-through device on the basis of an ICAM are given by Gray et al. (2006) and Musser et al. (2009), but the device has -to our knowledge- not been tested in the field yet. In this contribution, we present a flow-through device designed on the basis of the PSICAM (ft-PSICAM) which combines the ability of measuring spectral absorption coefficients without the influence from particle scattering with the possibility of obtaining high-resolution data on a temporal and spatial scale. It is designed to be connected to a FerryBox (Petersen et al. 2011) which collects other parameters important for the correction of the absorption coefficients like salt and temperature. Continuous, on-line measurements of total light absorption by all water constituents are performed with the ft-PSICAM in the field with a focus on the determination of $[\text{chl-a}]$ as concentration of chlorophyll-a and $[\text{TSM}]$ as concentration of total suspended matter. First, the performance of the ft-PSICAM is assessed by a comparison with measurements of discrete samples taken during stations using a controlled and continuously calibrated conventional PSICAM. Second, the necessary factors to convert the measured absorption
coefficients to [chl-a] and [TSM] are determined by discrete measurements of these parameters in parallel to the continuous measurements.

2. Materials and methods

2.1 Study area

All data were collected during two ship cruises with the R/V “Heincke” conducted in April and June 2011 (Fig. 1). The German Bight is affected by different water masses: (i) The southern and eastern part along the coasts is influenced by runoff from the major rivers Ems, Weser, and Elbe as well as by freshwater discharge from the hinterland via the Wadden Sea, while (ii) the offshore areas have more open ocean characteristics. Therefore, the study area provides a broad spectrum with strong inshore - offshore gradients of relevant parameters like TSM and CDOM. According to HPLC and gravimetric measurements conducted in this study (see below), concentrations of chl-a reached from 0.004 to 16 µg L-1, those for TSM were in a range from nearly 0 to 34 g m-3, just to give an impression of the diversity of the conditions.

2.2 Discrete measurements of chlorophyll, TSM, and absorption

During stations water samples were taken from the surface at a depth close to that of the water intake of the FerryBox system (ca. 4 m) by using a carousel water sampler (SBE 32, Sea-Bird Electronic, Inc.) equipped with seven 9-L sampling bottles (Hydrobios, Germany). The water samples were transferred from the sampling bottles into 10 L-PE containers and processed immediately for salinity (measured with WTW portable conductivity sensor), [chl-a], [TSM], and conventional PSICAM measurements.

2.2.1 Chlorophyll-a

Samples (1-5 L) for an analysis of [chl-a] were filtered onto 47 mm pre-combusted GF/F filters (Whatman, USA), flash-frozen in liquid nitrogen and stored at -80 °C. Later in the laboratory the pigments in these sample filters were extracted with 100 % acetone at – 30 °C for 24 h. The extract was cleaned by passing it through 0.2 µm syringe filters (Spartan, A13) and transferred into 2-mL glass-vials. The pigments were separated and analyzed by high performance liquid chromatography (HPLC) using a HPLC system from JASCO (Japan) and the method described in Zapata et al. (2000).

2.2.2 Suspended matter

The particle mass concentration was determined by filtration of large volumes (1-8 L) of the samples on pre-combusted, pre-washed and pre-weighted GF/F filter (ø 47 mm, Whatman)
as described by van der Linde (1998). Some additional precautions were taken to reduce the influence of remaining salt in the filter (Stavn et al. 2009): (i) Before filtration, the filters were wetted with purified water to fill the rim of the filter, reducing the amount of salt that cannot be washed out after filtration by subsequently applied purified water. (ii) For each sample, three filters with different sample volumes were prepared. This was used to determine the salt offset from a linear correlation of the particle mass on the filter and the sample volume. A constant salt offset (0.1-0.5 mg) that is independent on the sample volume was observed by a bias of the linear regression line. This offset was subtracted from the measured mass and the mass concentration (in mg L⁻¹) calculated afterwards.

2.2.3 Single-point absorption measurements (PSICAM)

At the different stations, absorption measurements of surface seawater were made by a conventional PSICAM as described at Röttgers et al. (2005) and Röttgers et al. (2007). The only change was the usage of a Hamamatsu C10083CA mini spectrometer (Hamamatsu Photonics, Japan). For details of the theoretical principles of a PSICAM and the equations for the calculation of reflectivity and absorption coefficients we refer to Kirk (1997), Leathers et al. (2000) and Lerebourg et al. (2002).

The PSICAM was calibrated once a day in triplicate using purified water (>18.2 MOhm) and nigrosine solution (an azine- and azo dye with relatively homogenous absorption coefficients over the visible spectrum) as described in Röttgers et al. (2005). The cavity was cleaned using a 0.1 % NaOCl (Riedel de Haën, Germany) solution for 15 min followed by rinsing of the cavity with purified water. The spectral absorption coefficients of the nigrosine solutions were measured in a liquid waveguide capillary cell (LWCC, path lengths: 0.5 and 2 m, WPI, USA) against purified water as a reference. All solutions with exception of those used for cleaning were adapted to the same (ambient air) temperature in a water bath prior to the measurements. Because the nigrosine solution is not stable over a longer period of time, for each calibration a fresh solution has been prepared.

All PSICAM measurements were performed in triplicate. Previous to each sample, a reference measurement was made using purified water. Subsequent to the sample measurement the PSICAM was rinsed with purified water to remove the residues of the sample from the cavity and the reference was measured again. Temperatures of both reference and sample were recorded and used together with the determined salinity of the sample to correct their influence on the pure water absorption as described in Röttgers & Doerffer (2007).
2.3 Continuous measurements

During the entire cruises (i.e. both at stations and during transects), continuous measurements of chlorophyll-a fluorescence and turbidity have been performed by the use of a FerryBox system like it is described in Petersen et al. (2011). Its water intake was in the moon pool of the research vessel in around 4 m depth. Furthermore, continuous determinations of spectral absorption coefficients were performed in parallel using the ft-PSICAM. Sample water was supplied via a bypass from the FerryBox.

2.3.1 Continuous absorption measurements (ft-PSICAM)

The design of the ft-PSICAM is similar to the design of the conventional PSICAM used by Röttgers et al. (2005), but with a cavity made from PTFE which has been equipped with water in- and outlets to enable flow-through operation. It was installed into a setup of pumps, valves and tubes for water supply, drainage and supply of other fluids needed for the experiments. A detailed overview of the assembly is given in figure 2. For illumination of the cavity a 150 W IT 3900 lamp (Illumination Technologies, USA) was used. Light leaving the cavity was detected by a Ramses UV/VIS-spectrometer (TriOs, Germany) in a range from 400 - 710 nm. Thus, the detector was different from the one used for the conventional PSICAM. Other fluids besides the sample were supplied from separate containers: (i) Deionized water, (ii) purified water (> 18.2 MOhm), (iii) nigrosine solution, (iv) cleaning solution, a mixture of 1 % Extran® (Merck, Germany) plus 10 % Ethanol, and (v) 0.5 % NaOCl (Riedel de Haën, Germany) solution. The entire system was operated by a custom-made control software programmed in LabVIEW (National Instruments, Germany), which provided the opportunity either to run the system automatically in a pre-defined instruction flow or to operate it manually by switching on the different pumps and valves. For the experiments performed for this study, the latter mode was used.

Due to the design as a flow-through device, all fluids were pumped into the cavity by activating the corresponding valves and pumps. No manual handling or opening of the cavity was necessary.

The principle of calibration and the equations for the calculation of spectral absorption coefficients are the same for both the ft-PSICAM and the conventional PSICAM. Calibration of the device was performed once a day in the same manner as for the conventional PSICAM, but with a modified cleaning step: In order to clean the system after each calibration measurement, first the cleaning solution was cycled from its container through the cavity for 2 min, afterwards the same was done with the bleaching solution for 5 min. Subsequently, the cavity was rinsed first with deionized water, then with purified water. Sample measurement in ft-PSICAM and conventional PSICAM differed from each other. In the latter case, a reference measurement was made right before each sample. In contrast,
as the flow-through system measured water samples continuously every five seconds during the time of the cruise (6 - 7 days), purified water as reference was only measured a few times a day, commonly at the stations (see below). To get artificial reference data for absorption coefficient calculation in the time between the stations, measured reference data were interpolated linearly. The calculated spectral absorption coefficients were corrected for the influence of salt and temperature in the same manner as described for the conventional PSICAM, but using the values measured by the FerryBox instead of manual measurements. Afterwards, the data were averaged in a one minute interval.

Measurements during the stations were used to assess the performance of the ft-PSICAM in comparison to the conventional one as well as to check the stability of the system under field conditions. Discrete sample measurements (1 min duration), were performed at the beginning and the end of every station. Each of the sample measurements were accompanied by a reference measurement. After the measurements at the beginning, the ft-PSICAM was cleaned and the measurements were repeated. The detailed measurement sequence at the stations was as follows: Sample measurement, reference measurement, cleaning, reference measurement, sample measurement. Sample data from the beginning and the end of each station were used to estimate the effect due to contamination of the system on the measurements.

2.3.2 Fluorescence and turbidity measurements
Chlorophyll-a fluorescence and turbidity have been continuously measured with a SCUFA-II-sensor (Turner Design, USA) mounted in the FerryBox system. It uses a prompt fluorescence method for chlorophyll-fluorescence. Turbidity is determined by measuring light scattering at 90°. Calibration of the sensor has been performed by the manufacturer and was checked before the cruise by using a solid fluorescence standard provided by the manufacturer.

3. Results and discussion
3.1 Assessment of the flow-through-PSICAM
Automatic absorption measurements with the ft-PSICAM were compared to measurements of discrete samples with the conventional PSICAM in the on-board laboratory. The conventional PSICAM was cleaned and calibrated very regularly, thereby it provided data with a measurement error < 3 % for the spectral range of 400 – 700 nm (Röttgers et al. 2005) and was used as reference method.
Figure 3 shows examples of spectral absorption coefficients of the seawater constituents \( a_{\text{p+cdom}} \), for the sum of particulate and dissolved fraction) from both cruises (April and June 2011) measured with both PSICAM versions. The data presented for the ft-PSICAM are from measurements at the beginning of each station, before the system had been cleaned. Samples are exemplarily shown from locations that strongly differ in the absolute light absorption, from values as low as 0.05 m\(^{-1}\) to up to 1 m\(^{-1}\) (Fig. 3). Both PSICAM systems showed a progression of the spectral absorption coefficients with wavelengths that is typical for marine waters: Highest absorption was observed in the blue portion of the light spectrum, mainly caused by phytoplankton pigments and CDOM, and the absorption decreased with increasing wavelength, with exception in the red part of the spectrum where an absorption peak around 675 nm related to chl-a was visible. Here, absorption is dominated by phytoplankton pigments and, to a lower extend, by non-phytoplankton particles, while CDOM absorbs only weakly (Roesler et al. 1989; Kirk 1994). As it can be seen from figure 3, the spectral absorption coefficients determined by both the ft-PSICAM and the conventional one were not identical in their values, but were similar in their progression. In the next section, the differences in the absolute values are quantified and the reasons are discussed.

### 3.1 Deviations of spectral absorption coefficients

It can be observed that the deviations - both on absolute and relative basis - varied on one hand between the cruise stations and, thus, with time and space, and on the other hand they were wavelength-dependent (Fig. 4). On average, the ft-PSICAM generated higher absorption coefficients than the conventional PSICAM; lower values for the whole spectrum were only measured for some stations, as well as for most of the stations in the red spectral region, predominantly above 700 nm. Because of the low absolute values at longer wavelengths, there was a tendency of higher relative deviations with increasing wavelength. Generally, the deviations were higher for the cruise in April than in June. Measurements at the beginning of the station were repeated after the ft-PSICAM had been cleaned. The aim was to evaluate the effect of the cleaning procedure that should remove possible contaminations of the instrument. It can be seen that the values measured at begin of the stations (uncleaned; Fig. 4A) differed more from the conventional PSICAM than those measured at the end (cleaned; Fig. 4B).

These deviations are the result of a composition of different factors affecting the ft-PSICAM measurements. In the following section, the deviations are explained in the context of factors influencing the measurement and calculation of the spectral absorption coefficients:

1. **Imprecision in the calibration procedure.** Determining the PSICAM’s cavity wall reflectivity during the calibration measurements is a critical factor for the retrieval of the spectral absorption coefficients. An error of 1 % in wall reflectivity induces an error in the absorption
determination by ca. 10 % (Leathers et al. 2000). Calibration of the ft-PSICAM has been performed once a day and several reflectivity spectra were obtained during both cruises. The reflectivity varied by 0.4 - 1.3 %, depending on the wavelength (Fig. 5). Besides actual measurement errors during calibration, the variations resulted mainly from contaminations inside the cavity. The differences in the spectral shape of the reflectivity between both cruises could not be readily explained, but they are likely related to the composition of contaminations remaining in the cavity. Because reflectivity determination has been performed irregularly and the ft-PSICAM has not been cleaned in advance, the observed variation is considered to cover the total range of wall reflectivity values for both cruises. A certain bias was introduced in the data by using a mean reflectivity value for the calculation of the spectral absorption coefficients instead of the (not known) true reflectivity at the time of the sample measurement. In order to estimate this effect, the absorption coefficients were recalculated with the mean reflectivity of each particular cruise and with reflectivity values of mean +/- standard deviation. Comparing the results, the spectral absorption coefficients varied between +/- 6 % in the blue spectral region and +/- 4 % in the red region for the cruise in April; for the cruise in June, the values were +/- 11 % and +/- 2 %, respectively. Hence, on a relative basis, these variations were generally lowest at longer wavelengths. This is expected as contamination by particles inside the cavity would lead to an influence proportional to their absorption which is typically low at longer and high at shorter wavelengths. Especially at the construction parts of the openings of the cavity it was observed that residues of particles were not always completely removed by water flow or the cleaning procedure and thus could have affected the reflectivity measurement. In addition, the design of the ft-PSICAM as an automated device without manual handling might also have had an influence on the calibration: When filling the cavity automatically, it is possible that a certain amount of air remained in the cavity during calibration measurements which could have biased the results. Furthermore, after draining the system, small leftovers of the previous measured liquid (nigrosine, purified water, cleaning or NaOCl solution) might have remained in the tubes and valves. This could have altered the absorption properties of the reference or the nigrosine solution in the subsequent measurement.

(ii) Degradation of the wall reflectivity by contamination. As shown in figure 4, the deviations between the conventional and ft-PSICAM measurements were smaller at the end of a station (Fig. 4B) than at the beginning (Fig. 4A). Because the two measurements were separated by two reference measurements and cleaning of the cavity, this change was most likely caused by contaminations that had accumulated at the cavity wall during the flow-through operation and that were removed by the cleaning procedure. To determine the effect of cleaning and to identify the type of particles contaminating the cavity, we examined the ratio of light intensity of the reference measurements before and after the cleaning as a function of wavelength.
The value would be 1 at a given wavelength, if no contamination was present in the cavity before cleaning, and decrease with amount of contamination. Depending on station and wavelength, we obtained values between 1 and 0.9. They were subtracted from 1 to get the relative light loss caused by removable contamination. In figure 6A, typical examples are given. As it can be seen, the progressions of the curves resemble phytoplankton absorption spectra, indicating that the contaminants that accumulated in the cavity were predominantly phytoplankton cells. In order to give a scale for the effect of this contamination-induced light loss, figure 6B shows the loss of light which occurs during normal sample measurements at the same stations for comparison. It is calculated from light intensity of a sample measurement divided by light intensity of the according reference measurement. This ratio is subtracted from 1 to get the light loss. Roughly, the loss of light due to contaminations was about 20% of that one during the according sample measurements. It is important to note that these effects are limited to that part of the contamination that was removable by cleaning: Thus, the cleaning was likely not exhaustive, since we still had differences between both PSICAM systems afterwards (Fig. 4B). Hence, there was probably a persistent contamination which has not been removed by the cleaning procedure.

(iii) Temperature effects. The sample measurements of the ft-PSICAM were corrected for the effect of temperature differences between sample and reference. For this purpose, temperature values obtained by the FerryBox as well as by hand-made temperature measurements (in case of the reference measurement with purified water) were used (Röttgers and Doerffer 2007). Nevertheless, high values for relative deviation between both PSICAMs above 590 nm with distinct features at 606 nm, 660 nm as well as above 690 nm (Fig. 4) indicated substantial errors in this correction due to a wrong determination of temperature, as at these wavelengths water absorption is especially influenced by temperature (Buiteveld et al. 1994; Röttgers and Doerffer 2007). A potential temperature change of 1 °C can alter the absorption by 0.00106 m\(^{-1}\) at 606 nm and 0.00085 m\(^{-1}\) at 710 nm, for example, which is in average about 3% of the total absorption measured at these wavelengths. The major source of errors regarding temperature was that it has not been directly measured inside the cavity, but in the FerryBox or – in case of the reference – in the storage container. On their way from the point of measurement to the cavity, one or both of the solutions probably changed their temperatures. In that case, the effective discrepancy in temperature between sample and reference were different from the measured one, which led to an improper correction of the data. This is also an explanation why deviations from the conventional PSICAM at the beginning of the station were higher in April than in June. In April, the average temperature over the whole cruise was ca. 6 °C for the samples and ca. 18 °C for the references, respectively. Thus, at the beginning of the station, the reference temperature has been determined in its container, but then the reference was pumped into
the (due to the previous continuous measurement) relatively cool flow through-system and might also have cooled down. In this case, the temperature difference between sample and reference would have been smaller in comparison to the previous determined difference. This led to an overcompensation of the temperature difference in the calculations. After the cleaning, the ft-PSICAM system was probably warmer and the reference has not been cooled down so much. Therefore, the real temperature difference was closer to the measured one and the differences between both PSICAMs were smaller. In contrast, the temperature difference between sample (average 14 °C) and reference (average 17 °C) were generally smaller in June. Consequently, this effect can be neglected for this cruise. In order to avoid such problems in the future, a temperature sensor should be installed directly in the cavity or at its inlet. This would also eliminate the need of manual temperature measurement of the reference water in its container.

(iv) Instability of light intensity. This paragraph discusses errors introduced in the measurements by instabilities in the optical setup of the ft-PSICAM. While fluctuations in overall light intensity of the lamp itself (e.g. due to aging) were detected via a sensor and corrected in our calculations, the light intensity within the cavity is also variable. It can vary due to contamination of the central light source’s surface or due to shifts of the glow filament within the lamp, which alter the amount of light getting into the fiber optic to the central light source. Both effects cause changes in light intensity observed by the detector, although the true absorption properties of the sample remain unchanged. Additionally, aging of the lamp could also have an effect on the spectral composition of the emitted light besides its overall intensity. The reference measurement of purified water corrects for these instabilities, thus, they are not crucial for the absorption coefficients measured at the stations. But they might play a role for the continuous measurements, where no references have been made over a longer period of time. Our approach was to assume a linear development of light instability related to setup-variations and therefore interpolate the values between the reference measurements made at the stations in a linear way. In reality, this development is unlikely, because there are probably short-time fluctuations in the light regime of the cavity (caused by the above named factors) in both ways. This is a problem inherent in the principle of continuous measurements performed with the ft-PSICAM. Thus, a determination of the error of the continuous measurement in relation to a certain time cannot be given in the context of this work, but we can assume that the error shortly after each station, when the system has been cleaned and a reference measurement was made, is relatively small but increases with time due to contaminations. However, a possible correction for setup-related light instabilities during the continuous measurements could be achieved by a permanent, direct determination of light leaving the central light source. For this, a direct connection from the light source’s surface to a photospectrometer is needed. But this would probably alter the
optical properties of the cavity and would require further testing. Moreover, changes caused by contamination of the light source’s surface would not be detected in this way, because the light source would not contaminate at the point of measurement.

(v) Variance in the detector response. During the cruise, also the light detection by the spectrometer used might have varied to a little extent. The detector signal can be influenced by changes in internal stray light, the nonlinearity response, and the dark offset due to changes in temperature. The influences of these parameters have not been investigated in particular during this study, but are considered as negligible.

