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

Juliette Ly [a], Catharina J.M. Philippart [b], Jacco C. Kromkamp [a,*]

[a] Department of Marine Microbiology, Royal Netherlands Institute for Sea Research, P.O. Box 140, 4400 AC Yerseke, The Netherlands
[b] Department of Marine Ecology, Royal Netherlands Institute for Sea Research, P.O. Box 59, 1790 AB Den Burg, The Netherlands

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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 is 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* (Chl*a*), 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 (F_v/F_m), in the expression of alkaline phosphatase activity (APA) measured with the fluorescent probe ELF-97, and in the ^{13}C 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.

Contents

* Corresponding author.
E-mail address: jacco.kromkamp@nioz.nl (J.C. Kromkamp).

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1. Introduction

The need for a better understanding of 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; Finkel et al., 2010; Malzahn et al., 2007). 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 (Beardall et al., 2001; Hecky and Kilham, 1988; Schindler, 2009), show that interpretation of the results obtained at small scales is 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* (Chl*a*) 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 Chl*a* 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 (Cadée and Hegeman, 2002; Loebl et al., 2009; Philippart et al., 2007). Although the results here consider the Marsdiep basin of the western Dutch Wadden Sea, many coastal systems have seen a decrease in nutrient loading as a result of changes in policy measures to combat eutrophication, and for this reasons the results applied here can probably serve as an example for other temperate coastal system which underwent similar reductions in nutrient loadings. These changes in absolute and relative nutrient loads coincided with major changes in phytoplankton community structure during the late 1970s and the late 1980s (Philippart et al., 2000) and were accompanied 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 then P-limited again thereafter (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 same 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 (Philippart et al., 2013).

Previous results on the nature and strength of nutrient limitation in the western Wadden Sea were all based on ambient nutrient concentrations, which are only weak indices of nutrient limitation because no information on uptake and mineralization is taken into account (Dodd et al., 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 performed bioassay experiments and physiological measurements and compare the results with conclusions drawn from ambient nutrient concentrations and ratios.

2. Material and methods

2.1. Sampling site and procedure

Water samples have been collected using a bucket at weekly intervals at high tide from the NIOZ-Jetty (53°00′06″ N; 4°47′21″ E) from 30th March to 30th April 2010 (Table 1). The NIOZ-Jetty is located in the Marsdiep basin near to the inlet between the North Sea and the Wadden Sea (Fig. 1). The average depth of the Marsdiep basin is approximately 4.5 m (Ridderinkhof, 1988). 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-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 derived from the NIOZ-Jetty samples is also indicative for trends in the western Wadden Sea.

In order to make sure that light availability did not influence our interpretation of the bioassays we estimated the underwater light climate in the Marsdiep basin during the period of our bioassay. Because the light attenuation coefficients (K_d; m^{-1}) were not measured during this period, we estimated K_d from Secchi depth measurements using the following empirical relationship:

$$K_d = a \cdot \sqrt{(\text{Secchi depth})} + b,$$

where $a = 5.377\ (m^{-1})$ and $b = 2.07\ (m^{-1})$ are fit constants obtained from a regression analysis ($r^2 = 0.71$; $n = 116$) of date obtained from the western part of the optically similar Oosterschelde estuary (Malkin and Kromkamp, unpublished results). These estimates of K_d varied

Table 1
Timing and start of the bioassays.

Bioassay	Start	End
B1	30th March 2010	5th April 2010
B2	10th April 2010	16th April 2010
B3	17thApril 2010	23rd April 2010
B4	24th April 2010	30th April 2010

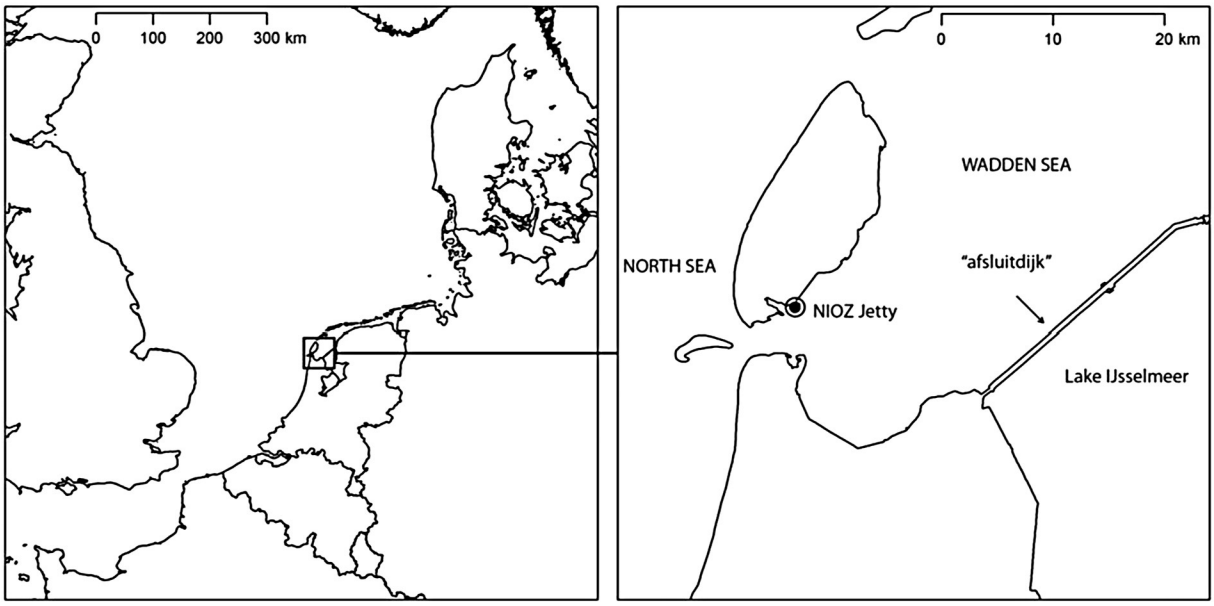

Fig. 1. The location at the NIOZ-Jetty sampling station in the western part of the Dutch Wadden Sea.

