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# FINE RESOLUTION OF PRIMARY PRODUCTION AND ITS LIMITATION IN PHYTOPLANKTON COMMUNITIES OF THE DARSS-ZINGST BODDEN CHAIN, A COASTAL LAGOON OF THE SOUTHERN BALTIC SEA

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**Abstract**

Evaluated direct methods for estimating primary production are both a precondition and a supplement for modeling scenarios in a eutrophicated and changing coastal ecosystem. Analyses of phytoplankton succession, integral primary production and potential limitation of phytoplankton growth of the shallow brackish Darss-Zingst Bodden chain indicate the extraordinary importance of colony-forming nanophytoplankton for primary productivity. Respective studies began in the 1990s. Exemplary studies focus on the contribution of individual aggregates and phytoplankton colonies to potential primary production as well as the microscale visualization and seasonal variability of extracellular enzymatic phosphate hydrolysis. Results from these studies suggest that an inhomogeneous distribution of productivity and limitation is relevant to the pelagic zones. This paper discusses the effect of heterogeneity on ecosystem behavior in this specific trophy succession state in the context of a future strategy to supplement bulk methods for productivity and limitation analyses especially for barely distinguishable phytoplankton species in euthropicated coastal waters.

**Key words:** nutrient limitation, primary production, size classes, aggregates

## INTRODUCTION

The German coast of the Baltic Sea is characterized by inner coastal estuaries that are not affected by tides. These estuaries represent important sinks for nutrient flows from terrestrial runoff. During the last few decades, these waters have been hotspots for eutrophication. The Darss-Zingst Bodden chain (DZBC) is one of the most intensively studied inner coastal waters of the German coast. Schiewer (2008) recently published a basic review of its ecology. The ecosystem underwent substantial changes in phytoplankton composition (Wasmund and Kell 1991, Schumann and

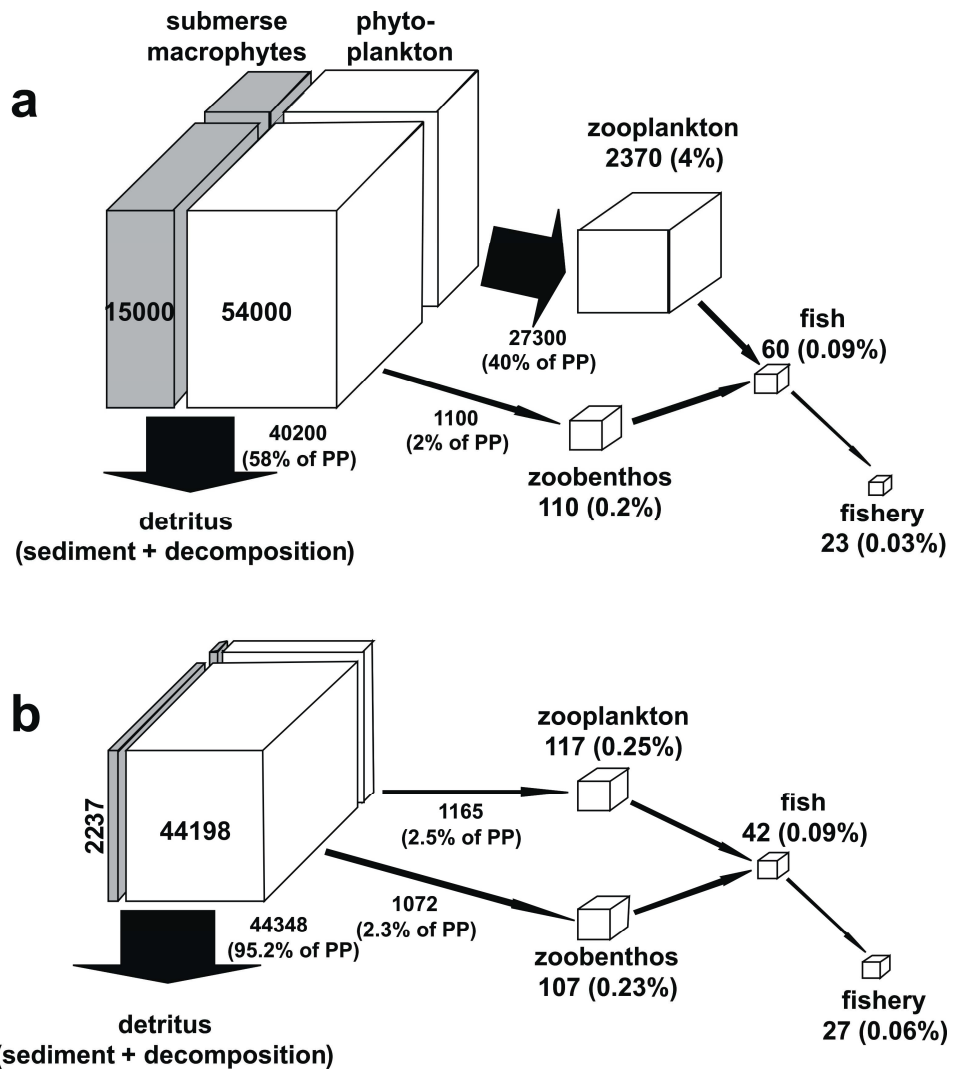



Fig. 1. Primary production and its basic transformation in the Darss-Zingst estuary (Barther Bodden) comprising the situation before 1981 (a) and after intensified eutrophication processes (b). Food web calculations based on biomass calculations. Units, kg $ha^{-1}$ $a^{-1}$; PP (primary production). Graph adapted from Schiewer (1998)

Karsten 2006), and phytoplankton primary production became dominant over phytobenthos (Schiewer 1998, Fig. 1). Primary production, the basis of eutrophication, requires closer inspection. High turbidity and irradiance attenuation in the pelagic region transformed photosynthetically active radiation (PAR) into a potentially limiting resource. The quantitative dynamics of PAR on short-term scales (e.g., Schubert et al. 1995) and the temporal irradiance acclimation behavior of phytoplankton (Schoor et al. 2008) are still unknown. The temporal and spatial resolution of factors

and resources limiting primary production in shallow coastal waters continue to increase in their complexity and sophistication.
A discrepancy between an increase in primary production and the estimation of eutrophication evolved. Nutrient load and chlorophyll *a* (Chl *a*) concentration dominate as relative and indirect measures of primary production, respectively. These parameters allow for the simple monitoring of environmental quality in applied ecology. The limitation of primary production is often investigated in a similar way, using common assumptions and approximations. Examples of these assumptions are the homogenous or proportional distribution of performance with biomass and Chl *a*. In fact, these relations are rarely verified.
Most limitation assays are based on Chl *a*, a measure of total biomass. However, different limitation states of species, organization forms (colonies, coenobia and filaments) and size classes remain undetected. Dominance changes in the natural environmental may prevent changes in total biomass. Moreover, colonies enveloped by large mucous coats may always have nutrient limitations and prosper under nutrient additions. Even the relatively basic relationship between phytoplankton biodiversity and planktonic primary production has barely been investigated (Striebel et al. 2009). Even if this relationship were elucidated, different marine coastal regions may exhibit opposite relationships between these two characteristics (e.g., Vadrucci et al. 2003). Tools that measure primary production in phytoplankton suspensions at very high spatial resolution, like microautoradiography (e.g., Watt 1971), have received minor attention in practice. This paper critiques estimates of primary production with respect to shallow brackish coastal waters and focuses on primary production as a central principle in ecosystem behavior. We address the inhomogeneity of primary production in the pelagic environment and the limitations of current methodical strategies. For the first time, potential primary production was evaluated by quantum yield analyses of photosystem II for individual colonies and phytoplankton aggregations. Additionally, it was measured seasonally and individually under in situ P limited conditions.

## MATERIAL AND METHODS

### Plankton samples, model aggregates and sampling sites

Nutrient limitation experiments were conducted mostly with Zingster Strom phytoplankton. The moderately eutrophic station Zingster Strom connects the two innermost basins of the DZBC and is the most investigated part of the system (Schumann et al. 2006). Mesocosm experiments conducted in June and July of the early 1980s provided additional original data. In the early 1970s, experiments with phytoplankton communities from other water basins of the DZBC were conducted. All these results were summarized in (Schiewer 1988).
Plankton samples were taken almost monthly from November 1991 through October 1992 at four stations of the DZBC to re-evaluate nutrient limitation in the early 1990s. Samples were collected along the nutrient and salinity gradients. In the

Grabow, Dabitz was the outermost site influenced by the Baltic Sea. The two sites with the greatest nutrient loads are situated in the Saaler Bodden (Dierhagen) and Ribnitzer See (Ribnitz). The Ribnitzer See is the site that is most influenced by freshwater. Biomass, species composition, Chl *a* contents and maximum primary production were investigated from February 1991 through October 1992. Biweekly, samples were taken from the upper 50 cm of the water column at the Zingster Strom and the Saaler Bodden (Dierhagen).
On 4 August 1997, aggregates for photosynthetic analyses were taken from the Saaler Bodden (Dändorf, salinity: 3.9 PSU, water temperature: 22.4 °C). At a water depth of 1 m, aggregates of the sediment/water interface were collected by subsequent use of a 50-ml syringe with a wide (5 mm) bore into 1-liter polyethylene bottles. Thirty liters of water were collected from the pelagic zone. Aggregates > 20 µm were concentrated (x100) by low-pressure reversed filtration using 20-µm gauze mounted on polyethylene cylinders. Aggregates were shaken carefully to be resuspended. At 5 sites within the DZBC, hydrolytic enzymes were measured from April 1996 through April 1997 on an almost monthly basis. Only the two earlier investigated stations, Zingster Strom and Dierhagen, are presented.
In order to obtain small-sized artificial aggregates for methodical evaluations of pulse-amplitude modulated (PAM) analyses of photosystem II (PSII) fluorescence, the cyanobacterium *Synechocystis* sp. PCC 6803 was grown axenically in cell culture plates (chamber diameter: 3 cm) at 25 °C in BG11 medium (Rippka et al. 1979) and under warm white light illumination (Osram, Lumilux DeLuxe, 150 µmol photons $m^{-2}$ $s^{-1}$). A rotary shaker moved the plates (150 rpm, amplitude: 2 cm). These cyanobacteria formed small aggregates within the center of the circular plate chambers, whereas common single cells were distributed within the whole chamber. The aggregates were sampled by a wide bore syringe (5 mm in diameter) without destruction and then gently shaken for resuspension.

