

Impact of ocean acidification on microbial degradation of organic matter

Dissertation

zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften

- Dr. rer. nat. -

der Mathematischen-Naturwissenschaftlichen Fakultät

der Christian-Albrechts-Universität zu Kiel

Vorgelegt von

Sonja Endres

Kiel, Januar 2013

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1 Summary

Marine bacteria play a key role in carbon and nutrient cycles of the ocean. The majority of organic carbon fixed during photosynthesis is rapidly recycled in the upper ocean via microbial degradation. The remaining organic carbon is sequestered in the deep sea making the ocean a net sink for atmospheric carbon dioxide (CO₂). Organic matter in the ocean is a complex mixture of organic compounds and a major portion is continuously transformed chemically from dissolved small molecules to polymers and gels, up to particles and broken back down to small dissolved monomers by microbial activities. Gel particles, in particular, are hotspots of microbial degradation as they provide food and a surface to attach to. Furthermore gel particles accelerate aggregation of particulate organic matter (POM) and play a major role in sedimentation processes in the sea. In order to utilize organic matter, bacteria have to produce extracellular enzymes that operate outside the cell to degrade polymers into smaller compounds. Many enzymes involved in the bacterial hydrolysis of organic matter were shown to be pH-sensitive. As CO₂ concentrations in the ocean rise due to anthropogenic CO₂ emissions, seawater pH decreases – a process known as ‘ocean acidification’. Since 1750, the ocean’s pH decreased by 0.1 units and a further decrease of around 0.15 - 0.35 units in the surface oceans by the year 2100 is projected.

Despite their importance, relatively little is known about in what way marine bacteria may be influenced by ocean acidification. This doctoral thesis addresses the question how microbial degradation of organic matter and organic matter composition may change in the acidifying ocean. The first part of the thesis investigates the response of a natural plankton community to CO₂ enrichment during a mesocosm study in a Norwegian fjord. The second part focuses specifically on the filamentous diazotrophic cyanobacterium *Nodularia spumigena*, which plays an important role in nitrogen and phosphorus cycling in the Baltic Sea.

In both studies, elevated CO₂ concentrations induced higher bacterial abundances as well as an enhanced accumulation of exopolymer substances such as gel particles and mucus. Stimulated carbon and nitrogen fixation of *Nodularia spumigena* resulted in higher biomass. Faster enzyme hydrolysis rates increased the microbial turn-over of organic matter in the natural plankton community as well as in the *Nodularia spumigena* cultures. Recycling of nitrogen or phosphorus from organic compounds by the extracellular enzymes alkaline phosphatase and leucine aminopeptidase was significantly accelerated with decreasing seawater pH, especially after nutrient depletion. This additionally supported microbial growth.

During the *Nodularia spumigena* study, mainly uncharacterized components dominated the decrease in the dissolved organic phosphorus (DOP) pool. Potential compositional changes in the DOP pool under elevated CO₂ concentrations could therefore not be detected due to

methodological constraints. During the mesocosm study, small compositional changes in amino acids under different CO₂ conditions were detected which may be related to stimulated bacterial degradation. These CO₂-induced changes in dissolved organic matter (DOM) composition may persist in the future ocean and change the bioavailability of DOM in the long-term.

Results of both studies indicate that marine microbial biomass and organic matter cycling will change due to ocean acidification. Enhanced enzymatic hydrolysis of organic matter and increased presence of gel particles may support higher bacterial cell numbers and degradation activities in the acidifying ocean. Generally, ocean acidification may improve the ability of bacteria to use various alternative nutrient sources by increasing nitrogen fixation or enzymatic recycling of organic nutrients. Particularly blooms of diazotrophic cyanobacteria such as *Nodularia spumigena* may intensify or expand in nitrogen-limited regions or periods in the future. This would provide additional bioavailable nitrogen and enhance the turnover of phosphorus within these regions. Enhanced microbial acquisition of nutrients by hydrolytic enzymes at lowered seawater pH in the surface ocean may decrease the flux of organic nutrients to the deep ocean.

In conclusion, I infer that ocean acidification will increase bacterial abundance and degradation activities resulting in faster heterotrophic turn-over of organic matter. This may increase the fraction of organic matter which is degraded at the surface and lower the efficiency of particle export and carbon sequestration fluxes to the ocean depth. However, changes in gel particle formation in the acidifying ocean may counteract this trend increasing particle export and supporting carbon storage in the ocean.

2 Zusammenfassung

Bakterien spielen im Ozean eine Schlüsselrolle in den Kreisläufen von Kohlenstoff und Nährstoffen wie Stickstoff und Phosphor. Der Großteil des organischen Kohlenstoffs, der photosynthetisch gebunden wurde, wird schnell wieder durch Bakterien im oberen Ozean abgebaut. Der verbleibende organische Kohlenstoff wird in der Tiefe gespeichert. Dies macht den Ozean zu einer Senke für atmosphärisches Kohlenstoffdioxid (CO₂). Das organische Material des Ozeans besteht aus vielen verschiedenen organischen Verbindungen, die ständig miteinander reagieren. Dabei entstehen Polymere und Gele bis hin zu größeren Partikeln, die durch mikrobielle Aktivität wieder zu kleineren Molekülen abgebaut werden. Insbesondere Gelpartikel zeichnen sich durch einen hohen mikrobiellen Abbau aus, da sie sowohl Nahrung als auch Oberfläche zur Ansiedlung bieten. Zudem beschleunigen Gelpartikel die Aggregation des partikulären organischen Materials (POM) und spielen eine wichtige Rolle in den Sedimentationsprozessen des Meeres. Um organisches Material nutzen zu können, müssen Bakterien extrazelluläre Enzyme produzieren, die Polymere in kleinere Verbindungen spalten. Viele Enzyme, die am bakteriellen Abbau des organischen Materials beteiligt sind, reagieren empfindlich auf Veränderungen des pH-Wertes. Während die atmosphärischen CO₂-Konzentrationen aufgrund anthropogener Emissionen stetig ansteigen, sinkt der pH-Wert des Ozeans kontinuierlich. Dieser Prozess wird als „Ozeanversauerung“ bezeichnet. Seit 1750 ist der pH-Wert des Ozeans um 0.1 Einheiten gesunken und Berechnungen gehen von einer weiteren Abnahme von 0.15 – 0.35 Einheiten im oberen Ozean bis zum Jahr 2100 aus.

Trotz ihrer großen Bedeutung, ist relativ wenig darüber bekannt, wie marine Bakterien durch Ozeanversauerung beeinflusst werden. Diese Dissertation beschäftigt sich mit der Frage wie der mikrobielle Abbau und die Zusammensetzung des organischen Materials sich ändern könnten, während der Ozean versauert. Der erste Teil dieser Arbeit untersucht die Veränderungen in einer natürlichen Planktongemeinschaft unter erhöhten CO₂-Konzentrationen während eines Mesokosmos-Experiments in einem norwegischen Fjord. Der zweite Teil konzentriert sich auf das fadenförmige stickstofffixierende Cyanobakterium *Nodularia spumigena*, welches eine wichtige Rolle im Stickstoff- und Phosphor-Kreislauf der Ostsee spielt.

In beiden Studien führten erhöhte CO₂-Konzentrationen zu einem Anstieg der Bakterien-Zellzahlen, sowie zu einer verstärkten Anreicherung exopolymerer Substanzen (Gelpartikel und Mucus). Die Stimulation von Kohlenstoff- und Stickstofffixierung in *Nodularia spumigena* ermöglichte den Aufbau von mehr Biomasse. Höhere Enzymraten steigerten den mikrobiellen Umsatz organischen Materials sowohl innerhalb der natürlichen Planktongemeinschaft als auch in den *Nodularia spumigena* – Kulturen. Die Regeneration von Stickstoff und Phosphor aus

organischen Verbindungen durch die extrazellulären Enzyme alkalische Phosphatase und Leucin-Aminopeptidase wurde deutlich durch den sinkenden pH-Wert des Seewassers beschleunigt, insbesondere während des Auftretens von Nährstoffknappheit. Dies unterstützte das mikrobielle Wachstum zusätzlich.

Während der Studie an *Nodularia spumigena* dominierten hauptsächlich nicht charakterisierbare Verbindungen den Abbau des gelösten organischen Phosphor (DOP)-Pools. Falls sich der DOP-Pool also in seiner Zusammensetzung durch die erhöhten CO₂-Konzentrationen geändert hatte, konnte das aufgrund methodischer Einschränkungen nicht nachgewiesen werden. Während der Mesokosmos-Studie wurden geringe Änderungen in der Aminosäure-Zusammensetzung bei verschiedenen CO₂-Konzentrationen festgestellt, die durch einen erhöhten bakteriellen Abbau entstanden sein könnten. Diese CO₂-bedingten Änderungen in der Zusammensetzung des gelösten organischen Materials (DOM) könnten in Zukunft bestehen und die Bioverfügbarkeit des DOM langfristig verändern.

Die Ergebnisse beider Studien deuten darauf hin, dass sich sowohl die mikrobielle Biomasse als auch Auf- und Abbau des organischen Materials im Ozean im Zuge der Ozeanversauerung verändern werden. Eine schnellere enzymatische Zersetzung organischen Materials und die Zunahme von Gelpartikeln könnten in Zukunft zu höheren Bakterienzellzahlen und zu einem schnellerem bakteriellen Abbau führen. Ozeanversauerung scheint die Fähigkeit von Bakterien zu verbessern alternative Nährstoffquellen zu nutzen, indem die Stickstofffixierung und die enzymatische Wiederverwertung organischer Nährstoffe stimuliert werden. Cyanobakterien-Blüten, wie z. B. von *Nodularia spumigena*, in Stickstoff-limitierten Regionen oder Situationen könnten sich dadurch in Zukunft verstärken. Dies würde allen Organismen zusätzlichen Stickstoff zur Verfügung stellen und den Umsatz von Phosphor erhöhen. Wenn im oberen Ozean bei niedrigerem pH-Wert mehr Nährstoffen durch hydrolytische Enzyme regeneriert werden, gelangen weniger organische Nährstoffe in die Tiefsee.

Zusammenfassend komme ich zu dem Schluss, dass sich die bakterielle Abundanz und deren Abbauraten durch Ozeanversauerung erhöhen werden, was einen schnelleren mikrobiellen Umsatz organischen Materials im oberen Ozean zur Folge haben wird. Dadurch könnte sich der relative Anteil des organischen Materials erhöhen, der an der Oberfläche abgebaut wird, und die Effizienz von Partikelexport und Kohlenstoffspeicherung in der Tiefsee verringern. Allerdings könnte eine Veränderung in der Bildung von Gelpartikeln diesem Trend entgegenwirken und den Partikelexport sowie die Kohlenstoffspeicherung im Ozean unterstützen.

3 Introduction

3.1 Role of marine bacteria in organic matter cycling

3.1.1 Marine organic matter

Organic matter in the ocean is a complex mixture of organic compounds which serve as carbon, nutrient, and energy sources for heterotrophic organism. Additionally, organic matter is a structural unit for bacteria to grow on. Nowadays, we know that there is a size continuum of organic matter from small molecules to colloids, macromolecules, polymers, macrogels, up to particles and eukaryotic cells (Verdugo et al. 2004). Traditionally, organic matter is categorized into dissolved organic matter (DOM; > 97%) that passes filters ($\sim 0.45\text{-}0.7\ \mu\text{m}$ pore size) and particulate organic matter (POM) that remains on filters ($> 0.7\ \mu\text{m}$) (Benner 2002; Hedges 2002).

With respect to size, DOM consists of about 70% low-molecular weight compounds (LMW, <1000 Da) and about 30% high-molecular weight compounds (>1000 Da) (Amon and Benner 1994; Benner 2002; Kujawinski 2011). Most LMW compounds are small enough to pass through the cell membrane for direct assimilation in marine microbes. However, the HMW DOM fraction is turned over more rapidly than the LMW DOM fraction (Amon and Benner 1994; Amon and Benner 1996). The turnover rates of DOM are in the range from minutes to thousands of years. Labile DOM is degraded within hours to days, while semilabile material accumulates in the ocean for month to years (Kirchman et al. 1991). The turnover time of refractory DOM is in the range of several thousands of years (Carlson and Ducklow 1995; Carlson 2002). Oldest DOM compounds are in average 6000 years old and were detected in the North Pacific (Williams and Druffel 1987). Around 80 - 90% of marine DOM is refractory and accumulates as it cannot be processed by bacteria (Ogawa et al. 2001). However, this is just a very small fraction of what has been produced and consumed over millions of years (Pomeroy et al. 2007).

The exact composition of marine DOM is largely unknown. To date several thousand molecular formulas have been detected via ultrahigh resolution mass spectrometry (Fourier transform ion cyclotron resonance mass spectrometry; FT-ICR MS). However, only 10 to 20% of the DOM in the surface ocean, and even less in the deep ocean, can be identified chemically. These include freshly produced compounds such as carbohydrates, amino acids, amino sugars, nucleic acids, and lipids (Ogawa et al. 2001; Kattner et al. 2011).

In total, the oceans contain approximately 38,000 Gt carbon (C) as dissolved inorganic carbon (DIC, $\sim 98\%$), dissolved organic carbon (DOC, $\sim 2\%$), and a minor fraction of particulate carbon (POC) (Siegenthaler and Sarmiento 1993). DOC concentrations equal the amount of carbon in atmospheric CO_2 (662 Gt C versus 750 Gt C, respectively) (Carlson 2002).

3.1.2 Exudation and formation of gel particles

Primary production in the euphotic zone is the main source of DOM to the open ocean. Autotrophic cells release organic carbon compounds passively through diffusion or actively through exudation depending on nutrient status and growth conditions (Carlson 2002). Furthermore, they release compounds for nutrient acquisition, communication, or chemical defense, and due to viral lysis, predation, or senescence (Kujawinski 2011).

When a critical concentration is reached, small organic compounds (LMW DOM) coagulate to form nanogels and microgels (Verdugo et al. 2004). Around 10% DOM in the surface ocean is estimated to self-assemble forming microgels (Chin et al. 1998) consisting primarily of polysaccharides, proteins, and nucleic acids chains. Nanogels and microgels can eventually aggregate further by continued annealing, forming gel particles, such as transparent exopolymeric particles (TEP) (Alldredge et al. 1993; Passow 2002a, 2002b) that contribute to the POM pool. TEP formation is an important mediator between the DOM and the POM pool (Verdugo et al. 2004). Some gel particles, referred to as Coomassie stainable particles (CSP), contain large amounts of peptides and proteins. Gel particles also may adsorb high amounts of inorganic nutrients providing food and surface for bacteria to attach and grow (Long and Azam 1996; Azam and Malfatti 2007). Consequently, gel particles are hotspots of microbial degradation with high substrate concentrations (Alldredge et al. 1993; Verdugo et al. 2004). Furthermore, gel particles play a major role in sedimentation processes and carbon cycling in the sea as particulate matter sticks to these gel particles thereby increasing the density of the aggregate which is then exported to the deep ocean (Alldredge et al. 1993; Logan et al. 1995; Passow 2002a, 2002b).

3.1.3 Marine bacteria and the carbon cycle

Heterotrophic bacterioplankton are the main consumers of DOM processing approximately 50 up to 90% of the net primary production. Marine bacteria thereby play a central role in the marine carbon cycle (Cho and Azam 1988; Cho and Azam 1990; Azam et al. 1993). The so-called 'microbial loop' is of major significance in the open ocean, especially in regions of little photosynthetic production where heterotrophic bacteria largely control organic matter fluxes (Gasol et al. 1997). The microbial loop describes the contribution of marine bacteria to carbon and nutrient cycling in the ocean, while the classic food chain refers to the POM flux from photosynthetic algae, to herbivores and on to higher trophic levels (Fig. 1). Heterotrophic bacteria utilize DOM released by autotrophic cells as carbon and energy source to form biomass. Small protozoans and flagellates feed on bacterial biomass returning POM into the classic pathway of carbon and energy flow (Azam et al. 1983). The efficiency of the microbial loop depends on a variety of abiotic and biotic factors such as the availability of additional inorganic nutrients (Kuparinen and Heinanen 1993), grazing by zooplankton (Wright and Coffin 1984), viral infection

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(Weinbauer and Höfle 1998), and quantity and quality of the organic matter (Billen et al. 1990; Kroer 1993).

LMW DOM such as amino acids and carbohydrates are easily assimilated into cells making them good indicators of microbial degradation processes. Free amino acid concentrations are quite low in marine DOM due to rapid microbial turnover (Fuhrman 1987; Keil and Kirchman 1999). Besides the cycling of these LMW compounds, heterotrophic bacteria are able to utilize large HMW DOM and POM with the help of cell-associated and extracellular enzymes (Hoppe et al. 2002). The ability of bacteria to solubilize and degrade even large particle has direct effects on the efficiency of the biological carbon pump which exports organic matter to the deep sea (Cho and Azam 1988; Smith et al. 1992). The efficiency and strength of the biological carbon pump depends upon the balance of organic matter production at the surface (< 100 m) and bacterial remineralization and particle dissolution in the surface and mesopelagic (100 – 1000 m) ocean (Passow and Carlson 2012). The more organic matter is degraded, the less is exported and sequestered in the deep ocean for 100 years and more. Furthermore, microbial processing modifies the molecular structure of DOM forming unknown organic compounds whose chemical structures are not identified yet and which may resist further degradation (Kattner et al. 2011). The production of refractory DOM via heterotrophic microbial processes is a potential sink for fixed carbon in the ocean (Ogawa et al. 2001) and has been termed the ‘microbial carbon pump’ (Jiao et al. 2010). Approximately one fourth of the bulk DOM pool is thought to be produced via the microbial carbon pump (Benner and Herndl 2011).