between 0.77 and 2.99 m^{-1} (median = 1.17 m^{-1}). In April, the median value of K_d was 0.91 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). Based upon these estimated PAR values, the average incident irradiance in the month of April was 380 μmol photons m^{-2} s^{-1}. Assuming that the photic depth equals the depth to which 1% of the surface irradiance penetrates and that the whole water column is mixed due to the tidal energy in the system, 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. 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 2010, the median water column irradiance equalled 96 μmol photons m^{-2} s^{-1}, which was very close to the irradiance value used (100 μmol photons m^{-2} s^{-1}) during the nutrient enrichment experiments.

2.2. Experimental design

Nutrient enrichment experiments with natural phytoplankton populations were performed on four different dates using a series of 8 liter polycarbonate bottles which were incubated under controlled light and temperature conditions for 6 days (Table 2). The water was filtered through a 100 μm net filter in order to minimize grazing by meso- and macrozooplankton on the phytoplankton during the bioassay incubation. The nutrient enriched bottles (see below) were incubated in the laboratory 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 (100 μmol L^{-1} NH_4NO_3 (final concentration); +P, inorganic phosphorus (10 μmol KH_2PO_4 L^{-1} (final concentration) and +Si (100 μmol $Na_2SiO_5{,}5H_2O$ L^{-1} (final concentration)). In total, five different nutrient addition treatments were assigned, each in triplicate: C (control without any additions of nutrients); +NP; +NSi; +PSi; +NPSi, in which all nutrients were added together (Table 2). In experiment B4 (see Table 1), due to an error in the laboratory, no +NSi treatments were added to the experimental design.

Table 2
Summary of the possible responses which might be observed in the bioassays.

Response to nutrient additions	Limiting nutrient(s)
NP, NSi and NPSi	Nitrogen
NP, PSi, and NPSi	Phosphorus
NSi, PSi and NPSi	Silicon
NP and NPSi	Phosphorus & nitrogen
NSi and NPSi	Nitrogen & silicon
PSi and NPSi	Phosphorus & silicon

2.3. Nutrient concentrations

Samples for dissolved nutrients (ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), phosphate (DIP) and silicate (Si)) in the water used for the bioassays were filtered through disposable filters (0.2 μm) and analyzed using an QuAAtro autoanalyser, with segmented flow analysis (Bran + Luebbe, Germany) according to the manufacturer's instruction (Hydes D 2010). Dissolved inorganic nitrogen (DIN) was calculated as the sum of ammonium, nitrate and nitrite. Total nitrogen (TN) and total phosphorus (TP) were measured 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 using Carlo Erba elemental analyzer (EA) coupled online to a Finnigan Delta S isotope ratio mass spectrometer (IRMS). PON and PP measurements were analyzed on the particulate material that was retained following filtration through Whatman GF/F glass-fiber filters (Whatman GF/F).

Total dissolved nitrogen (TDN) and total dissolved phosphorus (TDP) were estimated from the above measurements as TDN = TN − PON and TDP = TP − PP. Concentrations of dissolved organic nitrogen (DON) and dissolved organic phosphorus (DOP) were then calculated as DON = TDN − DIN and DOP = TDP − DIP. Several dissolved nutrient ratios were then calculated (DIN:DIP; DIN:TDP; TDN:TDP; Si:DIP, all by moles), in addition the biological available nitrogen (BAN) to phosphorus (BAP) ratio where BAN: BAP = ([DON + DIN] / [DOP + DIP]).

2.4. Chlorophyll-a

For each chlorophyll-*a* (Chl*a*) sample, a volume of 50 to 100 mL was filtered through a 47 mm Whatman GF/F filters. The filters were immediately placed in a glass container with 10 mL of 90% acetone and stored in the freezer (−20 °C) for at least 24 h, after which the Chl*a* 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 Chl*a*. Net specific phytoplankton growth rates (μ, day^{-1}) within the bioassay bottles were calculated from the observed daily changes in Chl*a* over time from day 1 to day 6 (t, days) as: $\mu = 1 / t \times \ln(Chla_{(t)} / Chla_{(0)})$.

2.6. Species counts

As part of the long-term field observation program from the NIOZ-Jetty, phytoplankton species composition was determined from surface water samples at a sampling frequency from once a month in mid-winter and twice per week during spring blooms. Phytoplankton samples were preserved with Lugol's iodine and the preserved 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 coarser taxonomic and size groups (e.g. small flagellates).