(vi) Setups and sampling procedures. At last, factors related to the setup and the different sampling procedures of both PSICAMs likely contributed to the differences in measured spectral absorption coefficients. Different detectors have been used for the conventional and the ft-PSICAM. The slight increase observed at \(a_{\text{p+cdom}}\) above 700 nm for the conventional PSICAM (compare Fig. 3) is probably an artifact related to a stronger internal stray light error of the used Hamamatsu-detector (Röttgers, unpublished). Additionally to the temperature-related effects, this contributes also to the deviations above 700 nm. Furthermore, the differences in the sampling procedure can also have a certain influence on the measurements. Water for the conventional PSICAM was sampled in Niskin bottles, while the FerryBox and the associated ft-PSICAM got their water via an impeller pump. For the conventional PSICAM there might have been subsampling effects, even if the samples used have been mixed very carefully. On the other side, shear effects caused by pumping the sample into the ft-PSICAM could also altered the optical properties in the sample, inasmuch as phytoplankton chains or cells might have been damaged. Furthermore, it should be mentioned that time and place of both sampling methods were not necessarily completely identical because of the different sampling procedure of both systems. It is possible that the water mass had changed during the time span of the station (10-25 minutes) due to ship movement, so that the ft-PSICAM indeed measured two slightly different water bodies at the beginning and the end of the particular station. An obvious example of a deviation between ft-PSICAM and conventional PSICAM resulting from a change in water mass can be seen at the station shown in brackets in figure 7B. As it can be seen, the station lies at the edge of an absorption peak. This results in a high deviation when the ft-PSICAM data for the beginning of the station are used; if the data from the end are used, the deviation is in the range of the fit shown in the inlay graphic. That this is not entirely due to the effect of the cleaning can be seen from the fluorescence data (not shown), where the same strong gradient for this station in visible. Thus, if a gradient is present, minor differences in the time of the sampling can result in high differences between both measurement systems. But this should be the exception; normally, differences according to changes in water properties during the measurements are considered to be of minor importance compared to those resulting from
contamination and calibration imprecisions. For verification of this assumption, the issue of the effect of different sampling procedures on the results should be incorporated in future tests of the ft-PSICAM.

Several of these factors are interacting with each other, thus, it is difficult to separate their exact contribution to an observed deviation between conventional and ft-PSICAM at a certain station. Despite all potential sources of errors, the general progression of the continuous measured data is in good agreement with the point measurements of the conventional PSICAM (exemplarily shown for $a_{p+cdom}$ at 676 nm in Fig. 7). The inlay in figure 7 shows the correlation between the data of both systems at the stations for this wavelength. The applied linear fit has a small offset, but the relative deviation represented by the slope of the fit of 14% in April and 20% in June is of more importance.

For other wavelengths, we also found good linear correlations with $r^2 > 0.91$ in all cases; slopes vary between 1.1 and 1.65, offsets between -0.1 and 0.01, depending on cruise and wavelength (Fig. 8). It can be seen that features related to the temperature effects and pure water absorption were visible in the progression of the slope over wavelengths, and, to a lower extent, in the progression of the offsets. This is an indication for a combined influence of relative (e.g. cavity wall reflectivity) and absolute (e.g. temperature effects) errors on the spectral absorption coefficients determined with the ft-PSICAM, whereupon the relative error is the major one.

3.2 Determination of chlorophyll-a and TSM based on absorption measurements

3.2.1 Chlorophyll-a determination

To determine [chl-a] on the basis of the phytoplankton spectral absorption coefficient ($a_\Phi$), often particular absorption ($a_p$) of a water sample is measured by the quantitative filter techniques according to Yentsch (1962). Subsequently, the proportion of non-algal particulate matter ($a_{nap}$) on $a_p$ is determined by extracting the pigments with organic solvents (e.g. Kishino et al. 1985) or by bleaching the sample (Tassan and Ferrari 1995). The difference between both results in $a_\Phi$. When using the ft-PSICAM, the absorption of the different water constituents cannot be separated. Therefore, to establish a relationship between absorption measurements and [chl-a] in a sample, we have to use the absorption coefficient at the red chl-a absorption maximum at 676 nm. At this wavelength, the absorption associated with CDOM is negligible, and the measured absorption of water constituents is only related to due to $a_p$. However, to get $a_\Phi$ at this wavelength, still $a_{nap}$ has to be subtracted.
As methods for $a_{\text{nap}}$ determination like bleaching or extraction of pigments are not suitable in our case, $a_{\text{nap}}$ at 676 nm had to be estimated. Since $a_{\text{nap}}$ is increasing with decreasing wavelength in a nonlinear manner, its values can theoretically be calculated using the appropriate slope and equations given e.g. in Bricaud et al. (1998) and Bowers & Binding (2006). The basis of such calculations would be measurements of $a_{\text{nap}}$ virtually free of the influence from phytoplankton pigment absorption. But such values would only be obtained at wavelengths considerably higher than 710 nm, the limit of reliable ft-PSICAM-measurements, because water absorption increases strongly in this region (Pope and Fry 1997). Since calculation of $a_{\text{nap}}$ at 676 nm is not applicable, we used an alternative approach to get a reliable value for $a_{\Phi}$ at 676 nm: The value of $a_{\text{nap}}$ at 676 nm was estimated by assuming that $a_{\Phi}$ at 700 nm (composed of $a_{\text{nap}}$ and a little portion of $a_{\Phi}$) is a reasonable representative of $a_{\text{nap}}$ at 676 nm, because its portion of $a_{\Phi}$ is equivalent to the real increase of $a_{\text{nap}}$ from this wavelength to 676 nm. In order to test this approach, we subtracted $a_{\Phi}$ at 700 from $a_{\Phi}$ at 676 nm to get estimated values for $a_{\Phi}$ at 676 nm. The latter ones were correlated with HPLC-measured [chl-a] and fitted in a linear way (Fig. 9A), assuming a linear relationship according to Lambert-Beers law. The basis for these considerations was the absorption coefficients obtained with the conventional PSICAM, because of its higher accuracy compared to the ft-PSICAM. The coefficient of determination was $r^2 = 0.84$, with a slope of 0.018 and an offset of 0.005. Thus, the absorption at 700 nm is a good estimation of $a_{\text{nap}}$ at 676 nm and we get a reasonable estimation for $a_{\Phi}$ at 676 nm, when we subtract it from $a_{\Phi}$ at 676 nm. A direct correlation of $a_{\Phi}$ at 676 nm with [chl-a] would result in a much weaker correlation ($r^2 = 0.63$) with a higher offset (0.019) and a different slope (0.026). This demonstrates the influence of $a_{\text{nap}}$ in the region of the red chl-a absorption maximum.

The variability in the linear relationship between $a_{\Phi}$ at 676 nm and [chl-a] observable in figure 9A is caused only to a minor degree by measurement errors of [chl-a] and absorption coefficients, because both HPLC and conventional PSICAM measurements have a high accuracy. Instead, the main factor is the natural variability of the in vivo chlorophyll-specific absorption coefficient. It is depended on the relationship between cell size and intracellular pigment concentration, the so called ‘pigment packaging effect’ (Kirk 1976; Morel and Bricaud 1981). Therefore, the same amount of pigment can result in different absorption coefficient values at a given wavelength. In addition, the in vivo chlorophyll-specific absorption coefficient can be altered due to changes in pigment composition (Hoepffner and Sathyendranath 1992). This affects predominantly the blue region of the spectrum, as most of phytoplankton pigments absorb to a considerable amount there. In the red region, pigment composition virtually affects the chlorophyll-specific absorption coefficient only in the presence of chlorophyll-b or phycocyanin. But as both pigments do not have an absorption maximum in this region, only high amounts of these pigments are expected to have an effect.
According to our HPLC measurements, the concentration of chlorophyll-\textit{b} is (with exception of one station) never higher than 20 % of [chl-\textit{a}] during the cruises (data not shown). Concentration of phycocyanin has not been determined. For our data, the values for the \textit{in vivo} chlorophyll-specific absorption coefficient at 676 nm were in a range of 0.01 – 0.04 m\textsuperscript{2} mg\textsuperscript{-1}. Since the maximal value caused by pigment packaging is considered to be 0.033 m\textsuperscript{2} mg\textsuperscript{-1} at this wavelength (Johnsen and Sakshaug 2007), the higher values indicate in fact an influence by pigment composition in addition to the packaging effect.

The high variability of the chlorophyll-specific absorption coefficient is probably linked to the broad spectrum of life conditions in our sampling area, which reached from almost oceanic to very turbid coastal and brackish waters. This results in a high diversity in phytoplankton community, including different species, pigment compositions and size classes. A regional variability of the chlorophyll-specific absorption coefficient according to different phytoplankton communities has been previously found by (e.g. Hoepffner and Sathyendranath 1992) in the western North Atlantic and by Staehr et al. (2004) in a transect from Danish coast to Greenland Sea. Bricaud et al. (1995) found a variability of the chlorophyll-specific absorption coefficient with a range from 0.01 to 0.06 m\textsuperscript{2} mg\textsuperscript{-1} at 675 nm in samples from different regions of the world, which is similar to our observations for the North Sea.

\subsection*{3.2.2 Total suspended matter determination}

In order to derive also a proxy for [TSM] from our absorption measurements, a similar simple approach as for [chl-\textit{a}] was used: We correlated the absorption coefficient at 700 nm with gravimetrically determined [TSM] data. The resulting correlation was quite strong with \(r^2 = 0.95\), a slope of 0.014 and an offset of 0.006 (Fig. 9B). Thus, a linear relationship between \(a_p\) at 700 nm and [TSM] in the water is approved, while the influence due to CDOM and pigments is negligible at this wavelength. Wavelengths above 700 nm could in principle also be used to establish a linear correlation with [TSM], but they were not used because of the stray light error of the Hamamatsu spectrometer in this region mentioned above.

A correlation between [TSM] and [chl-\textit{a}] (not shown) exhibited no linear relationship between both parameters. This indicates a considerably contribution of other particles than phytoplankton to the [TSM] in the water. This is not surprising, as a relatively large proportion of our study area is located close to the shore or is influenced by riverine input.

\subsection*{3.2.3 Comparison of [chl-\textit{a}] to both fluorescence and turbidity measurements}

The established relationships between absorption coefficients, [chl-\textit{a}] and [TSM] (Fig. 9) can now be applied to derive continuous [chl-\textit{a}] and [TSM] data from our ft-PSICAM measurements. However, for calculation purposes, we decided to force the appropriate fits
through zero, because it is physically true. Additionally, this avoids getting negative values when dealing with absorption coefficient values smaller than the offsets. This resulted in slightly altered slopes of 0.019 (formerly 0.018) for [chl-a] and of 0.015 (formerly 0.014) for [TSM] calculation, respectively. Furthermore, the obtained spectral absorption coefficients from the ft-PSICAM were corrected with the data of the conventional PSICAM measurements. For $a_\Phi$ at 700 nm, the slope and offset of the fit between both PSICAMs at the particular cruise for this wavelength was used (Fig. 8). In order to get corrected values also for $a_\Phi$ at 676 nm, we estimated them for the ft-PSICAM station measurements as described above for the conventional PSICAM. In a second step, they were correlated with the corresponding values obtained with the conventional PSICAM (Fig. 10). The offsets in the linear fits for both cruises are insignificantly small, thus the fits were again forced through zero; the final slopes used for the correction of $a_\Phi$ at 676 nm were 1.17 for April and 1.13 for June, respectively. This means that even the uncorrected ft-PSICAM data have only an error regarding [chl-a]-determination of max. 17 %.

The resulting progression of the calculated values for [chl-a] and [TSM] are in good agreement with chl-a fluorescence and turbidity measured by the FerryBox (Fig. 11). Although the values for fluorescence and turbidity are not calibrated for [chl-a] and [TSM] and the data are therefore not directly comparable, the similarities in the curves indicate that our measurements as well as our correction approaches were sufficient. Small fluctuations, as can be seen during the April cruise for ft-PSICAM-[TSM] at low concentrations, cannot be identified as real or artifacts due to temperature effects or reference interpolation. They have to be further investigated, especially as they did not appear during the cruise in June at similar concentrations.

4. Conclusion and outlook

In summary, absolute spectral absorption coefficients continuously measured with the ft-PSICAM differ from those obtained with the conventional PSICAM under laboratory conditions. Main factors influencing the determinations of the spectral absorption coefficients are: (i) Imprecision according to the calibration procedure, (ii) contamination-induced degradation of the cavity wall reflectivity during operation, (iii) instability in the reference light intensity (not at the station measurements), and (iv) improper temperature correction. The differences between conventional and ft-PSICAM vary with time and wavelength, but are primarily important on a relative basis, and can therefore be corrected to a large degree by some discrete validation measurements with the conventional PSICAM. But as such comparison measurements are not possible if the system is used as unattended device in
the FerryBox, for example, effort should be made to improve the system in order to reduce the observed deviation between ft-PSICAM and conventional PSICAM. This concerns setup modifications as well as changes in the measurement process. A major improvement would be the use of a solid object for calibration, because it would eliminate the need to prepare and handle the nigrosine solution. The duration of the calibration would be reduced, because the cleaning step after the use of the nigrosine solution could be omitted, which would allow a more frequent calibration. However, this would be a difficult technical challenge; a possible solution would be the installation of a small retractable bolt in the cavity wall. Furthermore, temperature should be measured directly in or close to the cavity, to avoid discrepancies between the measured and the real temperature in the cavity. Completing the automation of the system would allow a more regular measurement routine concerning regular cleaning intervals, reference measurements and calibrations, even during the night time. Furthermore, experiments should be made to invent a more effective cleaning procedure to prevent contamination of the cavity wall and the central light source.

Nevertheless, in conclusion and despite all possible improvements of the system, the general approach to develop a system for the continuous measurement of seawater absorption was already successful: The obtained continuous data are consistent with the pattern shown by the point measurements. Furthermore, when converting the appropriate absorption coefficients to [chl-a] and [TSM], the resulting curves are also confirmed by fluorescence- and turbidity-measurements of the FerryBox. Hence, the ft-PSICAM offers the possibility to determine relative changes of water constituent absorption in a very high temporal resolution. Fronts and trends in the optical properties of seawater can be observed in this way, which is not possible with single-point measurements. Because a whole absorption spectrum is measured instead of single absorption coefficients at defined wavelengths, the obtained data can be used not only for [chl-a] or [TSM] determination, but also for other purposes like the discrimination of phytoplankton groups due to their specific pigment absorption properties. Furthermore, as with absorption a direct physical property of chl-a is measured, the determination of [chl-a] based on this method is not biased by quenching effects resulting from the physiologic state of the photosynthetic apparatus, as it is the case when measuring chl-a fluorescence. The only variation is caused by the natural variability of the chlorophyll-specific absorption coefficient. In contrast to fluorimetric determinations, scattering effects are negligible using the ft-PSICAM at low chl-a concentrations even in high turbid waters and is supposed to be the preferred probe for investigations regarding [TSM] and [chl-a].
Appendix

Abbreviations used in the text

- $a_p$ absorption coefficient of particulate matter [m$^{-1}$]
- $a_{cdom}$ absorption coefficient of dissolved matter [m$^{-1}$]
- $a_{\Phi}$ absorption coefficient of phytoplankton pigments [m$^{-1}$]
- $a_{nap}$ absorption coefficient of nonalgal particulate matter [m$^{-1}$]
- CDOM chromophoric dissolved organic matter
- chl-a chlorophyll-a
- [chl-a] concentration of chlorophyll-a [mg m$^{-3}$]
- TSM total suspended matter
- [TSM] concentration of total suspended matter [g m$^{-3}$]

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Fig. 1: Map of standard cruise tracks and sampling sites in the German Bight
BE= Belgium, DK= Denmark, DE= Germany, IE= Ireland, NO= Norway, NL= Netherlands, SE= Sweden, UK= United Kingdom. The depth profile is represented by the different shades of grey (from light for shallow to dark for deep areas). Named stations are referring to the examples shown in figure 3. The map was created with Ocean Data View Software (Schlitzer 2011).
Fig. 2: Flowchart of the ft-PSICAM. V= valve.
Fig. 3: Spectral absorption coefficients obtained with both conventional (black line) and ft-PSICAM (grey line) in the range of 400 to 710 nm at different stations in the German Bight during cruises in April and June 2011. Note the different scale of ordinates for the stations.
Fig. 4: Deviation of the ft-PSICAM from the conventional PSICAM for the different stations of both cruises (April and June) depending on wavelength. Absolute values are displayed at the left side, relative values at the right side. (a) Deviations at the begin of the station (uncleaned system), (b) deviations at the end of the station (cleaned system)
Fig. 5: Measurements of the cavity-wall reflectivity for both cruises in April and June 2011. Single measurements are pictured in grey, the mean value in black with the standard deviation as error bars.
Fig. 6: Effect of cavity wall contamination on the measured light intensity. (a) Light loss caused by contaminations on the cavity wall, calculated from the reference measurements of purified water made before and after the cleaning (b) For comparison, light loss occurring during sample measurements on the same stations, calculated from the sample and the reference measurement before the cleaning. Note the different scales of both graphs.
Fig. 7: Values for $a_{p+cdom}$ at 676nm derived from ft-PSICAM (grey dots) and conventional PSICAM (black dots) in (a) April and (b) June 2011. Gaps in the timeline are due to maintenance work, calibrations or deletion of data points with obviously incorrect spectra. Inlay graphics show the correlation between the values from both systems at the cruise stations. The value in brackets was omitted.
Fig. 8: Results of linear fits applied to the station data of both PSICAMs. The fits were done for each wavelength separately. (a) Slope and (b) offset were plotted in dependence of wavelength.
Fig. 9: Linear fit between (a) $a_\phi$ at 676 nm and HPLC-measured [chl-a], (b) between $a_p$ at 700 nm and gravimetrically determined [TSM]. Both absorption coefficients were measured with the conventional PSICAM. $n =$ number of samples.
Fig. 10: Correlations with linear fits between estimated $a_\Phi$ at 676 nm derived from conventional and ft-PSICAM for both cruises in April and June, respectively. Fits are quite similar; therefore the lines of the fits are hardly distinguishable.
Fig. 11: Calculated values for (a) [chl-a] and (b) [TSM] on the basis of $a_{\Phi}$ at 676 nm and $a_p$ at 700 nm, respectively, measured by the ft-PSICAM. Calculations were done for both cruises and compared with chlorophyll-fluorescence and turbidity measurements, respectively.
Evaluation of different optical methods for the determination of chlorophyll-a and total suspended matter

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Abstract
The concentration of chlorophyll-a ([chl-a]) and total suspended matter ([TSM]) are important parameters in biological oceanography. [chl-a] is a commonly used proxy for estimating phytoplankton biomass while [TSM] also includes detrital material and mineral particles thus influencing light absorption and photosynthetic activity in the water column.

To characterize the distribution (patchiness) of the two parameters adequately over a longer time period, fast and effective measurement methods are needed that can also be applied in situ or continuously. Thus, alternatively to direct determination of [chl-a] and [TSM], optical proxy values, such as chl-a fluorescence, turbidity and absorption, are often measured.

In this study, we evaluate different optical proxies for determining [chl-a] and [TSM] by means of single in situ measurements and continuously measured transects. Data were collected in the German Bight (North Sea) in 2010 and 2011. For [chl-a], fluorescence measurements are compared with pigment absorption coefficient values at a wavelength of 676 nm ($a_{\Phi \text{676 nm}}$), while the [TSM]-proxies were turbidity and particle absorption at 700 nm ($a_{p \text{700 nm}}$). As reference data, HPLC-determined [chl-a] and gravimetrically determined [TSM] were used.

Our results showed linear relationships between [chl-a] and fluorescence or $a_{\Phi \text{676 nm}}$, respectively. Coefficients of determination ($r^2$) were in a range of 0.71 to 0.88, the higher values related to the absorption measurements. Furthermore, it was demonstrated that fluorescence underestimates [chl-a] depending on ambient photosynthetic active radiation (PAR). Linear relationships were also observed between [TSM] and its optical proxies with $r^2$ values between 0.93 and 0.98. Turbidity measurements were influenced to a greater extent
by the physical properties of the suspended material and therefore resulting in a slightly higher variability than the $a_{p,700\,nm}$ measurements.

Absorption measurements are therefore promising optical proxies for determining [TSM] and [chl-a] due to their lower variability compared with the other proxies. This improved accuracy could be already partially achieved also for continuous measurements. Moreover, a combination of the different optical methods has the potential to provide additional information besides concentration, such as the source of TSM in the water or physiological condition of the phytoplankton.

