Microalgal primary producers and their limiting resources

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CHAPTER 3

Phosphorus limitation during a phytoplankton spring bloom in the western Dutch Wadden Sea

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Abstract

Like many aquatic ecosystems, the western Dutch Wadden Sea has undergone eutrophication. Due to changes in management policy, nutrient loads, especially phosphorus decreased after the mid-80s. It is still under debate, however, whether nutrients or light are limiting phytoplankton production in the western Wadden Sea, as studies using monitoring data delivered sometimes opposite conclusions and outcomes were related to years, seasons and approaches used. Clearly, the monitoring data alone were not sufficient. We therefore examined the limiting factors for the phytoplankton spring bloom using different experimental approaches. During the spring bloom in April 2010, we investigated several nutrient regimes on natural phytoplankton assemblages at a long-term monitoring site, the NIOZ-Jetty sampling (Marsdiep, The Netherlands). Four bioassays, lasting 6 days each, were performed in controlled conditions. From changes in phytoplankton biomass, chlorophyll-a (Chla), we could conclude that the phytoplankton in general was mainly P-limited during this period, whereas a Si-P-co-limitation was likely for the diatom populations, when present. These results were confirmed by changes in the photosynthetic efficiency (Fv/Fm), in the expression of alkaline phosphatase activity (APA) measured with the fluorescent probe ELF-97, and in the 13C stable isotope incorporation in particulate organic carbon (POC). During our bioassay experiments, we observed a highly dynamic phytoplankton community with regard to species composition and growth rates. The considerable differences in net population growth rates, occurring under more or less similar environmental incubation conditions, suggest that phytoplankton species composition and grazing activity by small grazers were important structuring factors for net growth during this period.
1. Introduction

The need for better understanding the impacts of eutrophication on freshwater, coastal and marine ecosystems has been one of the main reasons to explore relationships between primary producer communities and fluctuations of nutrient concentrations (Cloern, 2001). Apart from influencing productivity levels, changes in ambient nutrient concentrations can also affect phytoplankton species composition, grazer activity and the trophic transfer to higher trophic levels (Brett and Muller-Navarra, 1997; Malzahn et al., 2007; Finkel et al., 2010). Studies on the response of phytoplankton communities to changes in nutrient loads at various scales, ranging from small-scale laboratory techniques, via field mesocosms to lakes and estuaries (Hecky and Kilham, 1988; Beardall et al., 2001; Schindler, 2009), show that interpretation of the results obtained at small scales are sometimes difficult to extrapolate to field conditions.

The widely accepted paradigm on nutrient limitation assumes that nitrogen (N) is the limiting nutrient for primary production in marine ecosystems, whereas phosphorus (P) is the limiting nutrient for primary production in lakes (Hecky and Kilham, 1988; Howarth and Marino, 2006). In both marine and freshwater ecosystems, however, chlorophyll-a (Chla) concentrations were found to be correlated with mean concentrations or loads of total nitrogen (TN) and total phosphorus (TP) (Heip et al., 1995; Smith et al., 2006). The study by Heip et al. (1995) also highlighted the importance of organic matter for primary production, whilst Monbet (1992) demonstrated the influence of the tidal regime on the relationship between N-availability and Chla concentrations. In addition, a meta-analysis on nutrient enrichments in a suite of habitats by Elser et al. (2007) revealed that freshwater systems can be frequently limited by N and marine habitats by P.

The Wadden Sea is one of the world’s largest coastal marine ecosystems which is strongly affected by changes in anthropogenic nutrient loads (Cloern, 2001). In the western part of this area, the concentrations of dissolved inorganic phosphorus (DIP) and dissolved inorganic nitrogen (DIN) increased during the 1970s and decreased after the mid-1980s as the result of changing riverine loads (Cadee and Hegeman, 2002; Philippart et al., 2007; Loebl et al., 2009). These changes in absolute and relative nutrient loads coincided with major changes in phytoplankton community structure during the late 1970’s and the late 1980’s (Philippart et al., 2000) and were followed each time by changes in community structures of macrozoobenthos, fish and estuarine birds (Philippart et al., 2007; Tulp et al., 2008).

Long-term trends in relative nutrient concentrations in the western Wadden Sea strongly suggest that phytoplankton production during the spring and summer blooms was P-limited in the 1970s, Si-limited (diatoms) or N-limited (flagellates) in the 1980s, and P-limited again hereafter (Philippart et al., 2007). Light limitation appears to play a minor role during the blooms. Whilst previous analyses indicated co-limitation by light (Colijn and Cadée, 2003), more recent results using the similar index (Cloern, 1999, 2001) suggested that nutrients were the main limiting resource during the growing season for phytoplankton in the Wadden Sea (Loebl et al., 2009). In addition, the turbidity of these waters was found to be highly variable during this
period but did not exhibit the long-term trends as was observed in phytoplankton biomass, productivity and species composition (Philippart et al., 2013).

Previous results on the nature and strength of nutrient limitation in the western Wadden Sea were all based on nutrient concentrations, which are only weak indices of nutrient limitation because no information on uptake and mineralization is taken into account (Dodd, 2003). To unambiguously determine the nature of the actual limiting nutrient, we performed nutrient enrichment experiments during the spring bloom in combination with several physiological measurements. To test the viability of historical statements on nutrient limitation in the western Wadden Sea, we compared the new results from the combination of techniques with the ratio’s in ambient nutrient concentrations.

2. Material and methods

2.1. Sampling procedure

Water samples have been collected using a bucket at weekly intervals at high tide from the NIOZ sampling jetty (53°00′06″ N; 4°47′21″ E) from 30th March to 30th April 2010 (Table 1). The NIOZ sampling jetty is located in the Marsdiep basin near to the inlet between the North Sea and the Wadden Sea (Fig. 1). Sampling was always performed at high tide (± 10 minutes) as predicted for the nearby city of Den Helder (www.getij.nl) in order to keep variation in parameter values as the result of tidal currents as limited as possible (Cadée, 1982). High tide at the NIOZ sampling Jetty falls approximately 30-45 minutes later than at Den Helder, which implies that sampling was just before high tide. Comparison with ferry box observations as determined from a ferry sailing across the Marsdiep tidal inlet during 11 years showed that turbidity at the NIOZ sampling jetty was correlated with total suspended matter concentrations in the Marsdiep tidal inlet (Philippart et al., 2013). This finding strongly suggests that information on trends as derived from the NIOZ sampling jetty samples is indicative for changes in the western Wadden Sea.