**Limitation assay**

For the limitation assays, the 4-liter samples were incubated in glass beakers for 8 days at 26 µmol photons $m^{-2}$ $s^{-1}$ (measured at the beaker bottom) with a light:dark cycle of 12:12 h. Temperatures were as low as 8 °C in the winter and as high as 20 °C in the summer. These temperatures exceeded *in situ* temperatures in winter by 11 K at maximum, but were rather similar to temperatures *in situ* for all other seasons. All samples were filtered through 200-µm gauze to remove mesozooplankton. Controls were not manipulated further. Another sample set was incubated in a light limited environment, at 1/3 of the photon flux. To a third set of samples, 1 ml of 50 mmol $KNO_3$ $l^{-1}$ and 1 ml of 5 mmol $KH_2PO_4$ were added per liter sample volume (nutrient addition), resulting in final concentration of 50 µmol N and 5 µmol P $l^{-1}$. This mixture remains slightly below the Redfield ratio of 16:1 for N:P. Chl a was extracted in 90% acetone (Jeffrey and Humphrey 1975, see below) and measured as a biomass parameter on days 0 and 8. Samples were assessed as light and/or temperature limited (L, T) when controls increased by > 30% from day 0 to day 8. If

phytoplankton growth, measured by the Chl *a* concentration at day 8, exceeded that of shaded samples by > 30% after nutrient additions, these samples were labeled as nutrient limited (N+P). This interpretation deviates from most limitation experiments (e.g., Hillebrand and Sommer 1999, Elser et al. 2007) and earlier investigations (Schiewer 1988), which rate nutrient enriched samples with a higher biomass than the controls under same conditions as (potentially) nutrient limited. This method of evaluation was chosen, because in most cases shaded samples developed more Chl *a* than the controls (see Discussion).

### Microscopic evaluation of species, colonies and aggregates

Phytoplankton cells were counted in Lugol fixed samples in 1-ml sedimentation chambers (Utermöhl 1958, Lund et al. 1958) at a magnification of 256-times with a JENAVAL microscope. If possible, eukaryotic species were determined at the genus or species level (Pankow 1990) and cyanobacteria after Komárek and Anagnosti-dis (1999, 2005). More than 90% of the total biomass was composed of some dominant species, which were counted with at least 100 individuals. Additionally, rod-shaped cyanobacteria of the pico-size class (< 2 µm) were quantified in glutaraldehyde-fixed samples (1.2% final concentration) under epifluorescence illumination (green light BP 545, magnification 1250-fold) with an Olympus BH2-RFCA. Two hundred cells were counted per sample. Biomass was calculated using a mean cell volume of 0.86 $\mu m^3$ (unpublished data). For the dominant cyanobacteria and chlorophyta, which form large spherical colonies, cell numbers were determined in 10 randomly chosen colonies per sample under green light and at a magnification of 1250-times. The total cell numbers per species were calculated using these mean cell numbers per colony. Cell diameters were measured for all larger species (> 5 µm). Assuming geometrical bodies, cell volumes were calculated (Edler 1979). Although a considerable amount of colonies were > 20 µm diameter, this cell class size was named colonies or nano-sized. Planktonic colonies or colony aggregates were typically 30 µm in diameter (ca. 0.007 $mm^2$), but grew as large as 60 µm (ca. 0.03 $mm^2$). These colonies and aggregates were at the lower end of PAM detectable objects (see below). Carbon content was estimated to be 11.25% carbon per fresh weight (Heerkloss and Vietinghoff 1981) at a specific density of 1.04 $g.cm^{-3}$.
Aggregate sizes were measured using the calibrated microscope (Ergaval, Carl Zeiss, Jena) with a ×16 eyepiece and corresponding objectives (×10, ×40). The shapes of aggregates collected at the sampling site were most accurately approximated using a rectangular representation.

### Chlorophyll *a*

Pigments were extracted with 90% acetone (Jeffrey and Humphrey 1975) in three subsamples of 30-100 ml. Mechanical homogenization improved the extraction efficiency. Variation coefficients of these replicates were < 3%. Chl *a* in the picoplank-

ton fraction was measured in 5-µm filtrates separated by gauze (Hydrobios, Germany). Besides the solitary picocyanobacteria, some destroyed Aphanothece colonies and other slender cells (*Limnothrix planctonica*, *Monoraphidium* spp.) passed through the gauze. The Chl *a* contents of aggregate suspensions were determined by absorbance measurements after dimethylformamide extraction (Suzuki and Ishimaru 1990) of aggregate-loaded filters (Whatman GF/F).

## PAM fluorometry

A common transmission light microscope (Ergaval, Carl Zeiss, Germany) was equipped with standard accessories of the pulse-amplitude modulated chlorophyll fluorometer PAM-100 (Walz, Germany). The measurement light emitting diode (101 ED) was mounted onto the condenser instead of the illumination unit, and it emitted red light directly. One eyepiece of the binocular was coupled to the photomultiplier detector unit (PM-101/D combined with a Schott RG8 filter) for the detection of chlorophyll fluorescence. The second eyepiece was used for size determination and for focusing the aggregates. The standard glass fiber of the PAM-100, mounted at 45° to the optical path beside the objectives of the microscope, delivered saturating light pulses and actinic light onto the object located within the light path of the microscope. A halogen lamp (Schott KL 1500) provided saturating light pulses of one-second duration at an effective (measured underneath the cover slide) irradiance of 5400 µmol photons $m^{-2}$ $s^{-1}$. The lamp was combined with two cut-off filters (FL103-F, Walz, Germany) that prevented saturation of the detector system by flash light wavelengths > 700 nm as described by Ting and Owens (1992). A slide projector (halogen bulb, 150W) combined with neutral density filters (Balzer, Liechtenstein) and two FL 103-F cut-off filters delivered different actinic light intensities for methodical tests. This combination ensured the correct estimation of the dark yield (maximum quantum yield of PSII, see below) parameter for individual aggregate analysis.
Suspended aggregates were transferred onto slides by a wide bore pipette and fixed by cover slides. Single aggregates were dark-adapted for 10 min before being moved into the measurement light path using the microscope table. The red measurement light was sufficient to observe single aggregates through the second eyepiece without a second light source. Thus, there were no problems in determining the initial fluorescence ($F_0$). Initial and maximum fluorescence ($F_m$) was determined by manual operation via the zoom options of the DA-100 software for PAM100 operation and fluorescence analysis instead of the automatic procedures averaging at least 1000 data points collected at 100 µs intervals.

## $^{14}C$ incorporation

Maximum primary production ($P_{max}$) was measured at approximately 20 °C. Measurements were taken after 1 ml $H^{14}CO_3^-$ (10 µCi) was added to a 100-ml sample and

exposed to a photon flux of 500-600 µmol $m^{-2}$ $s^{-1}$ for 90-120 min (Steemann-Nielsen 1952, modified after Schiewer 1982). Total primary production was measured in subsamples that were acidified (pH 3-3.5) and air bubbled for 20 min. By this procedure, microbial activity was stopped, and unused $H^{14}CO_3^-$ or $^{14}CO_2$ was expelled. Filtration (< 100 mbar) of acid treated samples through 3-µm polyester membranes (ROTRAC, Germany) produced the picoplankton fraction. Exudates, measured in 0.1-µm filtrates (polyester membranes, ROTRAC, Germany), were subtracted from both fractions. Dark $H^{14}CO_3^-$ uptake rates were also subtracted. All values were normalized to total $H^{14}CO_3^-$ concentrations (estimated immediately after its addition) and converted into total carbon uptake by an average (unlabelled) inorganic carbon concentration of 30 mg $l^{-1}$. Variation coefficients of triplicates were usually < 4%. In a small number of samples, variation coefficients were 10-13%.
Oxygen evolution rates of the aggregate suspensions were measured for a series of irradiances (0-395 µmol photons $m^{-2}$ $s^{-1}$ in 8 steps) using an automated Clark-type electrode system combined with a spectral corrected light dispenser (MK2, Illuminova, Sweden) in P/I analyses. Simultaneous measurements of dark yield and operational yield were obtained by a PAM-2000 (Walz, Germany) that was directly connected to the oxygen measurement chamber (volume: 7 ml) of the MK2 system via fiber optics. For dark yield, $F_v/F_m = (F_m-F_0)/F_m$, $F_m$ represents the PSII fluorescence yield when all plastoquinone $Q_a$ is reduced after a saturating light pulse applied after dark adaptation, and $F_0$ represents the minimal fluorescence yield when all $Q_a$ is oxidized after dark incubation. For operational yield, $(F_m'-F_0')/F_m'$, $F_0'$ represents the fluorescence yield when all $Q_a$ is oxidized, and $F_m'$ represents the fluorescence yield when all $Q_a$ is reduced after a saturating light pulse. Both parameters were measured after adaptation to a given irradiance. The maximum photosynthetic rates ($P_{max}$) were obtained by least squares fits of the data using the slightly modified (β-term omitted) exponential relationship of Walsby (1997): $P_c = P_{max}(1-\exp(-\alpha\ I/P_{max}))+R$. R represents the rate of respiratory oxygen uptake, α represents the gradient of P/I observed at light-limiting irradiance (light affinity) and $P_c$ represents the photosynthetic rate for a given irradiance (I). Relative electron transport rate (rETR) was calculated as the product of dark yield and photon flux density (PFD) of PAR at the given irradiance level.