**Keywords:** chlorophyll-a, total suspended matter, fluorescence, absorption, German Bight, method evaluation
1. Introduction

A large proportion of the world’s population lives close to coasts (Turner et al. 1995). The North Sea is, like most shelf and coastal seas, a highly productive ecosystem fostered by relatively shallow waters and a constant supply with nutrients by river discharge (Ducrotoy et al. 2000). The impact of e.g. fishing, shipping, pollution and energy production is high today and is likely to further increase in future. In enabling the detection of long-term changes driven by this increasing utilization as well as by climate changes, time series such as the Helgoland Roads (Wiltshire et al. 2010) or the Continuous Plankton Recorder (Reid et al. 2003) have shown their value. Information on phytoplankton, the basis of the marine food web, is of particular importance, as changes in this part of the ecosystem will probably also affect other parts. In this context, phytoplankton biomass is a key parameter and is needed for productivity and ecosystem model calculations. In addition, concentration of total suspended matter (hereafter referred to as [TSM]) in the water is another important parameter, because it is a factor governing, e.g., the light regime in the water and thereby influencing the photosynthetic activity of the phytoplankton. Both parameters can be measured by the analysis of discrete samples from fixed stations or occasional research cruises, but for many purposes, such investigations are insufficiently detailed because of the lack in spatial and temporal resolution. Instead, continuous or in situ measurements would be more appropriate. Optical methods offer a cost-effective, fast and convenient way to measure a variety of parameters relevant to marine researchers, and many of these methods can be applied on an in situ or continuously basis (Moore et al. 2009). [TSM] can be determined via measuring the amount of light scattered from a water sample (Sternberg et al. 1974; Vant 1990) using turbidimeters. Furthermore, certain particle absorption coefficients can also be used for this purpose (Wollschläger et al. 2013). In order to obtain a proxy for phytoplankton biomass, a wide-spread and an early introduced approach is to measure the concentration of the major algal pigment chlorophyll-a (referred to as [chl-a] hereafter) in the water (Harvey 1933; Mineeva 2011). However, the relation between both parameters is not constant. Different species show a different chl-a to biomass relation (Llewellyn and Gibb 2000), and therefore, the overall relationship between [chl-a] in the water and biomass can vary with changes in species composition. In addition and probably even more important are changes in the chl-a content of the cells due to physiological processes for example acclimatization to light conditions (Falkowski and Owens 1980; Llewellyn et al. 2005) or developmental stage (Llewellyn and Gibb 2000).

Nevertheless, [chl-a]—despite its limitations—is a commonly accepted and widely used proxy for photosynthetic biomass. Today, the most accurate method for direct [chl-a] measurement is high performance liquid chromatography (HPLC); however it is limited to the analysis of discrete samples and requires a relatively high effort. Less accurate alternatives are the
determination of [chl-a] via its fluorescence (Lorenzen 1966; Ostrowska et al. 2000) or its spectral absorption coefficients (Yentsch and Phinney 1989; Bricaud et al. 1995).

The origin of fluorescence is light energy, which is absorbed by the light harvesting complexes of the chloroplasts, but is not used for photochemistry or dissipated as heat. Since the amount of fluorescence depends amongst others on [chl-a] and this is in turn a proxy for phytoplankton biomass, the intensity of the fluorescence signal allows estimates about the phytoplankton biomass of a given sample. Because of the convenience of the measurements, this approach has been widely used for mapping horizontal and vertical phytoplankton distributions (e.g. Uehlinger 1985; Seppälä and Balode 1998; Wiltshire et al. 1998; Lazzara et al. 1996; Petersen et al. 2008). It has been shown that the fluorescence signal can be accurately calibrated against HPLC-determined [chl-a], but the calibration is not stable over longer periods of time or in changing environmental conditions (Wiltshire et al. 1998; Beutler et al. 2002; Beutler et al. 2004). This is caused by variability in the fluorescence to [chl-a] ratio, which is influenced e.g. by physiological state of the cells, species composition, and the variable fluorescence in dependence of the actual and short-time historic light conditions itself (Falkowski and Kiefer 1985; Cunningham 1996; Chekalyuk and Hafez 2011). Furthermore, discrete sampled and HPLC- analyzed [chl-a] data which can be used for calibration of fluorescence data are not always available, especially when using unattended devices for data acquisition (e.g. gliders or FerryBoxes; Petersen et al. 2011). In this cases, optical proxy values have to be converted into [chl-a] using a general relationship based on accumulated data of former experiments. Due to the high variability between fluorescence and [chl-a], this leads to potential high uncertainties in the resulting data.

Alternatively to fluorescence, [chl-a] can be determined by its absorption properties in the visible range of the light spectrum. The advantage is given by measuring a physical parameter of the cells instead of a more physiological value as it is the case for fluorescence. Thus, [chl-a] determination on the basis of absorption measurements could be more accurate and more stable than a determination via fluorescence, what would also reduce the potential errors when using a general relationship for conversion into [chl-a] in the absence of calibration data. Especially in remote sensing approaches, absorption coefficients, in the range of the blue absorption band of chl-a, are used for correlations (Bricaud et al. 1995; Bricaud et al. 1998; Trees et al. 2000). In this region of the spectrum, however, absorption is not just by chl-a, but commonly also due to other algal pigments (Hoepffner and Sathyendranath 1991) as well as to chromophoric dissolved organic matter (CDOM) and suspended matter. For oceanic waters, this is a minor problem, since the concentrations of the different constituents are well correlated to each other and appropriate correction procedures can be applied. In coastal waters, absorption due to suspended matter can be high due to mineral particles suspended in the water column. CDOM and phytoplankton...
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biomass are often independent from each other because of the additional riverine input of CDOM. Thus, for observational approaches in coastal regions, the measurement of absorption in the red absorption band of chl-a might be more suitable. At these wavelengths, absorption of CDOM is negligible to a large extent, while the proportion of TSM on total water constituent absorption can be estimated (Wollschläger et al. 2013). However, measurement of spectral absorption coefficients in seawater is more complicated than the measurement of chl-a fluorescence. With the exception of coastal areas, the content of absorbing material in seawater is relatively low and additional light loss due to scattering on particles requires the application of empirical scattering correction procedures. A variety of instruments is available for the determination of absorption coefficients (Pegau et al. 1995; Moore et al. 2009). Devices based upon the use of integrating cavities (Fry et al. 1992; Kirk 1997; Pope et al. 2000; Röttgers et al. 2005) seem to be the most promising approach, as they are highly sensitive and independent of the need for scattering corrections. Recently, efforts have been made to adapt these systems also for continuous measurements and tests in vitro (Musser et al. 2009) and in situ (Wollschläger et al. 2013).

In this study, we wanted to evaluate the performance of absorption coefficient measurements for the determination of [TSM] and [chl-a]. Thus, we compared this approach with traditional methods such as fluorescence and turbidity measurements. Because of the aforementioned advantages of these systems, the absorption coefficients were determined with a point source integrating cavity absorption meter (PSICAM) and its flow-through-version (ft-PSICAM). The evaluation was carried out on both discrete point measurements as well as on continuous data, in order to test whether potential advantages could be also achieved with continuous measurements using the ft-PSICAM device.

2. Materials and Methods

2.1 Discrete sampling and continuous measurements

Data were collected on several cruises with the R.V. ‘Heincke’ in 2010 and 2011 in the German Bight, North Sea (Fig. 1). At the hydrographic stations (dots in Fig. 1), discrete samples were taken from depths of around 4 to 5 meters using a sampling rosette (SBE 32, Sea-Bird Electronic, Inc.) equipped with seven 9-L Niskin sampling bottles (Hydrobios, Germany). The water was transferred into 10-L PE containers and processed subsequently for measurements of salinity, temperature, chl-a, turbidity, TSM, and absorption of water constituents. Continuous measurements of chl-a fluorescence and turbidity were performed on station and also along several transects by a FerryBox system as described in Petersen et al. (2011). The water intake for the FerryBox was in the moon pool of the research vessel at ca. 4 m depth, thus widely comparable to the sampling depth for the discrete
measurements. Continuous measurements of water constituent absorption were performed on the cruises in April and June 2011 using a flow-through-PSICAM (ft-PSICAM) as described in Wollschläger et al. (2013). The water for the continuous absorption measurements was provided from a bypass of the FerryBox water supply. This ensured that both devices got the same water samples and the results were comparable.

2.2 Determination of chlorophyll-a by HPLC
Concentration of chl-a was measured by high performance liquid chromatography (HPLC). Water samples (1-5 L) were filtered through pre-combusted GF/F filters (Whatman, USA, Ø 47 mm), subsequently, the filters were shock-frozen in liquid nitrogen and stored at -80 °C. Later in the laboratory, the pigments were extracted from the filters 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). The separation and analysis of the pigments was carried out by a HPLC system from JASCO (Japan) using the method described in Zapata et al. (2000).

2.3 Determination of total suspended matter by gravimetry
Concentration of total suspended matter 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). Prior to usage, the filters were wet with purified water to avoid saturation of the rim with sea water during filtration. This was done in order to reduce the amount of salt that cannot be washed out of the filter afterwards (Stavn et al. 2009). Additionally on each cruise, already filtered and therefore particle-free seawater was applied on empty filters like real samples, and the average mass increase due to the remaining salt was determined. It was subtracted from the mass values of all samples of the particular cruise before calculating total suspended matter concentration (in mg/L; see Stavn et al. 2009 for details).

2.4 Measurement of optical proxy-values
2.4.1 Chlorophyll-a fluorescence and turbidity
Chl-a fluorescence was continuously measured with SCUFA-II-sensors (Turner Design, USA) mounted in the FerryBox system. Calibration of the sensor was performed by the manufacturer. Sensitivity has been checked prior to the cruise by using a solid standard provided by the manufacturer. The same sensor was also used for continuous turbidity determination using a nephelometric method measuring light scattering at an angle of 90°. For turbidity, the sensor was calibrated before the cruises in the laboratory using different suspensions of formazine in a range of 0.5 to 35 formazine nephelometric units (FNU). The
turbidity of these suspensions were determined with a calibrated Hach 2100N IS turbidimeter (Hach, USA). The linear relationship between the SCUFA-II and the laboratory instrument was strong in all cases with $r^2 > 0.99$. For comparison with data from discrete samples, corresponding fluorescence and turbidity values were extracted from the continuous data set based on time of the sampling. In addition, manual turbidity measurements of samples have been done onboard using a Hach 2100P ISO turbidimeter (Hach, USA).

In preliminary analysis, an offset was visible –despite calibration– for both the turbidity and the fluorescence data measured with the SCUFA-II. After the cruises, for correction of these offsets, purified water was measured to obtain zero-values for both parameters. These values were then subtracted from the corresponding data. Since the fluorescence measurements are temperature depended, the purified water was maintained at 5 and 15 °C, because these temperatures were representative for the water measured in spring and summer/autumn, respectively. For turbidity, a mean value of 0.2 nephelometric turbidity units (NTU) was measured, for fluorescence a mean value of 0.55 and 0.54 arbitrary units (AU) for 5 °C and 15 °C, respectively. However, it should be noted that this correction was not sufficient for a complete elimination of the offsets.

### 2.4.2 Spectral absorption coefficient determination

Discrete absorption measurements were made using a PSICAM as described in Röttgers et al. (2005) and Röttgers & Doerffer (2007). It is referred to as ‘conventional PSICAM’ in the following. The only change in the setup of this device was the use of a Hamamatsu C10083CA mini-spectrometer (Hamamatsu, Japan) during the cruises in April and June 2011. The theoretical background as well as the equations for the calculation of spectral absorption coefficients or cavity wall reflectivity is given elsewhere (Kirk 1997; Leathers et al. 2000). Measurements of the cavity wall reflectivity for calibration of the PSICAM were performed daily in triplicate according to Röttgers et al. (2005) using an always freshly prepared solution of the dye nigrosine. Purified water (>18.2 MOhm) was used as reference. The cavity was cleaned after each calibration measurement with 0.1 % NaOCl solution (Riedel de Haën, Germany) for 15 min, followed by rinsing it with purified water. The spectral absorption coefficients of the nigrosine solution were determined using liquid waveguide capillary cells (LWCC; path lengths: 0.5 and 2 m; WPI, USA), again with purified water as reference. Prior to their use, purified water and nigrosine solution were brought to the same temperature in a water bath.

Sample measurements with the conventional PSICAM were also performed in triplicate. Prior to each sample, the reference was measured. Subsequent to the sample measurement, the cavity was rinsed with purified water for cleaning and then the reference was measured again. Temperature for both was recorded and used together with the determined salinity
(measured with a WTW 330i portable conductivity sensor; WTW, Germany) for correction of variations in pure water absorption due to temperature and salinity differences between sample and reference (Röttgers and Doerffer 2007).

Continuous measurements of spectral absorption coefficients were performed taking advantage of a custom-made ft-PSICAM (Wollschläger et al. 2013). For cavity wall reflectivity and spectral absorption coefficient calculation, the same equations as for the conventional PSICAM were used. Calibration was performed once a day in the same manner as described for the conventional PSICAM, but with a modified cleaning procedure: Subsequent to each nigrosine measurement, a cleaning solution consisting of 1 % Extran® (Merck, Germany) plus 10 % ethanol was pumped for 2 min cyclic from its container through the cavity. Afterwards, the same was done with 0.5 % NaOCl solution for 5 min. Finally, the cavity was rinsed with de-ionized and then with purified water. Sample water was measured every 5 seconds and averaged over one minute.

Since reference measurements were only performed at stations (in intervals of one to eight hours), spectral absorption coefficients on a transect line were calculated using linearly interpolated reference values. They were based on the data measured on the stations at the beginning and the end of the respective transect. Correction of the coefficients for the influence of salt and temperature were done according to the procedure used for the conventional PSICAM, but with temperature and salinity data obtained from the FerryBox. To maintain the performance of the system, the ft-PSICAM was cleaned in the way mentioned above for the calibration measurements at every station. However, the absorption coefficients obtained continuously with the ft-PSICAM were distorted by small contaminations and changes in the reference signal and were therefore corrected using the discrete data of the conventional PSICAM (see also Wollschläger et al. 2013). The particle absorption coefficient at 700 nm \( \text{a}_{p, 700 \text{ nm}} \) served as a proxy value for [TSM], while for [chl-a] the estimated absorption coefficient of phytoplankton pigments at 676 nm \( \text{a}_{\Phi, 676 \text{ nm}} \) was used (Wollschläger et al. 2013).

2.5. Additional data sets

In the evaluation of chl-a determination by fluorescence and absorption, additional datasets were used. The concentration of CDOM was qualitatively estimated by fluorescence using a Cyclops-7 sensor (WETLabs, USA) mounted in the FerryBox. Furthermore, for the cruises in April and June 2011, data on photosynthetic active radiation (PAR) was obtained from the hydrographic time series station ‘Wattenmeer’ (Badewien et al. 2009; Reuter et al. 2009).
3. Results & Discussion

3.1 Determination of total suspended matter

3.1.1 Comparison of station measurements

In order to evaluate the turbidity- and absorption-based approach regarding [TSM] determination, manually and continuously obtained turbidity data were compared with measurements of the particle absorption coefficient at 700 nm \((a_p \text{ } 700 \text{ } \text{nm})\). Turbidity was determined manually using the Hach turbidimeter and continuously by the SCUFA-II. Absorption coefficients were measured manually with the conventional PSICAM and continuously with the ft-PSICAM. Figure 2 shows the pooled station data obtained during all cruises from all four instruments used in relation to gravimetrically determined [TSM].

Turbidity values were observed in a range from 0.16 to 28 NTU and \(a_p \text{ } 700 \text{ } \text{nm}\) values from 0.001 to 0.4, corresponding to [TSM] of 0.4 to 26 g/m³. Even lower values were found during the transect measurements for both turbidity and \(a_p \text{ } 700 \text{ } \text{nm}\). Thus, our measurements span roughly two orders of magnitude. Most of the data (77 %) were derived from waters with lower [TSM] (< 5 g/m³).

In all cases, statistical significant linear relationships between the respective optical proxy value and [TSM] were observed (p<0.05, according to t-tests), but the variability in the data was different for the four methods: The strongest correlation was obtained for the PSICAM measurements of \(a_p \text{ } 700 \text{ } \text{nm}\) \((r^2=0.98, n=98; \text{fig. } 2E)\), followed by the Hach turbidity measurements \((r^2=0.95, n=97; \text{fig. } 2A)\). For the continuously obtained optical proxy values, the coefficients of determination were marginally lower than those of the respective manual measurements \((r^2=0.93, n=100 \text{ for the SCUFA-II and } r^2=0.94, n=42 \text{ for the ft-PSICAM measurements}; \text{fig. } 2C \text{ and } G, \text{ respectively})\). This was expected, since the continuous measurements were performed under less controlled conditions compared to the manual measurements. Furthermore, in the case of the continuous absorption measurements, the number of measurements is considerably lower and lack also higher [TSM] values.

The unexpectedly low slope of the continuous turbidity measurements indicates a problem with the SCUFA-II sensor, which is not readily explainable at this point. But as this affects only the absolute values, the data can still be used to evaluate the continuous turbidity measurements regarding their ability in determining [TSM].

In order to further quantify the accuracy of the different methods, the relative standard error of the slopes of the linear fits applied to the data was calculated. Regarding the manual measurements, it was 2.3 % for turbidity and 1.3 % for \(a_p \text{ } 700 \text{ } \text{nm}\). For the continuous measurements, the relative standard error of the slope was 2.7 % and 4.2 %, respectively.

Thus, although for the manual measurements the absorption-based approach is more accurate than the turbidity measurements, the same was not yet achieved for the continuous measurements. To a certain extent, this might be an effect of the relatively low sample size. 
and the smaller range available for the continuous absorption measurements. But a logarithmic transformation of the data (Fig. 2B, D, F, and H) also revealed increased scattering of the data around the applied linear fits at [TSM] < 3 g/m³. Although observable for all instruments, it was especially high for the ft-PSICAM and probably the reason for the higher error of the slope.

For a more detailed analysis of the performance of the different methods at low [TSM], we applied linear fits to a reduced data set, which contained only values up to 3 g/m³ TSM (data not shown). Higher scattering in the data and therefore smaller coefficients of determination were observed. They were 0.74 (n= 65), 0.77 (n= 66), 0.6 (n= 68) and 0.24 (n= 34) for manual measured turbidity, manual measured ap700 nm, continuous measured turbidity and continuous measured ap700 nm, respectively (p<0.05 according to t-tests). The weak linear relationship between [TSM] and ap700 nm measured by the ft-PSICAM suggest treating ap700 nm values measured continuously in this range with caution.

In summary, ap700 nm has shown to be in principle a slightly more accurate proxy for [TSM] determination than turbidity measurements. However, at low [TSM], this could not yet be achieved during continuous measurement. But considering the whole dataset, the performance of the ft-PSICAM is nevertheless promising, especially as there is room for improvement of the system as it is still at the developmental stage.

In principle, the observed variability in the relationship between [TSM] and the optical proxies can be explained by measurement errors and by natural variability. The measurement errors for the different methods used were estimated as follows: For both manual and continuous turbidity measurements, different concentrations of formazine (in a range of ca. 0.5 to 60 FNU) were measured by the respective instrument 3-5 times. The averaged standard error of the slope of linear fits applied to these data (0.249 FNU for the Hach turbidimeter and 0.368 FNU for the SCUFA-II) was considered as a measurement error of the instrument, including errors due to manual handling. The error associated with $a_{p 700 \text{ nm}}$ varied with the value of the respective measurement, since it is a combination of the error of the wall reflectivity determination and the precision of the instrument. For the conventional PSICAM, the values were ~2 % (Röttgers et al. 2005) and 0.001 m⁻¹ (Röttgers and Doerffer 2007). Values for the ft-PSICAM were higher in both cases (~5 % and 0.007 m⁻¹, unpublished data). Furthermore, uncertainties in the gravimetrically determination of [TSM] itself contributed to variability in the correlations. They are composed of errors in the determination of the mass retained on the filter (average 0.003 mg), of the salt-offset (average 0.3 mg) as well as from errors in the determination of the filtered volume (average 0.4 %). Since the filtered volume is not constant but depends on the amount of TSM in the water, the related error is variable for the different samples.
In figure 3, the calculated relative error of the different methods is shown in dependence of [TSM]. Generally, it was lowest for [TSM] itself and \( a_p \, \text{700 nm} \) measured by the PSICAM. The mean error was 5.5 % and 5.9 %, respectively, with maximal values of ca. 20 %. For the other optical proxies, the mean errors were considerably higher (20.5 % for Hach turbidity measurements, 27.4 % for SCUFA turbidity measurements, and 46.4 % for \( a_p \, \text{700 nm} \) measured by the ft-PSICAM), while the maximal error was in part higher than 100 %. Instrumental errors increase at [TSM] < 3 g/m³, indicating that they are primarily responsible for the observed variations in the relationships between [TSM] and optical proxies at low values.