2.2. Experimental design

The nutrient enrichment experiments with natural phytoplankton populations were performed in 8 liters polycarbonate bottles which were incubated under controlled light and temperature conditions during 6 days (Table 2). The water was collected and filtered over a 100 µm mesh size filter in order to minimize grazing by meso- and macrozooplankton on the phytoplankton. The enrichment bottles were incubated at in situ temperature conditions (8-10 °C). A light-dark cycle of 14:10 (L:D) was implemented using fluorescent tubes (Cool White 36 W, Philips) at an irradiance of 100 µmol photons m⁻² s⁻¹.

The experimental design incorporated three possible nutrient additions: +N, inorganic nitrogen (addition of NH₄NO₃ which would give a final concentration of 100 µmol L⁻¹ if no ambient DIN would be present); +P, inorganic phosphorus (10 µmol L⁻¹ (final) KH₂PO₄) and +Si
(100 µmol L\(^{-1}\) (final) \(\text{Na}_2\text{SiO}_3\cdot5\text{H}_2\text{O}\)). In total, four different nutrient treatments were composed, each in triplicate: C (control without any addition of nutrients); +NP; +NSi; +PSi; +NPSi, where all nutrients are added together (Table 2). In B4, due to an error in the laboratory, no +NSi treatments were added to the experimental design.

As we also performed \(^{13}\)C-labeling experiments and investigated particulate organic carbon (POC) bulk labeling (see below), no single additions were performed as this would increase the number of bottles substantially. Information on the effects of additions of a single nutrient were, therefore, derived by comparing the results of multiple nutrient additions. Water subsamples from the bioassays were taken at the beginning and end of each nutrient addition treatments for \(^{13}\)C stable isotope incubation. Water samples were enriched with \(^{13}\)C-\(\text{NaHCO}_3\) (99% \(^{13}\)C; Cambridge Isotope Laboratories, Inc.) for 2 hours with a concentration of 4% of the ambient dissolved inorganic carbon (DIC) concentration.

**2.3. Average irradiance depth at the Marsdiep tidal inlet**

We estimated the light climate in the Marsdiep basin during the period of our bioassays. As the light attenuation coefficients (\(K_d\; \text{m}^{-1}\)) were not available during this period we estimated this from the Secchi depths using the following empirical relationship:

\[
K_d = a \cdot \sqrt{\text{Secchi depth}} + b
\]

Where \(a = 5.377\; (\text{m}^{-1})\) and \(b = 2.07\; (\text{m}^{-1})\) are fit constants obtained from regression analysis \((r^2 = 0.71; \; n = 116)\) in the western part of the optically similar Oosterschelde estuary (Malkin and Kromkamp, unpublished results). \(K_d\) varied between 0.77 to 2.99 m\(^{-1}\) (median = 1.17 m\(^{-1}\)). Hourly irradiance (J cm\(^{-2}\)) data for the April months of 2009 -2012 were downloaded from the Dutch Meteorological Office (http://www.knmi.nl/klimatologie/) station De Kooy (located 8.6 km south from the sampling site) and converted into PAR (µmol photons m\(^{-2}\) s\(^{-1}\)) values using an empirical conversion factor of 5.2 (Kromkamp unpublished). According to the obtained values, the average incident irradiance value in the month of April was 380 µmol photons m\(^{-2}\) s\(^{-1}\). In April, the median value of \(K_d\) was 0.91 m\(^{-1}\). The average depth of the Marsdiep basin is approximately 4.5 m (Ridderinkhof, 1988).

Assuming the photic depth equals the depth to which 1% of the surface irradiance penetrates, it follows that the photic zone (\(z_{eu}\)) to mixing (\(z_m\)) ratio varies annually from 0.34 to 1.31 with a median value of 0.88, indicating that in nearly the complete water column primary production is taking place. The average \(z_{eu}/z_m\) ratio for April was 1.12. From \(K_d\), the average depth and the incident irradiance, we calculated that daylight averaged irradiance values in the water column varied between 27 and 112 µmol photons m\(^{-2}\) s\(^{-1}\) (median = 75 µmol photons m\(^{-2}\) s\(^{-1}\)). In April, the median water column irradiance equaled 96 µmol photons m\(^{-2}\) s\(^{-1}\), which was very close to the irradiance value used (100 µmol photons m\(^{-2}\) s\(^{-1}\)) during nutrient enrichment experiments.
2.4. Nutrient concentrations

Samples for dissolved nutrients (ammonium (NH$_4^+$), nitrate (NO$_3^-$), nitrite (NO$_2^-$), phosphate (DIP) and silicate (Si)) were filtered through a disposable filters (0.2 µm) and analysed using an QuAAtro autoanalyser, with segmented flow analysis (Bran+Luebbe, Germany) according to the manufacturers instruction (Hydes D 2010). Dissolved inorganic nitrogen (DIN) is the sum of ammonium, nitrate and nitrite. Total nitrogen (TN) and total phosphorus (TP) were extracted according to Valderrama (1981). In addition, particulate phosphorus (PP) contents were quantified with inductive coupled plasma spectroscopy (ICP-OES; Perkin Elmer Optima 3300 DV) on filtered samples (Nieuwenhuize and Poley-Vos, 1989). Particulate organic nitrogen (PON) samples were analyzed Carlo Erba elemental analyzer (EA) coupled online to a Finnigan Delta S isotope ratio mass spectrometer (IRMS). PON and PP were filtered onto glass-fiber filters (Whatman GF/F). Total dissolved nutrients were defined as TDN = TN – PON and TDP = TP – PP. Then, dissolved organic nitrogen (DON) and dissolved organic phosphorus (DOP) can be deducted; DON = TDN – DIN and DOP = TDP - DIP. Several dissolved nutrient ratios were calculated (DIN:DIP; DIN:TDP; TDN:TDP; Si:DIP), including the biological available nitrogen (BAN) to phosphorus (BAP) ratio where BAN: BAP = ([DON + DIN]/[DOP + DIP]).