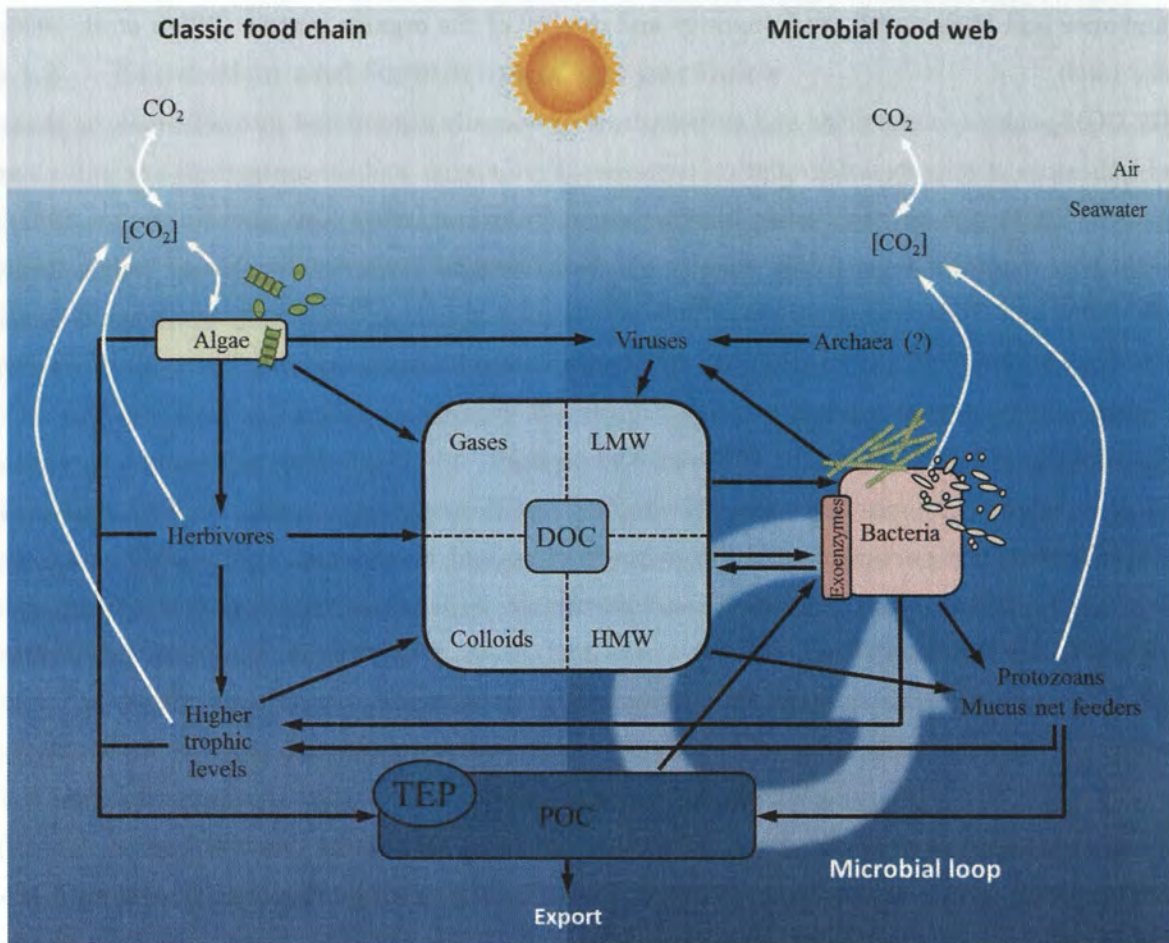


Figure 1 The classic food chain and the microbial food web: Simplified scheme of the ocean food web showing on the left the classic pathway of carbon flow through photosynthetic algae, to herbivores and on to higher trophic levels. The microbial food web is illustrated on the right including the microbial loop. Also shown in the microbial food web are Viruses and Archaea. The role of Archaea in the oceanic food web is not identified yet. White arrows indicate CO₂ fluxes. Shown at the bottom of this diagram is the downward export of particulate organic carbon (POC), which is now thought to fuel most processes in the deep ocean. (Modified from DeLong 2005)

3.1.4 Nitrogen fixation by diazotrophic cyanobacteria

The marine carbon cycle is closely linked with the cycles of the major nutrients nitrogen and phosphorus (Hutchins et al. 2009). Nitrogen is supplied to the upper ocean via input by rivers, atmospheric deposition, upwelling, and nitrogen fixation by marine nitrogen fixers (diazotrophs). Diazotrophic cyanobacteria, also known as blue-green algae, are the key organisms providing new organic nitrogen to the marine environment (Capone et al. 1997; Montoya et al. 2004). One of these important players is *Nodularia spumigena* which is widely distributed in the Baltic Sea. Cyanobacterial bloom development in the Baltic Sea is stimulated by low concentrations of dissolved inorganic nitrogen (DIN; below the detection limit) while dissolved inorganic

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phosphorus (DIP) is still available (Niemi 1979; Nausch et al. 2008). High concentrations of other nitrogen sources (ammonium, nitrate) may inhibit nitrogen fixation (Holl and Montoya 2005). Due to their ability to convert atmospheric nitrogen (N_2) into ammonium, primary production of cyanobacteria is independent of dissolved inorganic nitrogen (DIN) making cyanobacteria successful bloomers in nitrogen-depleted regions or periods. Some fraction of the fixed nitrogen is used to produce cyanobacterial biomass while the rest is often exuded as ammonium or dissolved organic nitrogen (DON; Fig. 2) (Gilbert and Bronk 1994; Wannicke et al. 2009; Ploug et al. 2011).

3.1.5 Inorganic nutrient limitation and recycling of organic nitrogen and phosphorus

Both, phytoplankton and heterotrophic bacteria use inorganic nitrogen and phosphorus as nutrient source. Heterotrophic bacteria may even be more successful in acquiring inorganic nutrients due to a higher surface-to-volume ratio or light limitation of the phytoplankton (Currie and Kalff 1984; Joint et al. 2002). Normally, nitrogen rather than phosphorus is the limiting factor for microbial growth in the sea (Tyrell 1999). Ammonium, nitrate, DON, and PON serve as nitrogen and energy source for heterotrophic bacteria. Ammonium is oxidized to nitrite and nitrate by bacterial nitrification (Brandes et al. 2007) or – under suboxic or anoxic conditions – by anammox (anaerobic ammonium oxidation) back to molecular N_2 (Kuypers 2005). Nitrate is converted to gaseous compounds (N_2O , N_2) through bacterial denitrification under suboxic conditions (Fig. 2).

Because of limited nutrient availability in the ocean and high competition for nutrients, microbes recycle these elements from organic matter by enzymatic hydrolysis (Nausch and Nausch 2004; Dyrman et al. 2007; Hutchins et al. 2009). DON is mainly recycled aerobically releasing ammonia while PON is either remineralized in the microbial loop or exported to the deep ocean (Hutchins et al. 2009). Nitrogen- and phosphorus-rich dissolved organic matter is thought to be preferentially degraded relative to carbon-rich material (Hopkinson and Vallino 2005). Dissolved amino acids are a central food source for heterotrophic bacteria supporting 4 – 41% of the bacterial nitrogen demand in the open ocean (Keil and Kirchman 1991, 1999).

To compensate for phosphorus limitation, many bacteria have very effective uptake systems for phosphate and store polyphosphates within the cell. In particular, cyanobacterial growth and N_2 fixation rates are regulated by the availability of phosphorus compounds (e.g. ATP, DNA; Sañudo-Wilhelmy et al. 2001; Degerholm et al. 2006). Hence, many cyanobacteria, but also other marine microbes, utilize dissolved organic phosphorus (DOP). This is indicated by increased alkaline phosphatase activity (APA), an enzyme cleaving orthophosphate groups from DOP molecules (Nausch 1998; Hoppe 2003; Degerholm et al. 2006).

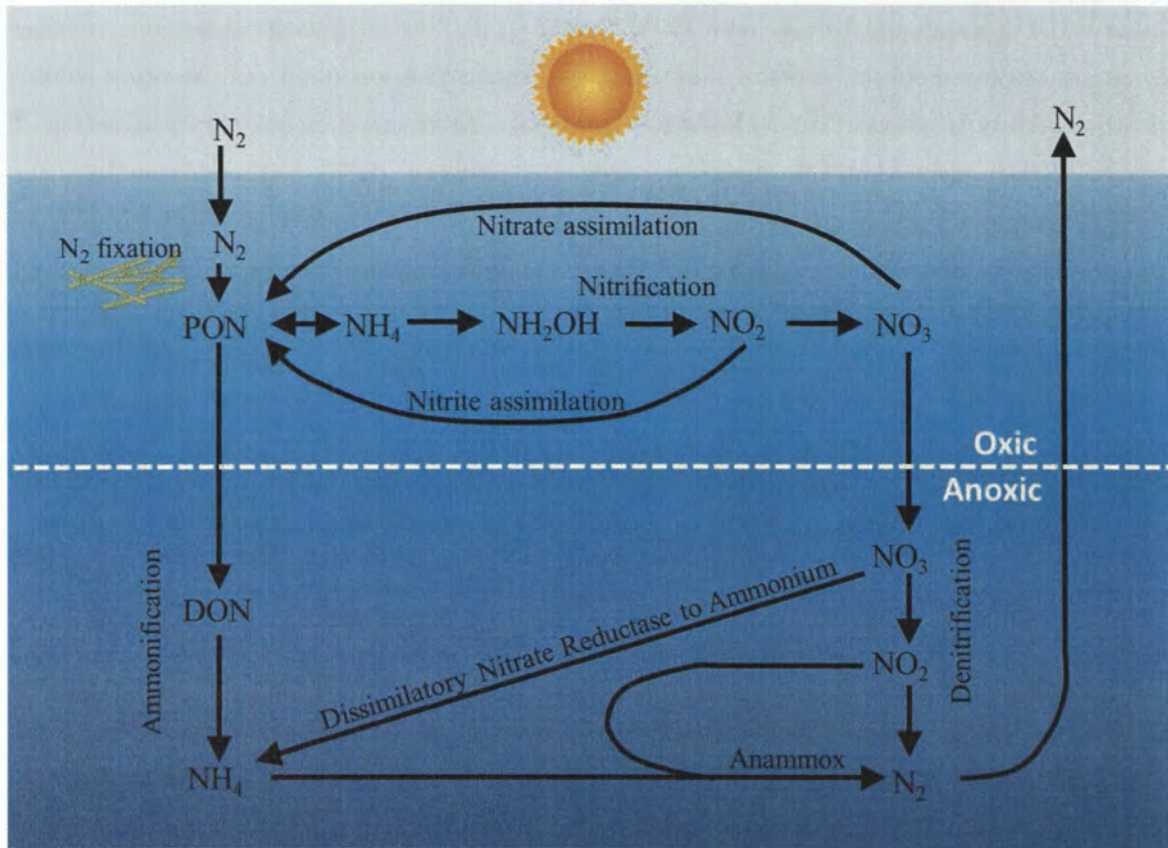


Figure 2 The marine nitrogen cycle. Atmospheric nitrogen (N_2) is fixed to particulate organic nitrogen (PON; including phytoplankton). Ammonium (NH_4) and nitrate (NO_3) are released as N_2 due to denitrification and anaerobic ammonium oxidation (anammox). Modified from Arrigo 2005.

3.2 Marine extracellular hydrolytic enzymes

3.2.1 Key enzymes and their role in carbon and nutrient cycling

Most bacteria can only transport small molecules (< 600 Da) into the cell via uptake porins in the cell membrane for further processing (Nikaido and Vaara 1985; Benz and Bauer 1988). Larger HMW compounds have to be hydrolyzed beforehand into smaller subunits by extracellular enzymes (Chrost 1991; Arnosti 2003). These extracellular enzymes may be cell-surface attached or released into the medium (Chrost 1991). The amount and activity of extracellular enzymes determine which molecules can be utilized as carbon or energy source by microbes and how fast organic matter is cycled by heterotrophic microbes. Hence, enzymatic hydrolysis is a central step in heterotrophic remineralization. Furthermore, extracellular enzymes solubilize POM releasing small compounds that diffuse out correspondingly reducing the sinking flux of the particle (Azam 1998). Structure, kinetic properties, and regulation of extracellular enzymes are largely unknown but extended knowledge would help to understand the fate of organic matter in the ocean (Arnosti 2011).

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Enzymes are typically classified based on the reactions that they catalyze. Key enzymes in marine carbon and nutrient cycling are α - and β -glucosidase, alkaline phosphatase, leucine aminopeptidase, lipase and chitinase. Each enzyme class includes a diverse range of enzymes types (Nagata 2008).

The β -glucosidase catalyzes the hydrolysis of β -linked polysaccharides. Most of the β -glucosidase activity is associated with bacteria (> 95%) (Hoppe 1983; Chrost et al. 1989; Chrost 1991). Eleven different β -glucosidases were detected during a coastal phytoplankton bloom revealing high β -glucosidase diversity in the ocean (Arrieta and Herndl 2002). β -glucosidases activity and diversity is regulated by the availability of utilizable substrates (Chrost 1991) and bacterial diversity (Arrieta and Herndl 2002).

Leucine aminopeptidase is one of several proteolytic enzymes required to hydrolyze proteins and peptides and is widely distributed in the ocean (Chrost 1991). Most of its activity is associated to heterotrophic bacteria and particles (Hoppe et al. 2002). Leucine aminopeptidase synthesis is stimulated by certain amino acids or peptides (Daatselaar and Harder 1974) and increased protein concentrations in the late stages of phytoplankton blooms. On the other hand, increased amino acid concentrations may suppress enzyme synthesis and decrease enzyme affinity (Chrost 1991).

The alkaline phosphatase catalyzes the hydrolysis of orthophosphate groups from larger molecules. The enzyme consists of two subunits which interact depending on pH (Chrost 1991). Alkaline phosphatase activity is used as indicator of phosphorous limitation in the ocean (Nausch 1998) because the enzyme is regulated by its end product (PO_4^{3-}); particularly by the intracellular PO_4^{3-} concentration and by the availability of DOP (Hoppe 2003). Phosphatase activity is associated to bacterial and algal cells but also detected in high amounts as free dissolved enzymes (Chrost 1991).

3.2.2 Enzyme kinetics and determination of enzyme activity

Heterotrophic bacteria use different enzyme types to hydrolyze complex substrates (Weiner et al. 2008) but concentrations of individual enzymes in the water are very low restricting the possibility to isolate single enzymes for direct investigations. Isoenzymes catalyzing the same chemical reaction may have diverse structures and amino acid sequences (Arrieta and Herndl 2002; Doman-Pytka and Bardowski 2004). Additionally, most of the marine bacteria have not yet been isolated in pure culture (Rappé and Giovannoni 2003). For this reason, small fluorogenic substrate proxies were used during the last 30 years to measure extracellular enzyme activities in the aquatic environments (Hoppe 1983). Substrate proxies are hydrolyzed by cell-attached as well as dissolved extracellular enzymes, so the relative contributions of both types cannot be distinguished with this method (Martinez and Azam 1993).

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In general, hydrolytic enzymes follow the Michaelis-Menten kinetics which means that hydrolysis rates (V) of a given substrate can be related to the ambient substrate concentration (S_n) by the following function:

$$V = V_{max} \frac{S_n}{K_m + S_n}$$

V_{max} is the maximal hydrolysis rate and K_m (Michaelis constant) is the substrate concentration at which the hydrolysis rate is $\frac{1}{2}V_{max}$ (Chróst and Siuda 2002; Nagata 2008). A low K_m value indicates the high enzyme affinity to the substrate while a high K_m value points to lower substrate affinity which means more unspecific binding of molecules to the active site of the enzyme. The plot of enzyme activities versus initial substrate concentrations follows a hyperbolic curve (Fig. 3). Usually, enzyme activities in literature are given as V_{max} of the enzyme.

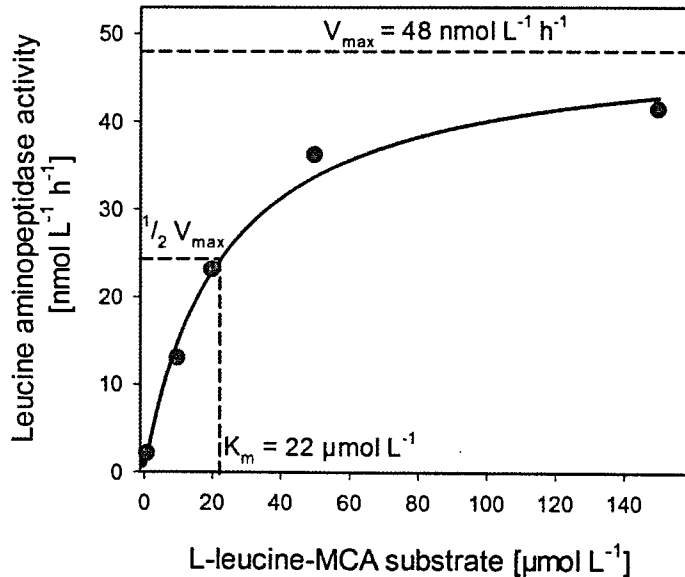


Figure 3 Leucine aminopeptidase activities at different substrate concentrations. V_{max} and K_m were estimated by model fitting the data to the standard Michaelis-Menten equation.

3.2.3 Regulation of extracellular enzyme activities

Enzyme synthesis within bacterial cells is generally regulated by the availability of organic substrate. Many enzymes are expressed in low concentrations and released to the environment acting as “sensor” to detect hydrolysable material. When appropriate substrate is present, LMW products accumulate, enter the cell and induce further enzyme synthesis (Chrost 1991; Chróst and Siuda 2002). Important abiotic factors influencing extracellular enzyme activities in the ocean and often also enzyme synthesis within the cell are temperature, pH, inorganic and organic nutrients, UV radiation and the presence or absence of inhibitors (Chróst and Siuda 2002; Hoppe 2003). Increasing temperature mostly stimulates enzyme activities and the optimal temperature is often higher than *in-situ* values (Chrost and Overbeck 1987; Cunha et al. 2010). Extracellular

enzymes are directly affected by seawater pH, because changes in hydrogen ion concentration modify the three-dimensional structure of the active site of the enzyme (Tipton et al. 2009). As for temperature optima, the pH optima of extracellular enzymes do not always match the *in-situ* pH (Münster 1991; Cunha et al. 2010). Some enzymes such as alkaline phosphatase or aminopeptidase are sensitive to environmental levels of UV light due to photodegradation of the enzyme (Hoppe 2003; Cunha et al. 2010). Enzymatic regulation influenced by temperature and pH gained attention recently as anthropogenic CO₂ emissions induce ocean warming and acidification.