## Hydrolytic enzyme activity

Total esterase and phosphatase activities were measured as the enzymatic hydrolysis of 4-methylumbelliferyl butyrate phosphate (Sigma-Aldrich, Germany). The procedure followed Hoppe (1983) with minor adaptations. The artificial substrates were dissolved in 100% ethanol and added to 2-ml subsamples, reaching a final concentration of 91 µM. All samples were buffered at a pH of 8.2 with TRIS-buffer (1 ml 50 mM TRIS/HCl per 10 ml sample). Three replicates per sample were incubated at room temperature (21 °C) and were recorded for the fluorescent hydrolysis product 3-times within 2 h for esterase and within 3-9 h for phosphatase activities in a HITACHI F4010 fluorometer (excitation 365, emission 451 nm, bandpass 1.5 nm,

response 2 s, averaged over 2 s). Blanks of sterile filtered, distilled water and the fluorogenic substrates were treated using the same method to correct for non-enzymatic hydrolysis. Standards of 0.1-50 µM 4-methylumbelliferone (= 7-hydroxy-4-methylcoumarin) in 5 mM TRIS-buffer (pH 8.2) were measured at each sampling date to calibrate hydrolysis rates for relative fluorescence units. Yellow-green crystals of ELF® marked the presence of extracellular phosphatase around individual colonies. This substance was cleaved enzymatically from 100 µM ELF® phosphate (Molecular Probes, USA) within 4 h. Microphotographs were taken under epifluorescence illumination (UV light UG-1, magnification 200-fold), and the FUJI Sensia 200 film was exposed for 5 s.

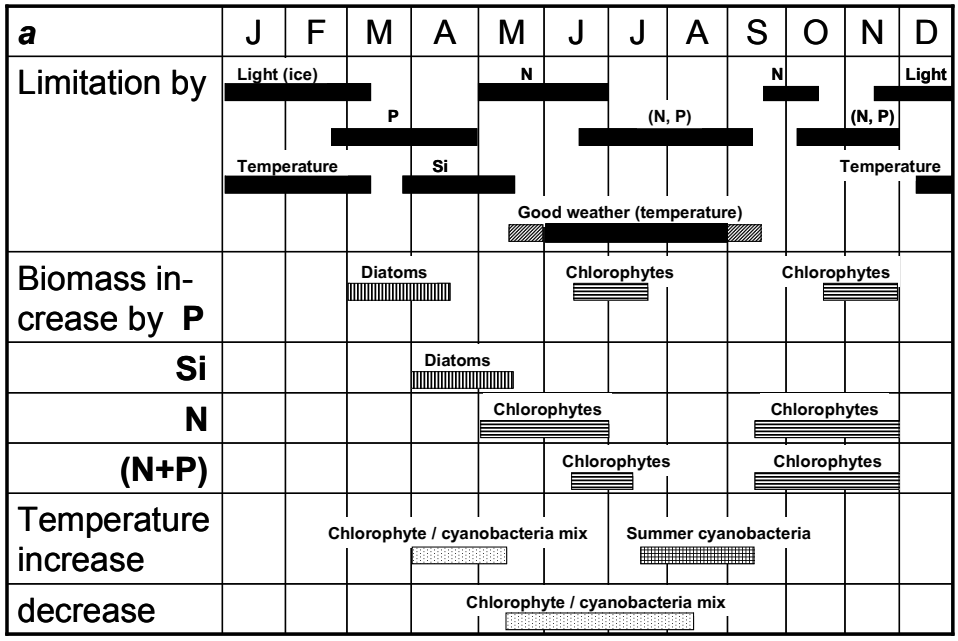
## RESULTS

### Annual periodicity of nutrient limitation

In many phytoplankton samples, nutrient additions increased primary production and biomass compared to controls (Fig. 2). Throughout the DZBC, seasonal patterns of P and N limitation were observed. P limitation occurred in the early spring, and N limitation followed in the late spring. In the summer and autumn, both P and N were limiting factors. Thus, nutrient limitation appeared to be independent of the specific trophic situation in each Bodden basin. While diatoms were favored by P concentrations in the early spring, chlorophyta benefited most from N in the summer and autumn. Cyanobacterial abundance increased after P additions in the summer. Unexpectedly, also warming events increased the biomass of cyanobacteria and chlorophyta in the early spring and summer. This description is summarising the situation in the 1980s, in which intensive agriculture combined with poorly cleaned municipal waste waters introduced higher and higher amounts of P and N into all surface waters.

In the early 1990s, a dramatic decrease in agricultural fertilizer usage began lowering the high nutrient input into surface waters. The implementation of waste-water treatment and a decrease in P-containing detergents and washing powders also altered these levels. At this time, nutrient limitation (only investigated by combined treatments; N+P) showed slightly different patterns for sites of contrasting trophic states (Fig. 2b). The two outermost sites investigated were nutrient limited in 5 and 6 out of 12 months, primarily falling in summer and autumn. The two innermost sites were limited 2 and 4 times. Unlike the results from earlier tests, nutrient limitation did not occur in spring (April to June). However, almost all samples (48 out of 52 samples) reached > 30% higher biomasses than the normal controls within one week. Thus, N+P treatments could be considered as nutrient limited if compared to controls grown under the same conditions and not to the shaded samples as carried out herein. This pattern deviates from the results in the 1980s that indicated a clear annual pattern of contrasting limitation situations. These results are surprising for a heavily eutrophic system. Unexpectedly, primary production in most samples from two sites, one eutrophic (Zingster Strom) and another polytrophic (Dierhagen), was

not stimulated in the winter by higher temperatures or improved light conditions compared to *in situ* conditions.



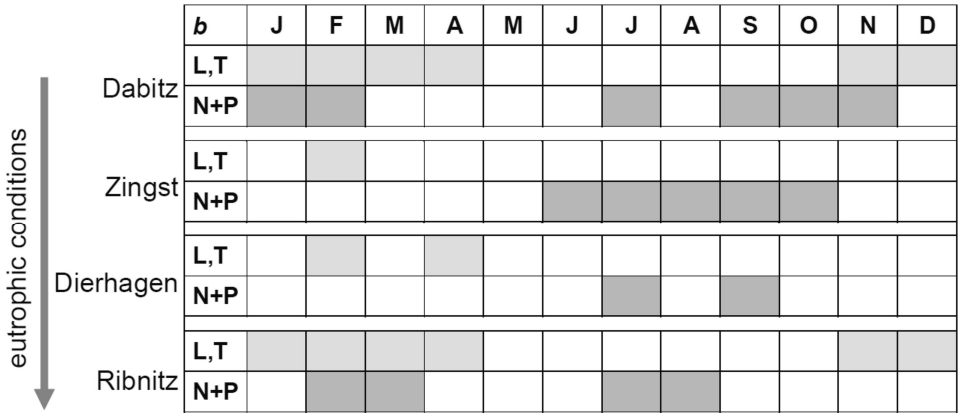




Fig. 2. Limitation of total phytoplankton and selected taxonomic groups throughout the Darss-Zingst Bodden Chain (a, adapted from Schiewer (1988) and limitation assays of 4 stations along a trophy gradient from November 1991 through October 1992 (b). L,T: potential light or temperature limitation derived from controls growing by > 30%, N + P: potential nutrient limitation estimated from > 30% more growth compared to shaded samples after N and P additions

## Phytoplankton composition and periodicity

The average phytoplankton biomass (2.8 mg C $l^{-1}$) at the more eutrophic site Dierhagen was 42% higher than at the Zingster Strom site. However, biomass composition was rather similar. The picoplankton fraction equaled 11 and 8% in Zingster Strom and Dierhagen, respectively (Fig. 3). Filamentous cyanobacteria, bacillariophyceae and flagellates together averaged 20 and 19% at both sites. Colony-forming cyanobacteria belonging to the order Chroococcales accounted for 27 and 30% of the

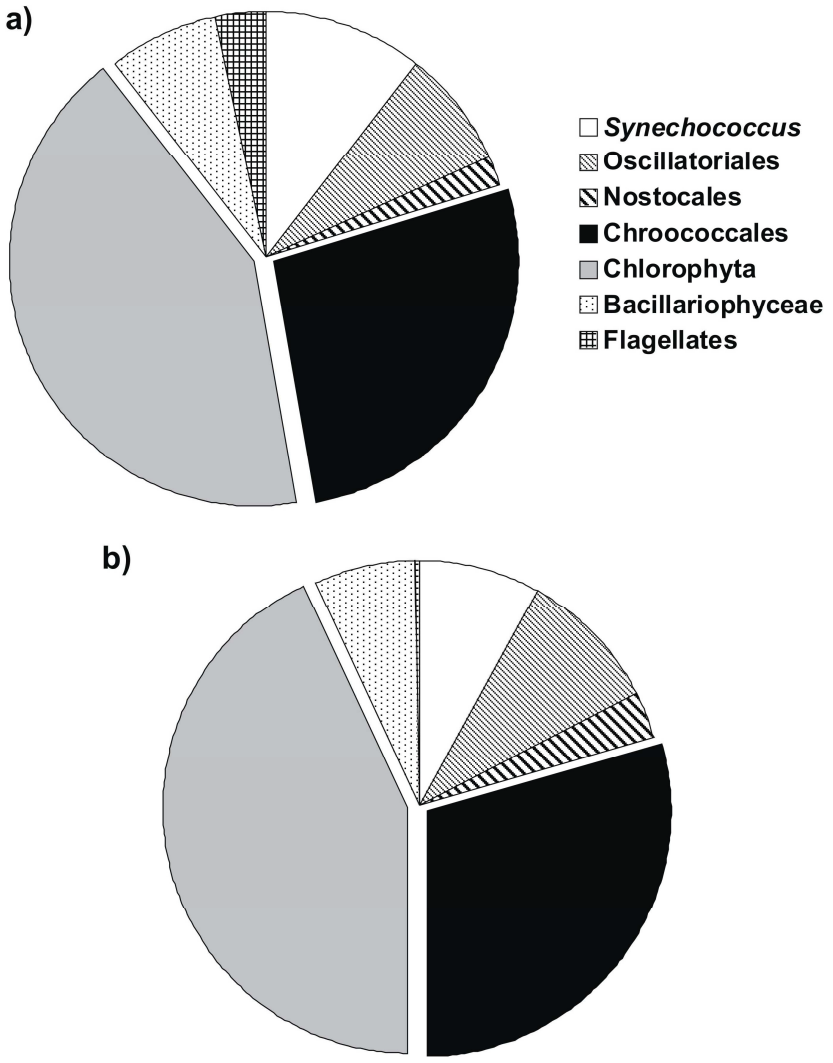



Fig. 3. Average phytoplankton composition (%) of 43 samples from the Zingster Strom (a) and 39 samples from Dierhagen (b) taken between February 1991 and October 1991

total biomass in Zingster Strom and Dierhagen, respectively. Dominant genera included *Aphanothece*, *Aphanocapsa*, *Cyanonephron*, *Snowella* and *Woronichinia*. Most of the chlorophyta were represented by *Tetrastrum triangulare, Oocystis* and *Scenedesmus* spp., which also formed larger (ca. 10-20 µm) colonies or at least coenobia. The percentage of chlorophytes was 42 and 43% at both sites, respectively.