2.7. Photosynthetic carbon incorporation using ^{13}C uptake into particulate organic carbon (POC)

Water samples of 1–2 l from the bioassays were taken at the beginning (day 0) and end (day 6) of each nutrient addition treatment for ^{13}C stable isotope incubation. These water samples were enriched with ^{13}C-$NaHCO_3$ (99% ^{13}C; Cambridge Isotope Laboratories, Inc.) for 2 h with a concentration of 4% of the ambient dissolved inorganic carbon (DIC) concentration. Samples for the ^{13}C incubation were filtered through precombusted glass-fiber filters (Whatman GF/F) and later analyzed with a Carlo Erba elemental analyzer (EA) coupled in-line to a Finnigan Delta S isotope ratio mass spectrometer (IRMS). 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 fraction of ^{13}C (μg C L^{-1}) in POC is expressed as the ^{13}C fraction ($^{13}C / (^{12}C + {}^{13}C) = R / (R + 1)$ derived from the delta notation (Middelburg et al., 2000). The ^{13}C uptake of POC (μmol 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 (μmol C L^{-1} h^{-1}) was obtained by dividing the uptake by the incubation time (2 h). 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 measured daily on a water sample from the bioassay using a Water-PAM fluorometer equipped with red LEDs (Heinz Walz, Effeltrich, Germany). The duration of the saturating flash was 800 ms. 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 ($F_v/F_m = (F_m - F_0) / F_m$). Although this time might be too short to obtain full relaxation of non-photochemical quenching (NPQ), the little amount of NPQ present and induced at the moderate growth light intensity relaxed quickly allowing a short relaxation time in order to evaluate changes in the F_v/F_m.

2.9. Alkaline phosphatase activity (APA)

At the beginning and end of each nutrient enrichment experiment, samples for cell-specific detection of APA were measured using a molecular probe, ELF-97 (Endogenous Phosphatase Detection Kit; E6601, Molecular Probes, Invitrogen, California). The phytoplankton biomass in a volume of 100–150 mL was reduced to approx. 5–10 mL 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. The concentrations were approximate because we were only interested in the proportion of ELF-97 labeled cells and not in cell concentrations. 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%) were added, and the preserved samples were stored at 4 °C. ELF samples were analyzed within one month. 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 which made it nearly impossible to detect individual cells as a recognizable species, making microscopy observations on single algal cells difficult. As the bright green fluorescence of the ELF stain was clearly visible within these 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*a*, 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 the 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 at each bioassay

Before nutrients were added in each bioassay, significant differences in ambient nutrient concentrations were found between all bioassays (B1, B2, B3 and B4) (one-way ANOVA, $p < 0.05$) and these will be discussed here. At the start of the first three bioassays (B1, B2 and B3), the ambient DIP concentrations ranged from 0.04 to 0.05 μmol 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 μmol L^{-1}. The concentrations of Si were below the detection limit at the start of B2 and B3, and low (0.15 μmol L^{-1} for B1 and 0.07 μmol L^{-1} for B4) but not significantly different at the start of the other two bioassays. From B1 to B3, NH_4^+ concentrations decreased significantly from 0.91 to 0.26 μmol L^{-1}. In B4, NH_4^+ concentrations had increased again to 0.67 μmol L^{-1}. NO_x was the main contributor of the DIN pool with NO_3 contributing around 97% of total NO_x, e.g. NO_x concentrations were already 40 times higher than NH_4^+ in B1. Concentrations of NO_x decreased from 40 μmol L^{-1} at the start of B1 to 17.5 μmol L^{-1} at the start of B4.

Ambient DOP increased from 0.5 μmol L^{-1} in B1 to 1 and 2 μmol L^{-1} in B2 and B3, respectively. In B4, DOP levels showed the highest values