3.3 Climate change and ocean acidification

3.3.1 Anthropogenic CO₂ emissions and seawater carbonate chemistry

Carbon dioxide (CO₂) is a major anthropogenic greenhouse gas that contributes to anthropogenic climate change (Karl 2003). Since the beginning of the Industrial Revolution in 1750, global atmospheric CO₂ concentrations have increased unprecedentedly due to combustion of fossil fuels and land use change (IPCC 2007) from around 280 µatm up to 390 µatm at present (Tans and Keeling 2012). The quantity and speed of the CO₂ increase has never been that high in the past (Honisch et al. 2012). CO₂ rapidly exchanges between atmosphere and the surface ocean leading to a corresponding increase in dissolved CO₂ concentrations in seawater.

Several different soluble compounds interact within the ocean carbonate system, all collectively known as dissolved inorganic carbon (DIC). When CO₂ dissolves in seawater around 1% forms carbonic acid (H₂CO₃), reacting further to bicarbonate (HCO₃⁻) and carbonate ions (CO₃²⁻), while releasing hydrogen ions (Wolf-Gladrow et al. 1999). The increased dissolution of anthropogenic CO₂ is consequently lowering seawater pH - a process commonly referred to as “ocean acidification”.

DIC is fixed photosynthetically or precipitated to calcium carbonate by calcifying organisms. It is exported from the surface to the deep ocean via sinking of organic matter and calcium carbonate (biological pump), or via downwelling of cold and CO₂ enriched water (physical pump) (Wolf-Gladrow et al. 1999). Consequently, the oceans are a net sink for CO₂. In the past 200 years, approximately one half of human-created CO₂ emissions have been absorbed by the ocean but positive feedbacks in ocean-atmosphere coupling may reduce the efficiency of CO₂ storage in the future ocean (Sabine 2004).

Direct measurements of oceanic carbon dioxide partial pressure ($p\text{CO}_2$) and pH from three subtropical time series stations (ESTOC, HOT, and BATS) reflect the constant change in the oceanic carbonate system over the last 20 years (Fig. 4). Surface oceanic $p\text{CO}_2$ increased between 1.6 and 1.9 µatm yr⁻¹ (IPCC 2007) to reach 393 µatm CO₂ in November 2012 (Conway and Tans

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2012). In average, surface oceans have a pH of about 8.1 ± 0.3 units. Since 1750, seawater pH decreased by 0.1 units (Fig. 4), which equals an increase in hydrogen ion concentration of 30% on the logarithmic pH scale (Caldeira and Wickett 2003; Feely et al. 2009).

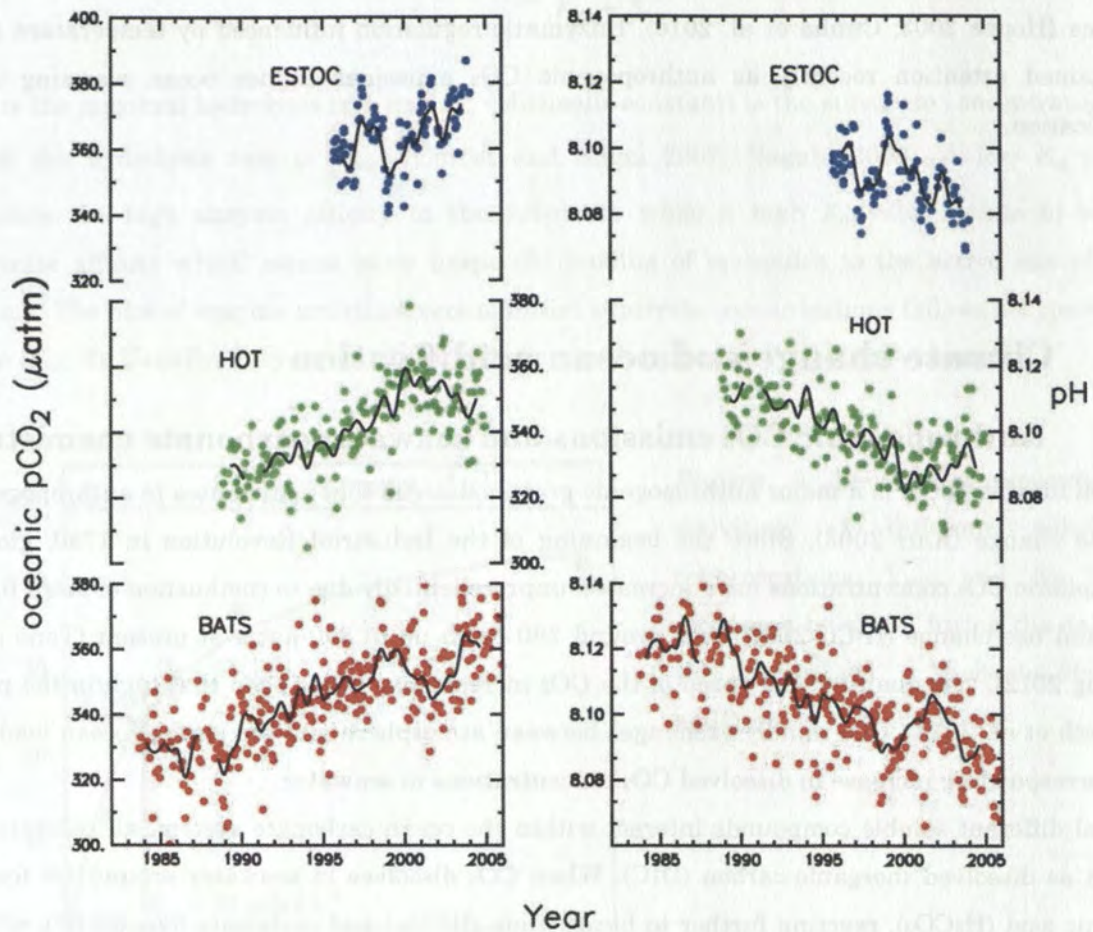


Figure 4 Changes in surface oceanic $p\text{CO}_2$ and pH from three time series stations: Blue: European Station for Time-series in the Ocean (ESTOC, Gonzalez-Dávila et al., 2003); green: Hawaii Ocean Time-Series (HOT, Dore et al., 2003); red: Bermuda Atlantic Time-series Study (BATS, Bates et al., 2002; Gruber et al., 2002). The mean seasonal cycle was removed from all data (from IPCC 2007).

The latest IPCC report (2007) makes projections on future atmospheric CO_2 concentrations and global ocean pH based on different CO_2 emission scenarios. These estimates based on different economic scenarios suggest that atmospheric $p\text{CO}_2$ could reach about 500 – 950 μatm by the year 2100 (Friedlingstein et al. 2006; IPCC 2007) which would lead to a decrease in seawater pH of around 0.15 - 0.35 units in the surface oceans (Fig. 5) (Orr et al. 2005; Feely et al. 2009).

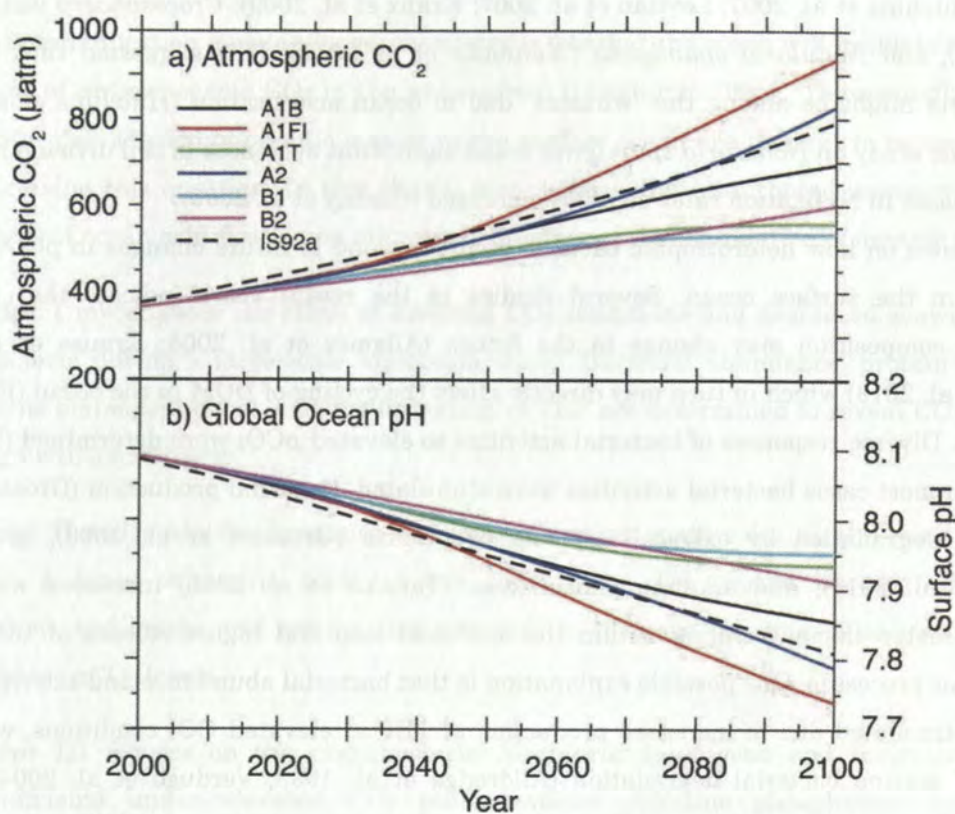


Figure 5 Changes in atmospheric CO₂ concentration and global average surface pH under various emission scenarios. Time series of (a) atmospheric CO₂ for the six illustrative emission scenarios and (b) projected global average surface pH (Plattner et al. 2001). Figure modified from IPCC 2007.

3.3.2 Biotic response to ocean acidification

Climate change and ocean acidification are supposed to have a variety of consequences for marine carbon and nutrient cycles which cannot completely be covered here. Therefore, I will focus on selected aspects relevant for this doctoral thesis.

Autotrophic organisms provide most of the organic matter used by the bacterial community (Field et al. 1998). So far, it has been found that doubling of the present atmospheric CO₂ concentration slightly stimulates the rate of photosynthesis in most marine algae tested (Beardall and Raven 2004; Schippers et al. 2004; Riebesell et al. 2007) while carbon fixation in the cyanobacteria *Trichodesmium* sp. is considerably stimulated (Hutchins et al. 2009). The rate of extracellular organic carbon production and formation of TEP increases at elevated CO₂ conditions (Engel 2002; Liu et al. 2010; Borchard and Engel 2012) and may additionally affect organic carbon fluxes in the future ocean.

Previous studies indicate that increasing $p\text{CO}_2$ lowers the energy demand for inorganic carbon acquisition. Thus, more energy is available for other processes such as N₂ fixation which was

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found to increase under elevated $p\text{CO}_2$ in cyanobacteria *Trichodesmium* sp. (Barcelos e Ramos et al. 2007; Hutchins et al. 2007; Levitan et al. 2007; Kranz et al. 2009), *Crocospaera watsonii* (Fu et al. 2008), and *Nodularia spumigena* (Wannicke et al. 2012). It is suggested that N_2 -fixing cyanobacteria might be among the “winners” due to ocean acidification (Hutchins et al. 2009). However, one study on *Nodularia spumigena* found significant decreases in cell division rates and slight decreases in N_2 fixation rates as $p\text{CO}_2$ increased (Czerny et al. 2009).

Little is known on how heterotrophic bacteria might respond to future changes in $p\text{CO}_2$ and pH conditions in the surface ocean. Several studies in the recent years indicate that bacterial community composition may change in the future (Allgaier et al. 2008; Krause et al. 2012; Sperling et al. 2013) which in turn may directly affect the cycling of DOM in the ocean (Kirchman et al. 2009). Diverse responses of bacterial activities to elevated $p\text{CO}_2$ were determined (Liu et al. 2010) but in most cases bacterial activities were stimulated. Bacterial production (Grossart et al. 2006), and degradation by extracellular aminopeptidase (Grossart et al. 2006), glucosidase (Piontek et al. 2010), and alkaline phosphatase (Tanaka et al. 2008) increased with $p\text{CO}_2$ suggesting faster element cycling within the microbial loop and higher release of CO_2 due to heterotrophic processes. One possible explanation is that bacterial abundance and activity may be indirectly stimulated due to increased production of TEP at elevated CO_2 conditions, which are hotspots of marine bacterial degradation (Alldredge et al. 1993; Verdugo et al. 2004). Direct effects of pH on enzyme activities, cell physiology and organic matter accessibility must also be considered.

3.4 Outline of the thesis

The most urgent question of ocean biogeochemistry is whether the ocean will mitigate or reinforce the increase of anthropogenic CO₂ in the atmosphere (Longhurst 1991). Heterotrophic bacteria and bacterial degradation of organic matter in the surface ocean are thought to be key elements when addressing this question. In this thesis, manuscripts and co-authored manuscripts report on the effects of ocean acidification on microbial abundance and degradation of organic matter.

Manuscript I investigates the effect of elevated CO₂ conditions and decreased seawater pH on marine bacteria during a large-scale mesocosm study. Bacterial abundance, protein hydrolysis rates (leucine aminopeptidase), and the formation of TEP are determined to reveal CO₂ effects on bacterial growth and activity.

Manuscript II addresses the dynamics of dissolved organic matter during the same mesocosm study with a focus on amino acids as key food source for marine bacteria. Bulk DOM concentrations and amino acid composition are used to compare the diagenetic status of DOM under different *p*CO₂ levels.

Manuscript III focuses on the cyanobacteria *Nodularia spumigena* and their strategies to acquire nutrients under elevated CO₂ concentrations. Alkaline phosphatase and leucine aminopeptidase activities are compared to bulk DOP and DON concentrations and formation of mucinous substances in *Nodularia* batch cultures under three different *p*CO₂ levels.

Additionally, two chapters (co-authored **Manuscripts IV and V**) focus on growth, biomass production as well as nitrogen and phosphorous cycling by *Nodularia spumigena* during the same batch experiment as in chapter III. Changes in carbon and nitrogen fixation rates, growth rates, elemental stoichiometry, organic matter and DOP-compound (ATP, DNA, RNA, and phospholipids) concentrations with elevated *p*CO₂ are studied.

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4 Manuscripts

4.1 List of manuscripts

This doctoral thesis is based on the following publications and manuscripts:

- I. S. Endres, L. Galgani, K.-G. Schulz, A. Engel, Marine bacteria thrive when the ocean acidifies, To be submitted
- II. S. Endres, R. Flerus, L. Galgani, A. Paul, J. Roa, K.-G. Schulz, A. Engel, Dynamics of dissolved organic matter and amino acid composition during a phytoplankton bloom under simulated ocean acidification. To be submitted
- III. S. Endres, J. Unger, N. Wannicke, M. Nausch, M. Voss, and A. Engel (2013), Response of *Nodularia spumigena* to $p\text{CO}_2$ – Part 2: Exudation and extracellular enzyme activities, *Biogeosciences*, 10 (1), 567-582, doi:10.5194/bg-10-567-2013

4.1.1 List of co-authorships:

- IV. N. Wannicke, S. Endres, A. Engel, H.-P. Grossart, M. Nausch, J. Unger, and M. Voss (2012), Response of *Nodularia spumigena* to $p\text{CO}_2$ – Part 1: Growth, production and nitrogen cycling, *Biogeosciences*, 9, 2973-2988, doi:10.5194/bg-9-2973-2012
- V. J. Unger, S. Endres, N. Wannicke, A. Engel, M. Voss, G. Nausch, M. Nausch (2013), Response of *Nodularia spumigena* to $p\text{CO}_2$ – Part 3: Turnover of phosphorus compounds, *Biogeosciences*, 10, 1483-1499, doi: 10.5194/bg-10-1483-2013

4.2 Declaration of the contribution of each manuscript

Manuscript I:

Data acquisition: Sonja Endres sampled and analyzed bacterial cell counts, gel particles and extracellular enzyme activities with support from Luisa Galgani, Anja Engel, the KOSMOS team (mesocosm logistics, sampling) and all other experiment participants. Kai Schulz provided pH and chlorophyll *a* data.

Data interpretation and preparation of manuscript: Sonja Endres with comments from all co-authors.

Manuscript II:

Data acquisition: Sonja Endres sampled and analyzed amino acids concentration and composition with the support from Luisa Galgani, Ruth Flerus, Anja Engel, the KOSMOS team (mesocosm logistics, sampling) and all other experiment participants. Allanah Paul sampled and Jon Roa analyzed dissolved organic carbon and dissolved organic nitrogen concentrations. Kai Schulz provided pH and chlorophyll *a* data.

Data interpretation and preparation of manuscript: Sonja Endres with comments from all co-authors.

Manuscript III:

Data acquisition: Sonja Endres sampled and analyzed gel particles, extracellular enzyme activities, dissolved organic carbon and dissolved organic nitrogen concentrations with support from all co-authors. Nicola Wannicke and Juliane Unger provided data on nutrients, carbonate chemistry, biomass development, and nitrogen fixation.

Data interpretation and preparation of manuscript: Sonja Endres with comments from all co-authors.

4.2.1 Contribution to each co-authored manuscripts:

Manuscript IV:

Data acquisition and interpretation: Nicola Wannicke sampled and analyzed nutrients, chlorophyll *a*, growth rates, nitrogen fixation rates, primary production and particulate organic matter concentrations with support from all co-authors. Sonja Endres and Juliane Unger provided data on dissolved organic matter concentrations.

Data interpretation and preparation of manuscript: Nicola Wannicke with comments from all co-authors.

Manuscript V:

Data acquisition and interpretation: Juliane Unger sampled and analyzed phosphorus compounds concentrations with support from all co-authors. Nicola Wannicke provided data on nutrients, carbonate chemistry, and biomass development. Monika Nausch provided ³³P-uptake rates.

Data interpretation and preparation of manuscript: Juliane Unger with comments from all co-authors.