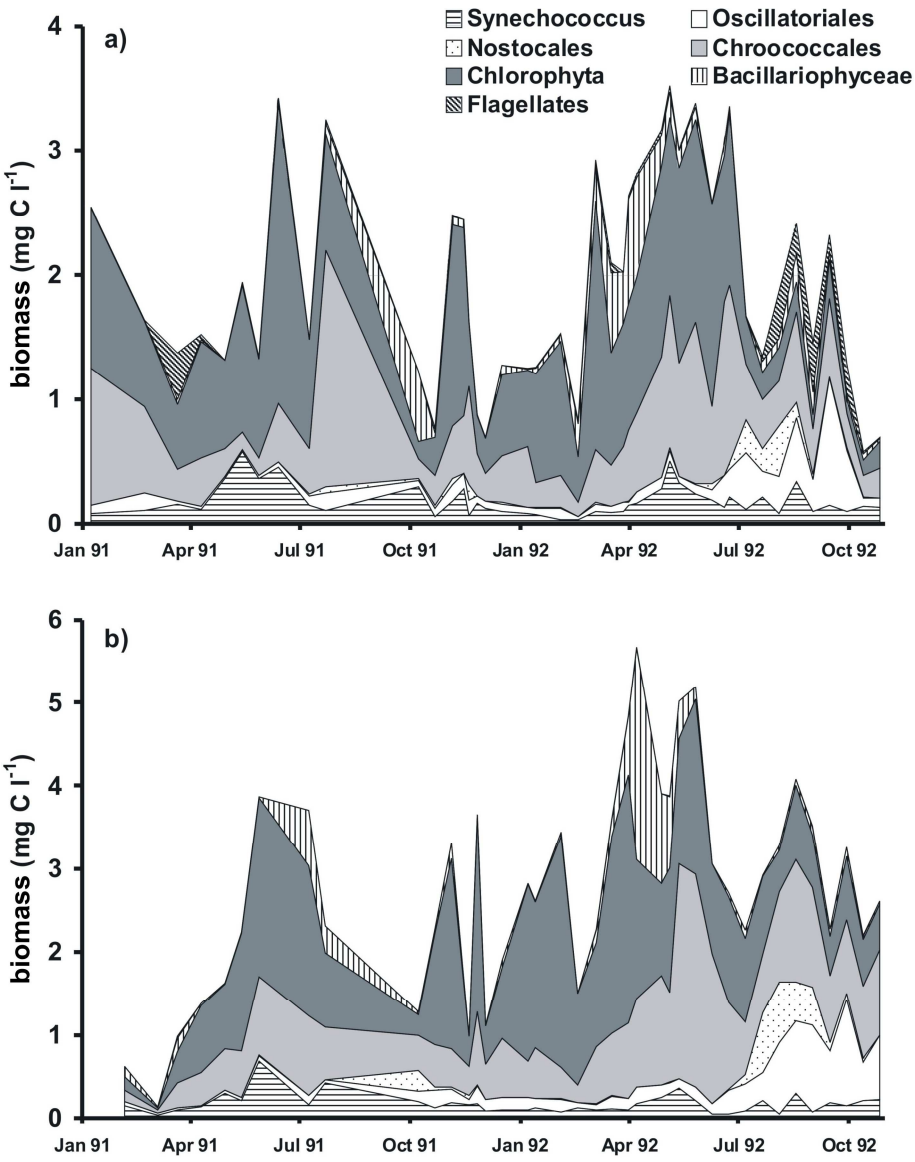



Fig. 4. Biomass (mg C $l^{-1}$) of different taxonomic groups in the Zingster Strom (a) and in Dierhagen (b) from February 1991 to October 1991

Altogether, more than 2/3 of the total biomass was composed of colony formers, which may suffer from nutrient limitation more than solitary cells of the same size. Both sites were characterized by fluctuations of total biomass, but they were more pronounced in the Zingster Strom (Fig. 4), in which different water masses may be sampled each time. Bacillariophyceae occurred mostly in spring, but never formed a bloom with respect to dominance as a percentage of biomass or as steep biomass increases. An autumn bloom of Oscillatoriales (*Limnothrix planctonica*, *L. redekei*, *Planktolyngbya contorta*) induced the only distinctly seasonal biomass peak.

**Photosynthetic performance of different phytoplankton aggregations**

A careful examination of a pulse-amplitude-modulated chlorophyll fluorometer combined with a transmission light microscope ensured the sensitivity and accuracy of the fluorescence yield determinations. With this approach, larger colonies (micro-size range) and aggregates formed out of phytoplankton colonies could be investigated. The otherwise described and dominating nano-sized planktonic colonies were too small for this method. The fluorescence curves of individual aggregates (data not shown) displayed good preconditions for the accurate determination of both $F_0$ and $F_m$. However, very low amounts of chlorophyll were present in small-sized objects, e.g., for two cells of *Melosira lineata* (0.0018 $mm^2$) or a single *Microcystis* colony (0.044 $mm^2$).

One prerequisite for determining the dark yield ($F_v/F_m$) is the prevention of any actinic effects of the measuring beam to ensure exact determination of $F_0$. The accuracy of $F_v/F_m$ has been verified at different measuring light intensity settings with an average of 20 individual aggregates. No decrease in $F_v/F_m$ was observed for the aggregates (data not presented) using a measuring beam intensity set to "8" on the PAM 101 device. This test indicated negligible actinic effects. The measuring beam intensity was then selected at level "4". The saturating pulse intensity necessary for a reliable determination of $F_m$ was determined in the next step. The results of the $F_v/F_m$ determination at different saturating pulse intensities led to the use of a 3 500 µmol photons $m^{-2}$ $s^{-1}$ saturating pulse intensity. This intensity was used for the complete saturation of the 50 aggregates as well as the colonies from the field samples. We used saturation pulse light intensities of 5 400 µmol photons $m^{-2}$ $s^{-1}$ as a conservative measure. Pre-screening tests indicated a measuring light intensity at "6" for determining $F_0$. In samples with high amounts of chlorophyll, the intensity was reduced back to "4". These settings corresponded to 2-4 measuring light intensity levels below the level, where actinic effects were first observed. Determinations of fluorescence parameters and aggregate size took several minutes. Therefore, we tested the stability of the results for the $F_v/F_m$ determination by repetitive measurements of 10 natural aggregates within 30 min. A data shift trend was not detected. However, prolonged exposition time on the microscope slide appeared to increase the standard deviation of the measurements. Clear trends were not observed for individual aggregates (data not shown). Therefore, we exchanged the sub-samples on microscopy slides every 15 minutes during the following measurements.

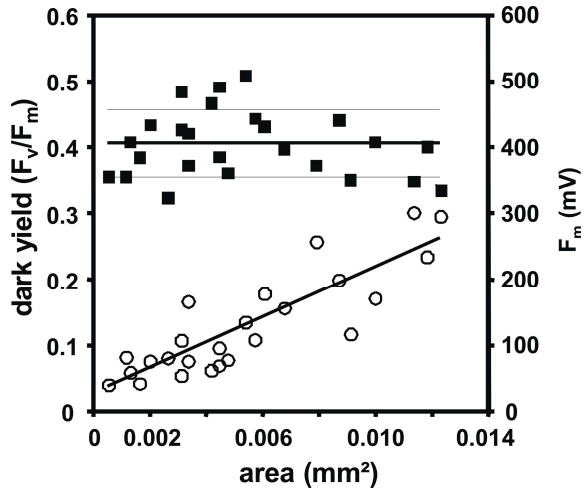



Fig. 5. Dark yield and $F_m$ versus size (area) plot for artificial *Synechocystis* aggregates. Filled squares denote the dark yield (thick line: mean dark yield of the whole data set; parallel thin lines: standard deviation oft the measurements). Open circles represent $F_m$ (regression line: $r = 0.863$, $p < 0.001$, $n = 25$). The magnification used was ×640. Instrument settings: measuring beam intensity "6"; saturation pulse: 1 s, 5 400 µmol photons $m^{-2}$ $s^{-1}$

Artificial aggregates were investigated within the size-range of 0.0008-0.012 mm² (Fig. 5) to determine whether the different amounts of chlorophyll present in smaller and larger aggregates result in incorrect fluorescence yield determination. A linear increase in $F_m$ with increasing aggregate size was observed. $F_v/F_m$ was nearly constant over the entire range of aggregate sizes.