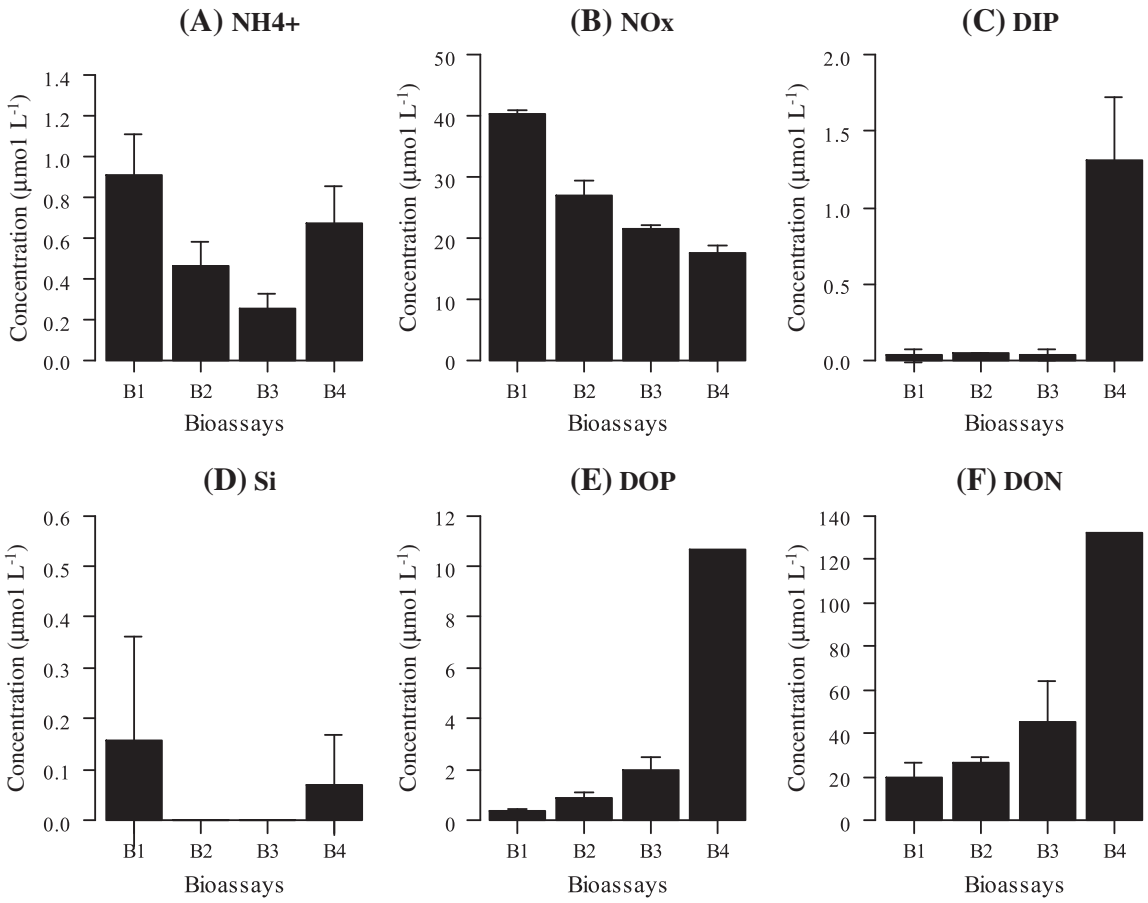

Fig. 2. Concentrations of (A) Ammonium (NH_4^+), (B) NO_x (nitrite (NO_2^-) + nitrate (NO_3^-)), (C) dissolved inorganic phosphorus (DIP), (D) Si, (E) dissolved organic phosphorus (DOP) and (F) dissolved organic nitrogen (DON) at the beginning of the experiments during weekly bioassays from 30th March (B1) to 24th April (B4) 2010. Values give an average ± standard deviation ($n = 2$).

of about 10 µmol L^{-1}. DON increased over the experimental period from 18 µmol L^{-1} in B1 to 40 µmol L^{-1} in B4. Significant differences in ambient nutrient ratios were found at the start of the four 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) at the start of the first three bioassays (B1–B3) and lower than 16 at the start of B4, which suggested P-limitation of the phytoplankton in the Marsdiep basin before 24 April and a relative N-shortage thereafter. The initial 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 experiment B1, all nutrient enrichment treatments resulted in an increase phytoplankton biomass during the day 6 incubation (Fig. 4). However, 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 µg 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 µg 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 4). Chl*a* concentrations in the +NP and +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, $p = 1.75 \cdot 10^{-3}$).

At the start of the incubation of B2, the initial water column 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 experiment B3, the initial water column 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 experiment B4, the responses of the phytoplankton community after the addition of the different nutrients were more difficult to interpret than for the other bioassays as we do not have information on the effects of +NSi addition. The initial ambient DIN concentrations,

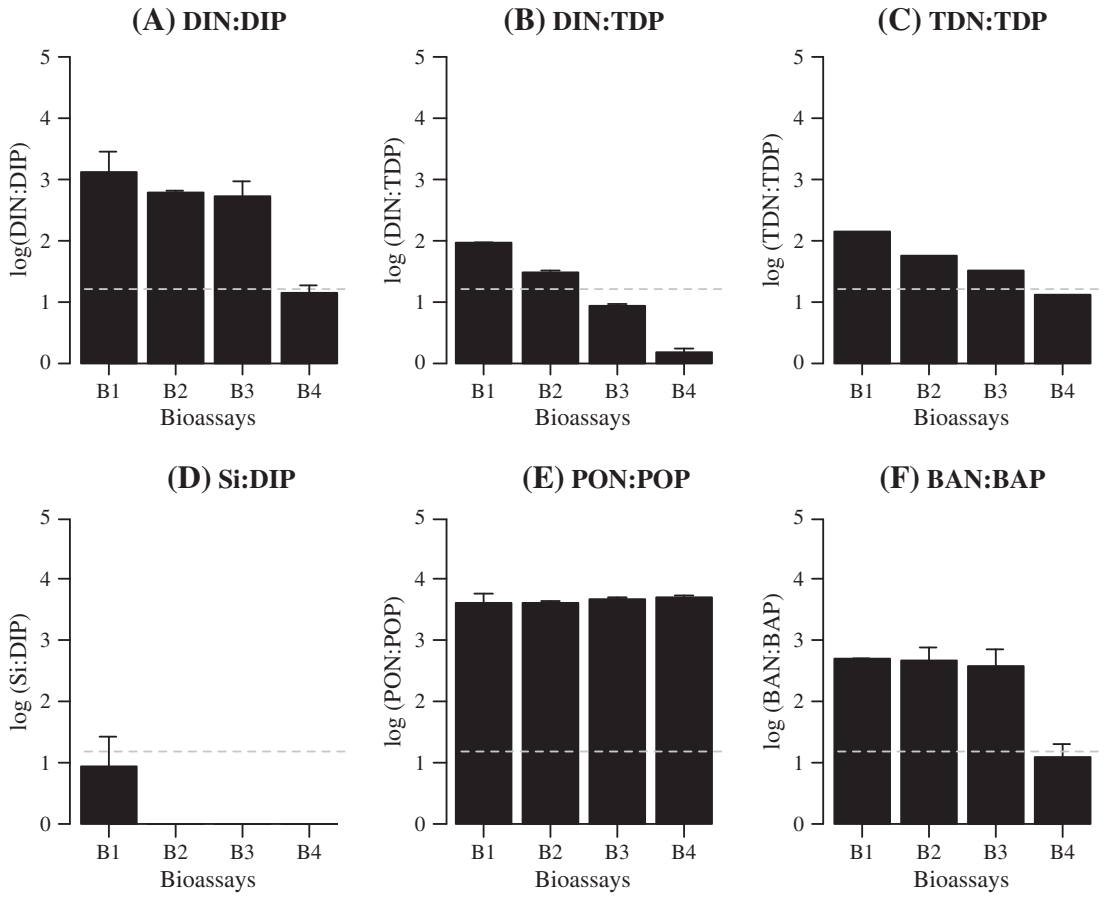

Fig. 3. Nutrient ratios (A) DIN:DIP, (B) DIN:TDP, (C) TDP:TDP, (D) Si:DIP, (E) PON:POP, and (F) BAN:BAP on a logarithmic scale in the four bioassays at the start of the incubations. Values are average ($n = 2$) and the bar indicates the upper bar of the standard deviation. Dashed line indicates the Redfield N:P ratio of 16 and the optimum Si:DIP ratio of 16.

however, seem high enough (~20 μmol L^{-1}) not to limit phytoplankton growth. Compared to the previous bioassays, the overall increase in Chl*a* was very limited with the 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 experiments B2, B3 and B4, the label incorporation showed a similar pattern as the changes in the Chl*a* concentrations, 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.