2.5. Chlorophyll-a

For each chlorophyll-a (Chla) sample, a volume of 50 to 100 mL was filtered through a 47 mm Whatman GF/F filters and put directly in a glass container with 10 mL of 90% acetone. The Chla samples were stored in the freezer (-20 °C) for at least 24 hours. Then, the Chla concentrations were measured with a fluorometer (Hitachi Fluorescence Spectrophotometer F-2500) with an excitation wavelength of 431 nm and emission wavelength of 671 nm. The fluorometer was calibrated with a known concentration of Chla. Specific growth rates (µ, day$^{-1}$) were calculated from changes in Chla over time (t; days) as µ = 1/t × ln (Chla (t)/Chla (0)).

2.6. Species counts

As part of the long-term field observation program from the NIOZ sampling jetty, phytoplankton species composition was determined from surface water samples at a sampling frequency from once a month in mid-winter to twice a week during spring blooms. Phytoplankton samples were preserved with Lugol and cells were counted with a Zeiss inverted microscope using 5-ml counting chambers (Philippart et al., in prep). Most algae were identified to species level; some were clustered into taxonomic and size groups (e.g. small flagellates).

2.7. Carbon incorporation using $^{13}$C uptake in particulate organic carbon (POC)

Samples for the $^{13}$C incubation were filtered over precombusted glass-fiber filters (Whatman GF/F) and later analyzed with a Carlo Erba elemental analyzer (EA) coupled online to a Finnigan Delta S isotope ratio mass spectrometer (IRMS). The fraction of $^{13}$C (μg C L$^{-1}$ h$^{-1}$) in
POC was calculated following specific isotopic calculations (Middelburg et al., 2000). Stable isotope data are expressed in the delta notation ($\delta^{13}C$) relative to carbon isotope ratio ($R = ^{13}C/^ {12}C$) of Vienna Pee Dee Belemnite ($R_{VPDB}= 0.0112372$): $\delta^{13}C = ([R_{sample}/ R_{VPDB}] - 1 \times 1000$). The $^{13}C$ uptake of POC ($\mu$mol C L$^{-1}$) was calculated from the difference of the fraction of $^{13}C$ at the start and at the end of the incubation, multiplied by the concentration of POC at the start of the incubation. After correction for the NaH$^{13}$CO$_3$ enrichment (4% added of ambient DIC), the rate of $^{13}C$-incorporation at the incubation conditions ($\mu$mol C L$^{-1}$ h$^{-1}$) was obtained by the incubation time (2 hours) The fraction of the POC was calculated as $^{13}C/ (^{13}C + ^ {12}C) = R/ (R+1)$. Total dissolved inorganic carbon (DIC) varied around 2.22 ± 0.06 mmol L$^{-1}$ (E. Epping, pers. comm).

2.8. Photosynthesis physiology

Chlorophyll fluorescence was measurement with a Water-PAM fluorometer (Heinz Walz, Effeltrich, Germany). For each sample, the minimum fluorescence yield ($F_0$) and the maximum fluorescence yield ($F_m$) were measured using a 1 min dark adaptation time before measurement in order to obtain the maximum PSII quantum efficiency ($F_v / F_m = (F_m - F_0)/F_m$).

2.9. Alkaline phosphatase activity (APA)

At the beginning and end of each nutrient treatment experiment, samples for specific detection of APA were measured using a molecular probe, ELF-97 (Endogenous Phosphatase Detection Kit; E6601, Molecular Probes, Invitrogen, California) following the instructions provided. The phytoplankton in a sample of 100-150 mL was concentrated using a membrane filter of 0.2 µm, and to 1 mL of the concentrated sample a freshly prepared ELF-97 working solution was added. After 30 min incubation in the dark, 100 µL of the phosphate buffer solution and 20 µL of a mixture of paraformaldehyde and glutaraldehyde (0.01:0.1%) was added. Samples were stored at 4 °C. The inoculated ELF samples were visualized using an epifluorescence microscope (Carl Zeiss axioplan 2 imaging microscope) connected to a LED illumination system (Colibri, Carl Zeiss SAS, Germany). Carl Zeiss Axiovision software was used to acquire and analyze the images. During the storage, however, the algal cells and other components in the water formed large aggregates making microscopy observations on single algal cells difficult. As the bright green fluorescence of the ELF stain was clearly visible within the aggregates, we decided to make a relative score of the “density” of the ELF stained particles in the aggregates, where an intense bright green fluorescence was scored as “high”, (, a pale green fluorescence as “low”, and the absence of any fluorescence as “zero”. In total, 100 cells or aggregates were scored for each sample. Only ELF fluorescence of intact algal cells was scored, i.e. only when the red chlorophyll autofluorescence was visible, the ELF fluorescence was included.
2.10. Statistics analysis

Parameters such as Chl$\alpha$, F$_v$/F$_m$ and C-incorporation via $^{13}$C labeling were analyzed with an analysis of variance (ANOVA), with time of incubation (6 days) and treatments as fixed factors for each bioassay (5 levels: control (C), +NP, +NSi, +PSi, +NPSi). A one way ANOVA was performed to test difference among the nutrient addition treatments for each bioassay at different times during the incubation. Assumptions of ANOVA (normality and homogeneity of variances) were tested. Analyses were undertaken using R version 2.12.0 (http://www.r-project.org/) with a level of significance of $p < 0.05$. 
3. Results

3.1. Starting conditions

For each nutrient concentration, significant differences were found between bioassays (one-way ANOVA, \( p < 0.05 \)). At the start of the first three bioassays (B1, B2 and B3), the DIP concentrations ranged from 0.04 to 0.05 \( \mu \text{mol L}^{-1} \) which was close to the detection limit for this nutrient (Fig. 2). At the start of B4, the DIP concentration was higher, reaching a concentration of 1.5 \( \mu \text{mol L}^{-1} \). The concentrations of Si were below the detection limit at the start of B2 and B3, and low (0.15 \( \mu \text{mol L}^{-1} \) for B1 and 0.07 \( \mu \text{mol L}^{-1} \) for B4) but not significantly different at the start of the other two bioassays. From B1 to B3, \( \text{NH}_4^+ \) concentrations decreased significantly from 0.91 to 0.26 \( \mu \text{mol L}^{-1} \). In B4, \( \text{NH}_4^+ \) concentrations had increased again to 0.67 \( \mu \text{mol L}^{-1} \). \( \text{NO}_x \) was the main contributor of the DIN pool with \( \text{NO}_3 \) contributing around 97% of total \( \text{NO}_x \), e.g. \( \text{NO}_x \) concentrations were already 40 times higher than \( \text{NH}_4^+ \) in B1. Concentrations of \( \text{NO}_x \) decreased from 40 \( \mu \text{mol L}^{-1} \) at the start of B1 to 17.5 \( \mu \text{mol L}^{-1} \) at the start of B4.