4.3 Manuscript I

Marine bacteria thrive when the ocean acidifies

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4.3.1 Abstract

Marine bacteria are the main consumers of organic matter in the ocean and largely control marine O₂ and CO₂ budgets. Many enzymatic processes involved in the bacterial digestion of organic compounds were shown to be pH sensitive in previous studies. Due to the continuous rise in atmospheric CO₂ concentration, seawater pH is presently decreasing at a rate unprecedented during the last 300 million years and with so-far unknown impact on microbial physiology, organic matter cycling and marine biogeochemistry. Here, we provide results from a large-scale mesocosm study indicating that marine bacteria benefit from decreasing pH. The combination of increased availability of gel particles as substrate and enhanced enzymatic hydrolysis of organic matter supported higher bacterial abundance in the low pH treatments. We infer that ocean acidification has the potential to change organic matter cycling and export in the ocean by favouring bacterial growth. As a consequence, ocean acidification may relate to further expansion of oxygen deficient zones and feedback on rising atmospheric CO₂ levels.

4.3.2 Introduction

Up to 96 % of the net marine primary production is routed into the microbial loop and respired to CO₂ by bacterioplankton (Martin et al. 1987; del Giorgio and Duarte 2002). Bacterial growth is regulated by abiotic factors (e.g. temperature) but also largely by the availability and accessibility of organic matter substrates (del Giorgio and Cole 1998). In order to utilize organic matter, bacteria produce extracellular enzymes that operate outside the cell degrading polymers into smaller compounds (Chrost et al. 1989). Dissolved polymers, like polysaccharides, can form a gel-like organic matrix such as transparent exopolymer particles (TEP), which comprise a substantial and highly dynamic fraction of the particulate organic matter pool (Verdugo 2012). Gel particles are a vehicle for the downward transport of organic matter in the water column and may serve as a food source for bacteria but also act as a structural unit to attach and grow (Verdugo 2012). Consequently, changes in the gelatinous pool may influence bacterial hydrolysis and growth; on the other hand, changes in bacterial hydrolysis rates may influence the biogeochemical fate of organic matter (Azam 1998). Generally, enzyme activities show strong pH dependency because changes in hydrogen ion concentration modify the three-dimensional structure of the active site of the enzyme (Tipton et al. 2009). As hydrolytic extracellular enzymes play a central role in organic matter remineralisation, their regulation needs to be examined in the context of ocean acidification. Recent studies reported on bacterial enzymatic responses to acidification (Piontek et al. 2010; Cunha et al. 2010; Yamada and Suzumura 2010; Grossart et al. 2006; Endres et al. 2013) but the answer whether or not marine bacteria may benefit from acidification by increasing abundance and degradation rates is still pending (Cunha et al. 2010).

4.3.3 Methods

Experimental set-up and bloom development

The experiment was conducted using the Kiel Off-Shore Mesocosms for Future Ocean Simulations (KOSMOS) that allow plankton dynamics to be followed over several weeks with minimal disturbance of the water body and under *in-situ* conditions. Experimental perturbations included CO₂ enrichment and nutrient additions. A detailed description of the experimental setup, its deployment, technical features and the sampling methods are described in Riebesell et al. (2012) and Schulz et al. (2013). Briefly, nine 25m-long, free-floating mesocosms with flexible thermoplastic polyurethane bags were deployed in the Raunefjord near Bergen in southern Norway. Seven mesocosms were adjusted over five days to target $p\text{CO}_2$ levels of 400 (M6), 600 (M8), 900 (M1), 1200 (M3), 1300 (M5), 2000 (M7) and 3000 (M9) μatm by stepwise addition of CO₂ saturated seawater. Two mesocosms (M2, M4) were used as control treatments at *in-situ* $p\text{CO}_2$ of around 300 μatm . Temperature varied between 6.8°C at the beginning (end of April) and 10.0°C at the end of the experiment in June. The average salinity in the mesocosm was 32.0 +/- 0.1 psu. During the sampling period, chlorophyll *a* concentrations in the fjord ranged between 0.5 and 1.9 $\mu\text{g L}^{-1}$ (data not shown). After CO₂ addition, samples of the entire enclosed water column were taken with a depth integrating water sampler (1-23m depth, Hydrobios Kiel, Germany) every other day for 35 days from all nine mesocosms and the fjord. The first sampling day before CO₂ addition was termed “day -1”.

We determined carbonate chemistry, chlorophyll *a* concentration, gel particles, bacterial cell counts, and extracellular enzyme activities (see below for a detailed description of the methods). The first phytoplankton bloom developed around day 5. On day 14, inorganic nutrients (5 $\mu\text{mol L}^{-1}$ nitrate and 0.1 $\mu\text{mol L}^{-1}$ phosphate) were added to the enclosed, nutrient-poor waters to stimulate a second phytoplankton bloom, which occurred at about day 20.

The average pH in the phase before nutrient addition ranged between pH 8.09 ± 0.01 in the control mesocosms and pH 7.38 ± 0.27 in the highest $p\text{CO}_2$ mesocosm. After nutrient addition, pH ranged between pH 8.11 ± 0.01 in the control mesocosms and pH 7.49 ± 0.14 in the highest $p\text{CO}_2$ mesocosm (supplementary Table S1).

pH measurements

pH was measured spectrophotometrically with a VARIA Cary 100 in 10 cm cuvette at 25°C as described in Dickson (2010) and then recalculated to *in-situ* pH. pH values are given relative to the total scale. The precision was typically better than 0.001 at high and 0.002 at low pH.

Chlorophyll a analysis

For chlorophyll *a* (Chl *a*) analysis 250 – 500 ml samples were filtered onto Whatman GF/F filters. Filters were stored at -80°C for at least 24 h and then homogenized with 90% acetone and glass beads (2 and 4 mm) in a cell mill. After centrifugation, chlorophyll *a* concentrations were determined with a TURNER 10-AU fluorometer as described in Welschmeyer (1994).

Bacteria cell counts

For bacterial cell counts, 4.5 ml samples were preserved with 200 μl 25% glutaraldehyde and stored at -20°C for up to three month until measurement. A stock solution of SybrGreen I (Invitrogen) was prepared by mixing 5 μl of the dye with 245 μl dimethyl sulfoxide (DMSO, Sigma Aldrich). 5 μl of the dye stock solution and 10 μl fluoresbrite microspheres (diameter 0.94 μm , Polysciences) were added to 200 μl of the thawed sample and incubated for 30 min in the dark. The samples were then analysed at low flow rate using a flow cytometer (FACS Calibur, Becton Dickinson) (Gasol and del Giorgio 2000). TruCount beads (Becton Dickinson) were used for calibration and in combination with Fluoresbrite YG microsphere beads (1.00 μm , Polysciences) for absolute volume calculation. Calculations were done using the software program “Cell Quest Pro”. Flow cytometry is a fast and highly reproducible method to determine bacterial cell counts. It may detect small bacteria ($<0.2\mu\text{m}$) that are difficult to distinguish with an epifluorescence microscope. However, it cannot detect large aggregates ($>30\mu\text{m}$). Therefore, determined bacterial cell counts refer mainly to free-living bacterial cells while particle-attached bacteria may be underestimated.

Leucine aminopeptidase activities

The leucine aminopeptidase (LAP) activity is frequently used as an indicator for microbial metabolic processes involved in the mineralization of peptides and proteins (Caruso and Zaccone 2000). The potential *in situ* activity of the LAP was determined by using the fluorogenic model substrates L-leucine-4-methyl-7-coumarinylamide (MCA) (Hoppe 1983). L-leucine-MCA was added to 180 μl samples and incubated in duplicates for 5 - 20 h in the dark at 11°C . Six different substrate concentrations ranging from 0 to 150 μM (0, 1, 10, 20, 50 and 150 μM) were tested. Sample fluorescence was measured in microtiter plates with a fluorometer (FLUOstar OMEGA, BMG Labtech, excitation 355 nm, emission 460 nm). Calibration was carried out with a dilution series of MCA. The fluorescent signal of MCA was tested not to be affected by pH. Detection limit for the LAP activity was 2 nmol L^{-1} . Distilled water or sterilized seawater was incubated with substrate at each of the concentrations as a control for background fluorescence and abiotic substrate hydrolysis. The activity of bacterial extracellular enzymes was calculated as the maximum hydrolysis rate (V_{max} ; i.e. the maximum rate achieved by the system at saturating substrate concentrations, see supplementary Figure S1) using the software SigmaPlot 12.0 (Systat).

Transparent exopolymer particles (TEP)

For photometric and microscopic analyses of acidic polysaccharide-containing transparent exopolymer particles (TEP), 20 to 60 ml samples were filtered onto 0.4 μm polycarbonate filters, stained with a calibrated Alcian Blue solution and rinsed with several ml of ultrapure water (Passow and Alldredge 1995). The filters were stored at -20°C for 2–6 weeks until spectrophotometric and microscopic analysis. The amount of Alcian Blue adsorption per sample was determined colorimetrically. Each filter was incubated for 3 h with 6 ml of 80% H_2SO_4 in order to dissolve the particles; The solution was measured at 787 nm with an UV-Vis spectrophotometer (Shimadzu UV-1700 PharmaSpec). The total concentration of TEP is given in xanthan gum equivalent (Xeq), as xanthan gum was used for calibration. The total area and particle abundance were determined microscopically by image analysis (Engel 2009). TEP size can be compared by calculating the equivalent spherical diameter (ESD) of each particle. The total area of TEP is the sum of the individual gel particle areas. Gel particles with an ESD $< 5\mu\text{m}$ were excluded from image analysis.

Data analysis and statistics

The daily deviation (AD_i) of each mesocosm was calculated by subtracting observation (X_i) from the average of all mesocosms (\bar{X}) on the specific sampling day ($AD_i = X_i - \bar{X}$). These daily deviations were then averaged over time according to $\frac{1}{N} \sum_{i=1}^N (AD_i)$ with N being the number of sampling days, in order to get the mean deviations (MD) of each mesocosm regarding a particular parameter (Engel et al. 2012). The mean deviations were tested against average pH of the different mesocosms by linear regression. The significance level of the ANOVA was set to $p < 0.05$. Statistical analysis was performed using the software package SigmaPlot 12.0 (Systat).

4.3.4 Results

Planktonic growth under pCO₂ perturbation in mesocosms

The purpose of our large-scale mesocosm experiment was to determine the response of a natural plankton community to CO₂-induced decrease in seawater pH. Here, we report on the effect of decreasing seawater pH on bacterial growth, gel particle production and bacterial extracellular enzyme activities.

A first phytoplankton bloom developed around day 3 with increasing chlorophyll *a* values of up to $3.5 \pm 0.3 \mu\text{g L}^{-1}$ (Fig. 1). Afterwards, chlorophyll *a* concentrations declined. The addition of inorganic nutrients to the mesocosms on day 14 initiated the development of a second algal bloom, which reached a maximum of $3.9 \pm 0.6 \mu\text{g L}^{-1}$ chlorophyll *a* between days 19 and 20, declining to $1.4 \pm 0.5 \mu\text{g L}^{-1}$ until day 34 (supplementary Table S2). In terms of biomass, both blooms were dominated by nano- and phycoflagellates, mainly *Plagioselmis prolunga* (Cryptophyta).

Initially, average bacterial cell numbers were $6.5 \pm 0.9 \times 10^5 \text{ mL}^{-1}$ (Fig. 1). We observed an increase of bacterial cell numbers within the first 25 days of the experiment. Cell numbers averaged over all mesocosms on day 25 were $2.0 \pm 1.1 \times 10^6 \text{ cells mL}^{-1}$. Towards the end of the experiment, bacterial abundance decreased in all treatments to $9.8 \pm 3.9 \times 10^5 \text{ mL}^{-1}$ (Fig. 1). Highest abundance was determined in the low pH mesocosms with up to $4.5 \times 10^6 \text{ cells mL}^{-1}$. To distinguish between a potential pH and nutrient effect, we analysed data of two phases separately: a) before nutrient addition (day 0 – 13) and b) after nutrient addition (day 14 – 34). pH significantly affected bacterial abundance within the mesocosms during both phases yielding bacterial numbers 18 – 23 % below average in the high pH mesocosms but 4.6 - 39.5 % above average in the two low pH mesocosms (Fig. 2).

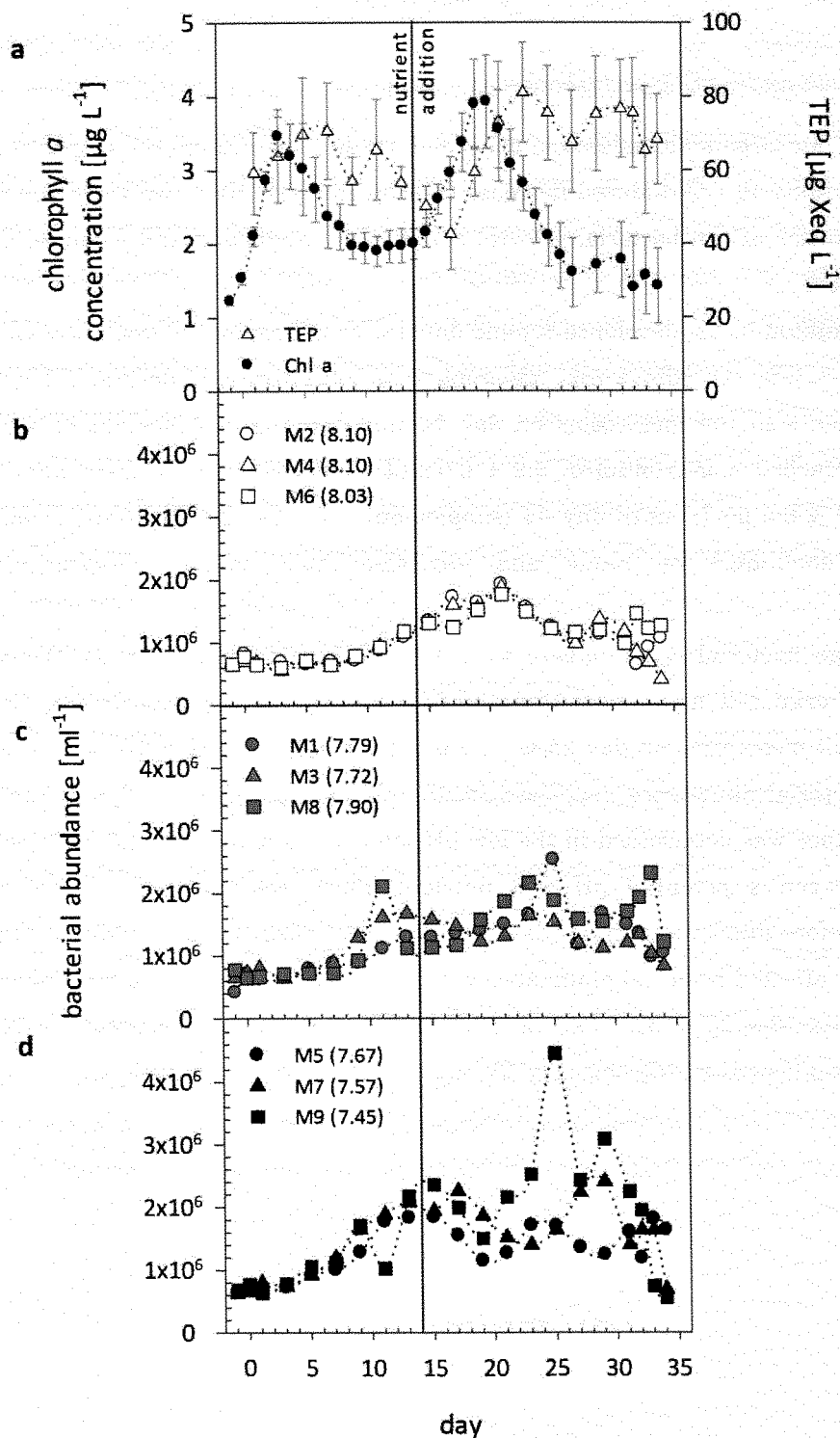


Figure 1, a-d Temporal development of average chlorophyll *a* and average TEP concentrations of all mesocosms (a) as well as free-living bacterial cell counts in the high (white, b), intermediate (grey, c) and low (black, d) pH treatments during the course of the experiment. Numbers in brackets give the mean pH value of each treatment over time. Error bars indicate standard deviation of nine mesocosms. Nutrients were added on day 14 to all nine mesocosms.

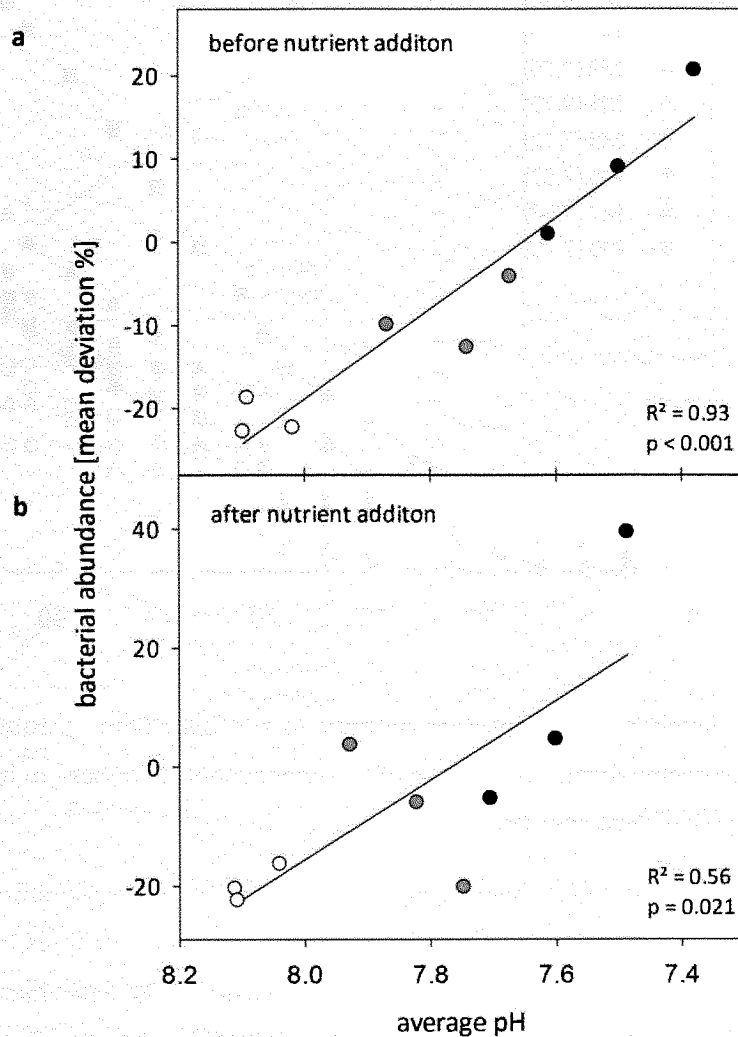


Figure 2, a-b Influence of pH on bacterial cell numbers. Symbols indicate the mean deviation of bacterial cell counts compared to the average value over time in the high (white), intermediate (grey) and low (black) pH mesocosms before (a) and after (b) nutrient addition.