Natural variability as the second factor influencing the relationship between optical proxies and [TSM] results from the fact that turbidity and particle absorption are not only functions of [TSM], but also of the size distribution and origin of the particles (e.g. organic or inorganic; Babin et al. 2003; Babin and Stramski 2004; Astoreca et al. 2012). A possible manifestation of this effect can be seen in the data displayed in figure 2A. Normally, based on the calibration of the instrument, the slope of the correlation between [TSM] and turbidity was expected to be close to 1. This was not the case, because at some onshore stations of the cruise in September 2010, we observed relatively low turbidity values although there were high [TSM] present (empty circles marked with arrows). Since the relative measurement errors are low at these high values (compare figure 3), it is likely that the reason for these stations being ‘outliers’ is because of different scattering properties of the TSM present in the water column. Indeed, during the time of the particular cruise, there was a storm event in the German Bight, and it is likely that this caused re-suspension of larger particles than usual present at onshore stations. Since large particles are less efficient in light scattering per mass than smaller particles (Stramski et al. 2007; Wozniak et al. 2010), this might be an explanation for these unusual low turbidity values relative to the measured [TSM]. The properties of TSM seem to influence \( a_p \, \text{700 nm} \) to a much lesser extent compared to turbidity, because in this dataset (Fig. 2E), the respective stations of September 2010 appear not as outliers. But also in the continuously measured turbidity values, they were not as clearly distinguishable from the others as in the manual measurements. It can be speculated that if the large particles were only aggregates of smaller ones, these aggregates might have been disrupted by the impeller pump of the FerryBox. Therefore, the scattering properties measured by the SCUFA-II might be more similar to those of the TSM commonly present in the water column at the onshore stations.

### 3.1.2 Comparison of continuous measurement methods

In order to evaluate the continuous measurement systems not only by means of the station data, the transect measurements of the optical proxies were converted to [TSM] and
compared. Only transects of the cruises in April and June 2011 were used, since for the other no ft-PSICAM data were available. The conversion was done using the relationships established in figures 2 C and G, respectively. As the offset is negligible for $a_p_{700 \text{ nm}}$ and it is more realistic to assume zero absorption at the absence of TSM, the fit was forced through zero previously to the calculation (resulting in no alteration in the slope). The resulting time series for calculated [TSM] of different origin are shown in figure 4, occasionally occurring negative [TSM] result from turbidity values below the offset. Although they do not comply with reality, they were included in the graph to show the relative progression of the data. It was similar for both proxies, especially in the more turbid waters. Nevertheless, besides a good match (e.g. 6th April, 8th to 9th April or 19th June), there were also differences visible, not only in the absolute values, but also in the relative progression of the calculated [TSM] data (e.g. 10th April, 12th April or 21st June). Since most of the differences were visible at low [TSM], the general higher variation of the ft-PSICAM in this region might have an effect (compare 3.1.1). However, as the differences were not simple scattering in the data but show a peak-like behavior, it is more likely that they were caused by errors in the calculation of the absorption coefficients during the transect measurements: In contrast to the stations, where reference values were measured, the reference was interpolated in a linear way during the transect measurements (compare 2.4.2). This assumption of a linear development of the reference values between two stations is of limited validity but was necessary for being able to calculate absorption coefficients from the continuous measurements. In reality, is likely that there occurred short-time fluctuations in reference light intensity due to instabilities in the light source or contaminations of the cavity. The influence of this error source is difficult to quantify, however, it is considered to be the explanation for the distinctive features in the relative progression of the continuous absorption-based [TSM]. To avoid this in the future, reference measurements have to be performed more frequently.

Differences in absolute values with retention of the relative progression, predominantly occurring in figure 4B (June cruise), are the result of calculating turbidity-based [TSM] using a relationship obtained over several cruises instead of a cruise-specific one. Comparing figure 2C, it can be seen that a fit applied only to the values of this cruise (black squares) would have a smaller slope compared to that calculated in the figure. As the manually taken turbidity data from this cruise do not differ significantly from those of the other cruises, the respective continuous data can be considered as being biased. Possibly, the sensor was contaminated on this cruise, thus underestimating turbidity in this case. However, as it was mentioned for the station measurements before, in general all observed deviations in the calculated [TSM] could also result at least in part from differences in the optical properties of the suspended material. But at low [TSM], where we observe most of the deviations, it is likely that most of this natural variation is masked by variation due to instrumental errors.
Thus, for further comparison of the turbidity- and absorption-based approach, additional data from areas with high [TSM] (estuaries, tidelands) would be useful.

3.2 Determination of chlorophyll-a

3.2.1 Comparison of station measurements

Fluorescence measurements and the pigment absorption coefficient at 676 nm ($a_{\Phi 676 \text{ nm}}$) were compared for their ability to serve as proxies for [chl-a] in the water. HPLC determined [chl-a] was in a range from 0.1 to 12.3 µg/L, spanning two orders of magnitudes, with the lower values sampled in the more offshore regions and the higher values near the coast.

Most of the values (87 %) were below a concentration of 5 µg/L.

[Chl-a] obtained on the different cruises were correlated with the measurements of the optical proxy values, and statistical significant linear relationships (p<0.05 in all cases according to t-tests) were found (Fig. 5). Similar to the turbidity measurements, we observed a considerable offset (0.547 +/- 0.092 AU) in the fluorescence measurements despite the correction applied on the data using fluorescence measurements of purified water as blank (see 2.4.1).

Because the fluorescence measurements are temperature depended, it is possible that an insufficient temperature correction of the sensor was the reason. Another explanation might be an incorrect separation of the excitation light from the emission light by the filter of the instrument. Thus, by internal reflection or scattering, minimal amounts of light might get through the filter and create a signal, even although there is no chlorophyll present in the sample. Linear correlations were in general stronger for absorption ($r^2= 0.88$, n= 110 for the manual and $r^2= 0.8$, n= 50 for the continuous measurements) than for the fluorescence measurements ($r^2 = 0.71$, n= 110).

In the linear fit of the continuous $a_{\Phi 676 \text{ nm}}$ data (Fig. 5G), the offset (0.011 +/- 0.004 m$^{-1}$) is higher than for the manual obtained data (0.004 +/- 0.002 m$^{-1}$), although this is only of low statistical significance (p= 0.08 derived from a t-test). Probably, the higher offset is caused by a lack in high [chl-a] measured by the ft-PSICAM, which distorted the regression line. Under the assumption that the offsets were negligibly small, the fits were forced through zero in the following. This is supposed to be realistic, because the absorption should be zero in the absence of chlorophyll. In this case, the slope of both PSICAMs was identical (0.0182 +/- 0.0004 for manual $a_{\Phi 676 \text{ nm}}$, and 0.0179 +/- 0.0008 for continuous $a_{\Phi 676 \text{ nm}}$, p= 0.7 according to a t-test). This equals a relative error of the slope of 2.2 % and 4.5 % respectively, which is less than for the fluorescence measurements (6.2 %). Based on these linear fits, the fluorescence measurements exhibit a higher variability in determining [chl-a] than the absorption measurements.

In order to emphasize the smaller values of which the dataset consists to a majority, the logarithm of the data (optical proxy-values and [chl-a]) was also taken and fitted in a linear
way. While the coefficient of determination for the $a_{\Phi_{676\,nm}}$ measurements increased ($r^2 = 0.91$ for manual measurements, $r^2 = 0.87$ for continuous measurements), it decreased for the fluorescence measurements ($r^2 = 0.6$), because of very high scattering in the data at smaller values. Thus, although the individual error could be high for both approaches, the stronger linearity of the relationship for both the normal and the log-data indicated that in general $a_{\Phi_{676\,nm}}$ is a more accurate proxy for [chl-a] than \textit{in situ} chl-a fluorescence, especially at lower [chl-a].

On the one hand, this might be caused by an influence of water turbidity on the fluorescence signal (TurnerDesigns 2004). On the other hand, and probably more important, fluorescence is strongly linked to the physiology of the phytoplankton (Falkowski and Kiefer 1985), and is therefore influenced by a variety of parameters. Firstly, there are the absorption properties of photosystem II (the mean optical cross section) which depend on the pigment composition of the cells. This composition is species specific, but can also vary due to long term adaptation of the cells to certain light conditions (Yentsch and Yentsch 1979; Soohoo et al. 1986; Lutz et al. 2001). Furthermore, self-shading of the chloroplasts (‘pigment-packaging’; Duysens 1956; Morel and Bricaud 1981; Kirk 1994) also influences light absorption and therefore the amount of energy which is supplied to the photosystem. The last and also important parameter is the variable fluorescence yield, which reflects the proportion of absorbed light energy the cell uses for photochemistry. It is in turn influenced for example by short-term acclimatization of the cells often involving non-photochemical quenching of the fluorescence signal (Szabo et al. 2005). This acclimatization can be triggered by actual available light (e.g. xanthophyll-cycle) or a diel rhythm (Prézelin and Ley 1980). Furthermore, the general condition of the photosynthetic apparatus with the associated enzymes also influences the fluorescence yield. For this reason, the nutrient state of the cells is also discussed as a parameter influencing the fluorescence yield and therefore the measured fluorescence signal (Kiefer 1973b; Parkhill et al. 2001).

The influence of light acclimatization on the measured fluorescence signal can also be seen in our measurements. When considering only the fluorescence values measured during the night, when the cells were some kind of ‘dark-adapted’ (Fig. 5C, D), the variability in the data is much lower compared to that observable in figure 5A and B, which also results in higher coefficients of determination ($r^2 = 0.82$ and $r^2 = 0.75$ for the log-transformed values, respectively; $n = 21$ in both cases). The value shown in brackets is considered as an outlier and not included in the calculation.

In contrast to the fluorescence signal, the chl-a specific absorption coefficient is less variable, because it is only marginally influenced by the physiology of the cells. It exhibits also a natural variation, but changes take place on larger timescales and result basically from only two factors: The pigment packaging effect (as also mentioned above for fluorescence) and
the pigment composition of the cell (Hoepffner and Sathyendranath 1991). The latter one is of importance only if measurements were conducted in the blue region of the light spectrum, where other pigments also absorb in addition to chl-a. For measurements in the red absorption maximum of chl-a, as done for this study, differences in the pigment composition can be neglected, because only chl-a and –to a lesser extent– chlorophyll-b and phycobilines absorb in this region. Since the concentration of chlorophyll-b was mostly below 20 % (mean value: 6 %) of that of chl-a according to the HPLC-measurements, and no blooms of cryptophytes or cyanobacteria containing phycobilines were observed, we can consider in most cases the whole absorption at 676 nm being due to chl-a. Thus, nearly all the natural variability we observed in the relation between [chl-a] and $a_{\Phi 676 \text{ nm}}$ is caused by the pigment packaging effect.

3.2.2. Comparison of continuous measurement methods

In order to get further information about the possibilities and limits of the fluorescence and absorption-based approach regarding determination of [chl-a], the transect measurements obtained by FerryBox (SCUFA-II) and ft-PSICAM were also used. Data of both optical proxies were converted into [chl-a] and compared. For fluorescence data, the equation given in figure 5A is used; for the $a_{\Phi 676 \text{ nm}}$ data, we used the slope of the linear regression forced through zero (0.018), as mentioned above.

The resulting time series for the two cruises are shown in figure 6A. Negative values for the fluorescence derived [chl-a] result from fluorescence values smaller than the offset; nevertheless they were shown in order to document the relative progression of the data. As expected from the correlations of the station data, the HPLC-measurements of [chl-a] fitted better to the absorption-derived [chl-a]. In part, there was also a good match between the [chl-a] calculated from the different proxies, both in relative progression and in absolute values. However, during other times of the cruises, the progression of both data sets remained similar, but the absolute values were considerably different.

To a certain extent, these differences were caused by light acclimatization (probably by non-photochemical quenching) of the phytoplankton, which resulted in a reduction of the fluorescence signal at high light intensities (Abbott et al. 1982). This led to an underestimation of the fluorescence-based [chl-a] compared to the absorption-based [chl-a]. These light induced differences can be seen in figure 6B, where the relation of [chl-a] derived from both optical proxies is shown together with the photosynthetically active radiation (PAR) measured outside the water for the respective period. During the night, the relation was close to 1 for most of the time, indicating a comparable performance of both methods in determining [chl-a]. But when PAR increased during the day, the relation increased to values above 8, caused by a clear drop in the fluorescence signal. This observed fluorescence
quenching at the sea surface around noon is a typically observed feature that alters the fluorescence / [chl-a] ratio (Babin et al. 1996; Kiefer 1973a).

When light availability is reduced as in the presence of high turbidity or high CDOM values (Fig. 6C) the relationship did not or only increases slightly, although PAR is high. Thus, it can be concluded that the observed increases were indeed light induced and not because of an inherent diel rhythm of the cells.

4. Summary and conclusion

It was shown that [TSM] and [chl-a], two important parameters in biological oceanography, can be reasonably well measured using optical proxy values even in a relatively heterogeneous environment like the German Bight and over a time span of several seasons. However, the performance of the different methods varied. Regarding [TSM] determination by analyzing discrete samples, both optical proxies (turbidity and absorption) were highly correlated with the amount of TSM in the water, but the absorption-based approach was slightly more stable over different regions and times/seasons. Furthermore, it seemed to be less influenced by differences in physical properties of the suspended material. This is indicated, in principle, also for the continuous measurements, but more data covering a wider range of different [TSM] are necessary for a final evaluation. By now, the ft-PSICAM measurements have to be improved with regard to fluctuations of the light regime in the cavity as well as to their accuracy at low [TSM]. Until this has been done, the continuous absorption measurements are, according to the limited set of measurements conducted with the ft-PSICAM, not of clear advantage over the continuous turbidity measurements regarding [TSM] determination. In contrast, for [chl-a] determination, the use of $a_{\Phi 676\;nm}$ was clearly shown to be more accurate than fluorescence because of its weaker linkage to the physiology of the phytoplankton. This higher accuracy was also achieved in continuous measurements.

Additional to their use for monitoring approaches, these more accurate and continuous measurements of [chl-a] might be valuable in terms of calibration and/or validation of remote sensing data. Furthermore, in combination with accurate oxygen measurements, productivity could be estimated more precisely. Although in situ fluorescence measurements are less accurate in the mere determination of [chl-a], they are nevertheless, especially because of the stronger connection of fluorescence to phytoplankton physiology, of great value for other investigations. Especially when combined with continuous measurements of $a_{\Phi 676\;nm}$, variations in the relation of absorption to fluorescence could be connected to different factors influencing the phytoplankton, as it has been demonstrated for PAR in this study. In a similar
way, measurement of turbidity and $a_p_{700\text{ nm}}$ in parallel could be used to study differences in the nature and/or origin of the particles in TSM present in the water column. The use of devices like the PSICAM and the ft-PSICAM for the determination of absorption coefficients has the additional advantage that not only the absorption coefficients important for [chl-a] and [TSM] determination are measured, but simultaneously the coefficients of the whole visible spectrum. Thus, these devices have the potential to provide additional data which can be, for example, used for the discrimination and identification of phytoplankton groups. This would be another set of valuable information for a variety of ecological questions.

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Figures

Fig. 1: Stations for the different cruises in the German Bight. Transects were carried out between the stations in different order, depending on weather conditions on the respective cruise. Different shades of grey represent the depth profile of the area (light areas= shallow, dark areas= deep). DE= Germany, DK= Denmark, NL= Netherlands. The map is based on Ocean Data View Software (Schlitzer 2011).
Fig. 2: Correlations between gravimetical determined [TSM] and optical proxy values. (A, B) Turbidity measured with the Hach turbidimeter, (C, D) turbidity measured with the SCUFA-II, (E, F) $a_p$ 700 nm obtained by the conventional PSICAM, (G, H) $a_p$ 700 nm obtained by the ft-PSICAM. Arrows in (A) mark stations with low turbidity in relation to the measured [TSM].
Fig. 3: Relative error of the different methods as a function of [TSM].
Fig. 4: Time series of [TSM] calculated from continuous turbidity and ap700 nm measurements taken in April 2011 (A), and June 2011 (B). Gravimetrically determined values are shown for comparison.
Fig. 5: Correlations between [chl-a] measured by HPLC and optical proxy values. (A, B) Fluorescence measurements, (C, D) fluorescence measurements made after sunset, (E, F) $a_{\phi 676\text{ nm}}$ measurements made with the conventional PSICAM, (G, H) $a_{\phi 676\text{ nm}}$ measurements made with the ft-PSICAM
Fig. 6: Time series of the cruises in April (left) and June (right). (A) [chl-a] derived from fluorescence, $a_{\text{ph}}$, and HPLC, (B) Relation of [chl-a] calculated from both proxies in dependence of PAR, (C) turbidity and CDOM.
3.5 Manuscript III

Assessment of North Sea phytoplankton via molecular sensing – A method evaluation

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Abstract
Phytoplankton community analysis is of importance in relation to the natural or human-induced changes in the marine environment. Due to high effort and limitations of traditional methods, the popularity of molecular sensing approaches continues to increase. Among others, microarray techniques targeting ribosomal 18S sequences have been successfully applied for phytoplankton investigation.

In this contribution, we compared the results of two microarray methods targeting 18S rDNA and 18S rRNA, respectively, with results obtained from microscopy, HPLC, and flow cytometry. On a qualitative basis, the microarrays performance was similar or potentially better than that of the non-molecular methods. Quantitatively, our data suggest that microarray signals obtained from 18S rDNA provide relatively rough estimates of phytoplankton abundance. In contrast, when targeting 18S rRNA instead, a robust linear relationship (r²= 0.68) between molecular sensed signal and microscopic cell counts could be demonstrated exemplarily for a probe specific to the genus Pseudo-nitzschia.

Thus, for both qualitative and quantitative purposes, microarray techniques can be valuable additions to traditional methods for phytoplankton analysis. Especially routine monitoring approaches could benefit from advantages such as reduced effort, higher taxonomical resolution, and a potential for automation.

Keywords: DNA microarray, method evaluation, cell counts, phylochip, molecular sensing
1. Introduction

Investigation of phytoplankton community structure is important with respect to natural or human-induced changes in marine environment. Moreover, the revelation of species abundance patterns contributes to an understanding of phytoplankton ecology. The most direct approach for a detailed analysis of phytoplankton community structures is microscopic observation of water samples. But this is time consuming, requires taxonomical experienced researchers and is therefore often inapplicable for larger numbers of samples. Furthermore, the picophytoplankton and also parts of the nanophytoplankton are, although being an important part of the community (Vaulot et al. 2008), not readily accessible by this method on a detailed taxonomical level. These cells are either hardly visible under the microscope or often lack morphological features for a reliable identification (Ebenezer et al. 2012). Thus, molecular biological methods relying instead on DNA-sequences (e.g. ribosomal sequences; Ebenezer et al. 2012) are considered as valuable additions or alternative approaches for investigating especially these types of phytoplankton. Moreover, their taxonomical resolution is potentially even higher than that of microscopy, allowing in principle also analyses below the species level. Finally, besides requiring in general less effort and taxonomical experience, they might also be automatable in the future (Diercks-Horn et al. 2011).

A variety of molecular biological techniques are available today in the field of plankton research (de Bruin et al. 2003) and probably even more methods are going to emerge in the future. Among these, microarrays (often called ‘phylochips’ when used for taxonomical investigation) have been shown to be valuable and frequently used tools. They were used for the assessment of surface cleanliness (Cooper et al. 2011) as well as for the analysis of rat guts (Nelson et al. 2011), soils (Sessitsch et al. 2006), or tooth pockets and lakes (Loy et al. 2002) microbial communities. In the past, emphasis on the application of the phylochip laid on the investigation of prokaryotes, but also marine eukaryotic communities have been analyzed by this method (Gescher et al. 2008a; Metfies et al. 2010).