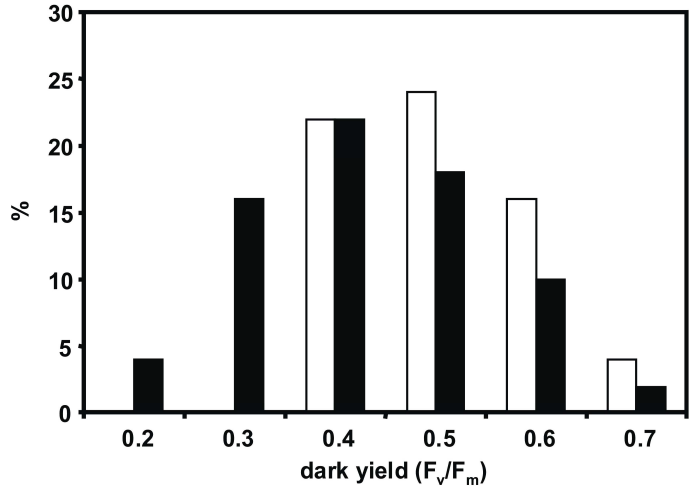



Fig. 6. Distribution of dark yield values for the samples from the pelagial (open bars) and the sediment / water interface (filled bars) of the Darss-Zingst estuary. Aggregates exhibiting no detectable fluorescence signals above the noise level are not displayed

Summarizing the dark yield determinations irrespective of aggregate size (Fig. 6), aggregate types, pelagic and those from the sediment-water interface, showed clearly different distribution of measurable values. The two classes of low dark yield values (0.100-0.399) were empty for aggregates from the pelagial. The inhomogeneity of values for aggregates from the pelagial was even more pronounced, since 6% of the pelagic and none of the sediment aggregates have not exhibited any fluorescence yield increase during saturating illumination ($F_v = 0$), although these aggregates contained chlorophyll. Aggregates without chlorophyll fluorescence or a dark yield were re-evaluated for fluorescence signals by changing the standard measuring conditions (change of measurement light intensity and/or saturating pulse light intensity, prolonged dark incubation) to overcome a missing $F_v$-value. Aggregates treated in this manner did not exhibit a single hidden signal. Results indicated that approximately one-third of the sampled pelagic aggregates did not contribute to primary production. The percentage of inactive aggregates at the sediment/water interface was surprisingly low (approximately 8%).

The histograms of aggregate sizes support markedly different distributions for the pelagic sample and sample from the sediment-water interface (Fig. 7a). Dark yield values of the pelagic sample did not appear to be dependent on aggregate size (Fig. 7b). Statistical analysis of the entire data set did not reveal significant relationships. The exclusion of all zero values (aggregates exhibiting no productivity in terms of $F_v$) also showed no correlation. In a third attempt, the data were sorted into five groups by decreasing order of aggregate size (Fig. 7b). Although standard deviations for the data of some groups remained very large, mean primary production capacity (dark yield) had a significant negative relationship to mean aggregate size ($r = 0.968$, $p < 0.01$, $n = 5$).

A second test for aggregate diversity was performed. $F_m$ was plotted against aggregate size as shown for the pelagic sample presented in Figure 7c. In a homogeneous sample with uniform species composition and negligible differences of the physiological status, $F_m$ depended only on the chlorophyll content. A relationship between $F_m$ and aggregate size was expected (Fig. 5). In the present case, three groups of aggregates were derived from the entire data set ($n = 50$). The first group ($n = 12$) contained aggregates of up to about 0.8 $mm^2$ as well as those exhibiting no or low maximum chlorophyll fluorescence yields. The second group comprised five aggregates. Four had high $F_m$-values, which were linearly related to aggregate size. These aggregates were *Microcystis* colonies mixed with traces of other phytoplankton. For the five colonies observed, $F_m$ and colony size were strongly correlated ($r = 0.995$, $p < 0.001$, slope: 1139, $F = 349.2$, $n = 5$). The third group included all of the heterogeneous aggregates exhibiting measurable chlorophyll fluorescence yields. This group showed a positive correlation between $F_m$ and aggregate size ($r = 0.811$, $p < 0.001$, slope: 359, $F = 59.3$, $n = 32$), but the size-dependent slope of $F_m$ was one-third of that calculated for the *Microcystis* colonies.

The interpretation of varying fluorescence yield data as a relative measure for primary production for phytoplankton and aggregate suspension requires the relative correspondence of rETR and oxygen evolution or carbon fixation. The measured results for total aggregate suspensions from the DZBC are summarized in Figure 8. In

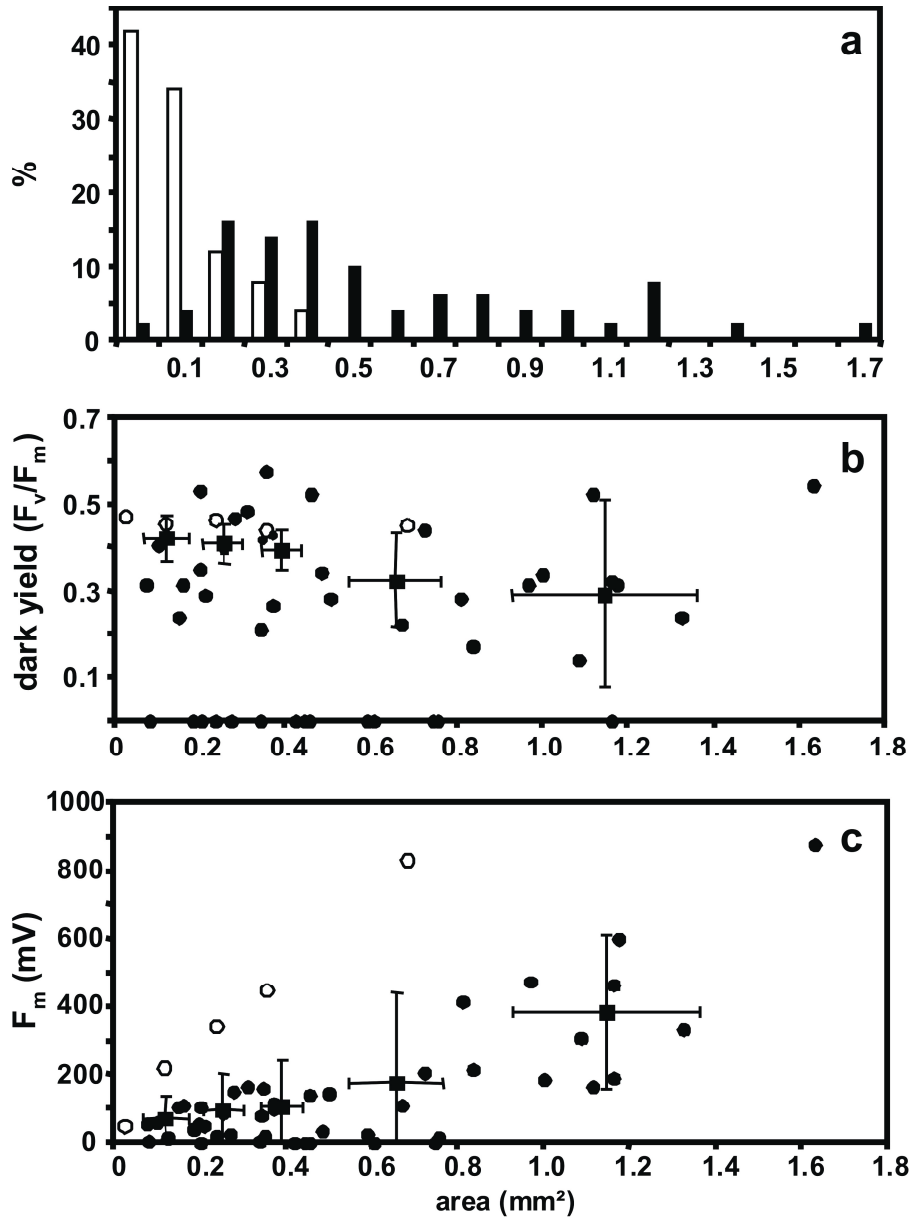



Fig. 7. Histograms of aggregate size (mm$^2$) in samples (pelagial: open bars; sediment / water interface: filled bars) of the Darss-Zingst estuary (a) and plots of dark yield (b) and $F_m$ (c) versus aggregate size for the pelagic sample. Filled squares represent mean values of groups of equal number of aggregates (sorted according to descending aggregate size, excluding zero values in the case of dark yield). Error bars denote corresponding standard deviations for dark yield (b) or $F_m$ (c) and aggregate size. Open circles represent aggregates dominated by a *Microcystis* colony

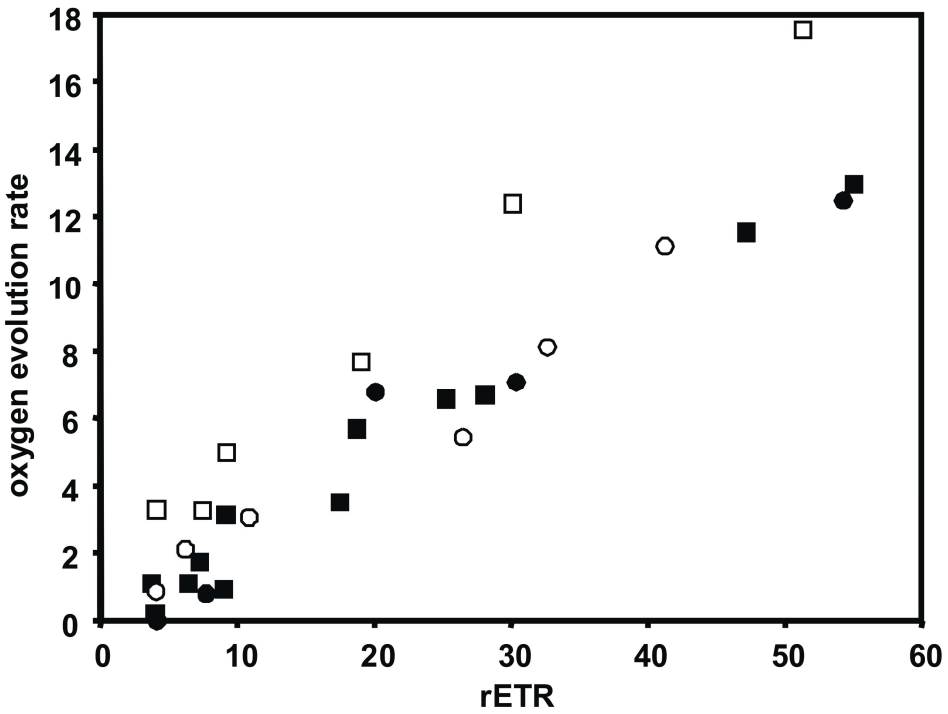



Fig. 8. Oxygen evolution rate (µmol $O_2$ mg chlorophyll $a^{-1}$ $h^{-1}$) and relative electron transport rate (rETR) of aggregate suspensions from the Darss-Zingst estuary (open symbols – pelagic samples, filled symbols – samples from the sediment / water interface, squares and circles represent different subsamples). Different rate levels were induced by variation of photosynthetically active radiation. Oxygen evolution and fluorescence yield were measured simultaneously in each sample

spite of the heterogeneous data set, oxygen evolution and rETR were closely correlated ($r = 0.915$, $p < 0.001$, $n = 47$). The correlation was stronger for each individual sample set (up to $r = 0.983$, $p < 0.001$, $n = 7$). However, this correlation does not hold up when nutrients are limiting, and carbon fixation should then be measured.