Total and cell-specific protein hydrolysis rates

LAP activities were low or even not detectable at the beginning of the experiment and increased over time. Maximal activities were measured between day 19 and day 29, when the second phytoplankton bloom declined (Fig. 3) yielding an average of $77.2 \pm 20.6 \text{ nmol L}^{-1} \text{ h}^{-1}$ equivalent to a substrate turnover time of $13.7 \text{ h } \mu\text{M}^{-1}$. Highest LAP activities were measured in the lowest pH treatment with $149.2 \text{ nmol L}^{-1} \text{ h}^{-1}$ on day 25.

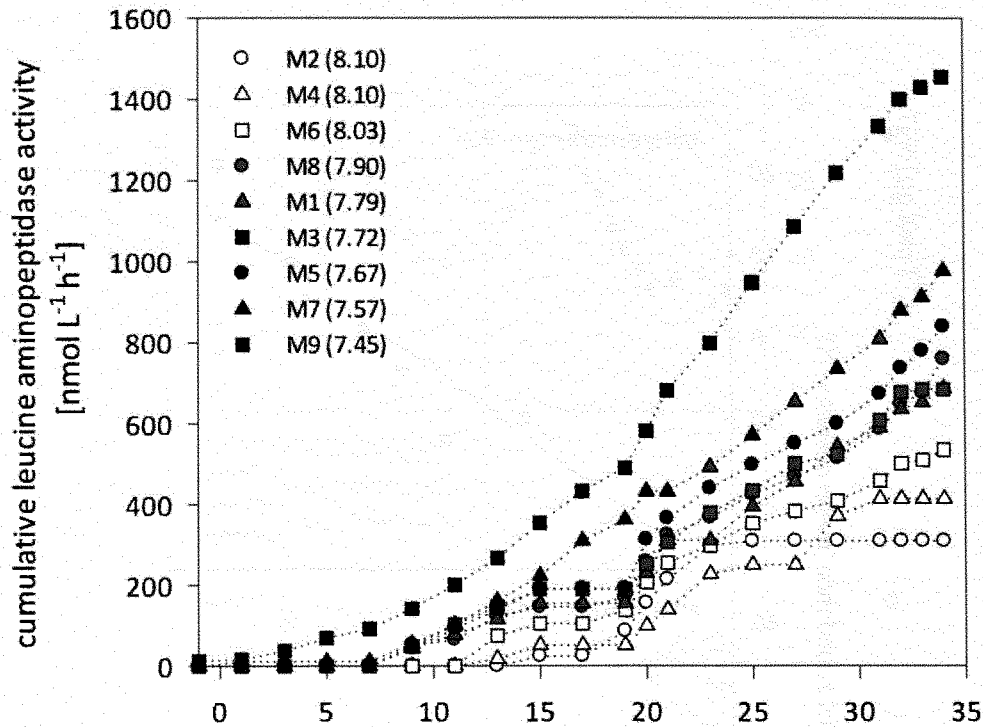


Figure 3 Cumulative leucine aminopeptidase activity in the high (white), intermediate (grey) and low (black) pH mesocosms during the course of the experiment. Numbers in brackets give the mean pH value of each treatment over time.

A pH effect on protein hydrolysis was significant in both phases of the experiment (Fig. 4). LAP activities were lowered by 12.3 – 24.2 nmol L⁻¹ h⁻¹ in the high pH mesocosms, but elevated by 5.6 – 43.5 nmol L⁻¹ h⁻¹ in the low pH mesocosms compared to the average mesocosm development. Enzyme activities also correlated with pH after normalization to cell abundance, indicating a direct pH effect (Fig. 4). Highest cell-specific activities of 47.2 ± 13.9 amol cell⁻¹ h⁻¹ were measured on day 20. Cell-specific activities were 38 - 68 % below average in the high pH and 57 - 71 % above average in the low pH mesocosms.

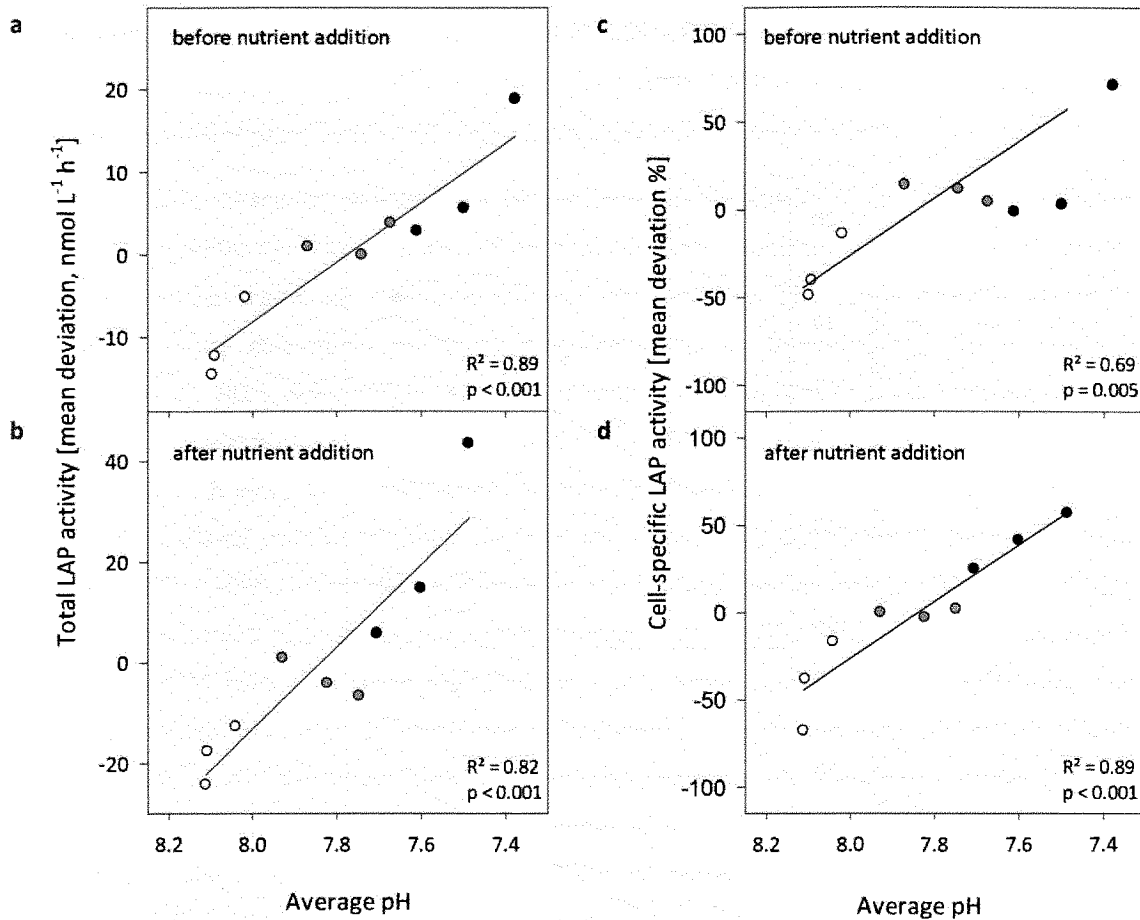


Figure 4, a-d Influence of pH on leucine aminopeptidase (LAP) activity. Symbols indicate the mean deviation of total (a, b) or cell-specific (c, d) LAP activity, respectively, in relation to the average pH value in the high (white), intermediate (grey) and low (black) pH mesocosms before (a, c) and after (b, d) nutrient addition.

Marine gel dynamics

Average TEP concentrations were $59.3 \pm 11.2 \mu\text{g Xeq L}^{-1}$ at the beginning of the experiment and increased over time forming two peaks. A first TEP peak was observed around day 6 with $70.6 \pm 13.1 \mu\text{g Xeq L}^{-1}$, while the second peak occurred around day 23 with $81.04 \pm 13.6 \mu\text{g Xeq L}^{-1}$. Both TEP peaks occurred two days after the chlorophyll α maxima (Fig. 1). In between the peaks, TEP concentration decreased to $42.7 \pm 9.8 \mu\text{g Xeq L}^{-1}$. The total area of TEP in the nine mesocosms increased from $46 \pm 14 \text{ mm}^2 \text{ L}^{-1}$ at the beginning to up to $234 \pm 121 \text{ mm}^2 \text{ L}^{-1}$ at the end of the experiment (data not shown). TEP size can be compared by calculating the equivalent spherical diameter (ESD) of each particle, which is the diameter of a round particle that has the same area as the analysed gel particle. The average ESD of TEP increased over time from $8.2 \pm 0.4 \mu\text{m ESD}$ up to $10.9 \pm 1.3 \mu\text{m ESD}$ (data not shown).

A significant pH effect was detected on TEP concentrations, TEP total area and TEP diameter (Fig. 5). In the high pH mesocosms, 10.5 - 11.7 % less TEP covering 8 - 29 % less total area and

with 1.6 - 5.6 % smaller particles were measured compared to the over-all mesocosm average. In comparison, the low pH mesocosms had 2.9 - 12.3 % more TEP resulting in 3 - 36 % more total TEP area with particles being 1.3 - 6.9 % larger.

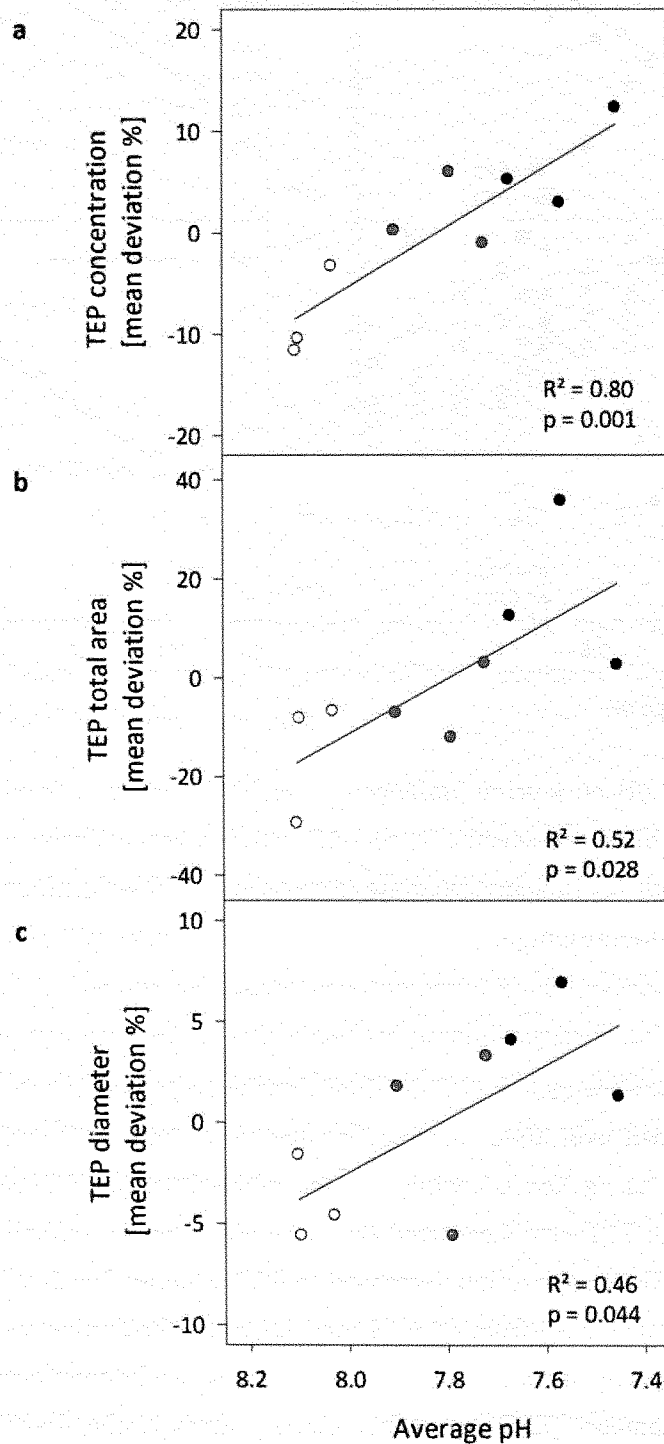


Figure 5, a-c Influence of pH on transparent exopolymer particles (TEP). Symbols indicate the mean deviation of gel particle concentration (a), total area (b) and size (c) relative to the average value in the high (white), intermediate (grey) and low (black) pH mesocosms.

4.3.5 Discussion

Increased bacterial abundance and degradation under low seawater pH

The large-scale mesocosm set-up used here allows manipulation of environmental factors (here CO₂ and nutrients) and the response of natural plankton communities and several trophic levels under close to natural conditions to be followed continuously. This study was the second full-scale experiment using this novel sea-going mesocosm system, following the Arctic study in 2011 (Riebesell et al. 2012; Schulz et al. 2013). Our results are the first to show a strong stimulation of the bacterial community due to ocean acidification. We found increasing bacterial abundance in combination with more TEP and higher cell-specific degradation activity at lower seawater pH.

For bacteria, direct uptake of substrate from the seawater is restricted to simple low-molecular-weight (LMW; <1000 Da) molecules. However, the high-molecular weight (HMW; >1000 Da) DOM fraction, which need to be hydrolysed prior to microbial uptake, is turned over more rapidly than the LMW DOM fraction (Amon and Benner 1994; Amon and Benner 1996). Hence enzymatic hydrolysis represents a rate-limiting step in bacterial nutrition and nutrient cycling (Cunha et al. 2010). In our study, we found highest enzymatic protein hydrolysis rates under low pH which leads to the hypothesis that limitation of bacterial growth due to enzymatic hydrolysis may be alleviated in the future ocean. Our results are in accordance with previous CO₂ perturbation studies that found increasing extracellular enzyme activities at high *p*CO₂ levels (Grossart et al. 2006; Piontek et al. 2010; Endres et al. 2013).

It is still unclear what exactly increases extracellular enzyme activities. Ocean acidification might lead to the production of more and/or different enzymes (effect on intracellular enzyme synthesis) or the enzyme assemblages remain the same but the enzymes can degrade faster under lower pH conditions (biochemical effect). Furthermore, we found an increase in TEP concentrations which could serve as a surface for dissolved enzymes to attach (physical effect). Attached enzymes have longer hydrolytic lifetimes than dissolved enzymes (Ziervogel et al. 2007). Nevertheless, at low pH, more substrate may be available for heterotrophic bacteria to gain energy and build-up biomass, reflected in higher bacterial cell numbers in the present experiment.

We further observed that more and larger TEP formed in the low pH treatments, corroborating earlier findings of higher TEP production at higher *p*CO₂ (Engel 2002; Borchard and Engel 2012). Large and more abundant TEP, used as a food source and suitable matrix for microbes, likely supported bacterial growth additionally.

Biogeochemical implications

It has been stated as null hypothesis that ocean acidification has little effect on major biogeochemical processes in the oceans because marine organisms always experienced variable pH conditions (Joint et al. 2010). In our study, however, we found evidence that marine bacteria

thrive under more acidic conditions. The combination of (1) increased availability of gel particles as food source and substrate to grow upon and (2) enhanced enzymatic hydrolysis of organic matter can explain higher bacterial cell abundances.

Increased bacterial growth may enhance competition with phytoplankton for inorganic nutrients. As bacteria are very efficient in nutrient acquisition, this would affect primary production in the ocean (Cunha et al. 2010). Just a minor fraction of the organic matter produced by photosynthesis in the ocean escapes bacterial respiration and is buried into deep sea sediments via the biological pump (Kirchman et al. 2009). However, this small leakage in the marine organic carbon cycle makes the ocean a net sink of CO₂ and is responsible for most of our atmospheric O₂ (Kasting 2002). In areas or at times when respiration rates exceed O₂ resupply, O₂-deficient water masses are formed (Wright et al. 2012). In the future ocean, increased microbial activity may add to oxygen deficiency. Microbial respiration provides one of the major natural sources for atmospheric CO₂ (del Giorgio and Duarte 2002). Enhanced CO₂ release from marine bacteria would diminish the ocean's capacity to act as a sink for anthropogenic CO₂ emissions. On the other hand, enhanced microbial activity may increase the transformation of fresh dissolved organic carbon (DOC) into refractory DOC that resides in the ocean for centuries to millennia. This 'microbial carbon pump' would increase the average residence time of the carbon in the ocean (Jiao et al. 2010).

Ocean acidification is only one aspect of climate change. Anthropogenic impacts are also expected on temperature, stratification, eutrophication and mixed-layer depth of the ocean (Boyd 2011). Similar to pH, ocean warming was found to increase enzyme activities, bacterial production and respiration rates (Cunha et al. 2010; Piontek et al. 2009; Sarmiento et al. 2010) as well as to enhance polysaccharide release and TEP formation rates (Borchard and Engel 2012). We therefore expect that ocean acidification and warming act in concert to promote or even amplify microbial processes and to reinforce the already dominant role of microbes in the marine biogeochemistry.