DOP increased from 0.5 \( \mu \text{mol L}^{-1} \) in B1 to 1 and 2 \( \mu \text{mol L}^{-1} \) in B2 and B3, respectively. In B4, DOP levels showed the highest values of about 10 \( \mu \text{mol L}^{-1} \). DON increased over the experimental period from 18 \( \mu \text{mol L}^{-1} \) in B1 to 40 \( \mu \text{mol L}^{-1} \) in B4. For each nutrient ratio, significant differences were found between bioassays (one-way ANOVA, \( p < 0.05 \)) (Fig. 3).

Within the study period, the N:P ratios of the dissolved nutrients (i.e., DIN:DIP, DIN:TDP, TDN:TDP and BAN:BAP) generally decreased. The DIN:DIP, the TDN:TDP and the BAN:BAP ratios were higher than the Redfield ratio (N:P = 16) during the first three bioassays (B1-B3) and lower than 16 during B4, which suggested P limitation before 24 April and a relative N-shortage hereafter. The PON:POP ratio, however, far exceeded the Redfield ratio and did not differ significantly between bioassays (one-way ANOVA, \( p = 0.6514 \)), suggesting P limitation during the full study period (B1-B4). The Si:DIP ratio was just below the Redfield ratio during B1 and showed very low values for the other bioassays (B2-B4), suggesting Si-limited growth of diatoms compared to P during the full studied period.

At the start of B1, Bacillariophyceae (diatoms) formed the dominant fraction in the phytoplankton community (dominated mainly by Thalassiosira spp) at the start of B2, a massive bloom of the haptophyte Phaeocystis globosa appeared, clearly visible by eye. At the start of B3, P. globosa continued to develop. At the start of B4, the P. globosa bloom declined (Table 3).

3.2. Phytoplankton growth rates

For B1, all treatments resulted in an increase phytoplankton biomass during incubation (Fig. 4). The largest increase in phytoplankton biomass was observed in +PSi and +NPSi treatments (Fig. 4). On the 3rd day of these two treatments, biomass approximately increased 3-fold up to 20 \( \mu \text{g L}^{-1} \). At the end of this bioassay, Chl\( a \) concentrations in these two treatments were more than 100 times higher than the initial value, reaching a value of 220 \( \mu \text{g L}^{-1} \) (Fig. 4). If the C:Chl\( a \) ratio was constant during the incubation period, then the net community growth rate
was approximately 0.64 day\(^{-1}\) (Table 2). Chl\(a\) concentrations in the +NP or +NSi treatments slightly increased to 24 and 30 µg L\(^{-1}\) at the end of the incubation period, respectively. During the whole duration of this bioassay the differences between treatments were significant (one-way ANOVA, \(p = 1.75 \times 10^{-3}\)).

At the start of the incubation of B2, the Chl\(a\) concentrations were approximately five times higher than at the start of B1 (Fig. 4). The +NP, +PSi and +NPSi treatments resulted in an increase in phytoplankton biomass during incubation, whilst biomass decreased for the +NSi treatment and the control (Fig. 4; Table 2). For B2, the largest increase in net growth rate of around 0.20 day\(^{-1}\) was found for the +NPSi treatment (Fig. 4; Table 4).

For B3, the initial Chl\(a\) concentration was lower than that at the onset of B2 and higher than during the start of B1. As for B2, the +NP, +PSi and +NPSi treatments resulted in an increase in phytoplankton biomass during incubation, whilst biomass decreased for the +NSi treatment and the control (Fig. 4; Table 2). The increase in phytoplankton biomass was highest for the +NPSi treatment. For those treatments at B3 where the biomass increased, net growth rates were higher than observed at B2 (Table 2).

For B4, the response of the phytoplankton community after the addition of the different nutrients was more difficult to interpret than for the other bioassays as we do not have information on the effects of +NSi addition. DIN concentrations, however, seem high sufficient (~20 µmol L\(^{-1}\)) not to limit phytoplankton growth. Compared to the previous bioassays, the overall increase in Chl\(a\) was very limited with highest net population growth rates of approximately 0.19 day\(^{-1}\) in the +NP, +PSi and +NPSi treatments (Fig. 4; Table 2).

### 3.3. Carbon incorporation rates

The C-incorporation rates as determined by \(^{13}\)C-label incorporation into POC differed between the various bioassays and the various treatments (Fig. 5). ANOVA analysis showed significant differences in \(^{13}\)C derived C-incorporation rates between the nutrient addition treatments at the end of incubation and compared to the \(t=0\) values (one-way ANOVA, \(p = 1.3 \times 10^{-14}\)). In B1, like in the Chl\(a\) results, C-incorporation only increased in the +PSi and +NPSi treatments and reached similar values of approximately 125 µg C L\(^{-1}\) h\(^{-1}\).

In B2, B3 and B4, the label incorporation showed a similar pattern as the Chl\(a\) results with a similar stimulation of C-incorporation in both the +NP and +PSi treatments and a higher rate of C-incorporation in the +NPSi. However, as we did not measure the C-incorporation rate every day, but only at the start and end of the bioassay, we cannot be sure about this.

### 3.4. Physiological properties

In the control experiments, \(F_v/F_m\) values slowly decreased during the 6 days of experiment in B2 and B3, whereas no changes were observed in B1 and B4 (Fig. 6). In all 4 bioassays, increases in \(F_v/F_m\) during incubation were noticeable for some treatments, with responses differing between the bioassays (two-way ANOVA, \(p = 1.1 \times 10^{-5}\) (bioassays) and \(p = 6.4 \times 10^{-11}\) (treatments)). In B1, the largest increase in \(F_v/F_m\) was observed in the +PSi and +NPSi
additions, whilst the response of +NP was limited. During the B2 and B3 experiments, $F_v/F_m$ values showed a more or less similar response to nutrient addition in the +NP, +PSi and +NPSi treatments.

In spite of the aggregate formation during storage, still two dominant algal groups could be distinguished by means of the epifluorescence microscopy, i.e. Thalassiosiraceae and Phaeocystis globosa. This is in agreement with the long-term field observations on phytoplankton species composition during this period (Philippart et al., in prep).