**Periodicity of pigmentation and production within different phytoplankton size classes**

Average Chl *a* concentrations corresponded to carbon biomass. In Dierhagen, concentrations of 131 µg $l^{-1}$ were 84% higher than in the Zingster Strom (Fig. 9). Due to the disproportionately high Chl *a* concentration, biomass-to-chlorophyll-ratios were on average 31 µg C µg Chl $a^{-1}$ in the Zingster Strom and 24 µg C µg Chl $a^{-1}$ in Dierhagen. The contribution of the colonies' size class to the total Chl *a* was 54 and 58%, respectively. The contribution to carbon biomass by the respective species led to higher average carbon to Chl *a* ratios of 57 and 82 µg C µg Chl $a^{-1}$. Solitary cells

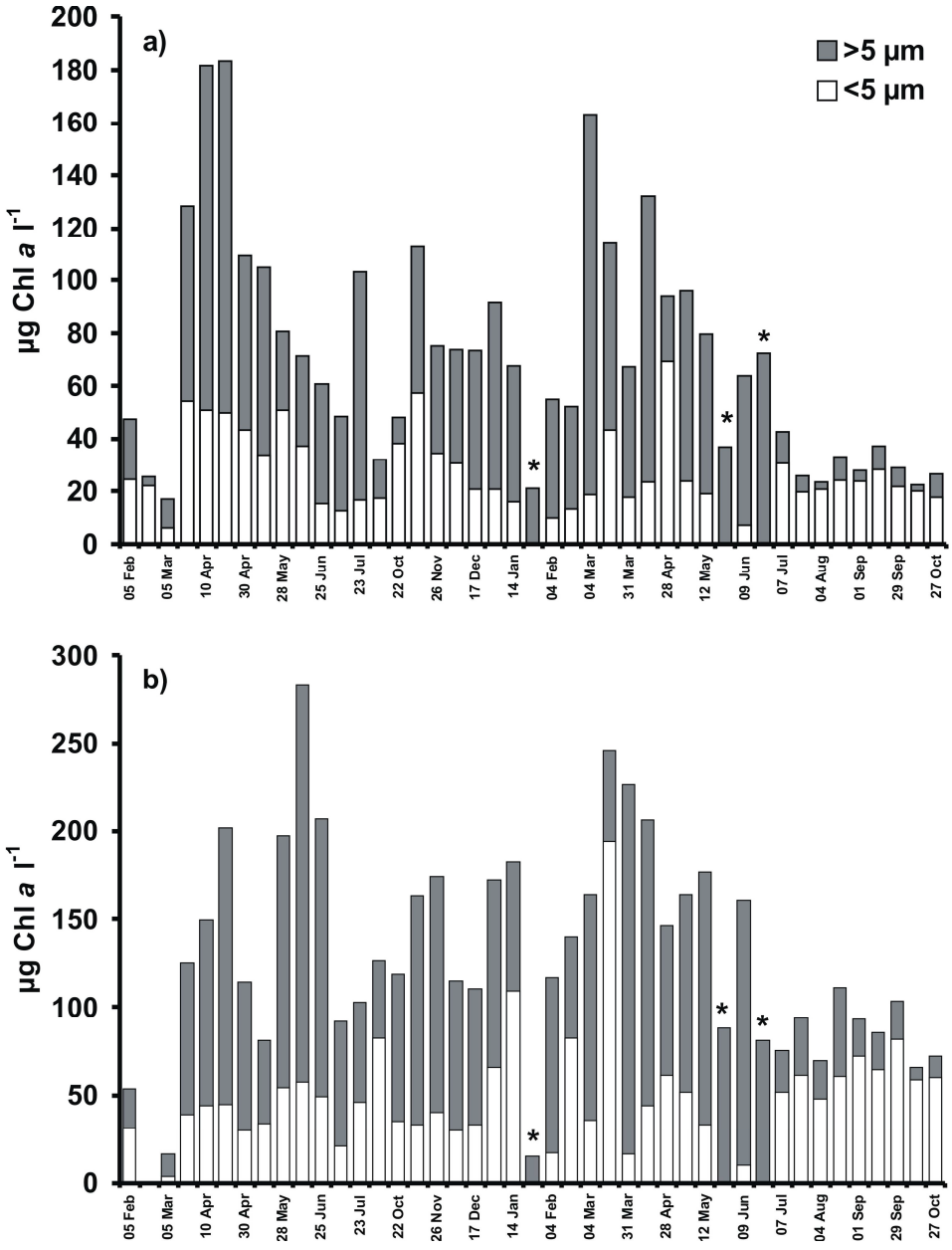



Fig. 9. Chlorophyll a concentrations (µg $l^{-1}$) in the Zingster Strom (a) and in Dierhagen (b) measured in two size classes (< and > 5 µm) from 5$^{th}$ Feb 1991 to 27$^{th}$ Oct 1992. * mark samples, from which only total Chl *a* was estimated

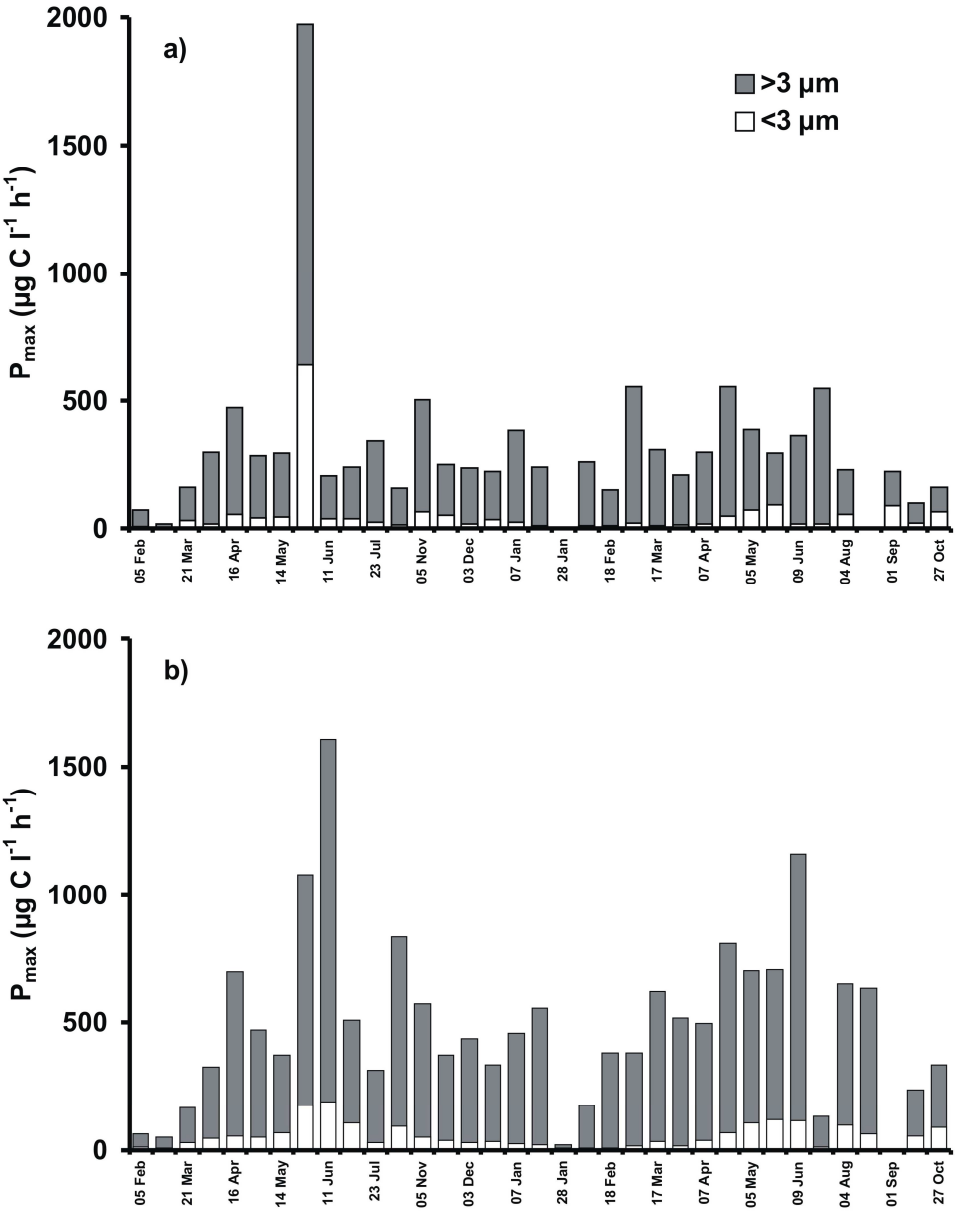



Fig. 10. Primary production $P_{max}$ (µg C $l^{-1}$ $h^{-1}$) in the Zingster Strom (a) and in Dierhagen (b) measured in two size classes (< and > 3 µm)