4.3.6 Supplementary material

Supplementary Tables

Table S1. Average pH values (AVG; and standard deviation, STDEV) in the nine mesocosms before and after nutrient addition on day 14.

	pH before nutrient addition		pH after nutrient addition	
	AVG	STDEV	AVG	STDEV
M2	8.10	0.01	8.11	0.01
M4	8.09	0.01	8.11	0.01
M6	8.02	0.02	8.04	0.01
M8	7.87	0.05	7.93	0.01
M1	7.74	0.10	7.82	0.02
M3	7.67	0.13	7.75	0.05
M5	7.61	0.16	7.71	0.06
M7	7.50	0.22	7.60	0.11
M9	7.38	0.27	7.49	0.14

Table S2. Determined chlorophyll α concentrations of the nine mesocosms and average concentration over time

Day	Chlorophyll α concentration [$\mu\text{g L}^{-1}$]									AVG	STDEV
	M1	M2	M3	M4	M5	M6	M7	M8	M9		
-1	1.15	1.24	1.15	1.26	1.21	1.21	1.32	1.24	1.27	1.23	0.06
0	1.40	1.51	1.54	1.63	1.54	1.58	1.65	1.42	1.58	1.54	0.08
1	1.81	2.12	2.17	2.18	2.02	2.22	2.17	2.22	2.08	2.11	0.13
2	3.02	3.08	2.79	2.93	2.71	2.71	2.89	2.76	2.86	2.86	0.13
3	3.85	3.07	3.50	3.42	3.42	3.14	3.73	3.32	3.71	3.46	0.27
4	3.30	2.54	3.21	3.07	3.30	2.95	3.69	2.73	3.95	3.19	0.44
5	3.14	2.25	2.99	2.89	3.39	2.47	3.67	2.29	4.07	3.02	0.62
6	2.77	2.17	2.71	2.51	2.89	2.57	3.40	2.27	3.41	2.74	0.44
7	2.28	2.04	2.34	2.27	2.36	1.98	2.99	1.92	3.14	2.37	0.43
8	2.49	1.90	2.36	2.22	2.19	1.77	2.68	1.97	2.54	2.24	0.31
9	2.32	1.75	1.86	2.03	1.96	2.03	2.04	1.79	1.97	1.97	0.17
10	2.18	1.76	2.06	2.08	2.24	1.95	1.61	1.85	1.79	1.95	0.21
11	2.08	1.64	2.07	2.06	2.09	1.95	1.71	1.92	1.59	1.90	0.20
12	2.19	1.80	2.07	2.20	2.04	2.07	1.74	1.97	1.55	1.96	0.22
13	2.20	1.67	2.06	2.06	2.13	2.13	1.81	2.13	1.55	1.97	0.23
14	2.20	2.18	1.75	2.07	2.09	2.15	1.81	2.09	1.64	2.00	0.21
15	2.47	1.92	2.31	2.07	2.23	2.20	2.15	2.18	1.82	2.15	0.20
16	2.78	2.39	2.66	2.63	2.87	2.66	2.61	2.63	2.19	2.60	0.20
17	3.14	2.88	2.97	2.75	2.95	3.08	3.03	3.29	2.54	2.96	0.22
18	3.95	3.55	3.45	3.46	3.23	3.55	3.08	3.57	2.54	3.37	0.40
19	4.65	4.27	3.89	4.00	3.13	4.00	3.69	4.59	2.84	3.90	0.61
20	4.85	4.10	4.09	3.82	3.32	4.22	3.64	4.55	2.75	3.93	0.64
21	3.88	3.55	3.95	3.61	3.15	3.87	3.08	4.33	2.60	3.56	0.53
22	3.96	2.89	3.56	3.07	2.87	3.16	2.34	3.19	2.67	3.08	0.48
23	3.29	2.24	3.08	2.46	3.24	2.59	2.78	3.10	2.59	2.82	0.37
24	2.87	1.69	2.50	2.09	2.70	2.15	2.27	2.49	2.70	2.38	0.37
25	2.34	1.32	2.17	1.80	2.55	1.75	2.15	2.30	2.57	2.11	0.41
26	1.99	0.96	1.85	1.42	2.19	1.64	1.99	2.02	2.46	1.84	0.45
27	1.74	0.71	1.32	1.22	1.82	1.54	1.90	1.98	2.27	1.61	0.47
29	2.07	1.12	1.41	1.30	1.70	1.64	1.86	2.03	2.25	1.71	0.38
31	2.53	1.19	1.37	1.16	1.93	1.88	1.45	2.36	2.17	1.78	0.51
32	0.99	0.39	0.52	1.10	1.95	1.73	1.45	2.26	2.24	1.40	0.70
33	2.07	1.07	1.20	1.01	1.65	1.40	1.14	2.24	2.31	1.57	0.52
34	2.03	1.11	1.11	0.80	1.45	1.08	1.14	1.83	2.29	1.43	0.51

Supplementary Figure

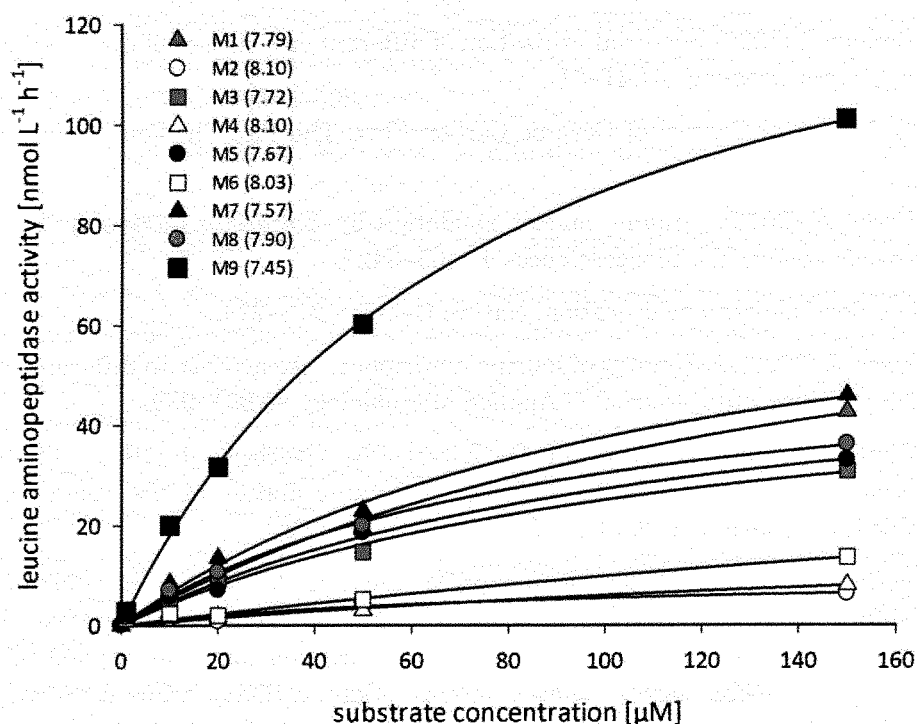


Figure S1. Enzyme saturation curves showing the relation between leucine aminopeptidase activity and substrate concentration in the high (white), intermediate (grey) and low (black) pH mesocosms measured on day 25; the maximum hydrolysis rate (V_{max}) reached at saturating substrate concentrations was calculated using Michaelis-Menten equation.

4.3.7 Acknowledgments

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4.4 Manuscript II

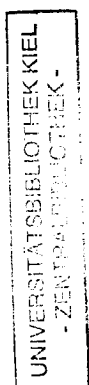
Dynamics of dissolved organic matter and amino acid composition during a phytoplankton bloom under simulated ocean acidification

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4.4.1 Abstract

Marine dissolved organic matter (DOM) is one of the largest carbon reservoirs in the ocean. Autotrophic production is the main source of marine DOM while heterotrophic degradation is the main sink. Most of the freshly produced DOM in the surface ocean is degraded in the water column and transformed into bacterial biomass or respired to inorganic carbon feeding back to atmospheric CO₂ concentrations. The increase in anthropogenic CO₂ emissions is constantly decreasing seawater pH. This ocean acidification is expected to affect primary production as well as bacterial degradation processes with potential consequences for DOM concentration and composition. We studied the effect of increased CO₂ concentration on DOM dynamics in a natural planktonic community during a large-scale mesocosm experiment in the Raunefjord (Norway). We determined the concentration of dissolved organic carbon (DOC) and nitrogen (DON) as well as amino acids composition. As heterotrophic bacteria preferentially use small reactive molecules, amino acids are indicators of the diagenetic status of DOM. Despite significant stimulation of microbial growth and enzymatic activity with increasing CO₂ concentration, no statistically significant CO₂ effect on absolute DOM concentration and bioavailability could be determined during this study. We identified CO₂-related changes in the concentration of four amino-acids (glycine, threonine, phenylalanine, and isoleucine). Interestingly, arginine concentrations were significantly cross-correlated with chlorophyll *a* development over time. Overall, compositional changes in amino acids were low (≤ 1 mol%). Further investigations are needed to elucidate the relationship between biological activities, species composition and amino acid dynamics. We suggest that small changes in amino acid composition have potential as early indicators of changes in autotrophic production and microbial degradation of organic matter under elevated CO₂ conditions.

4.4.2 Introduction

With around 662 Pg C, marine dissolved organic matter (DOM) is one of the major carbon reservoirs and plays an important role in the global carbon cycle (Hedges 1992; Hansell et al. 2009). The primary sources of marine DOM are photosynthesis, exudation, cell lysis, and grazing while heterotrophic degradation by bacteria is the main sink (Carlson 2002).

Marine DOM can be categorized by its bioavailability: labile compounds can be easily degraded within hours to weeks and constitute a very small fraction (< 1%) of DOM, whereas semi-labile material (15-20% of the primary production) remains in the water column for weeks to months until it is degraded by bacteria (Carlson et al. 2002; Hansell 2002; Hansell et al. 2009). According to estimations, 70 to 95% of the DOM is refractory which means it resists microbial or chemical degradation over thousands of years (Carlson and Ducklow 1995; Jiao et al. 2010). Labile and semi-labile fractions of DOM are more abundant in the surface oceans where they are produced by phytoplankton and other organisms.

The molecular composition of marine DOM is highly complex and varies depending on depth, season and region (Benner 2002). Thousands of different molecules can be distinguished by their elemental composition (Koch et al. 2008; Flerus et al. 2012) but only a few compounds can be identified in terms of structure by chemical analysis. These include reactive components, like carbohydrates, amino acids, proteins, peptides, lipids, and nucleic acids which are mainly enriched in freshly produced DOM (Meon and Kirchman 2001; Benner 2002; Carlson 2002). Amino acids only account for 1-3% of dissolved organic carbon (DOC) in the ocean (Benner 2002). The absolute and relative concentrations of amino acids in seawater are often used to estimate the freshness or the state of degradation of DOM (Amon et al. 2001; Benner 2002). DOC-normalized yields of amino acids are usually highest in freshly produced DOM but quickly decrease as degradation proceeds and bioavailability decreases (Cowie and Hedges 1994; Benner and Kaiser 2003). These yields are used as indicators of degradation especially during the early stages of DOM diagenesis (Davis et al. 2009; Kaiser and Benner 2009). Furthermore, the relative abundance of individual amino acids changes during degradation. This relation has been used to develop a degradation index based on amino acid composition (DI) in sediments (Dauwe and Middelburg 1998; Dauwe et al. 1999). The DI works most reliably on annual to decadal time scales of organic matter diagenesis (Davis et al. 2009). A modified degradation index (DI_{DAA}) was developed recently for DOM by Kaiser and Benner (2009) as amino acid composition as well as degradation processes in sediments differ for DOM in the water column.

The DOM pool is tightly linked to the particulate organic matter pool (POM). DOM compounds may act as precursors for gel particles such as the transparent exopolymer particles (TEP) through coagulation in the water (Chin et al. 1998; Engel et al. 2004b; Verdugo et al. 2004). TEP play an important role in aggregation and export of organic matter (Logan et al. 1995; Passow et al. 2001; Engel 2004) but also act as a structural unit for bacteria to attach and grow (Verdugo 2012).

Largest changes in organic matter composition occur during the early stages of heterotrophic degradation, when degradation rates are at a maximum and a lot of labile material is still available (Harvey et al. 1995). In total, up to 90% of the fresh DOM produced during photosynthesis is degraded by heterotrophic bacteria in the water column (Martin et al. 1987; Field et al. 1998; del Giorgio and Duarte 2002) and transformed into bacterial biomass or respired to inorganic carbon which potentially feeds back in atmospheric CO₂ concentrations (Suess 1980; Martin et al. 1987). Heterotrophic bacteria preferentially consume small compounds such as single amino acids and carbohydrates (Amon et al. 2001; Meon and Kirchman 2001; Benner 2002). In order to break-down larger molecules into single units that can be taken up by the cell, bacteria release hydrolytic enzymes, e.g. aminopeptidase, glucosidase and phosphatase (Hoppe 1991). During microbial degradation, small organic compounds may be produced that cannot be further degraded and remain in the water as refractory DOM (Kaiser and Benner 2009). The microbial carbon pump was proposed as an important mechanism for the sequestration of atmospheric CO₂ to the ocean as refractory DOM (Jiao et al. 2010). DOM that resisted degradation is exported from the surface ocean to deeper waters by mixing and down welling of water (Hansell et al. 2009).

The increase in atmospheric carbon dioxide (CO₂) concentration due to anthropogenic emissions is changing the ocean's carbonate chemistry (Zalasiewicz et al. 2008; Feely et al. 2009). As an indicator of ocean acidification, pH of ocean surface waters has decreased by about 0.1 since 1900 (Caldeira and Wickett 2003) and by the year 2100, a further decrease by 0.3-0.4 units from current conditions is expected (Wolf-Gladrow et al. 1999; Feely et al. 2009). Ocean acidification has potential effects on physiology of primary producers as well as heterotrophic organisms. Increasing CO₂ partial pressure (*p*CO₂) mostly enhances primary production (Hein and Sandjensen 1997; Riebesell et al. 2007; Egge et al. 2009) and decreasing pH stimulates the hydrolytic break-down of labile DOM by marine bacteria (Grossart et al. 2006; Cunha et al. 2010; Piontek et al. 2010; Endres et al. 2013; Endres et al. in prep.).

The response of a natural plankton community and biogeochemical processes to CO₂-enduced changes in seawater pH was determined during a large-scale mesocosm experiment in a Norwegian fjord. Plankton and DOM dynamics were followed in nine mesocosms over five weeks under *in situ* conditions. The combination of increased availability of TEP as substrate and enhanced enzymatic hydrolysis of organic matter supported higher bacterial abundance at elevated *p*CO₂ (Endres et al. in prep.). As heterotrophic bacteria are the main sink for fresh DOM, we focused on changes in the concentration, composition, and degradation of DOM during the mesocosm experiment.

The effects of increasing *p*CO₂ on DOM dynamics have only been rarely studied. No statistically significant effect of CO₂ on DOC concentrations was detected in earlier mesocosm experiments (Grossart et al. 2006; Kim et al. 2006; Schulz et al. 2008) although the amount of gel particles was shown to increase with *p*CO₂ (Engel et al. 2004a; Endres et al. in prep.). Amino acid accumulation was found to be slightly lower at elevated *p*CO₂ (Grossart et al. 2006) but was not affected by

rising temperature during another mesocosm experiment (Engel et al. 2011). However, so far no study has investigated compositional changes of amino acids in seawater in plankton communities under ocean acidification. In this study, concentrations of DOC, DON and amino acids were followed during the 35-day large-scale mesocosm study at the Marine Biological Station (University of Bergen). We focused on the response of amino acid composition and yields to increased $p\text{CO}_2$ levels in natural plankton community.

4.4.3 Methods

Experimental set-up and bloom development

A detailed description of the experimental setup, its deployment, technical features and the sampling methods are described by Riebesell et al. (2012) and Schulz et al. (2013). Briefly, nine 25m-long, free-floating Kiel Off-Shore Mesocosms for Future Ocean Simulations (KOSMOS) with flexible thermoplastic polyurethane bags were deployed in the Raunefjord near Bergen in southern Norway. The average salinity in the mesocosm was 32.0 ± 0.1 psu. Temperature varied between 6.8°C at the beginning (end of April) and 10.0°C at the end of the experiment in June. Seven mesocosms were adjusted over five days to $p\text{CO}_2$ target levels 400 (M6), 600 (M8), 900 (M1), 1200 (M3), 1300 (M5), 2000 (M7) and 3000 (M9) μatm by stepwise addition of CO_2 saturated seawater. Two mesocosms (M2, M4) were used as control treatments at *in situ* $p\text{CO}_2$ of around 300 μatm . The first sampling day before CO_2 addition was termed “day -1”. The first sampling day with CO_2 addition was “day 1”. After CO_2 addition, samples were taken with a depth-integrating water sampler (1-23m depth, Hydrobios Kiel, Germany) every day for 35 days from all nine mesocosms.

The first phytoplankton bloom developed around day 3 with increasing chlorophyll *a* (chl *a*) values of up to $3.5 \pm 0.3 \mu\text{g L}^{-1}$. Afterwards, chl *a* concentrations declined. On day 14, inorganic nutrients ($5 \mu\text{mol L}^{-1}$ nitrate and $0.1 \mu\text{mol L}^{-1}$ phosphate) were added to the enclosed, nutrient-poor waters to initiate a second phytoplankton bloom, which reached a maximum of $3.9 \pm 0.6 \mu\text{g L}^{-1}$ chl *a* between days 19 and 20, declining to $1.4 \pm 0.5 \mu\text{g L}^{-1}$ by day 34 (Figure 2). In terms of biomass, both blooms were dominated by nano- and phycoflagellates, mainly *Plagioselmis prolonga* (Cryptophyta). Bacterial abundance increased over time in all treatments up to $2.0 \pm 1.1 \times 10^6$ cells mL^{-1} on day 25. The average pH in the phase before nutrient addition ranged between $\text{pH } 8.09 \pm 0.01$ in the control mesocosms and $\text{pH } 7.38 \pm 0.27$ in the highest $p\text{CO}_2$ mesocosm. After nutrient addition, pH ranged between $\text{pH } 8.11 \pm 0.01$ in the control mesocosms and $\text{pH } 7.49 \pm 0.14$ in the highest $p\text{CO}_2$ mesocosm.

pH measurements

pH was determined in 10 cm cuvettes at 25°C with a VARIA Cary 100 spectrophotometer as described by Dickson (2010). The precision was typically better than 0.001 at high and 0.002 at low pH. Measured pH values were recalculated to *in situ* pH. pH values are given relative to the total scale.

Chlorophyll a analysis

For chlorophyll *a* (chl *a*) concentrations were measured as described by Welschmeyer (1994). Between 250 – 500 mL of sample was filtered onto Whatman GF/F filters and stored at -80°C for

at least 24 h. Samples were homogenized with 90% acetone and glass beads (2 and 4 mm) in a cell mill, centrifuged, and chl *a* concentrations were measured with a TURNER 10-AU fluorometer.