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).

Date	Rank	Species or taxonomic group	D/F	Cells mL^{-1}	(%)
31th of March	#1	Thalassiosiraceae (6–10 μm)	Diatom	3944	35
	#2	Thalassiosiraceae (10–30 μm)	Diatom	2597	23
	#3	Small colored flagellates (approx. 3 μm)	Flagellate	1539	13
6th of April	#1	Thalassiosiraceae (6–10 μm)	Diatom	2790	25
	#2	*Chaetoceros* species (<10 μm; colony cells)	Diatom	2405	22
	#3	Small colored flagellates (approx. 3 μm)	Flagellate	1154	10
14th of April	#1	*Phaeocystis globosa* (colony cells)	Flagellate	3463	37
	#2	Small colored flagellates (approx. 3 pm)	Flagellate	1347	14
	#3	Small colorless flagellates (<6 μm)	Flagellate	1058	11
21st of April	#1	*Phaeocystis globosa* (colony cells)	Flagellate	6734	31
	#2	*Phaeocystis globosa* (flagellate cells)	Flagellate	3752	17
	#3	*Chaetoceros* species (<10 μm; colony cells)	Diatom	2116	10
29th of April	#1	*Phaeocystis globosa* (flagellate cells)	Flagellate	5099	22
	#2	Small colored flagellates (approx. 3 μm)	Flagellate	3656	16
	#3	Small colorless flagellates (<6 μm)	Flagellate	2501	11

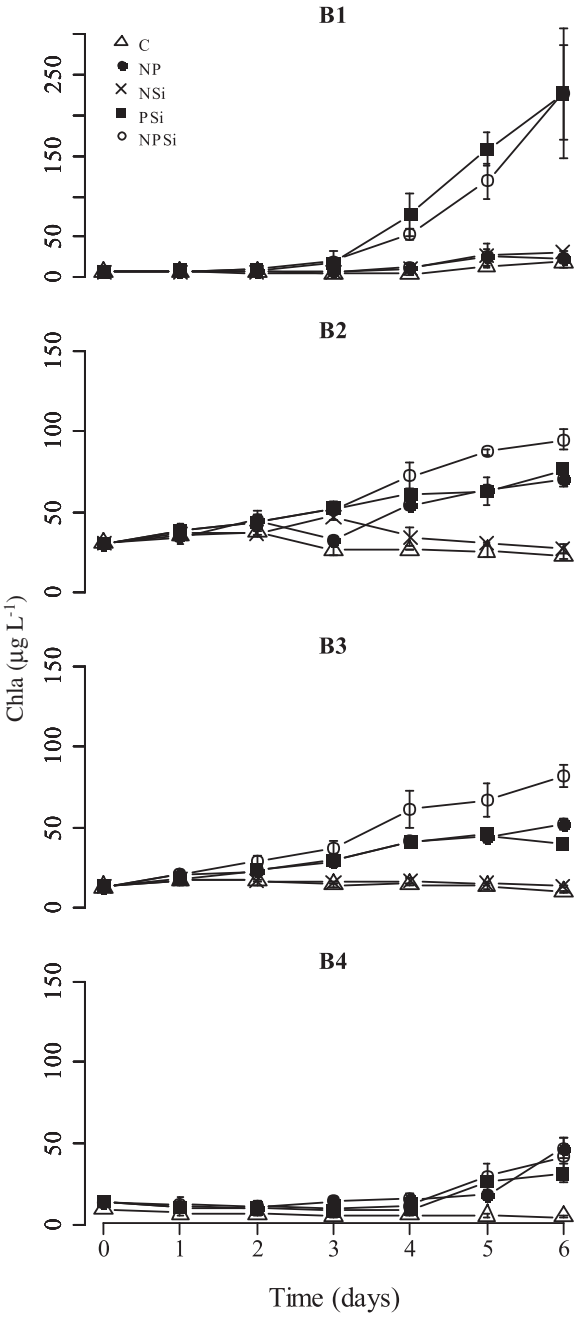

Fig. 4. Averages and standard deviation ($n = 3$) of bulk Chl*a* concentrations in C (control, no nutrient addition) and the different treatments +NP, +NSi, +PSi, +NPSi for the 4 different bioassays (B1, B2, B3, B4). Note the difference in scale for B1 compared to B2, B3 and B4.