At the start of B1, B2 and B3, every aggregate showed a high alkaline phosphatase activity (APA) as judged by the high ELF fluorescence (Fig. 7B). In B4, however, less than 40% of the aggregates showed high ELF and the rest low ELF fluorescence. During the incubations of the controls, the 100% high scores of ELF expression did not change for B1 and B3, and decreased from 100% high to 65% high and 35% low ELF expression in B2. For B4, the 30% high ELF expression increased to 90% during incubation, indicating that the cells depleted most of the DIP and DOP available at the start of the control treatment of this bioassay. For each treatment that included the addition of P (+NP, +PSi and +NPSi), the ELF fluorescence decreased, indicating a depressed APA activity. Only for some cases, aggregates without any APA activity were observed after incubation, e.g. +NP and +PSi addition in B1 where the aggregates showed a 20-30% of the aggregates lower ELF expression. Addition of nutrient mixtures without P (+NSi) also resulted in a lowered ELF expression in B1 and B2, although not as pronounced as when P was added. In the last bioassay (B4), high ELF fluorescence was only observed for less than 20% of the cells and the rest showed a low fluorescence.

In addition to the cells in the aggregates, we could detect single cells of pennate diatoms, which are associated with a benthic lifestyle (Fig. 7A and Fig. 8C and 8D). APA activity levels in the pennate diatoms were in general lower than found in the aggregates cells. In B1 and B2, no pennate diatoms were observed at the beginning of the bioassays. For those treatments in B2 where Si was added (+NSi, +PSi and +NPSi), however, pennate diatoms were observed at the end of the incubation period. Depending on the treatment, these diatoms showed no (+NPSi) to 25% high APA activity (+NSi). At the start of B3, 100% of the pennate diatoms showed a high APA activity, whilst only 60% of the pennate diatoms was comparably active at the start of B4. For B3 and B4, however, pennate diatoms were no longer found after incubation with +NP. The addition of P decreased the percentage of high ELF fluorescence in the diatoms cells in B3 (+PSi and +NPSi), but for B4 the situation was more complex as the +PSi addition did not show a decrease in APA activity, as was the case for the +NPSi treatment.
4. Discussion

4.1 Nutrient versus light limitation

The main aim of this research was to examine the nature of the limiting nutrient for phytoplankton production during a spring bloom in the western part of the Dutch Wadden Sea to aid to the understanding of contradictory results on nutrient limitation in the past. In turbid coastal ecosystems such as the Wadden Sea, light conditions during spring may also be a limiting factor for pelagic primary production (Heip et al., 1995; Cloern, 1999; Tillmann et al., 2000; Colijn and Cadée, 2003). For a correct interpretation of our results, we should know which role light limitation may have played in relation to nutrient limitation during our study period.

To examine the relative role of light versus nutrient limitation, Cloern (1999) developed a growth index based on ambient light conditions and concentrations. Applying this index to Marsdiep data from 1995/1996, Colijn and Cadée (2003) concluded that phytoplankton growth was mainly limited by light from August to April, co-limited by light and N (the only nutrient considered here) in June and July, and mainly N-limited in May. For the period from 1991–2005, (Loebl et al., 2009) argued that phytoplankton growth was generally limited by light from October to February, both by light and P in March and in September and mainly by P from April to July/August. Their result showed large interannual fluctuations in the timing and the nature of the nutrient limitation. For some years, for example, the index suggested limitation by Si or by N for one or two months per year.

In addition to ambient light and nutrient conditions, the values of the index are related to the parameter values of the half-saturation coefficients used to calculate the relative importance of the light ($K_l$) and nutrient ($K_X$) resources. Both calculations (Colijn and Cadée, 2003; Loebl et al., 2009) used the values for $K_l$ (2.4 mol photons m$^{-2}$ d$^{-1}$) and for $K_N$ (1.5 µmol L$^{-1}$) from Cloern (1999). With regard to nutrient limitation, however, Loebl et al. (2009) additionally took the possibility of nitrogen and silicate limitation into account, using $K_P = 0.5$ µmol L$^{-1}$ and $K_{Si} = 5$ µmol L$^{-1}$ for PO$_4^{3-}$ and Si uptake respectively. As the result of the fact that Colijn and Cadée (2003) based their conclusions on nutrient versus light limitation solely on nitrogen, they might have missed nutrient limitation for phytoplankton growth in those months where the availability of nutrients other than nitrogen was low, e.g. in April.

The calculated average irradiance experienced by the algae during the day in April in the Marsdiep tidal basin was approximately 96 µmol photons m$^{-2}$ s$^{-1}$ during the photoperiod. The irradiance used during our bioassays (100 µmol photons m$^{-2}$ s$^{-1}$) was comparable to the ambient light conditions in the field. This corresponds to a daily light dose of 4.8 to 5 photons m$^{-2}$ d$^{-1}$, which is higher larger than $K_l$ of 2.4 mol photons m$^{-2}$ d$^{-1}$ generally used for phytoplankton in coastal waters (Cloern, 1999; Colijn and Cadée, 2003; Loebl et al., 2009). The general absence of light limitation in April as previously observed (Loebl et al., 2009) and the relatively high light conditions during our study compared to $K_l$ (Cloern, 1999) strongly suggests that nutrients and not light was limiting phytoplankton growth during the period of our bioassays.
4.2. Bioassays and physiological indices of nutrient limitation

Within all bioassays, the addition of P resulted in an increase in Chl$\alpha$ concentrations, indicating that the ambient PO$_4^{3-}$ concentrations were not sufficient to support maximum growth of the phytoplankton community. For many of the bioassays, the biomass appeared not to respond immediately to nutrient addition. Such a lag phase is common in experiments where stimuli are applied, in particular when a limiting nutrient is added (Duarte, 1990; Scharek et al., 1997). Because the duration of the lag phase may vary for different algal species, a pulse of nutrients can potentially change the phytoplankton community structure, with relatively small and fast-growing algal species taking advantage over others (Duarte, 1990; Scharek et al., 1997).