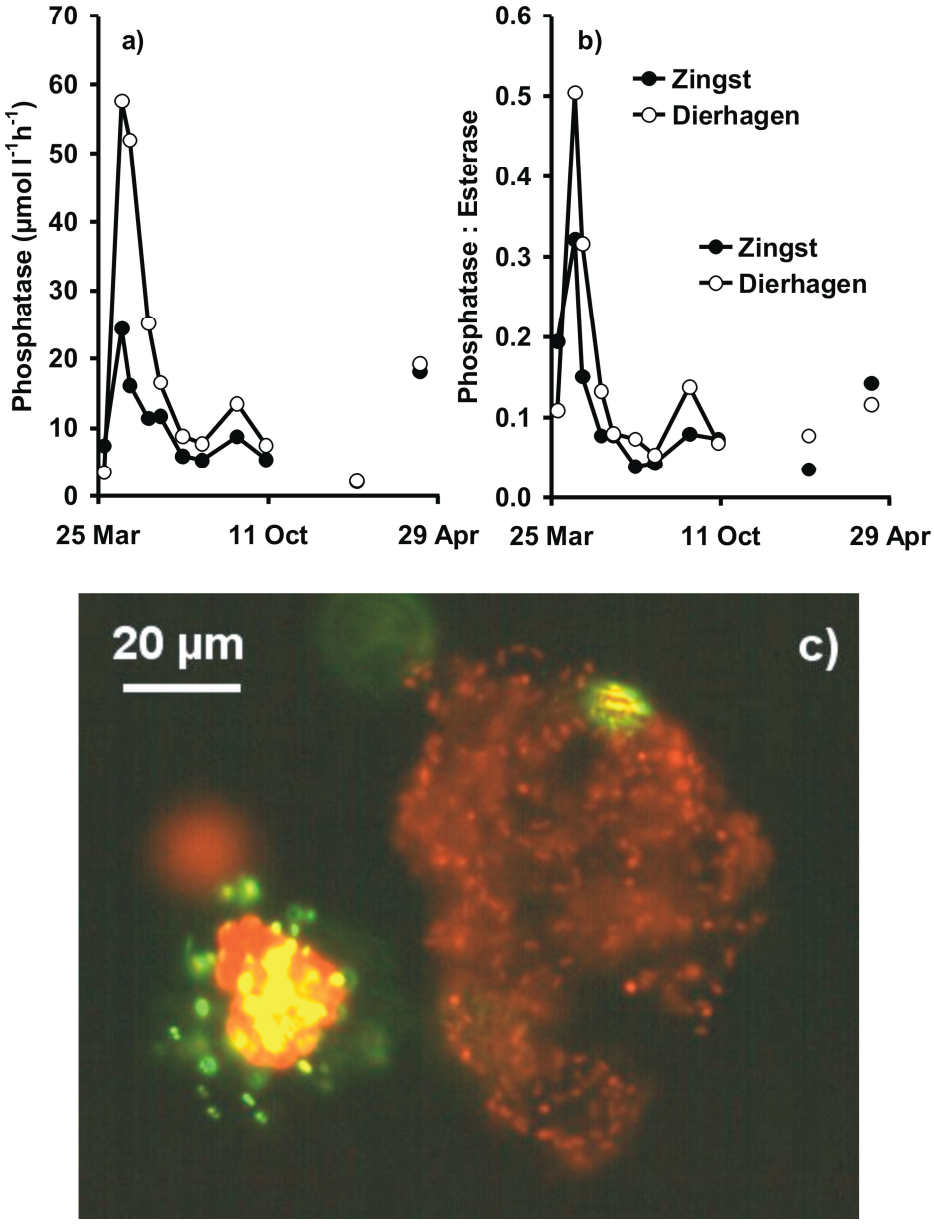



Fig. 11. Phosphatase activity (a, µmol $l^{-1}$ $h^{-1}$), activity ratios for phosphatase to esterase (b) in the Zingster Strom and in Dierhagen between March 1996 and April 1997 and extracellular phosphatase (c) at only one planktonic colony (left) (Zingster Strom 04.09.1997) marked by yellow-green crystals (ELF 97®) formed by enzymantic cleavage from 100 µM ELF 97®-phosphate (Molecular Probes). Olympus BH2-RFCA, 200x magnification, Beamsplitter UG-1 (UV), FUJI Sensia 200, exposure 5 s

(picoplankton) had a lower biomass-to-Chl *a* ratio of 7.7 and 4.3 µg C µg Chl $a^{-1}$ for Zingster Strom and Dierhagen, respectively. These species were rich in pigments above average.
The average production rates at light saturation were 349 and 530 µg C $l^{-1}$ $h^{-1}$ in Zingster Strom and Dierhagen, respectively. Production reached the highest values in May or June (Fig. 10). The contribution of the colonies' size class to particulate carbon production (i.e., without exudates) was disproportionately high (87% at both sites). Normalized to Chl *a*, the phytoplankton communities at both sites had a similar $P_{max}$ (without exudates) of 3.8 µg C µg Chl $a^{-1}$ $h^{-1}$. The average assimilation numbers in the colonies' fraction was 7.3 µg C µg Chl $a^{-1}$ $h^{-1}$. Due to the high production rates and slightly lower Chl *a* concentrations per biomass in the colonies' size class, this ratio was much higher than the assimilation numbers of the picoplankton fraction (1.6 µg C µg Chl $a^{-1}$ $h^{-1}$). Production rates normalized to carbon biomass (P/B ratios) differed far less between pico- and nano-sized fractions. The average total production rate was 0.21 and 0.24 $h^{-1}$ for the sites Zingster Strom and Dierhagen, respectively. Assuming saturated light conditions for 12 hours daily, these production rates would result in growth rates of >2 $d^{-1}$.

### Phosphatase activity as a marker of P limitation

If these potentially high $P_{max}$ rates lead also to *in situ* P limitation was investigated by phosphatase activity in 1996. Plankton samples from all five sites along the trophic gradient displayed a sharp increase in phosphatase activity in early May (exemplary data for Zingster Strom and Dierhagen, Fig. 11). Differences between the sites were proportional to biomass. The phosphatase:esterase ratio reflected these differences. This ratio normalizes a specific enzyme to a ubiquitously and abundantly occurring enzyme class, like esterases. At all stations, this ratio peaked sharply at the same time, like the absolute values. A certain P demand *in situ* in May is expected, when production rates are maximal. However, a high P demand does not necessarily indicate that all species suffer equally. Even, members of the same species also have different levels of vulnerability. Incubation with the ELF®-phosphatase substrate (Fig. 11c) revealed colonies of different limitation states (i.e., with and without detectable extracellular alkaline phosphatase activity) within one sample.

## DISCUSSION

### Special species composition in eutrophic Baltic coastal lagoons as a consequence of eutrophication

It is generally agreed that picoalgae dominate oligotrophic waters (Reynolds et al. 2002), whereas larger algae, especially bacillariophyceae and dinophyceae, require larger nutrient pulses. Thus, the high percentage of picocyanobacteria and the minimal presence of bacillariophyceae in the DZBC reflect low levels of nutrient avail-

ability. It remains unclear whether the highly abundant solitary picocyanobacteria in the DZBC (Klinkenberg and Schumann 1995) are actually *Synechococcus* spp. Uncultured DNA sequences support their presence (Geiss et al. 2004) and did not reveal typical colony forming species. This may be attributed to technical problems of PCR (polymerase chain reaction) approaches. The overwhelming majority of chroococcal sequences stem from this genus. Other poorly investigated genera and species with highly similar sequences (common marker fragments have a relatively low resolution power) are often underrepresented in common molecular databases. Less informative sequences from DZBC phytoplankton may be artificially classified as those of the "*Synechococcus* type" (Schlie 2009). In contrast, an unknown portion of small cyanobacteria (picoplankton fraction) may stem from the "colony type" contrasting or weakening an indication of poor nutrient conditions by picoplankton in these brackish waters.

Since 1990, the largest biomass fraction consisted of small but colony-forming species. The most important of these species across seasons and years were the cyanobacteria *Aphanothece clathrata* and *Snowella* spp. (formerly addressed as *Gomphosphaeria pusilla*) and the chlorophytes *Oocystis solitaria* and *Tetrastrum triangulare* (Schumann and Karsten 2006). Many of these species were mentioned in earlier years (Nasev 1980), but some could not be properly counted before the use of epifluorescence microscopy. Due to the large mucoid envelopes surrounding these colonies, > 50% of organic particle volumes consisted of this material (Schumann et al. 2001). This material may function like a cocoon and restrict nutrients from reaching the cells. Therefore, *Aphanothece* and *Aphanocapsa* spp. are typical for shallow waters and nutrient enrichment (Reynolds et al. 2002). The chlorophyte *Monoraphidium contortum* and most of all the filamentous cyanobacteria *Limnothrix redekei*, *Pseudanabaena limnetica* and *Planktolyngbya contorta* indicate the same conditions but do not form gelatinous colonies. *Woronichinia* and *Merismopedia* spp. belong to communities in mesotrophic systems, playing a seasonal role in the DZBC.

Although the colony-forming species appear to require higher ambient nutrient concentrations, they had higher assimilation numbers than picocyanobacteria. In general, picocyanobacteria are expected to use nutrients more efficiently. Across all size classes, productivity was related to biomass. The high efficiency of colonies is surprising because colonies are expected to suffer from obstructed nutrient uptake. Unexpectedly, small solitary cells were unable to benefit from experimentally improved light conditions. In the past, we did not reveal the diversity of the dominating Chroococcales, except for numerous sequences of uncultured *Synechococcus* spp. (Geiss et al. 2004, Schlie 2009). Isolates revealed the occurrence of phycoerythrin-rich *Synechococcus* (Johansen 2006). However, spectrofluorometric analyses of photosystem II excitation and phycobilin emission in different seasons (e.g., Pilkaitytë et al. 2004, Schoor et al. 2008) never indicated significant proportions of phycoerythrin-rich cyanobacteria in the DZBC phytoplankton. Recently, two stable isolates of colony-forming small rods has been obtained. Future investigation with isolates will help determine their potential niche, i.e., optima and tolerance limits for abiotic factors, nutrient demands and other resources. The interpretation of the spe-

cies' requirements from their annual periodicity, general occurrence and regional distribution may bias the interpretation of the species needs, because competition may determine the realized niche.