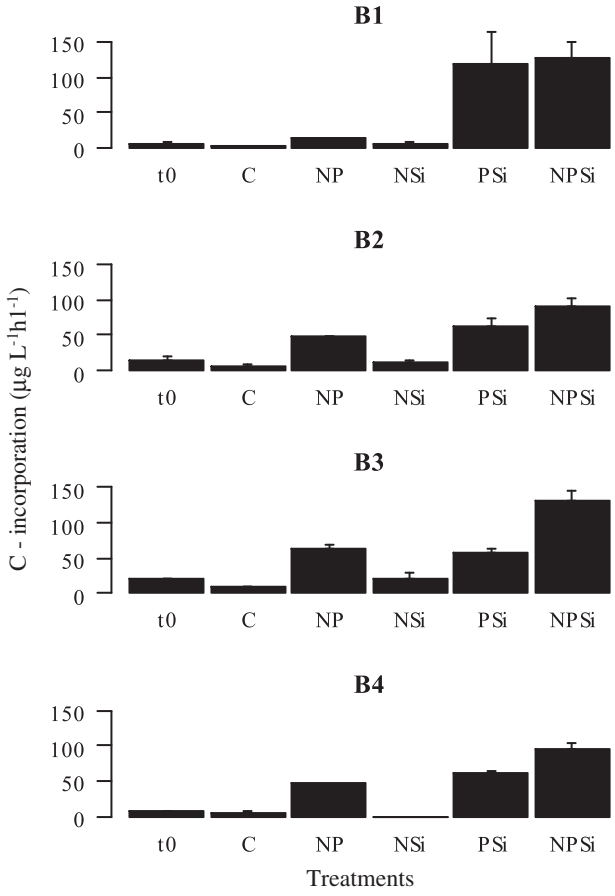

Fig. 5. Carbon incorporation into POC (µg C L^{-1} h^{-1}) in the different nutrient treatments from the four bioassays (B1-B4) at t = 0 and t = 6 (C, +NP, +NSi, +PSi and +NPSi) ($n = 2$). n.a. = data not available (B4, +NSi treatment).

3.4. Physiological properties: fluorescence and alkaline phosphatase activity

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, highly significant 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, two dominant algal groups could be distinguished by means of the epifluorescence microscopy, i.e. *Thalassiosiraceae* and *P. 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

Table 4
Net growth rate of the phytoplankton community in the bioassays (d^{-1}) based on changes in Chl*a* concentrations. The highest value within each bioassay is printed in bold, the closest values to the highest values are underlined (*na*: none available).

Bioassays	C	+NP	+NSi	+PSi	+NPSi
B1	+0.15 ± 0.088	+0.25 ± 0.045	+0.28 ± 0.077	**+0.67 ± 0.081**	+0.64 ± 0.063
B2	−0.06 ± 0.0.22	+0.14 ± 0.030	−0.027 ± 0.035	+0.15 ± 0.0094	**+0.20 ± 0.011**
B3	−0.054 ± 0.031	+0.22 ± 0.019	−0.011 ± 0.03	+0.21 ± 0.030	**+0.31 ± 0.022**
B4	−0.100 ± 0.018	**+0.19 ± 0.068**	*na*	+0.16 ± 0.086	**+0.19 ± 0.084**

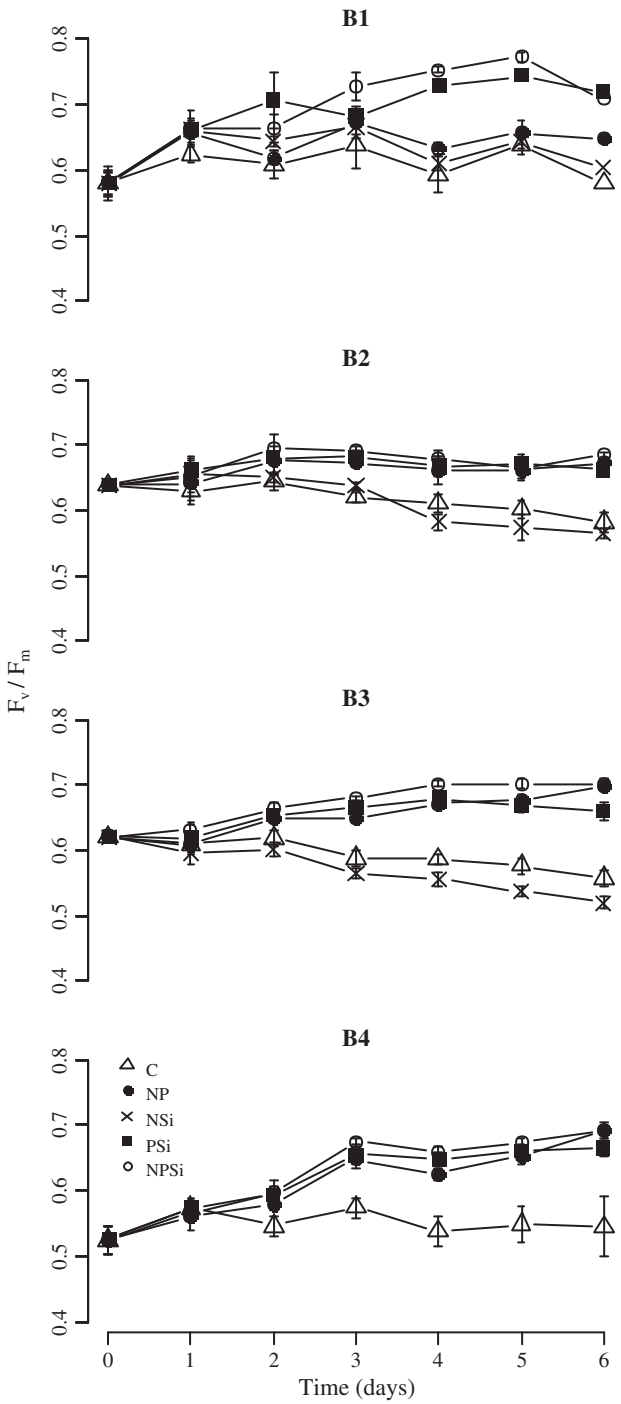

Fig. 6. Maximum PSII quantum efficiency (F_v/F_m) in C (control, no nutrient addition) and the different treatments +NP, +NSi, +PSi, +NPSi for the four different bioassays (B1, B2, B3, B4). Values are average ± standard deviation ($n = 3$).