Briefly, the principle of this method is as follows: Molecular oligonucleotid probes are designed specifically to a certain target nucleic acid sequence of the taxon of interest. These capture probes are fixated on the phylochip-surface and a solution containing biotin-labeled nucleic acid extracts of the community to be analyzed is applied on the chip. The complementary target sequences in the extract hybridize to the corresponding sequences on the chip, the rest is removed. The amount of nucleic acid bound to a specific probe is often estimated by fluorescence staining. An alternative method is the use of sandwich hybridization techniques which include the hybridization of an additional probe carrying the detectable signal to the target sequence (Ranki et al. 1983; Rautio et al. 2003; Metfies et al. 2005; Diercks-Horn et al. 2011).
Targets of the capture probes are commonly certain sequences of the 16S (prokaryotes) or the 18S (eukaryotes) rRNA. Ribosomal sequences provide the advantage that they are ubiquitous and highly abundant in the cells of all organisms, because they are essential for protein synthesis. Moreover, since harboring both conserved and variable regions, they are well suited and often used for taxonomical investigations (Moon-van der Staay et al. 2000; Díez et al. 2001; Moon-van der Staay et al. 2001). Capture probes can be designed specifically for these regions, enabling an analysis of the community of interest on different taxonomical levels (Metties and Medlin 2007). Alternatively to targeting the rRNA abundant in the cells directly, the genes coding for these RNAs can also be the targets of the capture probes. This requires amplification of the particular gene via PCR previous to the hybridization procedure. Using this approach, inactive organisms, which were not synthesizing rRNA, can also be detected.

The present contribution deals with the application of two molecular sensing methods, a DNA-microarray and an rRNA biosensor (Diercks et al. 2008a; Diercks et al. 2008c) on a set of natural phytoplankton samples collected during six cruises in the area of the German Bight (North Sea). Besides using capture probes which were applied before for the analysis of marine phytoplankton (Metties and Medlin 2004; Mettles and Medlin 2007; Gescher et al. 2008a; Gescher et al. 2008b), in this study an effort has also been made to develop additional probes in order to cover further important and commonly occurring taxa. After they were tested for specificity, these probes were included in the analysis. Focus of the study was the detection of a variety of taxa from different taxonomical levels of the micro- and nanophytoplankton fraction by means of these molecular sensing methods. These results were compared with other, more traditional methods like microscopic cell counts, pigment concentration, and pigment fluorescence obtained from flow cytometry.

2. Materials & Methods

2.1 Sample collection

Samples were obtained on six cruises with the R/V “Heincke” in the area of the German Bight. The cruises were conducted in May, July, and September 2010 as well as in April, June, and September 2011. Water samples were taken on cruise stations from 4-5 m depth using a carousel water sampler (SBE 32, Sea-Bird Electronic, Inc.) equipped with seven 9 L Niskin bottles. In order to obtain phytoplankton community samples for DNA-microarray analyses, 400-1500 mL of this seawater were filtered onto 0.2 µm Isopore GTTP membrane filter (Millipore, Germany). Subsequently, filters were shock-frozen in liquid nitrogen and stored at -20 °C. For pigment analyses, 1-5 L of seawater were filtered through pre-
combusted GF/F filters (Whatman, USA, Ø 47 mm). The filters were also shock-frozen in liquid nitrogen and stored at -80 °C until analysis. Samples (100 mL of seawater) intended for microscopic analyses were filled in brown glass bottles, fixated with 0.5 mL Lugol’s solution and stored at 4-8 °C. Finally, at each station, an aliquot of 3 mL seawater was fixated with glutaraldehyd (0.4 % final concentration) for flow cytometry. After an incubation period of 15 min, the sample was shock-frozen in liquid nitrogen and stored at -20 °C.

2.2 Cell counts by microscopy
Fixated phytoplankton samples were counted according to the method of Utermöhl (1958). Normally, 50 mL sample were filled in a sedimentation chamber overnight in order to let the phytoplankton settle. In case of higher concentration of debris, only 25 mL were used. The counting was performed using an inverted microscope (Olympus IX 51, Olympus, Japan), phase contrast, and 100x or 200x magnification.

2.3 HPLC-measurements
Phytoplankton pigments were separated and analyzed by means of high performance liquid chromatography (HPLC) using the method of Zapata et al. (2000) and a HPLC system from JASCO (Japan). Pigments were extracted from the filters with 100 % acetone for 24 h at -30 °C. Extracts were transferred into 2 mL glass vials and simultaneously cleaned from particles by passing them through 0.2 µm syringe filters (Spartan, A13, Whatman, USA).

2.4 Flow cytometry
Samples were analyzed using a FACSCalibur (BD Biosciences, USA) or an Accuri C6 Flow Cytometer (BD, Biosciences, USA). Autofluorescent phytoplankton was analyzed after excitation by a blue (488 nm) and a red laser (620 nm) of an electrical power of 20 mW, respectively. Gating was performed manually by visual inspection of 2D-density plots (orange vs. red emission and green emission vs. sidescatter, respectively). Yellow-green fluorescent latex beads (0.94 µm diameter, Polysciences, USA) were used for intercalibration between different samples and also served as a reference for the normalization of cellular optical properties. In case of the FACSCalibur, TruCount beads (Becton Dickinson, USA) were used for absolute volume calibration.

2.5 Specificity test of the hybridization probes
(i) Probe design: New probes have been designed for seven species using the probe design and probe match tool of the ARB software (Ludwig et al. 2004; Pruesse et al. 2007) and version 102 of the SILVA rRNA database released in February 2010 (www.arb-silva.de). If possible, a set of three different probes was designed for each of the target species. The
probes designed in silico had at least two mismatches against non-target species and if possible a length of 25 bases. The binding sites of the probes were located upstream of 1000 bp in the 18S rRNA. These criteria proved to positively influence specific binding of molecular probes to DNA-fragments of the 18S rRNA gene on the phylochip (Metfies et al. 2007; Metfies and Medlin 2008). The specificity of the probes on the phylochip was tested by *in vitro* by hybridization of 18S rDNA fragments amplified via PCR from laboratory cultures of both phylogenetically closely and more distantly related non-target species. The hybridization protocol was identical to the one for the environmental samples and is described in the next section. All probe sequences are listed in table 1.

(ii) *Culture conditions.* All algal strains for the specificity tests were cultured under sterile conditions in seawater-based F2- and K-media (Guillard and Ryther 1962; Keller et al. 1987) at 150 – 200 µmol photons m⁻² s⁻¹ with a light:dark cycle of 14:10 hours and at 15 or 20 °C.

### 2.6 DNA-microarray

(i) *Extraction of DNA.* Genomic DNA was extracted from the samples using the E.Z.N.A Plant DNA Mini Kit (Omega Bio-Tek, USA) accordingly to the instructions of the manufacturer. DNA-concentration in the obtained extracts was determined with a NanoDrop spectrophotometer (Thermo Scientific, USA).

(ii) *Amplification of the 18S rRNA gene.* As target for microarray analysis, a ca. 1400 bp fragment of the 18S rRNA gene was amplified via PCR using genomic DNA as template and primers universal for eukaryotic 18S rRNA. Normally, the forward-primer was 82F (5’ GTG AAA CTG CGA ATG GCT CAT 3’), only in some cases 328F (5’ACC TGG TTG ATC CTG CCA G 3’) was used because of better performance. The reverse primer was always 1055R (5’ CGG CCA TGC ACC ACC 3’). Per 50 µL PCR-reaction, 20 ng template-DNA were used. The PCR-protocol started with 94 °C for 5 minutes, followed by 34 cycles of 94 °C for 1 min, 58 °C for 2 min and 72 °C for 2 min. It finishes with 72 °C for 10 min; afterwards, the PCR-reaction was cooled down to 4 °C. Products were purified after manufacturer’s instructions taking advantage of the Quiquick PCR-purification kit (Quiagen, Germany). The only modification was the repetition of step 8 with the eluate to increase the yield of purified product. Finally, nucleic acid concentration was determined by the NanoDrop spectrophotometer.

(iii) *Labeling of the PCR fragments.* In preparation of the hybridization procedure, PCR-fragments were labeled with biotin using the BioLabel DNA Labeling Kit (Thermo Scientific, Germany) accordingly to manufacturer’s instructions. 200-1000 ng of PCR-product served as template for the reaction. The labeling turned out to fail with PCR-products refrigerated for several months, thus it was done contemporary to the PCR. The labeled fragments were
purified with the MinElute PCR Purification Kit (Qiagen) and DNA concentration was measured using the NanoDrop spectrophotometer.

(iv) Production of the microarray chips. Molecular probes as well as positive control were synthesized from Thermo Electron Corporation (Ulm, Germany) with a C6 MMT aminolink at the 5’ end. Probes and positive control were diluted to a final concentration of 1 mM with 3x saline sodium citrate buffer (3x SSC). Pure buffer served as negative control. Controls and probes were spotted onto epoxycoated glass slides (Nexterion Slide E, Peqlab Biotechnologie GmbH, Germany) using a pinprinter (VersArray ChipWriter Pro, Bio-Rad Laboratories GmbH, Germany) and split pins (Point Technologies Inc., USA). Probes as well as positive and negative controls were spotted several times on different locations on the chip. Afterwards, the spotted slides were incubated for 30 min at 58 °C.

(v) Hybridization protocol and staining. Hybridization was performed in duplicate for each sample. Microarray chips were pre-incubated for 60 min at 58 °C in STT-buffer (1M NaCl, 10 mM Tris pH 8, 0.005 % Triton x-100) containing 1 mg/mL BSA to block background noise. Subsequently, the chips were washed in deionized water and dried by centrifugation. Hybridization mixture (30 µL) was composed as follows: 2x hybridization buffer (2x STT with 1 mg/mL BSA and 0.2 µg/µL herring sperm-DNA), labeled PCR-fragment (650 ng), TBP (fragment of the TATA-box binding protein gene of Saccharomyces cervisiae, 150 ng) as positive control and purified water. Lifter cover slips were placed on the microarray-chip and the hybridization mixture was incubated at 94 °C for 5 minutes to denature the nucleic acid. Afterwards, the mixture was pipetted under the cover slips and dispersed by capillary action. Hybridization was carried out for 60 minutes at 58 °C in wet chambers, consisting of falcon tubes with wetted filter paper. After removal of the cover slips, excessive or non-specifically bound DNA was washed away by rinsing the chips in buffers with increasing stringency (10 min for each buffer): Wash-buffer 1 (2x SSC, 10 mM EDTA, 0.05 % SDS), wash buffer 2 (1x SSC, 10 mM EDTA) and wash buffer 3 (0.2x SSC, 10 mM EDTA). Finally, the chips were dried by centrifugation. The biotinylated target rDNA bound to the probes on the chip was stained using streptavidin-CY5 (Amersham Biosciences, Germany) in 1x hybridization buffer (at a final concentration of 0.01 µg/mL). Like the hybridization, the staining was performed in wet chambers, but only for 30 min at room temperature in a shaded environment, because of the sensitivity of the stain to excess light. Afterwards, the chips were washed again twice for 5 min with wash buffer 1 and once with wash buffer 2, followed by drying via centrifugation.

(vi) Evaluation of the fluorescence signal and data processing. Fluorescence signals of the DNA microarray were detected using a GenePix 4100 A microarray scanner (Axon Inc., USA) and the analysis software GenePix Pro 6.1. The recorded data were processed into signal-to-noise ratios taking advantage of the PhylochipAnalyzer-software (Metfies et al., 2008). The signals of all samples were normalized on the basis of the positive control signal,
the amount of DNA and the amount of TBP used in the hybridization. Finally, both replicates of a particular sample were averaged.

2.7 Nucleic acid biosensor

(i) Probe sets. In this study we used a set of capture and signal 18S-rDNA probes (PSNFRA: 5'-ATT CCA CCC AAA CAT GGC-3' and PNEXDEL: 5'-GCG CAA TCA CTC AAA GAG-3'), targeting the 18S-rRNA of the genus *Pseudo-nitzschia* (Diercks et al., 2008b). In order to allow immobilization on the gold electrodes of the sensor chip, probe PSNFRA was thiolated at the 5'-end, while the detector-probe PNEXDEL carried a digoxigenin-label at the 5'-end. The probes and the positive control were synthesized by Thermo Electron Corporation (Ulm, Germany).

(ii) Laboratory strains and culture conditions. The evaluation of the probe set targeting *Pseudo-nitzschia* spp. involved the analysis of field samples with microscopically determined cell concentrations of *P. seriata* or *P. delicatissima* and hybridizations of different cell concentrations of *P. subcurvata*. The culture of *P. subcurvata* was isolated by Philipp Assmy during R/V Polarstern cruise ANT-XXI/4 in April 2004 at 49° S, 02° E. It was cultured under sterile conditions in seawater-based media K (Keller et al. 1987) at 15 °C and 120 µmol photons m⁻² s⁻¹ with a light: dark cycle of 14:10 hours. Prior to the experiments, the cells were counted by light microscopy as described previously.

(iii) Spotting of multiprobe chips. Multiprobe chips (BST, Germany) were hand-spotted with 0.3 µL of thiolated capture probe (PSNFRA [10 µM in 0.5 mol/L HNa₂O₄P]) per electrode and incubated for 16 hours at room temperature. The sensors were stored in a moisture chamber for all incubation steps to protect the solutions from evaporating. Subsequently, unbound probe was removed by washing the sensor with 2x saline sodium citrate buffer (0.3 M NaCl / 0.03 M sodium citrate, pH 7). The multiprobe chips were blocked with 10 µL of 1 % [w/v] BSA and washed again with 2x saline sodium citrate buffer.

(iv) Sample preparation and analyses with the automated biosensor. Cells from a laboratory culture of *P. subcurvata* and field samples were collected on “Millipore”-cellulose acetate filters with a pore size of 0.4 µm (Merck, Germany). The sample material was preserved in 450 µL RLT buffer from the RNeasy Plant kit (Qiagen, Germany) and stored at -20°C until further analyses. Defined numbers of cells from *P. subcurvata* and samples that were known to contain *P. seriata* or *P. delicatissima* were subjected to the evaluation of the probe set with the automated biosensor device. The standard hybridization mixture for the analysis with the device contained 1x hybridization buffer (75 mM NaCl/20 mM Trizma base, pH 8.0/0.04 % SDS), 0.25 µg/µL herring sperm DNA, 0.1 pmol/µL dig-labeled probe PNEXDEL and the sample material in RLT-Puffer in a final volume of 1000 µL. The hybridization mixture was filtered through a 0.2 µm filter to remove cell debris prior to the insertion to the biosensor.
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system. All steps related to the detection of the target species in the sample material are carried out automatically in the biosensor system (Diercks et al. 2008a; Diercks-Horn et al. 2011). This involves denaturation by incubating the hybridization mixtures at 94 °C for 4 minutes, a hybridization at 46 °C for 30 minutes, washing the chips with POP buffer (50 mM NaH₂PO₄ × H₂O, pH 7.6/100 mM NaCl), incubation with an antibody solution (Anti-DIG-POD, 7.5 U/mL in PBS, pH 7.6/0.1 % BSA [w/v]/0.05 % Tween 20 [v/v]) and removal of unbound antibody-enzyme complex washing the multiprobe chip with POP buffer. The hybridization was detected during an incubation with substrate solution (4-aminophenylamine hydrochloride (ADPA) [44 µg/mL]/0.44 % ethanol [v/v]/0.048 % H₂O₂ [v/v]/50 mM NaH₂PO₄ × H₂O/100 mM NaCl). Electrochemical signals on the sensor chip were measured as negative values of the current [nA].

3. Results & Discussion

3.1 Specificity tests

A set of 23 newly designed probes targeting seven species (Ceratium fusus, Ceratium furca, Chaetoceros socialis, Chaetoceros debilis, Leptocylindrus minimus, Odontella aurita, Paralia sulcata, and Prorocentrum micans) was tested for probe specificity on a phylochip. This approach was necessary because previous studies revealed that probes that appeared to be specific in silico are not necessarily specific in vitro (Metfies and Medlin 2007). All probes and laboratory cultures used in this study are listed in table 1. A set of three probes was designed and tested for each of the target species. Overall, it has to be mentioned that it was difficult to find a set of three different probes for each target species, because we observed a high degree of similarity of the target species to other sequences in the database. However, based on the sets of three probes designed in silico it was possible to retrieve at least one probe for each target that displayed no cross-hybridization to non-target species in this study (Tab. 2). Probes that showed unspecific binding to non-target species in vitro were excluded from the method evaluation. For some molecular probes no cultures of the target species were available at the time of the specificity tests. If these probes did not display unspecific binding to closely related non-target species, it was assumed that a signal obtained for these probes from a field sample is a specific signal originating from the target species. This is a feasible assumption, because currently there is no definite proof for the specificity of molecular probes anyway. The specificity of molecular probes is strongly dependent on the amount and quality of sequence information available in the ribosomal databases at the time of the probe design. Although quality and number of sequences in ribosomal data bases is continuously increasing (Pruesse et al. 2007), they are currently far away from providing
comprehensive information on all ribosomal sequences occurring in the environment. Thus, the specificity of molecular probes has to be re-evaluated continuously in respect to new ribosomal sequences in the data bases.

3.2 Evaluation of the phylochip

In the first instance, evaluation of the phylochip-microarray was performed by a comparison in terms of presence/absence of the different taxa. The choice of the comparison parameters depended on the targets of the capture probes. For probes specific for certain species or genera, cell counts of the corresponding taxon were used. The same was done for probes targeting whole groups (bulk cryptophytes, bulk dinoflagellates), but also the concentration of group specific marker pigments (peridinin for dinoflagellates and alloxanthin for cryptophytes) in the sample, were used. Cryptophytes also frequently contain phycoerythrin, but since this pigment is difficult to measure by HPLC, flow cytometry measurements of orange fluorescence were used as proxy instead (Li and Dickie 2001). If more than one capture probe showed specificity for a certain taxa, the evaluation was performed for both probes. An overview of the investigated taxa, the used molecular capture probes, and the respective comparison parameters is given in table 3.

A second approach was the quantitative evaluation. This was done by correlating the strength of the signal-to-noise ratio obtained from the phylochip and the biosensor with quantitative data (e.g. number of cells per liter, concentration of pigment).

Presence/absence-matrices. When analyzing the microarray signals and the comparison parameters in terms of presence/absence, the result is a pattern of matches and mismatches (Tab. 3). Samples were considered as matching (light grey marks in figure 1), if either both methods showed a signal or if both lacked a signal at a certain station. For several taxa (C. fusus, O. aurita, bulk dinoflagellates and bulk cryptophytes), the accordance was higher than 70 %, indicating a good performance of the phylochip with respect to a qualitative assessment of phytoplankton occurrence. Mismatches were either caused by receiving no signal from the microarray, although the comparison parameter showed a signal (“false negative” signal of the phylochip, black marks in table 3), or by obtaining a microarray signal, although the comparison parameter lacked a signal (“false positive” signal of the phylochip, dark grey marks in table 3).

In the case of L. minimus, P. sulcata, Pseudo-nitzschia spp., and bulk chlorophytes, the main reason for mismatch was an underestimation (“false negative” signal) by the phylochip. Even though, there was at least 50 % agreement on the presence of these taxa between the phylochip data and the comparison parameter. These observations suggest that the molecular probes developed for theses target species bind in principle to their target species, but they do not bind to all different strains occurring in the field. This points out the need for
more culturing and extending ribosomal data bases in order to improve the probe development in silico.