**Nutrient limitation in eutrophic waters derived from incubation assays**

Because Chl *a* per biomass reacts to light climate changes, the Chl *a* increase during control incubations in limitation experiments may also result from the build up of the light harvesting apparatus instead of cell proliferation. This relationship is particularly important in shaded samples (L, T). The growing biomass with nutrient additions was compared to a shaded control in this investigation. Positive signals (potential nutrient limitation) were judged more strictly than in other limitation experiments. If all nutrient triggered growth (compared to equally irradiated controls) were considered as positive, almost all samples (48 out of 52) from all sites would be labeled as nutrient-limited. These results are surprising for a severely eutrophicated coastal ecosystem. Additionally, diffusive release from the sediments and resuspended sediment particles supply nutrients to the pelagic environment (Selig et al. 2005). Quantitatively, there were biomass accumulations of up to 3.5 times compared to the shaded control and 5.5 times compared to radiated controls, reported within one week in the early 1990s. These results may be evaluated as a strong fertilization response. In eight samples, the biomass was two times higher than that of the common control (not shaded) even though initial Chl *a* concentrations exceeded 100 µg $l^{-1}$.

One explanation (and drawback for limitation assays by Chl *a* increases) is the change of Chl *a* per cell with light acclimation (increase in L, T samples) or surplus of N (or the termination of N limitation in N+P samples). Both biomass increase responses may be biased by the assay or the observed biomass parameter. Initially, cellular Chl *a* content was unusually high at the two sites, indicating a sufficient N supply, light limitation and/or a unique species composition (cyanobacterial dominance). Underwater light is rather low due to high attenuation in the water column (Schubert et al. 2001). Only algae that are regularly transported to the water surface by wind-induced water movements face high light conditions. The causal relationship between low and, moreover, frequently changing levels of light in the water column and Chl *a* contents of phytoplankton requires further investigation. However, the picoplankton fraction had much higher Chl *a* to biomass ratios. A shift to this fraction could also explain the apparent growth.

Species shifts may also affect the observed frequent nutrient-driven biomass accumulations. After N and P additions in the early 1980s, there were changes in species composition. *Oscillatoria limnetica* (now named *Limnothrix planctonica*) almost disappeared from all mesocosms, which were inoculated with a June community (Börner and Kell 1982). In the last 15 years, this species bloomed in September. Perhaps, fertilization experiments early in the year documented its decreased competitiveness under poor conditions. Favored species were either small chlorophyta or cyanobacteria (e.g., *Merismopedia* spp.). In the following years, these species domi-

nated the community throughout the year. The initial dominance of species favored by high nutrient supply may have prevented community changes in long-term experiments (6 weeks) in the late 1980s (Schumann and Schiewer 2008). The high incidence of nutrient limitation from the incubation assay indicates that important phytoplankton members of the DZBC can use high nutrient pulses as long as the temperature and light suffice. Perhaps, phytoplankton in a eutrophic system is more nutrient-limited than typically previously considered.

*In situ*, high phytoplankton biomass, resuspended sediment particles and a high absorption by colored dissolved organic matter induce severe light attenuation (e.g., Schubert et al. 2001). These characteristics indicate that light must be the limiting resource. Attenuation leads to almost negligible photon flux densities for PAR in the deeper water layers of the very shallow water bodies, especially under disturbed insulation conditions. Potentially, grazing is an important limiting factor for net growth rates *in situ*. However, a significant occurrence of metazoa is usually restricted to a few summer months (Heerkloss and Schnese 1994). While zooplankton removed 76-94% of daily primary production in the outer regions of the DZBC that are influenced by the Baltic Sea, grazing losses were <10% lower in the inner basins (Heerkloss et al. 1980). This grazing pressure never led to a substantial reduction in standing stock. For example, clear water phases were not observed. Sedimentation is a more important loss process *in situ* (Nausch and Nausch 1980).

Several weeks after nutrient additions, phytoplankton biomass returned to the same values as natural communities in the surrounding water body. This quality indicates the high stability of the existing communities and food webs. Distinct changes in nutrient supplies cannot result in sustainable changes of the plankton community (Schiewer 1988). Microbial communities inhabiting the pelagial have high recycling capability and moderate grazing control. These communities are more or less independent from external nutrient supply by surface runoff or release from the sediment. The combination of limitation experiments with other parameters that indicate limitations, including extracellular hydrolases (Ranhofer et al. 2009), nutrient uptake (Riegman and Mur 1986), visualization of storage products (Mateo et al. 2006) or photosynthesis parameters (Dore and Priscu 2001), may reveal improved levels of temporal and spatial resolution for *in situ* nutrient states.

## Nutrient limitation evaluated without incubation

This paper provides a primary impression of the microspatial heterogeneity of extracellular phosphatase (Fig. 11c). The incubation protocol for this micrograph differs from the widely-used protocol of González-Gil et al. (1998). The protocol has more specific indication power because the former includes a permeabilization step, which leads to the detection of any phosphatase activity at and in a cell. Common housekeeping phosphatase activity provides background signals that interfere with those from enzyme activity in the extracellular space. Extracellular enzymes should be expressed specifically at a higher level of phosphate demand or limitation. Colonies of the same species with and without (more or less) extracellular phosphatase were pre-

sent, but quantitative results are not yet available. The total phosphatase peak in spring confirms a P limitation for the average phytoplankton at that time.
C/N/P ratios of particulate matter in the DZBC were moderately above the Redfield ratio indicating a moderate N limitation (Hecky et al. 1993) or much seston material, which is not phytoplankton or of cellular nature (Schumann et al. 2001). However, an isolated N limitation was rare in the 1980s and was only recently re-examined. In 2009, the addition of N by itself never induced more growth than in control incubations (unpublished data). Howver, coastal brackish waters are considered to be N limited in general (Howarth 1988). However, Hecky and Kilham (1988) weakened that opinion about marine systems because "a similarly rigorous demonstration" like the P limitation in lakes has not been observed. Nevertheless, the evaluation of nutrient ratios (Ryther and Dunstan 1971) or seston N/P ratios (Hecky et al. 1993) may be useful for determining the relative importance of N or P limitation at temporal and spatial scales. If potential limitations must be concluded from ambient nutrient concentrations, the element ratio of biomass may be particularly useful.
Photosynthesis parameters can be used to describe the state of phytoplankton individuals or communities on a shorter time scale. In rare cases, these performance parameters are recorded for different size classes or individual colonies. These few cases are important because individual (colony) size may influence its limitation state. For the first time, an asymmetric distribution of potential primary productivity (dark yield) for photosynthetically active aggregates and the occurrence of chlorophyll-containing aggregates (exhibiting $F_m$-values clearly above noise level) without potential primary productivity (no variable fluorescence detectable) have been documented for DZBC samples. Common assumptions on distribution of primary production in a defined volume of phytoplankton suspension from aquatic ecosystems are questioned. Normalization of total primary production to total chlorophyll suggests and calculates an average performance of phytoplankton cells. However, the result is biased in samples with an uneven distribution of performance. With skewed distributions or clearly separated distribution peaks of e.g. biovolume or biomass, integrated samples for photosynthesis measurements will only provide information on active and dominant aggregates. For example, it will deliver incorrectly lower values for active aggregates (and total errors for inactive aggregates). Although aggregates without chlorophyll can be excluded by transmission light and epifluorescence microscopy, error-producing inactive chlorophyll-containing aggregates will influence results calculated from integrated samples by conventional methods ($^{14}CO_2$ incorporation, oxygen evolution). The relative frequency of inactive aggregates was not constant in the different samples from the DZBC. Similar data for pelagic aggregates from different aquatic environments are not available. Thus far, general assumptions cannot be applied to other samples (different species, stations, seasons, smaller plankton colonies). We were unable to reduce the number of inactive aggregates by changes of the measurement settings. However, this result should not be applied *a priori* to other samples. The investigator may try reducing the measurement beam intensity and increasing saturation pulse intensity in the fluorescence analysis protocol to address inactive aggregate or colonies carefully.
Genty et al. (1989) described the background and the mechanism of the relationship

between oxygen evolution and ETR in detail. However, further investigations showed that the fluorescence yield can be influenced by several factors (e.g., species-dependent pigmentation, Strasser et al. 1995). As shown in this paper, more experiments with simultaneous determination of fluorescence yields and oxygen evolution rates are needed to gain confidence in analyses of field samples with different species compositions or of different history with respect to environmental factors and resources. Correlating data sets will allow for reliable analyses of potential primary production distributions in a sample using the dark yield parameter. Following the same method, a transformation of fluorescence measurements into absolute primary production values could be possible. For practical use, the absolute primary production of an aggregate should be calculated according to its size. The microscopic quantification of chlorophyll within a single aggregate is nearly impossible to perform. A knowledge of the inactive aggregates' distribution is required. Additionally, possible correlations between photosynthetic parameters and aggregate size given by an integrative calibration measurement must be considered. In summary, information about the primary production capacity of individual aggregates will be a necessary step towards defining the interaction between physiology and aggregate structure (e.g., species diversity, size, chemistry). This information will also elucidate the key factors (species, aggregations) that determine productivity and/or the limitation state in a particular volume of water. Our first investigation on colony-specific P supports these suggestions. The intensive hot spots of phosphatase activity in some chlorophyll-containing colonies and aggregates begin to orient the explanations of the severely uneven distribution of primary production in chlorophyll-containing aggregates to, e.g., different P starvation stress. Different aggregates of the same species may also contain similar heterogeneity. Our dark yield analyses of well-distinguished *Microcystis* colonies indicate the opposite result. For *Microcystis* colonies of different sizes, a constant dark yield was detected.

## Conclusions

Methods for investigating phytoplankton properties and activities with improved spatial and temporal resolution will help to explain and verify control factors for primary production. More highly resolved methods are needed for eutrophicated coastal waters like the DZBC, which is dominated by hardly distinguishable phytoplankton species. However, these methods will gain special importance if widely used parameters, like ambient nutrient concentration, element ratio, or incubation assays, provide inconclusive results. In the past, the latter often occurred especially in eutrophic and/or coastal waters. It is generally agreed that different species (and different colony sizes) suffer differently from nutrient (or light) limitation within the same water. Additionally, different nutrient demands lead to species succession. However, explanations and predictions for competition and succession depend on the ability to measure species, size class specific limitation and activity status in natural samples.

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