Microalgal primary producers and their limiting resources
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CHAPTER 1

Introduction
General background

Worldwide, coastal ecosystems and estuaries are facing increased anthropogenic pressure as more and more people live near the coastline. More than 40% of the world population is currently living within 100 km from the coastline (Martínez et al., 2007). Rivers bring nutrients and pollutants from their catchment area and concentrate them in the estuarine system. Estuaries are also important ecologically: they perform several ecosystem functions like providing nursery grounds for fish, sustaining shellfish fisheries industry, and estuaries are an important filter with respect to nutrient discharge into the coastal areas and oceans (Kaiser et al., 2005). Phytoplankton forms the basis of the aquatic food web. Through photosynthesis, phytoplankton produces new organic matter for their own growth as well as providing a food source for the higher trophic levels (Falkowski and Raven, 1997). In coastal systems with large intertidal flats or with a clear water column where light hits the bottom, microphytobenthos (MPB) is another important group of primary producers. There is often a positive linear relationship between MPB primary producers and macrozoobenthos density in intertidal sediments (Herman et al., 1999). In general, primary productivity is constraint by the physiology of the organisms caused on the one hand by limiting resources (i.e. bottom-up control), but on the other hand top-down control (grazing) also controls primary production and the standing stock of phytoplankton or MPB.

Bottom-up control of microalgal primary producers

Nutrient limitation

Photoautotrophic microorganisms require light, macro-nutrients (carbon (C) nitrogen (N), phosphorus (P), and in the case of diatoms, silicon (Si), and micro-nutrients (trace elements and vitamins)) for growth. Dissolved nutrient concentrations fluctuate in aquatic environments and their availabilities are affected by seasonal change in biotic and abiotic factors (see paragraph on seasonality in phytoplankton).

Phytoplankton species are characterized by their chemical composition, size and affinity for the diverse nutrients. The phytoplankton elemental stoichiometry was described by Redfield et al. (1958), who found that below the ocean thermocline the elemental composition of the organic matter has a ratio of C:N:P = 106:16:1, and that this ratio, which has become known as the Redfield ratio, was similar to the values obtained in other field studies. However, it has become clear that the Redfield ratio is less fixed than previously thought. The nutrient stoichiometry ratio can vary considerably amongst microalgae and between different environmental conditions (Geider and Roche, 2002). For example, nutrient limitation can cause a deviation of the nutrient stoichiometry from the Redfield ratio, and often this variation can be described according to the formulation of Droop (1983): $\mu = \mu_{\text{max}} (1 - Q_{\text{min},i}/Q_i)$ where $\mu_{\text{max}}$ is the maximum growth rate when $Q$ become infinite and $Q_{\text{min}}$ is the minimum quota of nutrient $i$ observed when $\mu = 0$. The empirical Droop equation shows that the theoretical internal stores of a nutrient can vary between a minimum and a maximum concentration, although luxury uptake,
which takes place when a limiting nutrient is suddenly supplied in excess, can result in an increase of the cell’s storage capacity. In the mixed layer of the water column the nutrient stoichiometry is variable, suggesting that factors such as the availability of light, temperature, nutrient and carbon dioxide can simultaneously affect the nutrient stoichiometry of the cells (De Baar, 1994).

Liebig’s law of the minimum, which is widely used in oceanography and phytoplankton ecology, from a single species to the community level (De Baar, 1994; Danger et al., 2008), states that control of the biomass accrual is determined by a single limiting resource (Liebig, 1842). Hence, macronutrients (N, P and Si for diatoms species) may impair the growth and physiology of photoautotrophic organisms. Liebig’s law of the minimum has been derived from agriculture, and is basically meant to control the maximum biomass (yield) which can be obtained from a limiting nutrient. Co-limitation by different resources can also disrupt cell growth (Arrigo, 2004), but usually indicates that one group in the phytoplankton community lacks of nutrient A, whereas the other population lacks of nutrient B. However, a restricted availability of a nutrient can also disrupt synthesis of components in the cell and decrease growth rates (Hansell and Carlson, 2002).

Seasonality in phytoplankton

Microalgae require at least two major resources in order to form a bloom namely: light and nutrients. Two main peaks of phytoplankton biomass occur in temperate marine systems (Winder and Cloern, 2010).