For the remaining probes, the majority of mismatches were due to “false positive” signals observed by the phylochip. On one hand, this kind of mismatch might be related to differences in the sampling volumes used for the different analyses. The sample volume counted by light microscopy (25 – 50 mL) or flow cytometry (3 mL) was significantly smaller than the sample volume analyzed with the phylochips (400-1500 mL). A low sample volume increases the likelihood of including rare species in the analysis and thus, it is possible that species might be missed by light microscopy or flow cytometry if they display low abundances. In this context, overestimations of the taxa C. fusus, C. furca, and P. micans might have been caused by this effect. As it can be seen in figure 1 from the median value of the cell counts, the abundance of these species was often quite low. For example, it was 80 cells/L for C. furca. Assuming the investigation of such an average sample, this is equivalent to 0.4 cells in a volume of 50 mL. Thus, it is quite likely that such rare individuals are missed during the sampling process or overlooked in the microscope. In contrast, in the microarray analysis, all 80 cells would be included assuming a sample volume of one liter. Although P. sulcata or O. aurita occurred in higher abundance, the “false positive” signals observed for these species might also be explained by such sampling effects. Since they are chain forming diatoms, relatively high abundances were created often by the presence of one or a few chains. These few chains can be missed in the same way as rare single cells. In addition to sample size effects, cells, although principally present in a sample, could be missed during microscopic observation. This probability increases with the rarity of cells, but also with decreasing cell size, especially in the presence of a high amount of debris (First and Drake 2012). In conclusion, the results indicate that the microarray is the more sensitive method and might reflect the true situation better because of the higher sample volume. On the other hand, “false positive” signals on the microarray might also be caused by unspecific binding of the probes to non-target species. This is an explanation that can never be excluded in the context of molecular probe applications. As explained before, the specificity of molecular probes strongly depends on the amount and quality of ribosomal sequences in public databases.

Molecular probes targeting cryptophytes displayed a high accordance with the reference parameter. The accordance between the phylochip data and the pigment based parameter (alloxanthin) was 80 %, while it was 94 % for the cell counts and 97 % for the fluorescence data. This high similarity between the different datasets points towards a high specificity of the molecular probe targeting the cryptophytes. In accordance to most of the other probes, mismatch between phylochip data and pigment data was mainly caused by “false positive” signals on the phylochip. As almost no “false positive” signals were observed on the
phylochip in comparison to the microscopy and fluorescence-based data, our observations suggest that alloxanthin is not a ubiquitously present pigment in cryptophytes. Thus, identification of phytoplankton with HPLC should be re-evaluated regularly with newly emerging methods. The phylochip data for the occurrence of dinoflagellates in the samples was also in good accordance to the reference parameter. Around 70% similarity was observed for the pigment data, while 94% of the positive signals could be confirmed by light microscopy. Again, the vast majority of mismatch between the phylochip and the pigment data was caused by “false positive” signals on the phylochip. As for the cryptophytes, the applicability of the HPLC-marker to target all dinoflagellates has to be re-evaluated.

In summary, mismatches in the dataset at which the microarray underestimates the occurrence of a taxon are probably based on insufficient binding of the molecular probes to all strains of the target taxon. In contrast, “false positive” signals on the phylochip are not necessarily caused by unspecific binding of the molecular probe to non-target species. This is certainly an important issue and molecular probes have to be re-evaluated constantly with newly released ribosomal sequences. However, “false positive” signals on the phylochip could also be explained by insufficiencies in the methods of the comparison parameters such as insufficient sampling volumes or insufficient marker pigments. In this regard, together with the often high percentage of match, the microarray can be considered as a reliable tool for indicating the presence or absence of its target-taxa in a sample.

**Linear relationships.** The correlation of the signals from DNA-microarray and comparison parameters for a certain taxon in quantitative terms was the second step of the evaluation. Since the range of the data often covered several orders of magnitude and were unlikely to be normally distributed, the logarithms of the data were used for this purpose.

Although the comparisons in section 3.2.1 indicated a good performance of the DNA-microarray in terms of presence/absence, linear correlations were only achieved for the probes specific for *C. fusus* and *C. socialis* (Fig. 2A, B). Furthermore, a correlation between the microarray signal for the bulk dinoflagellates and the measured peridinin-concentration was observed (Fig. 2C), but only if the data of the cruise in April 2010 (grey diamonds) were omitted. However, although statistically significant (p<0.05), the obtained linear correlations were weak with r² values in a range of 0.27 to 0.35. Regarding the other probes and their comparison parameters, the data were merely randomly distributed as it is shown exemplarily in figure 2D for *Pseudo-nitzschia* sp. (p= 0.548, probe PSNEXDELIB).

A separate consideration of the cruises yield similar results, some probes showed a weak linear relationship with the comparison parameter, others did not (data not shown). Interestingly, this result was independent of whether an overall correlation was found in the pooled dataset for the particular taxon or not. Thus, even for e.g. *C. fusus*, some of the single cruises showed no correlation at all. A similar lack of correlation was found by Cooper et al.
(2011) between phylochip signals and the results of a bacterial spore assay. Otherwise, to our knowledge, comparisons between microarray results and non-molecular methods are scarce in literature. A possible explanation for the results could be, at least for those taxa where a correlation is visible in the overall dataset, that the sample size is too small when only a single cruise is considered. Since the general relations between phylochip-signal and comparison parameter are already highly variable in the pooled dataset as suggested by the small coefficients of determination observed, they might be undetectable when using even smaller sets of data.

Furthermore, variability in the correlation can be also caused by the fact that microarray and comparison parameters have a different resolution or target different aspects of the cells. The microarray takes even those cells into account which were due to their small size not visible under the microscope, e.g. pico- and small nanophytoplankton cells. Thus, for example, the lack of correlation between the counted number of dinoflagellates and the signal-to-noise ratio of the DinoE-12 probe might be explained by the presence of a high percentage of picoplanktonic dinoflagellates. Indeed, they can be frequently abundant as it is shown by Vaulot et al. (2008). When using pigment concentration or pigment fluorescence, the problem is similar: These approaches detect only pigmented cells, but the microarray also non-pigmented. From this point of view, the misfit of the omitted data (grey symbols) of figure 2C in the rest of the dataset might indicate a high presence of heterotrophic (and therefore non-pigmented) dinoflagellates in some of these samples. However, in many cases, the molecular and non-molecular approach was exactly comparable. For example, cells of *C. fusus* or *Pseudo-nitzschia* sp. are readily countable and even though, correlations were weak or nonexistent. Thus, methodical biases of both the non-molecular approaches as well as the microarray method are likely to have contributed to these results. Regarding cell counts, the error is normally < 50 % (Lund et al. 1958), provided a sufficiently high number of cells per taxon have been counted as was the case in this study. For the used HPLC pigment measurements, an overall error of ca. 10 % was calculated based on repeated measurements of samples, as well as on chlorophyll-a standards (unpublished data). The error associated with the measurement of the orange fluorescence via flow cytometry was not determined, but fluorescence in general is a relatively variable parameter, since it depends on a variety of physiological factors (Falkowski and Kiefer 1985). However, since logarithmized values were used, the effect of these errors can be considered as relatively small. Besides the comparison parameters, also the microarray, although a frequently used tool in microbiological ecology, still suffers from certain biases, which may have contributed to the observed results. Probably the most important issue is not directly related with the DNA-microarray itself, but with the need to amplify the target gene via PCR.
Normally, it is assumed that the relative contribution of a certain taxon to the whole community present in a sample remains unchanged during the PCR. But there is evidence that in mixed samples, PCR can lead to alterations in the original DNA-ratio (Suzuki and Giovannoni 1996; Polz and Cavanaugh 1998). Amplification efficiency of sequences is influenced by their G-C content (Reysenbach et al. 1992; Dutton et al. 1993) and also by their concentration in the PCR reaction. At higher concentrations, products tend to hybridize with each other rather than with a primer and their amplification efficiency decreases (Ruano et al. 1991). Products whose templates were present in high amounts already at the beginning of the PCR reach this plateau phase earlier than those which occurred at lower concentrations. Thereby, depending on the number of cycles used, originally present concentration differences were compensated to a certain degree (Suzuki and Giovannoni 1996). Furthermore, the hybridization itself can be influenced by the position of a certain probe on the chip surface (Koren et al. 2007; Steger et al. 2011), but the performance of the hybridization in wet chambers during this study should have prevented spatial biases caused by convection effects to a large extend. Unusual high fluorescence signals on the edges of the microarray were not observed in our samples. Finally, regarding the fluorescence staining of the microarray, the concentration of ozone in the ambient air has been shown to be a critical factor (Fare et al. 2003; Branham et al. 2007; Byerly et al. 2009). Apart from a swift handling of the wet slides to minimize the exposure time to ambient air, no special precautions were taken regarding this issue. Thus, it is possible that varying concentrations of ozone might have biased our fluorescence signals, but the extent of the effect is difficult to estimate.

In conclusion, molecular probes specific for taxa which include a great proportion of small cells that cannot be observed directly but are only accessible by using bulk indicators (pigments or fluorescence), are generally only of limited suitability for a comparison with other, also molecular, data. In addition, the relatively high number of steps during the microarray analysis potentially introduces a high methodological bias, which also was probably the main reason for weak linear correlations for taxa that are readily countable under the microscope.

3.3 Evaluation of the nucleic acid biosensor

As it has been shown, PCR-based assays such as the phylochip have a high potential to introduce biases into the analysis of phytoplankton communities. In order to evaluate whether the low quantitative correlation between phylochip signals and cell counts are primarily based on PCR-related biases, an additional molecular method was evaluated that directly targets the 18S rRNA in a cell. Thus, we evaluated an automated nucleic acid biosensor (Diercks et al. 2008a; Diercks et al. 2008c) for its capability to quantify
phytoplankton. The automated nucleic acid biosensor circumvents the isolation of nucleic acids from a sample, PCR-amplification of the ribosomal sequences, labeling of PCR-fragments and staining of the hybridized nucleic acids on the chips. After filtration, the cells in the samples were lysed. Subsequent to the lysis, cell debris was removed by an additional filtration, before the sample was subjected to the automated analyses device. The lowest correlation in respect to the quantification of a taxon with the phylochip was observed for the *Pseudo-nitzschia* spp. genus level probe. Therefore, we addressed for this genus whether a direct detection of 18S rRNA with the automated nucleic acid biosensor would improve molecular based quantitative assessments. The sequence of the capture probe used for the biosensor assay was identical to the sequence of the molecular probe targeting the genus *Pseudo-nitzschia* on the phylochip. Evaluation of the rRNA biosensor involved quantification of defined cell numbers from a laboratory culture (*P. subcurvata*) and the surveillance of *Pseudo-nitzschia* spp. (*P. seriata* and *P. delicatissima*) in field samples. The resulting dataset showed a significant linear correlation ($r^2= 0.68$, $n= 11$, $p= 0.0018$) between the signals measured with the biosensor and the cell numbers of the different species of *Pseudo-nitzschia* spp. (Fig. 3). Thus, signaling and linearity of the biosensor measurement was independent of the species that was targeted by the genus level probe. The main reason for the only moderately high $r^2$-value was probably the small number of samples investigated, and a repetition of the analysis including a larger number of samples would most likely result in an even better correlation. To a certain extent, errors related to cell counting have also to be taken into account, as well as variations of the rRNA content per cell depending on their physiological state. However, the results suggest a considerable improvement of quantitative taxon detection by the direct targeting of the 18S rRNA compared to the assessment via 18S rDNA so this approach should be the preffered method with respect to assessing changes within the phytoplankton community.

4. Conclusions

In summary, a relatively high agreement was observed between the microarray signals and the signals of the comparison parameters in terms of presence/absence. Furthermore, many of the mismatches can be explained by insufficiencies of the reference methods and were not necessarily the result of biases in the microarray method. Even though, the phylochip could be a source for biases related to the specificity of the molecular probes. However, if more than one reference method was used, there was indication that the microarray results might be even more reliable than some of the other reference methods, e.g. pigment data.
In contrast, a quantitative comparison of the signal obtained from rDNA microarrays with other parameters was difficult. On one hand, this was because of certain sources of bias inherent in the microarray; on the other hand, it was due to limits in the compared methods or by the fact that microarray and comparison parameter often did not exactly refer to the same target. But in cases like *C. fusus*, which is easily identified and where the counts are reliable because of its sufficient size, linear relationships could be established. But the weakness of the correlation and the need for a logarithmic transformation of the data to achieve the correlations demonstrated the high impact of the different error sources on the phylochip results. Therefore, relative changes detected in species abundance are burdened with high variability and should therefore be considered only as rough estimates. If quantitative data are the aim of the investigation, the direct targeting of 18S rRNA sequences is indicated as the more promising approach.

Generally, a drawback of both molecular sensing approaches is the need for the development and specificity testing of molecular probes, which requires a lot of time and effort. Furthermore, diversity-analysis using this approach is limited by the set of probes, because the species which are going to be investigated have to be defined in advance. Thus, for such analyses, other molecular approaches, for example pyrosequencing (Margulies et al. 2005) of 18S rDNA, might be more suitable, it directly reads the sequences of the taxa abundant in the sample and does not rely on molecular probes.

Nevertheless, the strength of the microarray method is its rapidity once the molecular probes are available, and it is, regardless of all biases, very useful to allow a quick overview of a large dataset on the basis of key taxa. In this way, samples of interest for a specific scientific question could be identified with relatively low effort and analyzed subsequently with other methods (e.g. microscopy or pyrosequencing). But also routine monitoring can be performed in a fast and reliable way, if primarily the presence or absence of a species is of interest, for example in the case of toxic phytoplankton species which are harmful even at low concentrations. However, more emphasis should be laid on rRNA-based microarrays, since they appear to be less prone to errors. However further evaluations by comparison with cell counts would be reasonable in order to support the first results we obtained in this study.

Moreover, since they bear the potential for a complete automation, molecular sensor-based methods might be incorporated in unattended systems, like the FerryBox (Petersen et al. 2011) in the future. Even though microarrays might not yet be a replacement for other methods, they do have potential for improvement and are within their limits already a valuable addition to the toolbox of marine ecologists.
Acknowledgements

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References


Nelson TA, Holmes S, Alekseyenko AV, Shenoy M, Desantis T, Wu CH, Andersen GL,


### Tables

**Tab. 1:** Target taxa and sequences of molecular probes used in the present study. *The probe sequences for C. debilis target also C. curvisetus, C. muelleri and C. gracilis.*

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Tab.2: Results of the specificity tests for the newly designed molecular probes. “+” indicates signals obtained from the hybridization, “−” indicates no signal. Signals obtained from non-target taxa (false positives) are shown in brackets. These probes were not used in the further study.

B= Bacillariophyceae, H= Haptophyceae, D= Dinophyceae, C= Chlorophyceae

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Tab. 3: Matrix of match and mismatch between phylochip and the different comparison parameters for all stations of the different cruises. Light grey squares indicate a match between phylochip signal and signal of the comparison parameter. Dark grey squares indicate a signal from the phylochip, but no signal from the comparison method, while it is the other way around for the black squares. White squares mean that no comparison has been made. Counts are given in cells/L, pigment concentrations in µg/L and fluorescence obtained by flow cytometry in arbitrary units. FC= flow cytometry, CP= comparison parameter, MA= microarray.
Tab. 3: continued

| Match [%] | 71 | 49 | 55 | 49 | 60 | 63 | 73 | 71 | 73 | 54 | 57 | 49 | 60 | 63 | 73 | 71 | 73 | 54 | 51 | 51 | 70 | 94 | 80 | 94 | 97 | 66 |
|-----------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| over-estimation by MA [%] | 29 | 31 | 33 | 43 | 29 | 23 | 16 | 24 | 27 | 24 | 10 | 14 | 10 | 14 | 75 | 11 | 10 | 25 | 0 | 17 | 4 | 0 | 0 | 10 |
| under-estimation by MA [%] | 0 | 19 | 12 | 4 | 23 | 17 | 22 | 2 | 2 | 2 | 36 | 34 | 36 | 1 | 39 | 40 | 5 | 6 | 3 | 2 | 3 | 24 |
| CP median value | 200 | 80 | 1668 | 1668 | 1668 | 2480 | 12041 | 12041 | 12041 | 1940 | 1940 | 1940 | 1940 | 590 | 590 | 590 | 590 | 80 | 1223 | 1223 | 0.05 | 0.05 | 1694 | 2.7E+08 | 0.08 |
Fig. 1: Correlations between the signal/noise-ratio of the phylochip and different comparison parameters. (A) Ceratium fusus, compared with cell counts, (B) Chaetoceros socialis, compared with cell counts, (C) dinoflagellates, compared with concentration of peridinin (stations represented by grey diamonds are omitted from the calculation of the linear fit), (D) Pseudo-nitzschia sp. (probe PSNEXDELIB), compared with cell counts.
Fig. 2: Correlation between the electrochemical current obtained by the automated biosensor (probe PSNEXDELIB) and microscopically obtained cell count of *Pseudo-nitzschia* spp.
3.6 Manuscript IV

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 ecology is crucial for an understanding of the whole marine ecosystem due to its pivotal role as primary producer. In the North Sea, research on the biodiversity and ecology of phytoplankton is usually carried out using microscopic observations. Due to limitations of this approach regarding detection and identification of nano- and picophytoplankton, these investigations are generally focused on the microphytoplankton. In the last decades, a variety of methods have evolved based on optical and molecular biological approaches which enable (i) a more rapid and convenient analysis of phytoplankton samples and (ii) allow an assessment of small phytoplankton in more detail.

In this study, a selection of these methods (in situ fluorescence, flow cytometry, genetic fingerprinting, and DNA microarray) were used in addition to traditional microscopy to analyze biomass distribution and the complete community structure of phytoplankton in the German Bight. Investigations were carried out on the level of the whole community level as well as separately for the different phytoplankton size classes. The study time period was 2010 and 2011, covering spring, summer, and autumn, to investigate seasonal variation of the community. Furthermore, the cruises covered nearly the whole German Bight to also analyze spatial variability.

A wide patchiness in biomass distribution was found, and microphytoplankton was identified as the major contributor in all seasons, followed by the nanophytoplankton. In contrast,
picophytoplankton was negligible in terms of biomass. The overall contribution of small phytoplankton was higher in offshore areas and also in areas exhibiting higher turbidity. In terms of community structure, differences between the small phytoplankton community and the microphytoplankton were found. The latter exhibited a seasonal pattern regarding number of occurring taxa, 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 primary producers in marine ecosystems and constitute the basis of the marine food web. Moreover, although representing less than 1% of global biomass, they account for roughly 50% of global carbon fixation and are thus a crucial factor in the carbon cycle (Field et al. 1998). Especially coasts are highly productive areas due to mixing of water columns, a comparably low water depth and increased nutrient input by upwelling or adjacent rivers. The North Sea is an example for such a coastal ecosystem. Like many other coastal areas in the world, it is highly utilized by the surrounding countries (Ducrotoy et al. 2000) and is subjected to pollution and increased nutrient input. In such a human influenced system it is important to understand the spatiotemporal distribution and biodiversity of phytoplankton, because changes in this community are likely to also affect higher trophic levels.

Furthermore, detailed investigations are necessary for the detection and – where necessary – prevention of negative biodiversity shifts, which potentially reduce the ability of the ecosystem to cope with environmental changes or human induced stress (Yachi and Loreau 1999).

A lot of knowledge on the structure and variability of the phytoplankton community in the North Sea has been obtained by microscopic observations, either as part of time series (Reid et al. 1990; Tillmann and Rick 2003; Wiltshire et al. 2010) or during occasional research cruises. These analyses require a high effort, often resulting in a reduced temporal or spatial coverage of the investigations. Furthermore, the focus of most studies using microscopy is primarily on microphytoplankton (20-200 µm), since nanophytoplankton (2-20 µm) and especially picophytoplankton (0.2-2 µm) is difficult to count microscopically and often lack morphological features for a reliable identification. Thus, for microphytoplankton in the North Sea, information is available regarding general seasonal succession patterns (Hagmeier and Bauerfeind 1990; Reid et al. 1990), response to environmental factors (Hickel 1998; Freund et al. 2012; Schlüter et al. 2012), and diversity (Hoppenrath 2004; Wiltshire and Dürselen 2004; Hoppenrath et al. 2007). Although attempts have been made to also assess the diversity of smaller phytoplankton (e.g. Novarino et al. 1997; Knefelkamp 2009), these are relatively scarce due to the high effort of the investigation. However, for a thorough understanding of phytoplankton ecology, information on nano- and picophytoplankton of comparable quality to the information available for microphytoplankton would be advantageous.