If the response of the phytoplankton community following the nutrient additions resulted in an increase of the ratio between Chl$\alpha$ and carbon, then Chl$\alpha$-change based growth rates are overestimating actual growth rates. In spite of the fact that we measured POC and Chl$\alpha$, we cannot determine the Chl$\alpha$:C ratio because the algal C fraction is only a small fraction of the total POC. The length of the lag phase of several days further suggests that internal P stores in the phytoplankton were depleted at the start of the incubations, corroborating P limiting growth conditions in the Marsdiep in April 2010 as derived from the biomass response in the bioassays. Although questioned in several studies (Parkhill et al., 2001; Kruskopf and Flynn, 2005), the F$_v$/F$_m$ ratio has proven to be a trustworthy indicator of nutrient limitation in other cases (Flameling and Kromkamp, 1998; Kolber et al., 1988; Lippemeier et al., 1999; Beardall et al., 2001). Our results clearly showed that when the P limitation of phytoplankton growth was relieved by adding P (i.e., the +NP, +PSi, and +NPSi treatments), the maximum PSII efficiency increased. Furthermore, the conclusions drawn from patterns of change in F$_v$/F$_m$ were very similar to those from changes in phytoplankton biomass, showing that F$_v$/F$_m$ can be applied as a reliable indicator of nutrient limitation in shallow coastal ecosystems such as the Wadden Sea.

Our results for B4, where Si limitation appeared to have lowered F$_v$/F$_m$, is in agreement with the findings for diatom cultures (Lippemeier et al., 1999) but never observed during field studies before. Theoretically, a lowered F$_v$/F$_m$ can be caused by an interference of phycobilin fluorescence from cyanobacteria (Campbell et al., 1998). According to our pigment data (not shown in this study), however, no zeaxanthin, a pigment biomarker for cyanobacteria, was detected. Therefore, we can conclude that the signal is from eukaryotic phytoplankton. In spite of that fact that Si is no structural component of the photosynthetic apparatus, we think it likely that when cells harvest more light than they can use for growth and formation of storage products, this will cause backpressure on photosystem II, lowering the PSII quantum efficiency. Only when the cells have reduced their pigment contents to cover their need, F$_v$/F$_m$ will recover.

Alkaline phosphatase activity (APA) is used by many authors as an indicator of P limitation of phytoplankton (González-Gil et al., 1998; Dyhrman and Palenik, 2001; Rengefors et al., 2003; Duhamel et al., 2010). In marine ecosystems, DOP is often present in higher concentrations than DIP (Dyhrman et al., 2007). APA is generally considered to be able to hydrolyse the phosphate group from DOP only when the phosphate group is ester-bound (C-O-P bond). So-called phosphonates, which are ether-bound organic P compounds (C-P bond), were
thought not to be used by phytoplankton until the recent observation of phosphonate utilization by the marine cyanobacterium *Trichodesmium* sp. (Dyhrman et al., 2006). The characterization of DOP in combination with the capacity of photoautotrophs to utilize the several forms of DOP requires further study.

Some studies suggest that APA is regulated by external phosphate concentrations (Jochem, 2000; Lomas et al., 2004) whereas others indicate that it is the internal P content which regulates APA activity (Rouzic and Bertru, 1997; Lomas et al., 2004; Ranhofer et al., 2009). Although we did not measure internal P storage, our results showed that ELF is a useful indicator of P-limitation, as it corroborates with the results from the changes in Chla, Fv/Fm, extracellular P concentrations, nutrient N:P ratios and C-incorporation rates.

During the bioassays, we noticed that pennate diatoms repressed APA faster than the phytoplankton cells in the aggregates upon P-addition. At the end of the bioassays in which P was added, most cells still expressed AP activity although phosphate concentrations varied then between 2 and 8 µmol L\(^{-1}\) (indicating that growth was not P-limited anymore). These observations suggest that the APA life time varies between functional groups, and that algal species or even cells may differ in their APA regulatory response.

Comparison of the bioassays results with ambient nutrient concentrations revealed that the dissolved nutrient ratios did predict the nature of the limiting nutrient well as long as the concentrations were potentially limiting. The particulate N:P (PON:POP) ratio, however, did not give any conclusive information as it remained constant and did not capture the changes in concentrations at the start of B4. Our results suggest that the DIN:DIP ratio gave the best prediction, as the DIN:TDP, TDN:TDP or BAN:BAP ratios did not show P limitation in B3, whereas the other measures showed a P-Si-co-limitation for B3. The BAN:BAP ratio was less accurate than the DIN:DIP ratio suggesting that not all DOP might be biological available. The Si:P ratio is an indicator for competition and succession of diatoms, with ratios being highest in B1 where diatoms were the most dominant group in phytoplankton population (see § 4.3).

### 4.3. Interactions between nutrient limitation and phytoplankton succession

The variation in external inorganic nutrient concentrations during the spring bloom of 2010 coincided with changes in the phytoplankton community composition. At the beginning of our experimental period, i.e. at the end of March, the phytoplankton was dominated by diatoms, in particular by *Thalassiosira* species. The increase in Chla concentrations, C-incorporation and maximum photosynthesis quantum yields (Fv/Fm) were the highest in the treatments when Si and P were added (+NPSi and +PSi), indicating that P and Si were the limiting nutrients for phytoplankton growth. In order to build their silica frustules, diatoms require a sufficient amount of silicate in the system to complete their cell cycle (Claquin et al., 2002). Si limitation affects species composition and cells size (Rousseau et al., 2002; Martin-Jézéquel et al., 2003), and a decline in Si availability is shown to select for diatoms with a low Si-requirement (Bakker et al., 1994).
At the start of the second bioassay, colonies of the haptophyte *Phaeocystis globosa* had become very abundant, whilst phosphate concentrations were still very low. Possibly the development of this *Phaeocystis* bloom was supported by a P-flux from labile P in the sediment into the water column. Alternatively, *P. globosa* might have been able to utilize DOP as its P resource (Schoemann et al., 2005) or made use of a phosphate reserves stored in the polysaccharide matrix of the colonies (Veldhuis et al., 1991; Schoemann et al., 2005; Beardall et al., 2008). When Si concentrations are high (>2 µmol L\(^{-1}\)), *P. globosa* will rarely dominate the plankton community (Breton et al. 2006; Peperzak et al. 1998), but as Si concentrations < 0.15 µmol L\(^{-1}\), this situation did not occur during B2. Although Si concentrations were not detectable at the start of B2 and B3, pennate diatoms species were found in the phytoplankton community; but remained a minor fraction of population. Bottom-dwelling pennate diatoms might have profited from the available Si-store in the sediment pore water (Rousseau et al., 2002). The addition of Si did not show an enhancement of planktonic diatom growth, indicating that a viable centric diatom population was lacking or that they were only present in very low numbers at the start of B2. This is in agreement with the observation that addition of Si did only stimulate the growth of pennate diatoms.