Winter phytoplankton should be able to grow under low light and temperature (Zingone et al., 2010). Several species can develop resting stages (e.g. spores) that help to survive the winter and return to an active vegetative stage when conditions become more favorable. During the winter period, the mixed layer is deeper than the critical depth, and, hence, the net daily production in the mixed layer becomes insufficient to sustain growth of phytoplankton. In winter, growth of phytoplankton depends on physical factors rather than on grazing or supply of nutrients. Physical and meteorological conditions during winter such as strong winds will cause mixing of the water column and there will be no stratification (thermocline). Although phytoplankton is present during winter and nutrients are distributed evenly in the water column, photosynthesis is hampered by the short day length and the low light intensity. Due to remineralization in winter and the low growth of phytoplankton, high nutrient concentrations are found in the water column before the spring bloom starts. The increase of phytoplankton biomass coincides with the available resources (light and nutrients) present at the water surface that trigger the onset of phytoplankton growth and result in the spring bloom. The spring bloom of phytoplankton is one of the most conspicuous seasonal features in pelagic systems. According to Sverdrup’s hypothesis, the spring phytoplankton bloom is initiated when the mixed layer depth is shallower than the critical depth (water depth when integrated daily photosynthetic carbon assimilation is compensated by the integrated daily respiratory carbon losses) in stratified systems, the net primary production (total photosynthetic carbon assimilation – respiration carbon losses) becomes positive (Sverdrup, 1953). In systems without stratification, the
phytoplankton bloom starts when respiratory losses are smaller than gross photosynthesis (defined as the light dependent rate of electron flow from the water to the terminal electron acceptors in the absence of any respiratory losses) due to an increase of day length, higher incident irradiance, and a higher transparency of the water column because of less strong winds. Consequently, the phytoplankton biomass accumulates at the surface or in the mixed layer and conditions (light and nutrients) allow phytoplankton growth. Alternative, to the Sverdrup model, the dilution-recoupling hypothesis suggests that the onset of phytoplankton bloom in spring is caused by a dilution of grazers in deeper mixing layers during winter rather than from nutrient supply (Behrenfeld, 2010). Grazing pressure by zooplankton or depletion of nutrients may cause the breakdown of the spring bloom.

In late summer and early autumn, a second bloom may occur when regeneration processes result in increasing nutrient concentrations in the water column. The autumn phytoplankton bloom is usually less intense than the spring bloom, because light limits phytoplankton growth. The mixed layer migrates to greater depth. As a result, the nutrient concentrations become available at greater depth. In coastal areas, the changes in phytoplankton composition and biomass are tuned to the season but may also be affected by local conditions, such as point sources for nutrient loadings. It is the interplay of light, nutrient availability, physical conditions and biological interactions in the water column that leads to the complex seasonal phytoplankton dynamics (Winder and Cloern, 2010).

**Seasonality in microphytobenthos**

Unicellular benthic primary producers, MPB, can be important primary producers in coastal systems. In large tidal flat ecosystems, MPB can be responsible for up to 50% of the total primary production of the whole estuary (Underwood and Kromkamp, 1999). Like in the pelagic system, a myriad of physical and biogeochemical factors cause fluctuations in the light intensity, temperature, and nutrient concentrations. The sediment grain size is one of the factors that determine MPB biomass and its spatial distribution (Underwood, 2010). Across different ecosystems, MPB seasonality can vary due to the sediment type and available resources (Billerbeck et al., 2007). Some estuarine ecosystems display seasonal MPB patterns between spring and summer due to high light availability and high temperature (De Jonge and van Beusekom, 1995; Ubertini et al., 2012), while other estuaries show no apparent MPB seasonality (Thornton et al., 2002). Only when grazers are present or when bioturbation by benthic macrofauna takes place, the sediment may be disrupted the integrity of the MPB biofilm.

**Tidal basin ecosystem**

**Wadden Sea ecology**

The Wadden Sea is a shallow coastal area bordering the North Sea of the Netherlands, Germany and Denmark and is an important international nature reserve (Lotze et al., 2005). The Wadden Sea is the largest coastal area in northern Europe with extended tidal flats (450 km),
stretching from Den Helder in the Netherlands to Esbjerg in Denmark. In 2009, the Dutch and German Wadden Sea entered the UNESCO World Heritage list due to the unique biodiversity of migratory birds, important benthos stocks, extensive seagrass meadows, and the important habitat for fish and seals. This thesis focuses on the westernmost part of the Dutch Wadden Sea, the Marsdiep basin. The Marsdiep basin is a shallow system of barrier islands and tidal inlets. The main sources of nutrients in this area are from the river Rhine and the North Sea, as well as from irregular discharges of freshwater from Lake IJsselmeer (fed by the river IJssel, a branch of the river Rhine) (Van Raaphorst and De Jonge, 2004). Regular tidal exchanges and sediment transport from the North Sea are entering the Marsdiep basin through the Texel inlet (Postma, 1981; Van Heteren et al., 2006). As a consequence, the Wadden Sea is a dynamic coastal ecosystem that is regularly receiving riverine and North Sea inputs.