Over the past decades, a number of methods have emerged to assess various aspects of the nano- and picophytoplankton community. A common one is flow cytometry which allows a fast and accurate determination of cell size distributions in a sample (Olson et al. 1985; Phinney and Cucci 1989; Vives-Rego et al. 2000). Furthermore, molecular biological approaches have become popular, because since they rely on nucleic acid sequences, they
are especially useful for obtaining taxonomic information about small cells which would otherwise not be identifiable. An overview of the most common molecular methods is given by de Bruin et al. (2003), but frequently used and convenient techniques are community fingerprints and DNA-microarrays. A common fingerprinting technique is automated ribosomal intergenic spacer analysis (ARISA). It is often applied for analysis of prokaryotic communities (Danovaro et al. 2006; Kovacs et al. 2010), but it has also been applied for eukaryotes (Fechner et al. 2010; Wolf et al. 2013). Although such fingerprints are not suitable for an absolute assessment of biodiversity (Bent et al. 2007), they allow a rapid comparison of community composition between samples (Knefelkamp 2009; Wolf et al. 2013).

In contrast, DNA-microarrays can provide taxon specific information about the phytoplankton community in a sample (Kochzius et al. 2007). They are based on the detection of certain DNA-sequences in a sample by the use of taxon-specific, complementary oligonucleotide molecular probes. These probes often target regions in the 18S-rRNA gene in the ribosomal operon, because these genes are very suitable for investigations of different taxonomical levels (Díez et al. 2001; Moon-van der Staay et al. 2001). Microarrays have frequently been used for the investigation of prokaryotic communities of various origin (Sessitsch et al. 2006; Nelson et al. 2011), but also for cryptophytes and prasinophytes in the German Bight (Gescher et al. 2008; Metfies et al. 2010).

The aim of the present study was a characterization of the phytoplankton community in the German Bight on the level of micro-, nano-, and picophytoplankton in order to get an as detailed overview as possible. To achieve this, the above mentioned techniques flow cytometry, molecular fingerprinting and DNA microarray were used complementary to traditional microscopic observations. The investigation was carried out on several cruises over two years covering numerous transects in the German Bight in order to analyze the composition of the phytoplankton with respect to seasonal, but also spatial differences. Simultaneous measurement of abiotic parameters, were used to identify factors influencing biomass distribution and biodiversity.

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). The study area covered the region of the German Bight in the North Sea and cruise stations as well as transects are given in figure 1. Due to the weather, the order of stations was not always the same and in September, the most offshore stations could not be sampled. On each cruise, “extra” stations were integrated along the transect lines, but their frequency and position varied between the
cruises, thus they were not displayed on the map. 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 depth of 4-5 m 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 different methods described below.

2.2 Discrete measurements of chl-a and total suspended matter
Chl-a concentration was measured by High Performance Liquid Chromatography (HPLC) according to 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 the pigments was carried out by a HPLC system from JASCO (Japan).

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 of the rim with sea water during filtration. This was done in order to reduce the amount of salt that cannot be washed out of the filter afterwards (Stavn et al. 2009). To correct for still remaining salt, on each cruise, filtered seawater was applied to empty filters, and their average salt induced weight increase was 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 fluorescence
Continuous measurements (at 1 min intervals) of temperature, salinity, chromophoric dissolved organic matter (CDOM), turbidity, and chlorophyll-a fluorescence were performed taking advantage of a FerryBox system as described in Petersen et al. (2011). Its water inflow was in the moon pool of the ship at a depth of ca. 4 m, comparable to the depth of the water sampling. The sensors mounted in the FerryBox are listed in table 1. Furthermore, concentrations of nitrate and phosphate were measured using a Systea µMac nutrient analyzer (Systea, Italy) attached to the FerryBox. Sample water for the nutrient analyzer was provided from a bypass of the FerryBox and filtered by a cross-flow filter (MiniKros, pore size 0.2 µm, Spectrum Laboratories, USA) previous to analysis. Measurement intervals were
approx. 50 min. In between these measurements, values for the total duration of the respective cruise were interpolated. Furthermore, discrete water samples were taken behind the cross-flow filter, frozen and stored at -20 °C. They were analyzed in the laboratory for nitrate and phosphate using an AutoAnalyzer 3 (Bran+Luebbe, Germany), and the methods from Grasshoff et al. (1983). These measurements were used to correct the field measurements subsequent to the cruise.

2.4 Microscopic cell counts

At each station, 100 mL of seawater were filled in brown glass bottles, fixed with 0.5 mL Lugol’s solution, and stored at 4-8 °C until analysis according to the method of Utermöhl (1958). Normally, 50 mLs of sample were filled in an Utermöhl chamber and cells settled for 24h. When water was full of particulate matter, only 25 mLs of sample were analyzed for optical reasons. Cells belonging to the microphytoplankton (>20 µm) were counted using an inverted microscope (Olympus IX 51, Olympus, Japan), phase contrast and 100x or 200x magnification. No regular replicate counting of samples was made, since in most cases, a single count is considered to be sufficient (Lund et al. 1958). Random re-counts of single species in different samples showed that the counting error averaged 11 % (with the highest value of 22 %) in the present study. Cells were identified to species or genus level, but at least to class level. Separately for every cruise, cell dimensions were recorded for every counted taxon based on pictures taken during microscopic analysis. If the abundance in a sample was high enough, at least 25 individuals per taxon were measured. Afterwards, biovolume per cell was calculated using the equations given in Hillebrandt et al. (1999) and the mean value of the respective cell dimensions. If dimensions of a certain taxon could not be measured in a particular sample, the average cell dimensions of this taxon from the other cruises were used instead to calculate the biovolume. The introduced error is smaller than it would be if the taxon was to be completely omitted from the sample. However, this was done only in exceptions and mostly for rare taxa which only constituted a small fraction of the whole abundance in the sample.

Cell-specific biovolume was converted into cell-specific carbon content by using the equations given in Menden-Deuer & Lessard (2000) for diatoms and other cells, respectively. Total carbon concentration per taxon at a particular station was obtained by multiplying its abundance with its cell specific carbon content.

2.5 Flow cytometry

For flow cytometry analyses, 3 mL seawater was fixated with glutaraldehyde (0.4 % final concentration) and incubated for 15 min. Subsequently, the sample was shock-frozen in liquid nitrogen and stored at -20 °C. Sample analysis was carried out using a FACSCalibur...
Autofluorescence of phytoplankton was excited by blue light (488 nm) emitted by a 20 mW-laser. In order to isolate eukaryotic nano- and picophytoplankton, gating 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 also served as reference for the normalization of cellular optical properties. In case of the FACSCalibur, TruCount beads (Becton Dickinson, USA) were used for absolute volume calibration. For both phytoplankton fractions obtained parameter were cell counts, average cell size (based on side scatter) as well as red and orange fluorescence intensity.

Biovolume of nano- and picoplankton was calculated 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 the 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 analysis was identical to the one described in Wollschläger et al. (2013), 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 used probes is given in table 2.

**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). 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).

3. Results & discussion
3.1. Abiotic parameters and biomass distribution
Figure 2 gives an overview of results obtained for temperature, salinity, CDOM, TSM, nitrate, phosphate, N:P-ratio, and chl-a concentrations from continuous measurements of the FerryBox. TSM and chl-a concentrations were calculated on the basis of continuous turbidity and chl-a fluorescence measurements using the coefficients and the equation given in table 3. They are the results of linear regressions between discrete TSM and chl-a measurements obtained at the stations and values for the corresponding optical proxy data extracted from the continuous data set. The color scaling of the figure is non-linear to present differences more clearly. Nutrient values smaller than 0.05 µmol/L can be considered to be below the detection limit, they were shown here, however, to illustrate distributions. They were omitted for the calculation of N:P ratios, in order to avoid the creation of unrealistically high or low ratios. 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 seen in terms of lower salinity, coupled with higher concentrations of CDOM and nutrients in these areas. Nitrate levels were higher in the earlier periods of the year (April and May, but also June). Remarkably low values of phosphate, were detected in May 2010, while between the other cruises, differences 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. Phytoplankton biomass (expressed as chl-a concentration) was highest in May 2010, reaching up to 60 µg/L; on the other cruises, the maximum values were ca. 15 µg/L, with exception of the June cruise in 2011 (ca. 30 µg/L). Generally, the biomass pattern was quite patchy, emphasizing the value of continuous measurements for mapping phytoplankton distribution. Regarding the accuracy of the chl-a
concentrations in absolute terms, it has to be taken into account that they were based on fluorescence measurements, which exhibit a relatively large variation in their relationship to chl-a (Falkowski and Kiefer 1985; see also the r²-values shown in table 3). However, in the course of this study, the relative distribution instead of the absolute values was of primary interest.

Although coastal waters are considered to be beneficial for phytoplankton growth due to high loads of nutrients (Radach 1992) and growth promoting humic organic substances (Prakash and Rashid 1968; Carlsson and Granéli 1993) originating from riverine input, these parameters could not explain directly the biomass distribution in the German Bight. Also the biomass peaks could not be related to especially high CDOM values or concentrations of nitrate or phosphate. Only a weak linear relationship between biomass and general coastal characteristics (using salinity as proxy) was observed (tab. 4). This is probably caused by a time-lag in the response of phytoplankton to changing nutrient conditions. Cells are able to build up internal reservoirs (Dortch 1982), so low actual nutrient availability is not necessarily an instant impediment for growth. Moreover, once nutrients were taken up, they have to be assimilated into organic molecules (Wheeler 1983), before they can be used for growth processes. Thus, interpretation of phytoplankton growth by nutrient situation is difficult when only a snapshot of the situation is available. This approach is more suitable for time series, where the development of both parameters can be tracked over a longer period.

Furthermore, nitrate and phosphate are not the only nutrients important for phytoplankton growth, for example, silicon is required by diatoms for frustule formation. Another crucial point is the ratio of nutrients, since imbalance in nutrient availability can also limit phytoplankton growth (Tilman et al. 1982). In this study, calculated N:P-ratios showed a potentially phosphorus limitation near the coast at least at the beginning of the year (April – June). Later in the year, and generally in the offshore regions, nitrogen appeared to be the limiting factor. But besides nutrient limitation, also unfavorable light conditions have shown to inhibit phytoplankton growth (Loebl et al. 2009), as well as biotic factors such as zooplankton grazing (e.g. Calbet and Landry 2004) or degradation by viruses (Brussaard 2004; Rhodes et al. 2008).

### 3.2 Biomass distribution by size class

Carbon biomass (referred to as “biomass” in the following) was calculated for each size class separately on the basis of cell size measurements. Only autotrophic cells were taken into account. Cell dimensions were either determined by microscopic observation (microphytoplankton) or flow cytometric analyses (nano- and picophytoplankton). The cruise in May 2010 was omitted from the analysis, since data for microphytoplankton were not available.
Total autotrophic biomass on the basis of these calculations ranged from 9 – 533 µg/L in July 2010 and from 62 – 621 µg/L in September 2010. In 2011, the ranges were 10 – 566 µg/L (April), 21 – 603 µg/L (June), and 17 – 359 µg/L (September).

A comparison of these calculated biomass values with available chl-a concentrations is shown in figure 3. Since the conversion factor between chl-a and biomass is usually between 20 and 50 for healthy cells of diatoms, dinoflagellates, and microflagellates (Reid et al. 1990), the obtained slope of 37.89 indicates that the calculations are in general reasonable. However, the coefficient of determination is relatively low ($r^2= 0.6$, p<0.001), which is caused on the one hand by the well-known natural variability between biomass and chl-a (Banse 1977; Hallegraeff 1977; Geider 1987; Jiménez et al. 1987; Llewellyn and Gibb 2000), but on the other hand probably also by difficulties associated with biomass calculation. The calculations are influenced by accuracy of cell size measurements as well as by errors in biovolume calculation and carbon conversion. In the case of the present study, especially the biomass values obtained for the nano- and picophytoplankton are likely to a certain extent to be biased, because for their calculation, station-specific average size values for the respective size class were used and the cells were assumed to be spherical. However, although being a simplification, these assumptions were necessary for biomass calculations based on the data provided by flow cytometry, and the introduced error should be small enough not to interfere with the main conclusions drawn from the data.

On average, microphytoplankton constituted the major part of autotrophic biomass in most seasons (Fig. 4) followed by the nanophytoplankton which contributed on average over all cruises approx. 40 % to total biomass. In contrast, the contribution of picophytoplankton was almost negligible with average values below 5 %. This low impact of picoplankton on bulk biomass is characteristic for productive waters (Agawin et al. 2000), because when nutrients are not the main limiting factor, the picophytoplankton has no competitive advantage over larger cells due to its more efficient nutrient uptake and usage capacities (Fogg 1991; Chisholm 1992). When the cruise results were sorted by month irrespective of the year (Fig. 4), it could be observed that the proportion of nanophytoplankton was high in spring and early summer, decreased in late summer and increased again in autumn.

A similar development has been found for nanophytoplankton in the Barents Sea (Druzhkova and Makarevich 2008); however, they showed also a high dynamic in the development of the nanophytoplankton biomass over the year. Since our observations are only based on data from two years and the temporal coverage within a year was also low, further investigations are necessary for a more scientifically sound analysis regarding the pattern of nanophytoplankton contribution to total biomass during the year in the German Bight.

On a spatial scale, contribution of nanophytoplankton was patchy, but not completely randomly distributed (Fig. 5). As a tendency, higher proportions could be observed both in
most offshore areas and close to the coast, especially near Borkum and the North-Frisian Islands, in the areas in between, microphytoplankton dominated biomass. The higher contribution in the offshore regions is in accordance with observations by Sabetta et al. (2008), and is possibly related to the aforementioned advantage of smaller cells in dealing with low nutrient concentrations (Fogg 1991; Chisholm 1992). The occurrence of a higher proportion of nanoflagellates off the coast of the North-Frisian Islands was also found by Hesse et al. (1989), who attributed it to water turbidity. Small cells are more efficient in light absorption than larger ones due to a lower “pigment packaging effect” (Morel and Bricaud 1981; Kirk 1994). Thus, the higher contribution of nanophytoplankton to total autotrophic biomass in the coastal areas might be caused by light limitation in the water. Furthermore, biotic factors like zooplankton distribution and species composition can also have an effect in shaping the biomass proportions between the different size classes, for example by selective grazing (Riegman et al. 1993; Gaul and Antia 2001; Lindén and Kuosa 2004), although their impact was not assessed in the course of this study. However, in summary, the results emphasize the generally high importance of nanophytoplankton in the German Bight and the need to further characterize this size class properly in its temporal and spatial distribution.

3.3 Taxonomic structure of the phytoplankton community

3.3.1 General community patterns

In a first step, the whole phytoplankton communities of the stations from the different cruises were investigated by obtaining 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. 6A): The first group included all cruises from 2010, with no clear differences between the seasons, while the second group was a tight cluster consisting of the stations in 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 (temperature, salinity, CDOM, and turbidity) available for the stations. The resulting pattern was considerably different from the one obtained from ARISA-data (Fig. 6B): Spring cruises in 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 ($r = -0.11$, $p = 0.974$). Nevertheless, the pattern in the ARISA data suggested a major change in the phytoplankton community between 2010 and the first half of 2011, and a second one at the end of the growing season. However, without supplementary data, no conclusions could be
drawn about the kind of change that had taken place and if all parts of the community were involved equally. Thus, these parts were investigated separately in the following.

3.3.2 Microphytoplankton

Analysis of the microphytoplankton fraction was conducted on the basis of microscopic cell counts, since this size class is readily countable and identifiable by this method. The first aim was to determine its general biodiversity in the German Bight at the time of the different cruises. Firstly, the number of occurring taxa was compared by the creation of rarefaction curves (Fig. 7). Interpolation of the curves to an equal number of samples suggested that during summer, less taxa were present than during spring and the highest number of taxa occurred in autumn. Furthermore, they showed that 15-25 samples were enough to cover the majority of taxa abundant in the German Bight, since most curves nearly reached saturation at this level. In September 2010, some taxa were probably missed, because fewer samples were evaluable due to high debris loads induced by a storm. Moreover, due to the higher presence of non-algae particulate matter, some of the smaller taxa in the remaining samples were probably overlooked (First and Drake 2012).

Since not only the number of occurring taxa is a descriptor of biodiversity, but the abundance per taxon also has to be taken into account, the Simpson index ‘1-D’ (Magurran 2004) was calculated for all stations. It ranges from 0 to 1, with increasing values to more ‘even’ communities with several equally contributing taxa. On average, it was 0.52 in July 2010, 0.63 in June 2011, and 0.67 in April 2011. The September cruises showed statistically significant higher values in both years (p<0.05 according to an ANOVA) with 0.8 in 2010 and 0.85 in 2011. Differences between seasons 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 the September of both years (1.13 in 2010 and 1.4 in 2011).

In a next step, the community composition of the different cruises was analyzed in more detail by means of canonical correspondence analysis (CCA; Fig. 8). For clarity reasons, only the most abundant taxa were considered. All dinoflagellate taxa were included which contributed to 90 % of total dinoflagellate abundance on a cruise. For non-dinoflagellates, this threshold was set to a lower value (80 %) because of the higher number of taxa present. 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 position of the different taxa in
relation to the environmental variables was used to draw conclusions about their preferred occurrence.

Diatom taxa were found in all environments throughout the year. Especially the genus Chaetoceros with various members was an important element of the diatom community. Most of them appeared to prefer clearer waters, only Chaetoceros pseudocurvisetus was found in more turbid regions. Also the majority of the pennate forms (e.g. Navicula spp., Bacillaria paxillifer, Pseudo-nitzschia seriata) were found there, probably because they are often associated with solid substrates (Hoppenrath et al. 2009). Mediopyxis helysia, a species newly recorded in the German Bight, was also found in the course of this study. According to its position in the CCA, it appeared to prefer colder, coastal waters, which is in accordance with previous observations made for this species (Kraberg et al. 2012). The genera Guinardia and Leptocylindrus are both represented in the dataset with two species. Interestingly, in both cases, the smaller one was more common in turbid waters while the larger one had its main distribution in clearer waters. It could be speculated that this distribution might again be a result of differences in light absorption efficiency between smaller and larger cells.

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 characteristic of the summer periods. Indeed, dinoflagellates dominated the community both in terms of cell number and biomass during this time, with exception of the coastal areas (Fig. 9). This observation is in accordance with results published by e.g. Hagmeier & Bauerfeind (1990), Peeters & Peperzak (1990) and Hickel (1998). Under stratified, more oligotrophic conditions, dinoflagellates have competitive advantages over diatoms (Fogg 1991). They require no silicate for the formation of their cell walls, and are also able to exploit nutrient rich water near or below the thermocline due to diurnal migrations (Cullen 1985; MacIntyre et al. 1997). Besides small thecate and athecate dinoflagellates, Dinophysis acuminata was frequently found as well as several members of the genus Ceratium. The increased occurrence and contribution to biomass of this genus is a typical feature of the North Sea in the second half of the year (Reid et al. 1990).

Considering the distribution of the stations, the CCA showed a difference between spring, summer and autumn communities. The stations of corresponding seasons were positioned relatively close to each other, indicating similar communities in both years. Minor variations between 2010 and 2011, as observable for the summer cruises, are probably related to differences in phytoplankton succession stage caused by mismatches in the time of the sampling.

However, in conclusion, neither the general investigation of biodiversity nor the CCA showed a pattern which is similar to the one seen in the ARISA data. No evidence for a sudden
change in microphytoplankton community composition between 2010 and 2011 was observable.

3.3.3 Nano- and picophytoplankton

The first approach to assess the eukaryotic nano- and picophytoplankton community was flow cytometry. Since orange fluorescence (originating from phycoerythin) can be used as indicator for the presence of cryptophytes (Li and Dickie 2001) and the red fluorescence (originating from chl-a) as proxy for total biomass, the ratio between both was used in this study to estimate the proportion of cryptophytes in the respective size class (Fig. 10). However, it is a relatively crude parameter, allowing only semiquantitative estimations due to the variability inherent in fluorescence measurements (Falkowski and Kiefer 1985). Cyanobacteria, which also show orange fluorescence, were omitted from the analysis on the basis of their lower side-scatter. For both nano- and picophytoplankton, 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, the ratio indicated a relatively constant proportion of cryptophytes in the picophytoplankton in 2010 in all seasons, while in April and June 2011, the drop in the ratio suggested a considerably higher contribution of this class. In September 2011, the proportion of cryptophytes was smaller again, but more variable between the stations. For the nanophytoplankton, a similar, but much less pronounced pattern could be observed.