Dissolved organic carbon and nitrogen

Samples for dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) analysis were filtered through combusted GF/F filters, collected in 20 mL combusted (8 h, 500°C) glass ampoules and acidified with 80 µL of 85% phosphoric acid. Samples were stored at 0-2°C for 12 months until analysis. DOC and TDN concentrations were determined simultaneously in the filtrate by high temperature catalytic oxidation with a Shimadzu TOC-VCSH analyzer equipped with a Shimadzu TNM-1 module. DOC and TDN concentrations are average values of quadruplicate measurements. Values of TDN are corrected for nitrate, nitrite, and ammonium, and thereafter referred to as dissolved organic nitrogen (DON). Total organic carbon (TOC) values were calculated by summing DOC and particulate organic carbon (POC, data provided by K. Schulz).

Total and dissolved amino acid composition

Total (TAA) and dissolved (DAA) amino acid concentration and composition were determined on day 0 and every other day from day 1 until day 31, except for DAA on day 21. For TAA analysis, 6mL samples were filled in combusted (8h, 500°C) glass vials and stored at -20°C. Samples for DAA analysis were filtered through 0.45 µm filters (GHP membrane, Acrodisk, Pall Corporation) before storage as for TAA. Amino acids were analysed according to Dittmar et al. (2009) and Lindroth & Mopper (1979). 1mL of sample and 1mL of 30% hydrochloric acid (Merck, suprapure) were hydrolysed in sealed ampoules at 100°C for 20h. The hydrolysate was dried in a microwave under nitrogen atmosphere and was washed twice with 0.5mL of ultrapure water to remove the HCl. Finally the samples were re-dissolved in 1mL ultrapure water. Amino acids were separated by HPLC (1260, Agilent) equipped with a C18 column (Phenomenex Kinetex, 2.6µm, 150 x 4.6mm) after in-line derivatization (2 min.) with o-phthaldialdehyde and mercaptoethanol. For solvent A 0.1 M sodiumdihydrogenphosphate was adjusted to pH 7 with sodium hydroxide and premixed with acetonitrile (19:1 v/v). Solvent B was acetonitrile. A linear gradient was run starting from 6% solvent B to 27% solvent B in 40 min at a flow rate of 0.8 mL/min. Standards were used for asparagine + aspartic acid (AsX), glutamine + glutamic acid (GIX), serine (Ser), glycine (Gly), threonine (Thr), arginine (Arg), alanine (Ala), tyrosine (Tyr), valine (Val), isoleucine (Ileu), phenylalanine (Phe), and leucine (Leu). Standards were run in the beginning and after each 5th sample. Single amino acid concentrations are given in mol percentage (mol%) of the total amount of amino acids. The degradation index (DI_{DAA}) of DOM was calculated according to Dauwe & Middelburgh (1999) with modified correlation factors to make the DI applicable to DOM (Kaiser and Benner 2009; and personal communication). The carbon content of DAA (DAA-C) was

normalized to the amount of dissolved organic carbon and nitrogen and is reported as %OC and %ON, respectively.

Data analysis and statistics

The temporal increase in DOC and DON concentrations was evaluated by linear regression analysis. The relation between chl *a* and Arg concentration was assessed by the Pearson Product Moment Correlation analysis and cross correlation analysis. Cross-correlations are useful for determining the time delay between two signals. For cross-correlation analysis, a lag between -8 and +8 days was tested. The pairs of variables with positive cross-correlation coefficients tend to increase together. After calculating the cross-correlation between the two signals for different time shifts, the maximal cross-correlation coefficient indicates the time shift where the signals are best aligned. Best fit was calculated for lag of -4 days. The relation between DI_{DAA} and DOC-normalized yield of amino acids (DAA %OC) was analyzed by linear regression analysis. To test for a pH effect on DOM concentrations, the daily deviation (AD_i) of each mesocosm was calculated by subtracting observed concentration (X_i) from the average concentration of all mesocosms (\bar{X}) on the specific sampling day ($AD_i = X_i - \bar{X}$). These daily deviations were then averaged over time according to $\frac{1}{N} \sum_{i=1}^N (AD_i)$, with N being the number of sampling days, in order to get the mean deviations (MD) of each mesocosm regarding a particular parameter. The mean deviations were tested against average pH of the different mesocosms by linear regression. The significance level of the ANOVA was set to $p < 0.05$. Statistical analysis and illustration of the data were performed using the software packages WinSTAT (R. Fitch Software) and SigmaPlot 12.0 (Systat).

4.4.4 Results

Temporal trends in bulk DOM and amino acid concentrations

Dissolved organic carbon (DOC) and nitrogen (DON) values were determined daily from day -1 to day 33. Initial values in the nine mesocosms on day -1 were in average $99.4 \pm 3.6 \mu\text{M}$ DOC and $6.9 \pm 0.5 \mu\text{M}$ DON (Figure 1a,b). DOC concentrations decreased to $93.2 \pm 2.1 \mu\text{M}$ on day 1 and then constantly increased up to $131.1 \pm 4.4 \mu\text{M}$ on day 31 (Figure 1a). Highest increase was observed in M6 with $44.8 \mu\text{M}$ over 30 days. Two periods with high DOC accumulation were found. From day 1 until day 13, corresponding to the phase of the first phytoplankton bloom, DOC increased by $1.6 \pm 0.2 \mu\text{M C d}^{-1}$ ($p < 0.001$). After the chlorophyll *a* peak of the second phytoplankton bloom, between day 21 and 31, DOC concentrations increased by $1.3 \pm 0.3 \mu\text{M C d}^{-1}$ ($p = 0.006$). In between these two phases no significant DOC accumulation could be detected. DON concentrations were slightly increased by $1.8 \pm 0.9 \mu\text{M}$ until day 31, but variations between sampling days were higher than the overall increase (Figure 1b). Highest average DON concentrations were measured on day 13 with $8.9 \pm 0.8 \mu\text{M}$ and on day 32 with $8.8 \pm 0.9 \mu\text{M}$. On the other hand, low DON concentrations were determined on day 3 with $6.2 \pm 0.5 \mu\text{M}$ and day 17 with $5.4 \pm 0.7 \mu\text{M}$.

On day 0, TAA concentrations in the nine mesocosms were $397 \pm 45 \text{ nM}$ (Table 1). TAA concentrations increased in all treatments (Figure 1c) by $14 \pm 3 \text{ nM d}^{-1}$ ($p < 0.001$) reaching highest values on day 21 with $837 \pm 104 \text{ nM}$ and decreased to $679 \pm 109 \text{ nM}$ on day 31 (Table 1). DAA concentrations were $287 \pm 30 \text{ nM}$ on day 0 and significantly increased from day 13 on by around $19 \pm 2 \text{ nM d}^{-1}$ ($p = 0.001$) until reaching a maximum of $422 \pm 111 \text{ nM}$ on day 23. Afterwards, concentrations decreased to $383 \pm 11 \text{ nM}$ (Table 1). However, variation between sampling days was higher than the increase in DAA per day (Figure 1d). The carbon and nitrogen contents of TAA and DAA were calculated and compared to the organic carbon and nitrogen pool. TAA accounted for 0.4 - 0.7 %OC and 3.3 - 13.1 %ON. Both yields were highest during the phytoplankton blooms (day 2-5 and day 17-21) indicating an enrichment of amino acids in the new biomass. DAA accounted for 0.8 - 1.3 %OC and 3.4 - 7.3 %ON, respectively (Table 1). These yields were relatively stable over time compared to the yields of the total fraction.

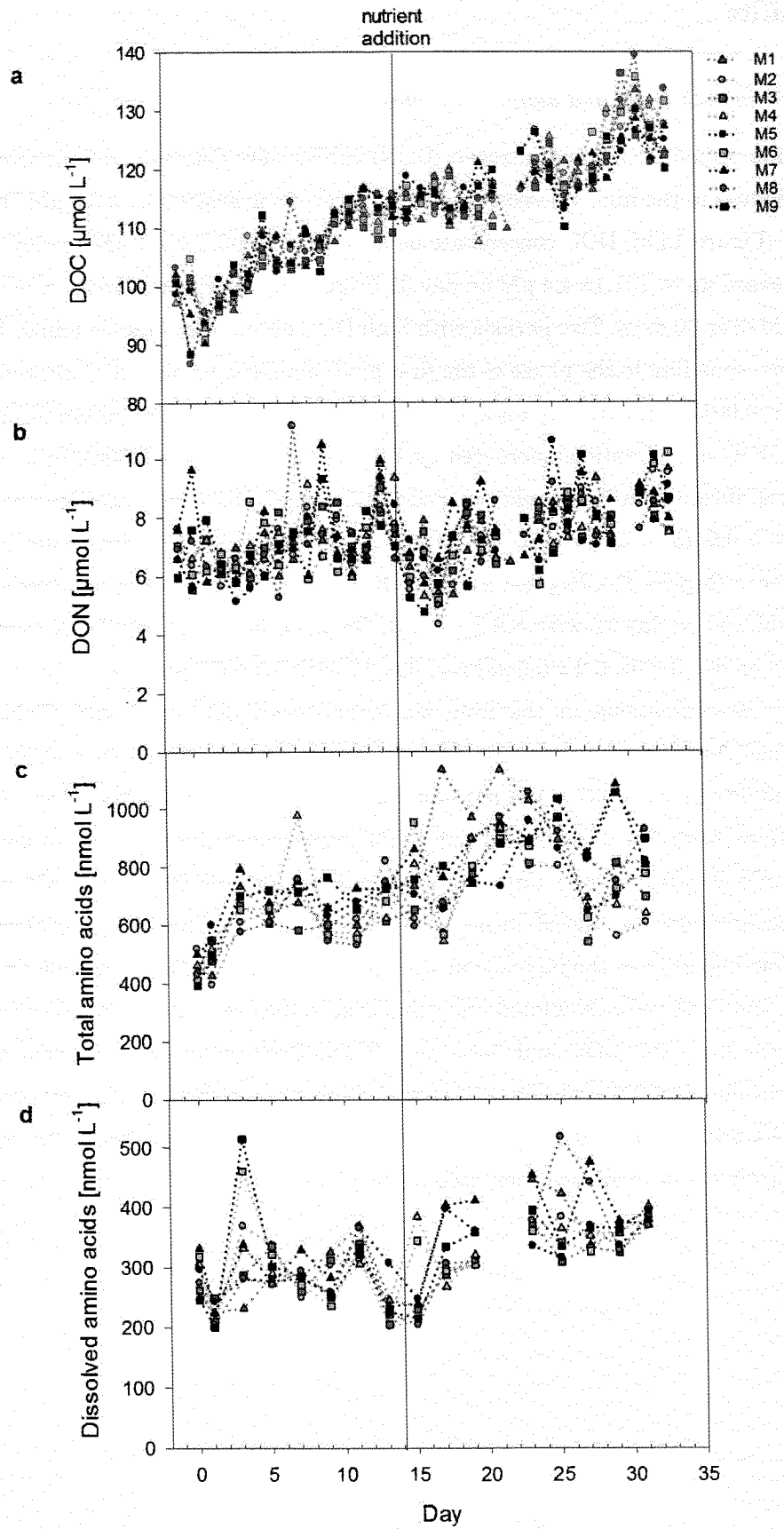


Figure 1 Temporal development of dissolved organic carbon (DOC), dissolved organic nitrogen (DON), total and dissolved amino acid (TAA and DAA) concentrations.

Table 1 Average concentration, carbon and nitrogen contribution (%), and mol percentages (mol%) of total and dissolved amino acids over time.

Values give the mean and standard deviation of all nine mesocosms.

	Day	concentration nM	%OC %	%ON %	AsX mol%	GIX	Ser	Gly	Thr	Arg	Ala	Tyr	Val	Ileu	Phe	Leu
TAA	0	397 ± 45	0.4 ± 0	7.1 ± 1	15 ± 0	13 ± 0	8 ± 0	25 ± 1	7 ± 0	3 ± 0	11 ± 0	2 ± 0	4 ± 0	3 ± 0	2 ± 0	5 ± 0
	1	444 ± 64	0.5 ± 0.1	7.8 ± 1.5	15 ± 0	14 ± 0	8 ± 1	24 ± 1	6 ± 0	3 ± 0	11 ± 0	3 ± 0	4 ± 0	3 ± 0	3 ± 0	5 ± 0
	3	601 ± 65	0.6 ± 0.1	3.3 ± 0.9	16 ± 0	16 ± 1	8 ± 1	20 ± 0	6 ± 0	5 ± 0	11 ± 0	3 ± 0	5 ± 0	3 ± 0	3 ± 0	5 ± 0
	5	565 ± 38	0.5 ± 0	9.5 ± 1.1	15 ± 1	16 ± 1	9 ± 1	21 ± 2	6 ± 0	5 ± 0	11 ± 1	3 ± 0	4 ± 0	3 ± 0	3 ± 0	5 ± 0
	7	652 ± 111	0.6 ± 0.1	11.7 ± 2.5	15 ± 1	14 ± 0	8 ± 1	22 ± 1	5 ± 0	6 ± 0	10 ± 2	3 ± 0	4 ± 1	3 ± 0	3 ± 0	5 ± 0
	9	524 ± 70	0.5 ± 0.1	8.7 ± 0.7	15 ± 1	16 ± 0	8 ± 0	21 ± 1	6 ± 0	6 ± 0	11 ± 0	3 ± 0	5 ± 0	3 ± 0	3 ± 0	5 ± 0
	11	549 ± 64	0.5 ± 0.1	9.4 ± 1	14 ± 0	15 ± 0	8 ± 0	24 ± 1	7 ± 0	4 ± 0	11 ± 0	3 ± 0	2 ± 0	3 ± 0	3 ± 0	5 ± 0
	13	633 ± 65	0.6 ± 0.1	8.7 ± 1.3	15 ± 1	15 ± 0	8 ± 1	20 ± 2	7 ± 1	4 ± 0	12 ± 0	3 ± 0	4 ± 0	4 ± 0	3 ± 0	5 ± 0
	15	660 ± 117	0.6 ± 0.1	11.9 ± 1.9	15 ± 1	15 ± 1	8 ± 1	22 ± 1	7 ± 0	4 ± 0	11 ± 1	3 ± 0	4 ± 0	4 ± 0	3 ± 0	5 ± 0
	17	621 ± 182	0.6 ± 0.1	12.1 ± 2.8	15 ± 0	16 ± 0	7 ± 1	21 ± 1	7 ± 0	4 ± 0	11 ± 0	2 ± 0	4 ± 0	4 ± 0	3 ± 0	6 ± 0
	19	750 ± 84	0.7 ± 0.1	12.9 ± 6.2	15 ± 1	16 ± 1	7 ± 1	20 ± 1	7 ± 0	4 ± 1	11 ± 0	3 ± 0	4 ± 1	5 ± 1	3 ± 0	6 ± 0
	21	837 ± 104	0.7 ± 0.1	13.1 ± 1.7	15 ± 1	16 ± 1	7 ± 1	19 ± 2	6 ± 0	6 ± 0	10 ± 0	3 ± 0	5 ± 0	3 ± 0	3 ± 0	5 ± 0
	23	813 ± 88	0.7 ± 0.1	13 ± 1.5	16 ± 1	16 ± 0	7 ± 0	19 ± 1	7 ± 0	6 ± 0	10 ± 0	3 ± 0	4 ± 0	3 ± 0	3 ± 0	5 ± 0
	25	776 ± 77	0.7 ± 0.1	11.7 ± 1.8	15 ± 1	15 ± 1	9 ± 2	21 ± 2	7 ± 1	6 ± 1	10 ± 0	3 ± 0	5 ± 1	3 ± 0	3 ± 0	5 ± 0
	27	612 ± 108	0.5 ± 0.1	8.7 ± 0.7	14 ± 1	14 ± 1	9 ± 1	22 ± 2	7 ± 0	4 ± 0	11 ± 1	3 ± 0	4 ± 0	3 ± 0	3 ± 0	6 ± 0
	29	680 ± 173	0.6 ± 0.1	10.7 ± 1.9	15 ± 1	15 ± 1	9 ± 0	21 ± 2	7 ± 1	4 ± 0	12 ± 1	3 ± 1	4 ± 0	3 ± 1	3 ± 0	5 ± 0
	31	679 ± 109	0.6 ± 0.1	9.6 ± 1.5	15 ± 1	15 ± 1	9 ± 0	21 ± 1	7 ± 1	5 ± 0	11 ± 0	3 ± 0	4 ± 0	3 ± 0	3 ± 0	6 ± 0
DAA	0	287 ± 30	1 ± 0.1	5.4 ± 0.9	14 ± 1	12 ± 1	11 ± 1	32 ± 2	8 ± 1	3 ± 0	11 ± 1	2 ± 0	3 ± 1	2 ± 1	1 ± 0	2 ± 1
	1	220 ± 17	0.9 ± 0.1	3.9 ± 0.6	12 ± 0	9 ± 1	8 ± 1	29 ± 1	7 ± 0	3 ± 0	17 ± 1	2 ± 0	3 ± 1	2 ± 1	2 ± 0	4 ± 1
	3	344 ± 91	1.3 ± 0.4	6.8 ± 2.1	15 ± 1	12 ± 1	11 ± 1	29 ± 2	7 ± 1	3 ± 1	11 ± 1	2 ± 0	3 ± 0	2 ± 0	2 ± 0	3 ± 1
	5	299 ± 26	1 ± 0.1	5.3 ± 0.7	14 ± 1	13 ± 1	10 ± 1	28 ± 1	7 ± 1	3 ± 0	13 ± 1	2 ± 0	3 ± 0	2 ± 0	2 ± 0	3 ± 0
	7	282 ± 22	1 ± 0.1	4.6 ± 0.7	14 ± 1	12 ± 1	10 ± 1	29 ± 1	9 ± 1	2 ± 0	13 ± 0	1 ± 0	3 ± 1	2 ± 1	2 ± 0	2 ± 0
	9	275 ± 32	1 ± 0.1	4.4 ± 0.8	14 ± 1	10 ± 1	6 ± 2	32 ± 1	8 ± 1	4 ± 0	12 ± 0	2 ± 0	3 ± 1	2 ± 0	2 ± 0	4 ± 1
	11	338 ± 24	1.1 ± 0.1	6.1 ± 0.6	13 ± 1	10 ± 1	10 ± 1	31 ± 1	8 ± 1	2 ± 0	12 ± 1	2 ± 0	2 ± 0	3 ± 0	2 ± 0	3 ± 0
	13	235 ± 31	0.8 ± 0.1	3.4 ± 0.4	17 ± 1	12 ± 1	11 ± 1	26 ± 1	8 ± 0	4 ± 0	12 ± 1	1 ± 1	4 ± 1	2 ± 1	1 ± 0	2 ± 1
	15	257 ± 63	0.8 ± 0.2	5.3 ± 1.3	16 ± 1	11 ± 2	10 ± 3	31 ± 2	8 ± 1	3 ± 0	11 ± 1	1 ± 1	3 ± 1	1 ± 1	0 ± 0	2 ± 0
	17	319 ± 49	1 ± 0.2	7.3 ± 0.6	14 ± 1	11 ± 1	9 ± 1	30 ± 1	8 ± 0	3 ± 0	12 ± 1	2 ± 1	4 ± 1	2 ± 1	1 ± 0	2 ± 0
	19	331 ± 37	1.1 ± 0.1	5.4 ± 1.1	15 ± 1	12 ± 1	9 ± 1	30 ± 1	8 ± 0	3 ± 0	12 ± 1	2 ± 0	3 ± 1	2 ± 1	1 ± 0	2 ± 0
	23	422 ± 111	1.3 ± 0.1	7.1 ± 1.1	16 ± 3	11 ± 2	10 ± 3	27 ± 1	9 ± 1	3 ± 0	11 ± 1	2 ± 0	4 ± 1	2 ± 1	2 ± 0	3 ± 1
	25	369 ± 65	1.1 ± 0.2	5.8 ± 1	17 ± 1	12 ± 1	9 ± 1	27 ± 2	8 ± 0	3 ± 0	11 ± 1	2 ± 0	4 ± 1	2 ± 1	2 ± 0	2 ± 0
	27	375 ± 54	1.2 ± 0.2	5.3 ± 0.6	14 ± 1	11 ± 1	11 ± 2	27 ± 1	10 ± 1	2 ± 0	12 ± 1	1 ± 0	3 ± 1	2 ± 1	2 ± 0	3 ± 1
	29	350 ± 20	1.1 ± 0.1	5.8 ± 0.4	15 ± 1	11 ± 1	9 ± 0	27 ± 1	11 ± 1	4 ± 0	11 ± 0	2 ± 0	3 ± 1	2 ± 1	2 ± 0	3 ± 1
31	383 ± 11	1.1 ± 0	5.4 ± 0.3	14 ± 1	11 ± 1	10 ± 1	28 ± 1	9 ± 0	2 ± 0	12 ± 0	2 ± 0	4 ± 1	2 ± 1	2 ± 0	3 ± 0	