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. Only for some cases, aggregates without any APA 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 (Figs. 7A and B8C and D). APA 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 (+NSi). At the start of B3, 100% of the pennate diatoms showed a high APA, whilst only 60% of the pennate diatoms were 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, 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 be a potential limiting factor for pelagic primary production (Cloern, 1999; Colijn and Cadée, 2003; Heip et al., 1995; Tillmann et al., 2000). For a correct interpretation of our results, we should know whether light limitation may have played a role in our interpretation of nutrient enrichment experiments 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 nutrient 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 to 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_I) and nutrient (K_X) resources. Both calculations (Colijn and Cadée, 2003; Loebl et al., 2009) used the same values for K_I (2.4 mol photons m^{-2} d^{-1}) and for K_N (1.5 μmol L^{-1}), taken from Cloern (1999). With regard to nutrient limitation, however, Loebl et al. (2009) additionally took the possibility of phosphate 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 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 mol photons m^{-2} d^{-1}, which is larger than

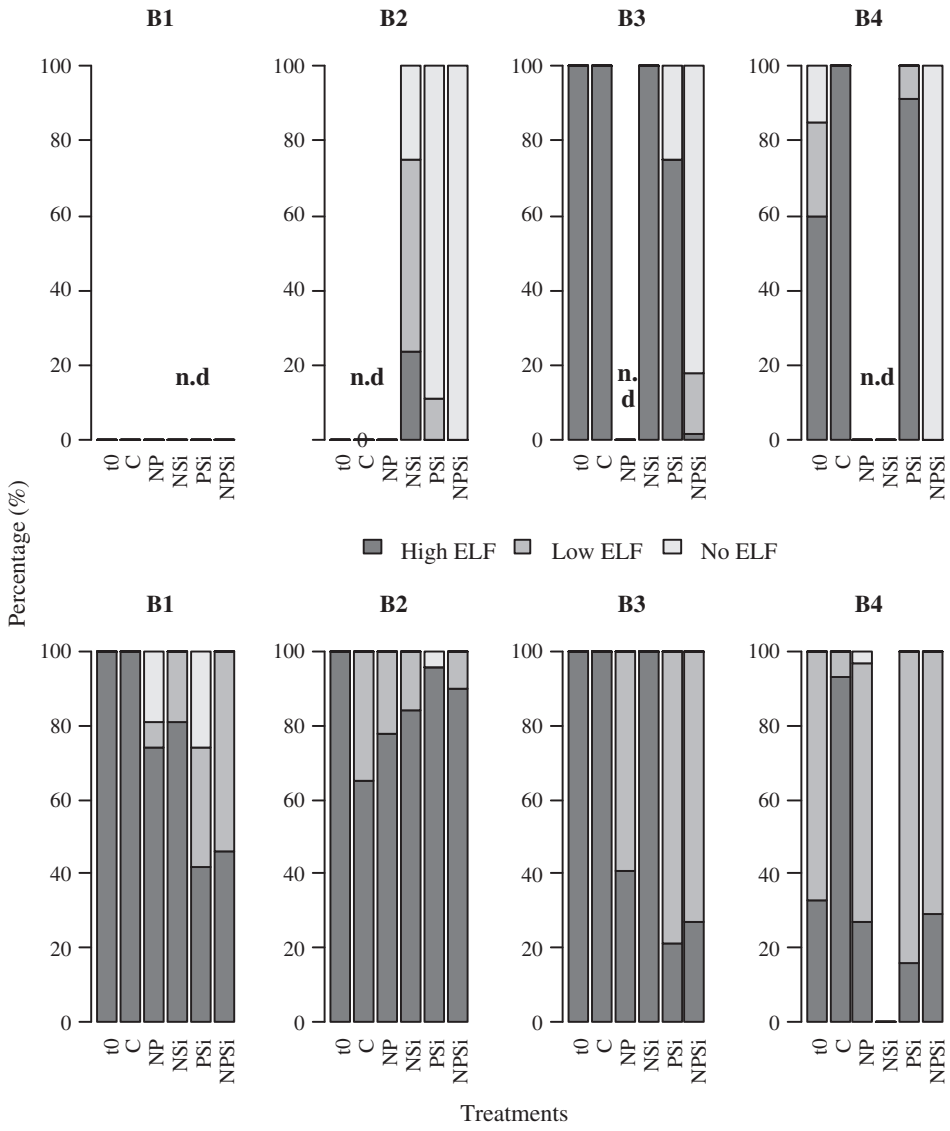

Fig. 7. Percentage of cells showing ELF fluorescence: (A) in the pennate diatoms and (B) in cells in the aggregates in the different bioassays before (t = 0) and after (t = 6 days) incubation under different nutrient conditions, i.e. C (control, no nutrient addition), +NP, +NSi, +PSi and +NPSi. Three 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) no available data.

a K_I of 2.4 mol photons m^{-2} d^{-1} 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_I (Cloern, 1999) strongly suggest that nutrients and not light were 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*a* 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*a* and carbon, then Chl*a*-change based growth rates are overestimating actual growth rates. In spite of the fact that we measured POC and Chl*a*, we cannot determine the Chl*a*: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 (Kruskopf and Flynn, 2005; Parkhill et al., 2001), the F_v/F_m ratio has proven to be a trustworthy indicator of nutrient limitation in other cases (Beardall et al., 2001; Flameling and Kromkamp, 1998; Kolber et al., 1988; Lippemeier et al., 1999). 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.