The second approach for a closer investigation of the nano- and picophytoplankton community was the use of a DNA-microarray. It targeted different clades of cryptophytes and prasinophytes, because both classes have been shown to be important contributors to the small phytoplankton community in the German Bight (Gescher et al. 2008; Metfies et al. 2010). Because samples were not size fractionated in advance, nano- and picophytoplankton were handled together, and representatives of cryptophytes and prasinophytes larger than 20 µm could also have been included in the analysis. However, such large members are in general comparably scarce within the targeted clades, and were also not especially apparent in the microscopic observations. Since the obtained signal intensity of a DNA-microarray can be biased from several sources (Medlin et al. 2006; Wollschläger et al. 2013), the data were only interpreted with respect to the presence or absence of the different clades in this study. Figure 11 shows the relative abundance of signals obtained for the various clades on the different cruises. Regarding the cryptophytes (Fig. 11A), it can be seen that the probe which was specific for the whole class gave a signal at nearly all stations (compare also Wollschläger et al. 2013). This indicated that they were ubiquitous members of the phytoplankton community during all seasons in the German Bight, which is in accordance with results from Metfies et al. (2010). The presence of the different clades was relatively
similar in 2010, only clade 4, 5 and 6 were less frequent in July 2010. Using the average number of signal-giving probes per station as an index for cryptophyte biodiversity, it was 3.7 (May), 2.8 (July) and 3.4 (September), respectively. Thus, in 2010, the diversity within this class was relatively similar.

In contrast, at the beginning of 2011, the presence of all targeted clades was considerably lower. Although increasing again to the end of the year, the values remained below those observed for 2010, with exception of clade 4 and 6, which fully recovered. Consequently, this was accompanied by a reduction in cryptophyte biodiversity: On average, only 1.3 (April), 1.9 (June) and 2.4 (September) probes per station gave a signal in 2011. A similar development was observed for the prasinophytes (Fig. 11B): The relative abundance of signals from the different probes dropped remarkably in 2011 compared to 2010, and also their average number per station. In 2010, it was 7.7 (May), 6 (July), and 7.6 (September), while it was 1.4 (April), 3.8 (June), and 4.7 (September) in 2011.

In order to evaluate not only the temporal, but also the spatial heterogeneity in cryptophyte and prasinophyte distribution, the average number of signal-giving probes was plotted on a map (Fig. 12). The results showed a high accordance of cryptophyte and prasinophyte diversity. Furthermore, the diversity distribution was patchy, with high and low values occurring both offshore and near the coast. Direct correlations with environmental parameters were not found, but some tendencies could be deduced from the figure: Commonly, high diversity occurred in the region of the East Frisian Islands as well as off the coast of Sylt. These were also the turbid areas, where nano- and picophytoplankton exhibited often high biomass proportions (see 3.2), indicating an environment generally suitable for small phytoplankton. In contrast, a lesser degree of diversity was frequently observed near the Elbe estuary, and at the inner parts of the German Bight.

In summary, although referring to different aspects, both flow cytometry and DNA-microarray showed a change in the small phytoplankton community between 2010 and 2011, and a second in September 2011. There was no clear evidence for seasonality as observed for the microphytoplankton community. Thus, small phytoplankton appears to respond different to the given physicochemical parameters, as it has also been observed for picophytoplankton by Not et al. (2007). However, the patterns observed by the two methods widely resemble the one seen in the ARISA data (section 3.3.1). 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; Not et al. 2007; Vaulot et al. 2008; Knefelkamp 2009). Furthermore, fingerprinting methods tend to neglect rare species (Liu et al. 1997), and in terms of cell number, nano- and picophytoplankton was ca. 100 times more abundant than microphytoplankton (data not
shown). Thus, for a more detailed analysis of the three size classes by ARISA, the samples should be size-fractionated in advance. The reason for the observed change remained unexplained; temperature and salinity data obtained from routine FerryBox measurements in the German Bight during the winter showed no distinctive features compared with other years (data not shown). Furthermore, as only the data from two years were available, it could not be evaluated whether such an abrupt change in the community is common or an exception.

4. Conclusions
Phytoplankton in the German Bight was investigated on various levels using a selection of methods ranging from traditional microscopy to methods based on optical proxy measurement and molecular techniques. Biomass distribution was patchy in all seasons showing no strong linkage to the measured environmental parameters. Besides the microphytoplankton, the nanophytoplankton contributed largely to autotrophic biomass, and even dominated in certain areas. This emphasizes the importance of this size class in the German Bight and the need for its more detailed investigation. In contrast, picophytoplankton played a minor role with respect to biomass in this coastal ecosystem.

With respect to community structure, seasonal patterns were observed for the microphytoplankton (e.g. dinoflagellate dominance in summer, a higher biodiversity in autumn). In contrast, the nano- and picophytoplankton community behaved different: It was similar in the whole year 2010, changed at the beginning of 2011, and again at the end of 2011. This was visible in terms of the relative proportion of cryptophytes as well as in cryptophyte and prasinophyte diversity. However, no corresponding changes in the physicochemical parameters of the German Bight could be observed. On a spatial scale, the distribution of cryptophyte and prasinophyte diversity was similar. In total, it was patchy, but although not always harboring the highest biodiversity, turbid coastal areas showed regularly high diversities on all cruises. The factors governing the observed changes and also the distribution of diversity in the small phytoplankton remain largely unknown, and due to the lack in data from this region for comparison, the interpretation of the observed patterns is often difficult. However, the finding of these large differences in the development of microphytoplankton and small phytoplankton community emphasizes again both the value but also the requirement of using additional methods to routinely microscopic observation. The available database has to be broadened, especially with respect to the large contribution of nanophytoplankton to total biomass in the German Bight.

Flow cytometry, DNA-microarray and ARISA have shown to provide useful information in this context both on taxonomical and quantitative level by requiring simultaneously low effort.
Thus, for routine usage, they are more suitable than other approaches, e.g. the direct sequencing of 18S rDNA. Moreover, the performance of the microarray approach can be relatively easy expanded by the development of further molecular probes specific for other taxa.

References


**Tables**

**Tab. 1:** Parameters measured by the FerryBox used in this study.

<table>
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<td>Citadel</td>
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<td>°C</td>
<td>Thermosalinograph CT</td>
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<td>SCUFA-II / ECO FLNTU</td>
<td>Turner Designs, USA / WetLabs, USA</td>
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<td>fluorescence</td>
<td>AU</td>
<td>Cyclops-7</td>
<td>Turner Designs, USA</td>
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<td>AU</td>
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<td>probe</td>
<td>reference</td>
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Tab. 3: Coefficients obtained from linear regression used for the conversion of chl-a fluorescence into chl-a concentration and turbidity into TSM concentration, respectively. The used equation was \( \text{parameter} = (\text{optical proxy value} - \text{offset}) / \text{slope} \). *In this cruise, the continuous measurements were conducted by the ECO FLNTU sensor instead of the SCUFA-II.

<table>
<thead>
<tr>
<th>cruise</th>
<th>slope</th>
<th>offset</th>
<th>( r^2 )</th>
</tr>
</thead>
</table>
| chl-a
| May 2010    | 0.35  | 0.88   | 0.94      |
| July 2010*  | 149.42| 136.19 | 0.77      |
| September 2010 | 0.5  | 0.23   | 0.68      |
| April 2011  | 0.49  | 1.13   | 0.76      |
| June 2011   | 0.44  | 1.17   | 0.81      |
| September 2011 | 0.36 | 1.25   | 0.7       |
| TSM
| May 2010    | 0.43  | -0.62  | 0.97      |
| July 2010*  | 0.36  | 0.28   | 0.93      |
| September 2010 | 0.34 | 0.97   | 0.87      |
| April 2011  | 0.45  | 0.68   | 0.98      |
| June 2011   | 0.28  | 0.81   | 0.77      |
| September 2011 | 0.38 | 1.16   | 0.99      |
**Tab. 4:** Linear regressions between biomass (expressed as chl-a concentration) and salinity. With the exception of June 2011, p<0.05 in all cases.

<table>
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<th>slope</th>
<th>offset</th>
<th>$r^2$</th>
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<td>0.36</td>
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<td>0.19</td>
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Figures

**Fig. 1:** Stations and transects in the study area in the German Bight. Map is based on Ocean Data View Software (Schlitzer 2011).
Fig. 2: Map plots of continuously measured environmental variables and fluorescence-derived chl-a concentrations. Maps are based on Ocean Data View Software (Schlitzer 2011).
Fig. 3: Correlation between chl-a concentrations measured at cruise stations by HPLC and corresponding total calculated carbon based on cell size measurements by microscopy and flow cytometry. The dotted lines represent the 95 % confidence interval of the linear fit.
Fig. 4: Contribution of the different phytoplankton size classes to total carbon biomass averaged for all cruises as well as for the single cruises.
Fig. 5: Spatial distribution of the proportion of nanophytoplankton on total calculated carbon biomass. Maps are based on 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.
Fig. 6: Non-metric multidimensional scaling of fragment patterns obtained by ARISA (A) and environmental data available for the stations (temperature, salinity, CDOM, and turbidity; B).
**Fig. 7:** Rarefaction curves for the different cruises based on microscopic observations of the microphytoplankton fraction.
Fig. 8: 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. 9: Distribution of diatoms and dinoflagellates in terms of cell numbers (left) and carbon biomass (right). Maps are based on 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.
**Fig. 10:** Ratio of red fluorescence to orange fluorescence measured for the nanophytoplankton fraction (left) and the picophytoplankton fraction (right) for the stations of the different cruises.
Fig. 11: Relative abundance of positive signals from molecular probes specific for different clades of cryptophytes (A) and prasinophytes (B). For cryptophytes, more than one probe was specific for a certain clade. The order of columns shown is identical to the order of the probes given in table 2.
Fig. 12: Number of probes giving a positive signal per station, shown for the different cruises. Maps are based on 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.
4. Synthesis

Traditional routine monitoring of phytoplankton in the North Sea relies to a large extend on determination of bulk biomass by the measurement of chl-a via HPLC, while community composition is assessed by microscopic observations. These methods require a high manual effort, are time consuming, often restricted to the analysis of discrete samples, and hardly automatable. Furthermore, emphasis is on the investigation of microphytoplankton; smaller phytoplankton is not readily accessible by this method and thus, the wax and wane of this part of the community is often only insufficiently characterized. In order to overcome these drawbacks, optical and molecular biological approaches were used, and meanwhile, a variety of methods and devices have been evolved (de Bruin et al. 2003; Moore et al. 2009). The aim of this thesis was the evaluation of a selection of methods with focus on a routine use in phytoplankton investigation. Furthermore, the various methods were used to characterize the phytoplankton community in the German Bight. Based on these studies, recommendations will be given for their further use in routine monitoring.

4.1 Method evaluation

In manuscript I, the development of a system for the continuous measurement of total water constituent absorption on the basis of a point-source integrating cavity absorption meter (PSICAM) is described. Further to this, field test results of this flow through-PSICAM (ft-PSICAM) are presented and compared with results of a manually operated, conventional PSICAM. In absolute values, wavelength specific deviations occurred between both instruments, caused by contamination of the ft-PSICAM with phytoplankton particles during operation. However, general progression of the continuously obtained data was in accordance with the discrete measurements. Thus, the main goal, a temporally high resolution of relative changes in water constituent absorption over the whole spectrum of visible light, could be achieved using this system. Additionally, the general possibility of determining chl-a and TSM concentrations in the water by measurement of absorption coefficients was demonstrated. Moreover, it could be shown that this approach of chl-a determination was less variable than determination by in situ fluorescence measurements (manuscript II), especially at lower chl-a concentrations. However, the continuous absorption coefficient measurements performed less well than manual ones, probably because of the aforementioned contamination effects. The higher variability in the traditionally used fluorescence/chl-a relationship was attributed to a stronger linkage of fluorescence with the physiology of the cells. Comparison of chl-a concentrations calculated on the basis of both optical proxies (fluorescence and absorption coefficient) revealed differences related to light conditions, demonstrating the influence of short term light history and quenching mechanisms on the fluorescence measurements. With respect to TSM
concentration, the absorption based approach turned out to be also more stable when compared to the determination by turbidity, but the difference was generally smaller than for chl-a. Moreover, the variability of the continuous absorption measurements was even higher at low concentrations than it was for the turbidity based approach. In conclusion, absorption measurements are principally advantageous for the determination of the bulk parameters chl-a and TSM concentration, but with respect to continuous measurements, the results showed that the ft-PSICAM is not yet as accurate as the manually operated system. However, continuous or in situ measurement systems are often a tradeoff between accuracy and high temporal resolution, and since the ft-PSICAM was just a first approach for conducting absorption measurements in this way, there remains a high potential for further improvement.

In manuscript III, microarrays were evaluated as a fast and convenient tool for obtaining information about phytoplankton community structure on a taxonomical level, whereby the focus lay on the DNA-microarray. Its results were compared to those of different non-molecular methods (microscopic cell counts, HPLC derived pigment concentrations, and fluorescence measurements obtained by flow cytometry), depending on the target of the considered molecular probes. Qualitatively, thus in terms of presence or absence of a target, there was good accordance between microarray and comparison parameters for many taxa. Although some of the remaining deviations had to be related to shortcomings in the used molecular probes, the majority could be explained by insufficiencies of the comparison parameters. The DNA-microarray detected signals where the comparison parameter often did not. This overestimation occurred primarily when target taxa abundance was low. For this reason, it was not simply considered as an effect of low probe specificity, but as an effect of different sample size. Because sample size was higher for the microarray than for e.g. microscopy, there was also a higher chance of finding rare taxa. Hence, the observed “overestimation” was more likely a higher sensitivity of the microarray, and therefore, in terms of presence/absence, it could even be considered to be the more reliable method. Quantitatively, the agreement between DNA-microarray and comparison parameters was considerably lower. Only for a few taxa, weak linear relationships were observed. This was explained by the fact that various factors influence the microarray analysis, for example biases introduced by amplification of the target gene via PCR. In conclusion, the DNA-microarray can be considered as a tool for the rapid and convenient detection of the presence or absence of taxa, even in the otherwise taxonomically less analyzable nano- and picophytoplankton part of the community (manuscript IV). However, the obtained signal intensity should be treated only as very rough estimate of abundance. For qualitative purposes, the RNA-microarray appeared to be more suitable indicated by the good correlation between acquired signal and microscopically obtained cell counts (manuscript III).
Flow cytometry and ARISA were not directly evaluated by comparison with other methods. Nevertheless, according to its usage in \textit{manuscript IV}, flow cytometry has shown to provide data about the nano- and picophytoplankton which are in accordance with the results of other methods used. Furthermore, it was the only method which provided comprehensive quantitative data for these cell size classes. Thus, it can be considered as an important method which is also very convenient in usage. The same convenience can be attributed to the ARISA method, which allowed a rapid detection of general changes in the community. Visible differences can be considered as reliable, because they were consistent with observations made using flow cytometry and DNA-microarrays. However, ARISA seemed to reflect primarily changes in the nano- and picophytoplankton community, while seasonal patterns in the community composition as observed for the microphytoplankton were not detected. This was explained by the fact that the small cells are dominant by numbers, what gave them more weight in the analysis. Thus, for a more detailed analysis of the samples by ARISA, samples should be size fractionated in advance.

In conclusion, the evaluated optical and molecular methods have improved the analysis of the phytoplankton community, providing a more detailed picture by complementing the data of traditional microscopy (\textit{manuscript IV}). Nevertheless, microscopy will remain an important method, because especially in quantitative terms, it is not yet readily replaceable by the newer approaches.

\textbf{4.2 Suggestions for application of the evaluated methods in routinely investigation of North Sea phytoplankton}

As mentioned before, analysis of the phytoplankton community includes two aspects: Assessment of biodiversity and determination of biomass. Due to its patchiness (\textit{manuscript IV}), biomass distribution should be estimated via an optical proxy in continuous manner. Although this is not as accurate as HPLC-based chl-a measurement or direct biomass determination by microscopy, it requires much less effort and enables a sampling frequency high enough to map spatial and/or temporal differences in sufficient resolution. Absorption coefficient measurements should be the preferred proxy due to their lower variability in relationship to chl-a (\textit{manuscript I and II}). However, this requires the further improvement of the ft-PSICAM (or similar devices) in advance. In the light of the general low effort of continuous measurements, it would be even more advantageous to conduct parallel measurements of absorption and fluorescence. Since both parameters are likely to be affected differently by variability in phytoplankton composition or physiological state, their ratio might be useful for detection of changes within the community regarding these aspects.

Phytoplankton biodiversity should be primarily analyzed by the combination of methods used in \textit{manuscript IV}, because microscopy, flow cytometry and DNA-microarray provide...
complementary data for the whole community, from the microphytoplankton level down to the picophytoplankton level. Possibly, ARISA might be left out, because general changes in the community are probably also visible in the results of the other methods. However, although with this combination more information is gained compared to microscopy alone, effort is comparably high or even higher due to the more methods used. This is a major hindrance for routine observation. One possibility for effort reduction would be a lower sampling frequency. In this case, resulting loss in resolution could be partially compensated for by additional selective sampling based on e.g. extraordinary high/low values observed in the continuous biomass proxy data, which would mean potentially interesting short time events would not be lost even at a reduced sampling rate.

Another way for performing routinely monitoring with a simultaneous reduction in effort would be that all samples were merely analyzed in the first instance by the convenient and rapid methods (ARISA, flow cytometry and DNA-microarray). Only a selection of these samples (especially those showing distinctive features) would then be analyzed quantitatively in the second instance via microscopy. For such an approach, however, the taxonomical resolution of the microarray has to be increased by the design of probes for a larger number of taxa (or at least for important key species).

Probably the best compromise between low effort and gain of information is the RNA-microarray approach. Because for this type of microarray an amplification step is omitted, the quantitative correlation was much better than those of the DNA-microarray (manuscript III). If this can be confirmed by further tests and for other probes, qualitative and quantitative changes within the community could be conveniently tracked using this method. Therefore, it has the potential for high frequency analysis of samples on a level of detail comparable to that of microscopy, and would therefore being very suitable for the use in a time series. However, for microarrays in general, the possibilities for observation are limited by the number and specificity of the available probes. Species uncommon for a certain region might be not detected due to the lack of a corresponding probe. For this reason, microscopic observation with all its drawbacks should still remain a part of every routine observation, although in reduced frequency.

4.3 Future perspectives

High resolution data are becoming increasingly important in all fields of science, and also in phytoplankton ecology. Methods based on optical proxy measurement as well as molecular sensing provide the ability to obtain such information relatively easy by either allowing continuous measurements, or at least facilitate a rapid analysis of large sample numbers. However, some measures are required to further improve the performance of the methods used in this study.
In order to achieve the same accuracy as for the manually operated PSICAM, the cleaning procedure of the ft-PSICAM has to be optimized to prevent contamination of the system, especially the cavity. Furthermore, since the absorption in the whole visible spectrum is measured, the usage of these measurements for a continuous identification of phytoplankton classes by their specific absorption properties should be evaluated. This would be a very valuable extension to the mere mapping of phytoplankton biomass distribution.

Another crucial point is the number of available molecular probes for the microarrays. It has to be increased in order to cover a larger part of the phytoplankton community in the analysis, especially important key species. Special emphasis should be put on the RNA-microarray because of the better quantitative performance. Moreover, this type of microarray was already performed by a semi-automated device, indicating further potential for a complete automation. Automation should be a general aim for all methodological approaches, because this would offer the opportunity to use them as unattended devices, eventually connected to a platform like the FerryBox. When mounted in ships-of-opportunity, spatial and temporal coverage of investigations could be further increased. In this context, as flow cytometry have shown to be a very valuable tool in the course of this study, the long-term use of continuously operating flow cytometry devices like the FlowCAM or the CytoBuoy as addition to unattended systems should be evaluated.

In general, in addition to the improvement or automation of the already available methods, effort should be made in the identification and adaptation of additional promising laboratory techniques for in situ or continuous operation to further enhance their value in phytoplankton research.
5. References


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6. Statutory Declaration

I, Jochen Wollschläger, hereby declare that I have written this PhD thesis independently, unless where clearly stated otherwise. I have used only the sources, the data and the support that I have clearly mentioned. This PhD thesis has not been submitted for conferral of degree elsewhere.

Bremen, October, 2013

Signature:______________________________________