At the start of the fourth bioassay, the ambient nutrient concentrations had changed with Si reaching values of 0.07 µmol L\(^{-1}\), DIP of more than 1.3 µmol L\(^{-1}\), and DOP approximately 7-fold higher than the DIP concentrations. The rise of available Si concentration coincided with a development of diatoms. All this suggest that nutrient limitation was less intense than in previous bioassays. The increase in DIP coincided with a decrease in the percentage of cells showing a high APA.

Nevertheless, all cells still showed ELF fluorescence, apart from a small fraction of pennate diatoms. \(F_v/F_m\) also showed a significant increase upon addition of the limiting nutrient(s). Similarly, the C-incorporation rates were enhanced in all nutrient additions scenarios, with the +NPSi addition given the highest rates of C-fixation. This suggests that the diatoms in the phytoplankton community were still Si-limited. But this can only be part of the explanation as also the +NP addition showed an increase in \(F_v/F_m\), and this addition showed a similar response with regard to the increase in Chla as the other additions. Hence, the most likely explanation for these results seemed to be that the increase in DIP and DOP was very recent and that the algae had not yet completely acclimated to the increase in P availability.

This phytoplankton succession in spring from diatoms to *P. globosa* is a common pattern observed in the North Sea coast and Wadden Sea (Egge and Aksnes, 1992; Cadee and Hegeman, 2002; Rousseau et al., 2002). The ability of *P. globosa* to form colonies allows them to escape grazing (Peperzak et al., 1998). The consequence of it is that a larger fraction of the primary production will enter the microbial foodweb though ciliates and other microzooplankton are considered as trophic intermediate between small preys and larger predators. Phytoplankton species composition will influence the composition of the grazer community, but vice versa, grazing by microzooplankton will also impact the phytoplankton community structure, especially during *Phaeocystis* blooms (Stelfox-Widdicombe et al., 2004; Loebl and Van Beusekom, 2008).
Diatoms and *Phaeocystis* usually co-exist during initial phase of the spring bloom, but the grazing activity on the two different phytoplankton groups are different. Large phytoplankton species such as diatoms are generally preyed upon by large grazers like copepods (Loebl and Van Beusekom, 2008). Solitary cells of *P. globosa* are eaten by smaller grazers, whilst the grazing impact on *P. globosa* colonies is low. However, as we filtered the field samples using a 100 µm mesh size sieve, we removed the large grazers (and possibly some of the *Phaeocystis* sp. colonies) but not the microzooplankton. Because *P. globosa* can reach high growth rates (Schoemann et al., 2005), it seems likely that grazing by microzooplankton caused the lower specific growth rates based on the rate of increase in the Chla-concentration in B2 to B4 (Egge and Aksnes, 1992; Schoemann et al., 2005).

5. Conclusion

During the spring bloom of 2010, bioassays and physiological indices consistently showed that phytoplankton growth in the Marsdiep tidal inlet was limited by P, with co-limitation of P and Si for diatom growth. This corroborates previous general findings on the nature of the limiting nutrient for this part of the Wadden Sea based on dissolved nutrient concentrations (e.g., Philippart et al., 2007; Loebl et al., 2009). Results further underline the importance of knowledge of nutrient affinities of algal species, of conditions of algal cells (e.g., depletion of reserves), of nutrient sources (e.g., P-fluxes from the sediment) and of selective grazing to fully understand phytoplankton succession in shallow coastal waters. If P concentrations in the Wadden Sea further decrease, an increase in the intensity and duration of P limitation is likely and a shift from larger to smaller organisms with a better affinity for phosphate possible. Such a change in the strength of P limitation will not only shape phytoplankton communities but will also have an impact on total primary production, transfer of energy and carbon to higher tropic levels, and ecosystem services such as fishery yields.

Acknowledgements

This project was funded by the Coast and Sea Program (ZKO) of the Netherlands Organization for Scientific Research (NWO) projects P-reduce (grant nº 839.08.340) and IN PLACE (grant nº 839.08.210).
Tables

Table 1. Timing and start of the bioassays

<table>
<thead>
<tr>
<th>Bioassay</th>
<th>Start</th>
<th>End</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>30th March 2010</td>
<td>5th April 2010</td>
</tr>
<tr>
<td>B2</td>
<td>10th April 2010</td>
<td>16th April 2010</td>
</tr>
<tr>
<td>B3</td>
<td>17th April 2010</td>
<td>23rd April 2010</td>
</tr>
<tr>
<td>B4</td>
<td>24th April 2010</td>
<td>30th April 2010</td>
</tr>
</tbody>
</table>

Table 2. Relationships between phytoplankton responses towards nutrient additions and limiting nutrients.

<table>
<thead>
<tr>
<th>IF</th>
<th>THEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response to nutrient</td>
<td>Limiting nutrient(s)</td>
</tr>
<tr>
<td>additions</td>
<td></td>
</tr>
<tr>
<td>(\uparrow)NP, (\uparrow)NSi and (\uparrow)NPSi</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>(\uparrow)NP, (\uparrow)PSi, and (\uparrow)NPSi</td>
<td>Phosphorus</td>
</tr>
<tr>
<td>(\uparrow)NSi, (\uparrow)PSi and (\uparrow)NPSi</td>
<td>Silicon</td>
</tr>
<tr>
<td>(\uparrow)NP and (\uparrow)NPSi</td>
<td>Phosphorus &amp; Nitrogen</td>
</tr>
<tr>
<td>(\uparrow)NSi and (\uparrow)NPSi</td>
<td>Nitrogen &amp; Silicon</td>
</tr>
<tr>
<td>(\uparrow)PSi and (\uparrow)NPSi</td>
<td>Phosphorus &amp; Silicon</td>
</tr>
</tbody>
</table>

Table 3. Rank, functional group (diatoms or flagellates), abundance (cells ml\(^{-1}\)) and contribution to total density (%) of most dominant phytoplankton species in the Marsdiep tidal inlet during the period at which the bioassays were performed (Philippart et al., unpublished)
Table 4. Net growth rate of the phytoplankton community in the bioassays (d^{-1}) based on changes in Chl{\text{a}} concentrations. The highest value within each bioassay is printed in bold, the closest values to the highest values are underlined (na: none available).