**Eutrophication**

Eutrophication is defined as an increase of nutrient loadings to an ecosystem (Nixon, 1995). In order to manage ecosystems perturbed by anthropogenic nutrients, strategies to decrease the P- and N loadings are necessary to alleviate symptoms of eutrophication (Howarth et al., 2011). Phytoplankton is at the base of the food web. Long-term monitoring programs have shown that phytoplankton growth is controlled by nutrient availability in the water column. Nutrient concentrations may be elevated due to external loads and phytoplankton responds directly to such increase in nutrient concentration. Phytoplankton is sensitive to fluctuations in the nutrient concentrations (Paerl et al., 2003). According to a long-term dataset of almost 30 years (1974-2003) of nutrient concentrations and composition of phytoplankton, macrozoobenthos and estuarine birds (Cadée and Hegeman, 2002; Philippart et al., 2007), three distinct periods were identified. In the first period, soluble reactive phosphorus (SRP) concentration and load increased until the mid-1980s and then a decline was observed during the second period around the 1990s. Si concentrations increased during both periods. Then, after the 1990s was identified as the third period that was characterized by low concentrations of SRP and high dissolved inorganic nitrogen (DIN):SRP and silicate (Si):SRP ratios, compared to the two previous two periods. Nitrogen concentrations did not decrease significantly during these three periods (Philippart et al., 2007).

In order to increase the knowledge of nutrient concentration changes since 2003, data were retrieved from the international institute for coastal and marine management, Rijkswaterstaat (RWS) (Fig. 1). Data of DIN (sum of nitrate, nitrite and ammonium), SRP and Si were extracted from station Noordwijk, located 2 km out of the coast (52° 15’10.29’’N, 4° 24’19.88’’), and under the influence of the river Rhine. Starting from the 1990s, molar DIN:SRP and Si:SRP ratios increased and were above the Redfield ratio of 16, indicating a potential P limitation. Since 1995, the annual median values from the DIN:SRP ratio did not show a decline. The pattern of molar Si:SRP ratios are more complex: between 1990 and 1995, this ratio increased and then appeared to reach a constant value, as was the case with the DIN:SRP ratio. That ratio was just below the Redfield ratio of 16, suggesting a near balance in the Si and SRP demands. However, the molar Si:SRP ratio jumped to values >16 in 1998 and varied slightly...
from year to year, until 2010, when the ratio decreased. The data thus suggest that, apart from a recent change in Si concentration, the Si:SRP ratios were close to the Redfield ratio in the late 1990s, early 2000s. As shown for the Noordwijk station data, the decrease of P load from river discharge into the Wadden Sea led to a decrease of P availability in the system. Based on monitoring data, P had been identified as the most likely limiting nutrient for phytoplankton (Si is only limiting for diatoms) (Philippart et al., 2007).
Figure 1. Soluble reactive phosphorus (SRP), silicate (Si) and molar DIN:SRP and Si:SRP ratios from 1975 to 2012 at station Noordwijk (2 km off the coast). Data were retrieved from the database of Rijkswaterstaat. Dashed lines represented DIN:SRP Redfield ratio 16 and Si:SRP optimum ratio 16.
Methodology

Several types of measurements and experiments can be used to test nutrient limitation in aquatic systems (Beardall et al., 2001). The responses of phytoplankton to a variation in nutrient conditions are often measured as the change in chlorophyll-a (Chl$\alpha$) as a proxy of phytoplankton biomass. Chl$\alpha$ is a universal pigment found in all algae classes and easy to measure (Jeffrey et al., 1997). However, routine measurements of Chl$\alpha$ are not always suitable to estimate phytoplankton biomass for instance because changes in phytoplankton composition may obscure these measurements. The use of Chl$\alpha$ gives only “bulk” information on phytoplankton biomass whereas more insights can be obtained if also information is obtained on the changes in the different functional types or different species. The responses of phytoplankton communities and shifts in their taxonomic composition to changing environmental conditions need to be understood.