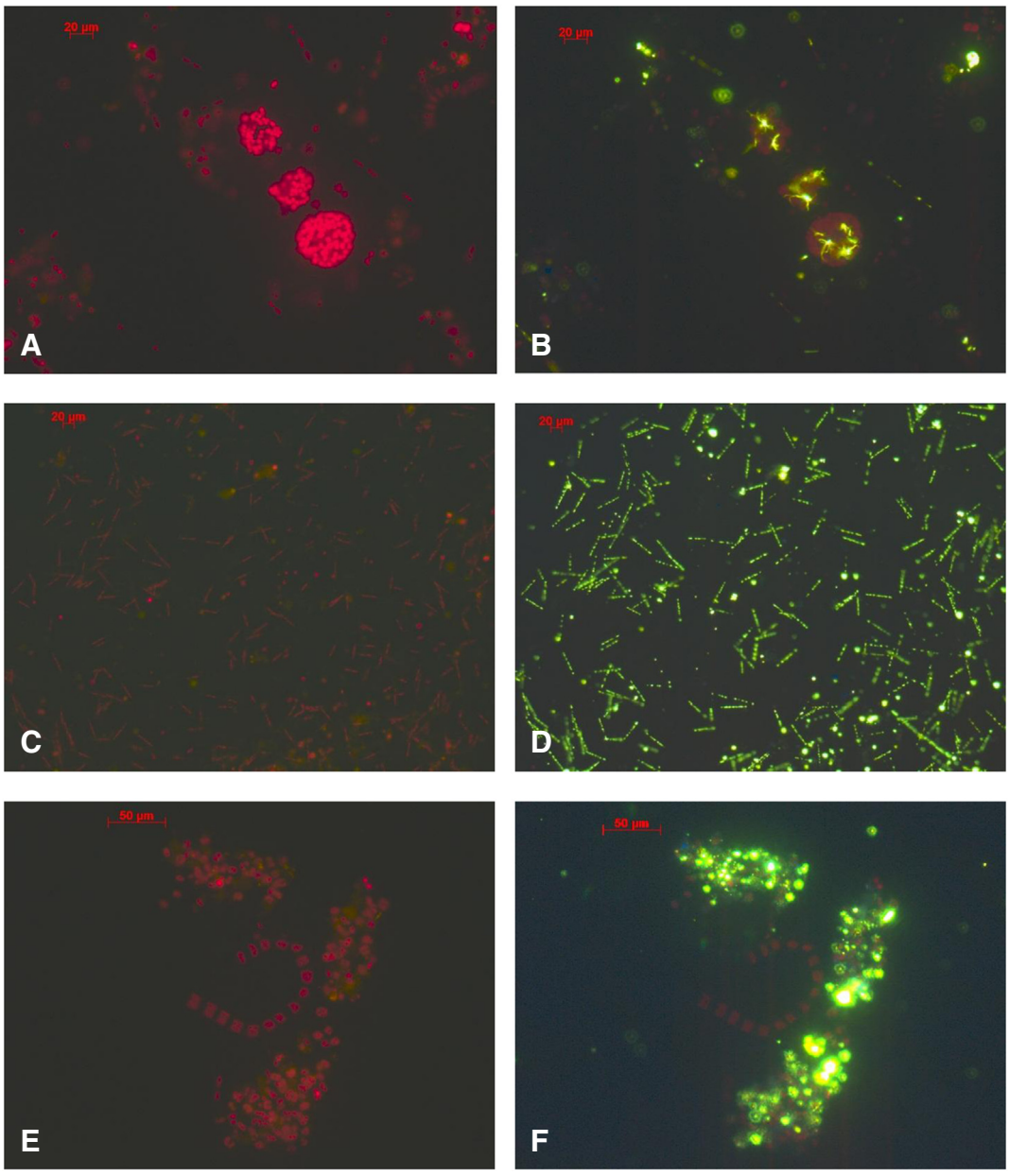

Fig. 8. Selected pictures of ELF stained samples. Images showing: red chlorophyll autofluorescence (left images) and green fluorescence indicating the alkaline phosphatase activity (right images) for *Mediopyxis helysia* (A & B), for pennate diatoms (C & D) and for aggregated cells and a chain-forming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

Our results for B4, where Si limitation appeared to have lowered F_v/F_m, are 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 not a 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 (Duhamel et al., 2010; Dyhrman and Palenik, 2001; González-Gil et al., 1998; Rengefors et al., 2003). In marine ecosystems, DOP is often present in higher concentrations than DIP (Dyhrman et al., 2007). APA is generally considered to be able to hydrolyze 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 (Lomas et al., 2004; Ranhofer et al., 2009; Rouzic and Bertru, 1997). 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 Chl*a*, F_v/F_m, 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 AP life time varies between functional groups, and that algal species or even cells may differ in their APA regulatory response.

Comparison of the four bioassay results with ambient nutrient concentrations revealed that the dissolved nutrient ratios did indeed 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 suggest 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 or DON 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 Section 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 Chl*a* concentrations, C-incorporation and maximum photosynthesis quantum yields (F_v/F_m) was 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 cell size (Martin-Jézéquel et al., 2003; Rousseau et al., 2002), 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 *P. 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 (Beardall et al., 2008; Schoemann et al., 2005; Veldhuis et al., 1991). 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 Chl*a* 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 (Cadée and Hegeman, 2002; Egge and Aksnes, 1992; 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 (Loebl and Van Beusekom, 2008; Stelfox-Widdicombe et al., 2004).

Diatoms and *Phaeocystis* usually co-exist during the initial phase of the spring bloom, but the grazing activity on the two different phytoplankton groups is 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 possible that grazing by microzooplankton caused the lower specific growth rates based on the rate of increase in the Chl*a*-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., Loebl et al., 2009; Philippart et al., 2007). 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 (Philippart and Epping, 2010).

Acknowledgments

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