<table>
<thead>
<tr>
<th>Date</th>
<th>Rank</th>
<th>Species or taxonomic group</th>
<th>D/F</th>
<th>cells ml^{-1} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>31th of March</td>
<td>#1</td>
<td>Thalassiosiraeae (6-10 μm) diatom</td>
<td>3944</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>#2</td>
<td>Thalassiosiraeae (10-30 μm) diatom</td>
<td>2597</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>#3</td>
<td>Small colored flagellates (approx. 3 μm) flagellate</td>
<td>1539</td>
<td>13</td>
</tr>
<tr>
<td>6th of April</td>
<td>#1</td>
<td>Thalassiosiraeae (6-10 μm) diatom</td>
<td>2790</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>#2</td>
<td>Chaetoceros species (&lt;10 μm; colony cells)</td>
<td>2405</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>#3</td>
<td>Small colored flagellates (approx. 3 μm) flagellate</td>
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<td>10</td>
</tr>
<tr>
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<td>Phaeocystis globosa (colony cells) flagellate</td>
<td>3463</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>#2</td>
<td>Small colored flagellates (approx. 3 μm) flagellate</td>
<td>1347</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>#3</td>
<td>Small colorless flagellates (&lt; 6 μm) flagellate</td>
<td>1058</td>
<td>11</td>
</tr>
<tr>
<td>21st of April</td>
<td>#1</td>
<td>Phaeocystis globosa (colony cells) flagellate</td>
<td>6734</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>#2</td>
<td>Phaeocystis globosa (flagellate cells) flagellate</td>
<td>3752</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>#3</td>
<td>Chaetoceros species (&lt;10 μm; colony cells)</td>
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<td>10</td>
</tr>
<tr>
<td>29th of April</td>
<td>#1</td>
<td>Phaeocystis globosa (flagellate cells) flagellate</td>
<td>5099</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>#2</td>
<td>Small colored flagellates (approx. 3 μm) flagellate</td>
<td>3656</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>#3</td>
<td>Small colorless flagellates (&lt; 6 μm) flagellate</td>
<td>2501</td>
<td>11</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Bioassays</th>
<th>C</th>
<th>+NP</th>
<th>+NSi</th>
<th>+PSi</th>
<th>+NPSi</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>+0.15±0.088</td>
<td>+0.25±0.045</td>
<td>+0.28±0.077</td>
<td><strong>+0.67±0.081</strong></td>
<td>+0.64±0.063</td>
</tr>
<tr>
<td>B2</td>
<td>-0.06±0.022</td>
<td>+0.14±0.030</td>
<td>-0.027±0.035</td>
<td>+0.15±0.0094</td>
<td><strong>+0.20±0.011</strong></td>
</tr>
<tr>
<td>B3</td>
<td>-0.054±0.031</td>
<td>+0.22±0.019</td>
<td>-0.011±0.03</td>
<td>+0.21±0.030</td>
<td><strong>+0.31±0.022</strong></td>
</tr>
<tr>
<td>B4</td>
<td>-0.100±0.018</td>
<td><strong>+0.19±0.068</strong></td>
<td><em>na</em></td>
<td>+0.16±0.086</td>
<td><strong>+0.19±0.084</strong></td>
</tr>
</tbody>
</table>
Figures

Fig. 1. The location at the NIOZ sampling jetty station in the western part of the Dutch Wadden Sea.

Fig. 2. Concentrations of (A) Ammonium (NH$_4^+$), (B) NO$_x$ (nitrite (NO$_2^-$)+ nitrate (NO$_3^-$)), (C) dissolved inorganic phosphorus (DIP), (D) Si, (E) dissolved organic phosphorus (DOP) and (F) dissolved organic nitrogen (DON) at the beginning of the experiments during weekly bioassays from 30$^{\text{th}}$ March (B1) to 24$^{\text{th}}$ April (B4) 2010. Values give an average ± standard deviation ($n=2$).
Fig. 3. Nutrient ratios (A) DIN:DIP, (B) DIN:TDP, (C) TDP:TDP, (D) Si:DIP, (E) PON:POP, and (F) BAN:BAP on a logarithmic scale in the four bioassays at the start of the incubations. Values are average \( (n=2) \) and the bar indicates the upper bar of the standard deviation. Dashed line indicates the Redfield N:P ratio of 16 and the optimum Si:DIP ratio of 16.
Fig. 4. Averages and standard deviation ($n = 3$) of bulk Chla concentrations in C (control, no nutrient addition) and the different treatments +NP, +NSi, +PSi, +NPSi for the 4 different bioassays (B1, B2, B3, B4). Note the difference in scale for B1 compared to B2, B3 and B4.
Fig. 5. Carbon incorporation into POC (µg C L\(^{-1}\) h\(^{-1}\)) in the different nutrient treatments from the four bioassays (B1-B4) at t=0 and t=6 (C, +NP, +NSi, +PSi and +NPSi) (n = 2). n.a. = data not available (B4, +NSi treatment).
Fig. 6. Maximum PSII quantum efficiency ($F_v/F_m$) in C (control, no nutrient addition) and the different treatments +NP, +NSi, +PSi, +NPSi for the four different bioassays (B1, B2, B3, B4). Values are average ± standard deviation ($n = 3$).
Fig. 7. Percentage of cells showing ELF fluorescence: (A) in the pennates diatoms and (B) in cells in the aggregates in the different bioassays before (t=0) and after (t=6 days) incubation under different nutrient conditions, i.e. C (control, no nutrient addition), +NP, +NSi, +PSi and +NPSi. Three categories of ELF fluorescence were distinguished, i.e. high ELF (intense bright
green fluorescence), low ELF (pale green fluorescence), and no fluorescence at all. n.d. (none detected) pennate diatoms, (*NSi in B4) none available data.

Fig. 8. Selected pictures of ELF stained samples. Comparison of fluorescence microscopy images with a green excitation filters UV excitation. Images showing: red chlorophyll autofluorescence (left images) and green fluorescence indicating the alkaline phosphatase activity (right images) for *Mediopyxis helysia* (A & B), for pennate diatoms (C & D) and for aggregated cells and a chain-forming *Chaetoceros* species (E & F).