Therefore, the research described in this thesis used phospholipid fatty acid (PLFA) as a chemotaxonomic biomarker in combination with $^{13}$C stable isotope labeling to describe the changes in abundance and activity of specific groups of primary producers (Dijkman et al., 2009) (Fig. 2). PLFA constitutes a major part of the lipid pool and it is an important compound of living cells. It is an ubiquitous compound with short turnover time, and therefore PLFA provides a good indicator of living biomass (Boschker and Middelburg, 2002; Bianchi and Canuel, 2011). Application of PLFA as a biomarker has proven to be a useful indicator of changes in the composition of lower trophic levels (eukaryotic algae, bacteria, fungi and actinomycetes) and the physiological status of the cells because most PLFAs are synthesized de novo (Sargent, 1997; Müller-Navarra et al., 2000). The variability in the PLFA composition results from changes in the metabolism and in species composition that are caused by environmental parameters crucial for phytoplankton primary productivity such as light, temperature, and nutrient concentrations (Dalsgaard et al., 2003; Piepho et al., 2012). Overall, the use of PLFA has been successfully used for the interpretation of changes of specific groups of phytoplankton (Brett and Muller-Navarra, 1997; Middelburg et al., 2000; Dalsgaard et al., 2003; Dijkman and Kromkamp, 2006; Kürten et al., 2013).
Figure 2. Major biosynthetic fatty acid pathways in marine algae. Desaturase enzymes Δ12 and Δ15 are only found in primary producers. Examples of specific abundant PLFAs in diatoms, green algae or flagellates. Figure taken from (Bergé and Barnathan, 2005).

**Research hypotheses**

The main objective of this thesis was to improve our knowledge of the bottom-up control of pelagic and benthic primary producers. For this research, the Marsdiep tidal basin, western Dutch Wadden Sea, was chosen as the location of the investigation. The investigation addressed the major limiting resources (nutrients and light) of phytoplankton growth. According to long-term data series, it has been hypothesized that P is the most important limiting factor for phytoplankton growth following decrease in nutrient concentrations after de-eutrophication in the Marsdiep basin. The conclusion of P limitation was based on nutrient concentrations and ratios. However, few studies have in fact proven that P was the limiting resource for phytoplankton in the Marsdiep tidal basin or have investigated the effects of this limitation on the phytoplankton community. P limitation will select for phytoplankton species that have a high affinity for P and/or a low internal P storage capacity. Hence, it can be expected that this would lead to changes in the composition of the phytoplankton community. Because the Marsdiep basin is a shallow and dynamic tidal basin with large intertidal flats, it was expected that MPB plays a role in P release in the water column. This thesis was set out to confirm and prove P limitation in phytoplankton in the Marsdiep tidal basin and improve our knowledge of the relationship between P and phytoplankton composition.
Outline of the thesis

The research questions and hypotheses were answered through a number of field surveys. During these field surveys the limiting resources were investigated in the Marsdiep basin of the western Dutch Wadden Sea at different temporal and spatial scales and related to the phytoplankton community composition. In addition, nutrient enrichment experiments were carried out with natural phytoplankton assemblages.

In chapter 2, the main objective was to test whether spatio-temporal distribution of phytoplankton was under the influence of episodic freshwater discharge into the Marsdiep basin. The spatial variability of abiotic parameters and distribution of the phytoplankton community at different stages of the phytoplankton seasonal cycle were investigated at different locations characteristic for the western Dutch Wadden Sea.

In chapter 3, the limiting nutrients for the phytoplankton biomass were determined with a series of short term nutrient enrichment experiments under controlled light and temperature during the spring bloom at the NIOZ sampling jetty. In addition to phytoplankton biomass (chlα), phytoplankton physiological indices were also measured (maximum quantum efficiency of photosystem II and alkaline phosphatase activity).

In chapter 4, the influence of phosphate limitation on phytoplankton natural assemblages was examined at three locations in the Marsdiep basin from mid-spring to early autumn. The effect of phosphate supply in phytoplankton community was measured by a change in C-fixation using 13C stable isotope incorporation into PLFA.

In chapter 5, it was tested whether MPB was suspended into the water column. To answer this hypothesis, the benthic and pelagic primary producers were described and primary production was estimated at different sampling seasons across three pelagic stations and two benthic stations by comparing two methods: a molecular fingerprint, denaturing gradient gel electrophoresis (DGGE) and a chemotaxonomic biomarker, PLFA. In addition, MPB primary production was estimated.

In chapter 6, a two dimensional analysis using imaging Pulse Amplitude Modulated (PAM) fluorescence was developed to study photosynthetic activity and vertical migration of MPB in muddy and sandy sediments.

Finally, in chapter 7, a synthesis of the results obtained this thesis is presented. It shows that the limiting nutrients have an impact on phytoplankton community composition and the consequences of these changes for consumers in the Wadden Sea ecosystem. In conclusion, the implications of the results are explained in the light of the data obtained from the long-term monitoring studies at the NIOZ sampling jetty.