Amino acid composition

The most abundant amino acid in both, total and dissolved, fractions was Gly with 29 to 32 mol%, followed by AsX and Glx with 12 – 17 mol% and 9 – 16 mol%, respectively. Phe and Tyr were the least abundant amino acids with 1-3 mol% (Table 1). Amino acid composition in the nine mesocosms showed only minor variations over time except for Arg, Phe, Thr, Ileu, and Gly.

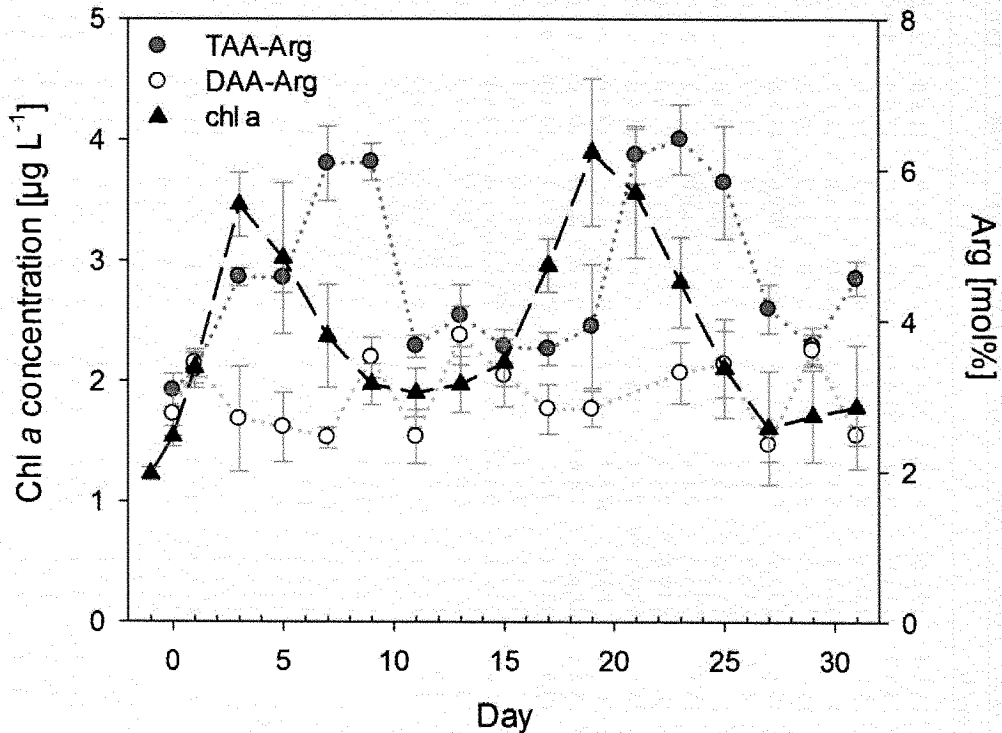


Figure 2 Temporal developments of chlorophyll a as well as total and dissolved arginine (TAA-Arg and DAA-Arg) concentrations. Symbols indicate the mean of nine mesocosms and standard deviations.

Total Arg in all mesocosms ranged between 3 and 6 mol% during the experiment. A first peak of 6 ± 0 mol% was observed around day 7, followed by a decrease until day 17 to 4 ± 0 mol%. The second peak was measured around day 25 reaching 6 ± 1 mol% (Table 1). Arg was not directly correlated with chl *a*, but total Arg peaks followed the chl *a* development with some days delay (Figure 2). We performed a cross-correlation analysis and determined a strong correlation between both data sets when shifted by minus 4 days (Table 2) indicating that Arg followed the chl *a* development with a four day delay. The significance of this correlation was confirmed by Pearson Moment Correlation with transformed Arg data ($p < 0.02$, $n=16$). We did not observe these trends and correlations in the dissolved fraction (Figure 2).

Table 2 Cross-correlation analysis of chlorophyll α concentration and mol percentage of total arginine. A time shift of the arginine data (lag) between -10 and + 10 days was tested. The pairs of variables with positive cross-correlation coefficients tend to increase together. Highest correlation (highlighted) was calculated for lag = -4 days.

1st variable:	chl α	lag [d]	cross-correlation coefficient									
			M1	M2	M3	M4	M5	M6	M7	M8	M9	Mean
		-8	-0.48	-0.37	-0.40	-0.41	-0.31	-0.15	-0.40	-0.25	-0.47	-0.39
		-6	0.24	0.40	0.13	0.20	0.17	0.37	0.25	0.30	0.03	0.30
		-4	0.75	0.72	0.69	0.87	0.76	0.71	0.81	0.64	0.60	0.83
		-2	0.63	0.69	0.69	0.62	0.59	0.50	0.73	0.46	0.67	0.69
		0	0.27	0.33	0.31	0.13	0.28	0.03	0.17	0.05	0.32	0.19
		2	-0.36	-0.32	-0.23	-0.31	-0.28	-0.55	-0.39	-0.44	-0.18	-0.43
		4	-0.64	-0.57	-0.64	-0.60	-0.72	-0.67	-0.66	-0.52	-0.50	-0.71
		6	-0.55	-0.57	-0.67	-0.59	-0.76	-0.47	-0.71	-0.46	-0.72	-0.66
		8	-0.30	-0.42	-0.32	-0.34	-0.31	-0.13	-0.28	-0.12	-0.30	-0.28

The proportion of dissolved Thr increased over time in all treatments from 8 ± 1 mol% on day 0 up to 11 ± 1 mol% on day 29 and then decreased to 9 ± 0 mol% until day 31. In contrast, Thr was consumed or degraded more compared to other DAAs in the total fraction during the first phytoplankton bloom until day 7 but then accumulated again up to 7 ± 0 mol% by the end of the experiment (Figure 3a). The proportion of Phe in the total fraction remained stable around 2-3 mol% during the experiment. However, Phe increased slightly from 1 ± 0 mol% on day 0 up to 2 ± 0 mol% on day 9 in the dissolved fraction. Afterwards the proportion of Phe decreased until it almost disappeared on day 15 (0 ± 0 mol%), but then increased again up to 2 ± 0 mol% on day 31 (Figure 3b). As highest dissolved Phe values were measured in the post-bloom phases, we assume that it may be a product of cell lysis or during heterotrophic degradation. The amount of Ileu in the total fraction was around 3 ± 0 mol% except for the second phytoplankton bloom phase between day 13 to 19 when Ileu concentrations reached up to 5 ± 1 mol% (Table 1, Figure 3c). In the dissolved fraction, Ileu concentrations ranged between 1-3 mol% and variations were relatively high so no clear peak could be observed.

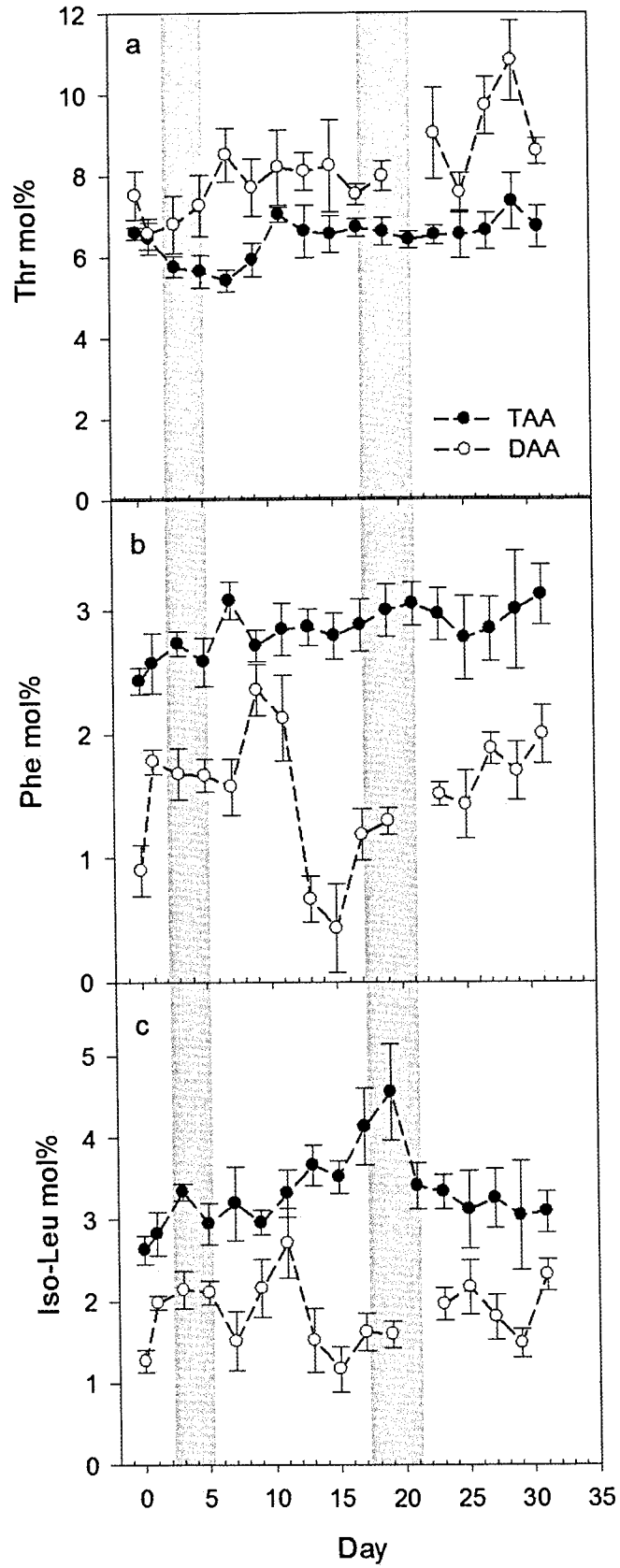


Figure 3 Temporal development of total and dissolved threonine (Thr), phenylalanine (Phe), isoleucine (Ileu). Symbols show the mean of nine mesocosms and standard deviation. Shaded areas indicate the maximum of chlorophyll a concentrations.

Diagenetic status of DOM

Two amino acid-based indicators for DOM diagenesis were compared (Figure 4). The degradation index (DI_{DAA}) for DOM was calculated according to Kaiser and Benner (2009) following the calculations of Dauwe et al. (1999). This index is based on the molecular composition of amino acids because amino acids vary in their diagenetic reactivity (Dauwe et al., 1999). Negative values indicate degraded material, while positive values indicate freshly produced DOM. To determine bioavailability, DOC-normalized yields of DAA (DAA %OC) were calculated as the percentage of DOC detected in amino acids. Degraded DOM has an average DOC-normalized yield of amino acid of 0.7% (Davis and Benner 2007). This fraction of amino acids resists microbial degradation and indicates refractory organic matter. DOM with yields greater than 0.7% DOC is assumed to be (semi-)labile and accessible to degradation (Shen et al. 2012).

Average DI_{DAA} values ranged between 0.47 – 1.22 on day 0. DOC-normalized DAA yields were 0.88 - 1.27 DAA %OC. The freshness of the DOM increased during the first chl a peak up to 2.06 (DI) and 1.90 DAA %OC on day 3. The DI remained high until day 13 and then dropped to -0.12 – 1.13 on day 15. When the second phytoplankton peak developed, DI increased up to 1.20 – 2.46 on day 23 and declined slightly afterwards (Figure 4). DOC-normalized DAA yields decreased from day 3 on until day 13 to 0.66 – 1.00 DAA %OC. Afterwards they increased, while the second phytoplankton bloom developed, reaching 1.20-1.46% DOC on day 23 (Figure 4). Calculated values of both methods correlated significantly (Figure 5, $r = 0.41$, $p < 0.001$), suggesting that both can be used to describe the diagenetic status of fresh DOM in surface waters and in the context of phytoplankton blooms.

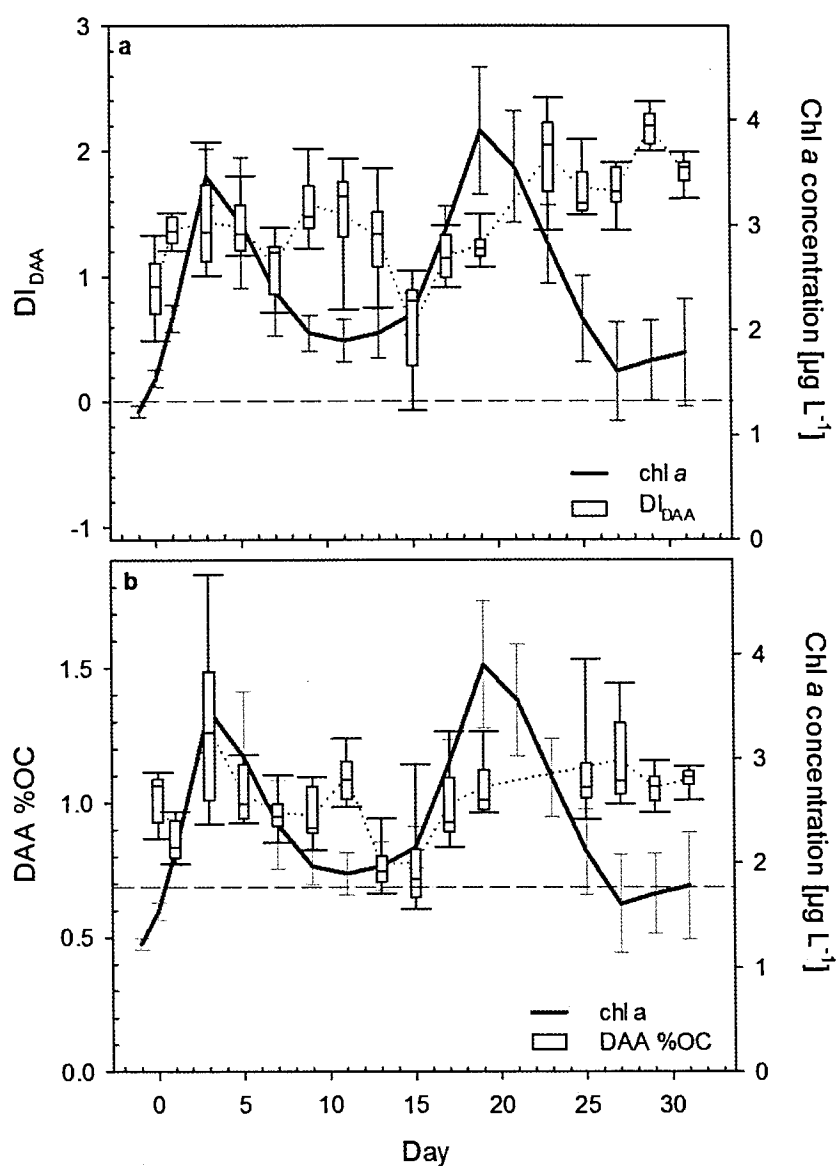


Figure 4 Box Plot illustration of the temporal development of the amino-acid based degradation index (DI_{DAA}) and the carbon-normalized yield of dissolved amino acids (DAA %OC) compared to the average chlorophyll a development (solid line). The horizontal line within the box marks the median, and the length of the box represents the interquartile range which is the difference between the 75th and 25th percentiles so that 50% of the cases have values within the box. Whiskers (error bars) above and below the box indicate the 95th and 5th percentiles. Dashed lines in the graphs mark the lower border for fresh (bioavailable) organic matter.

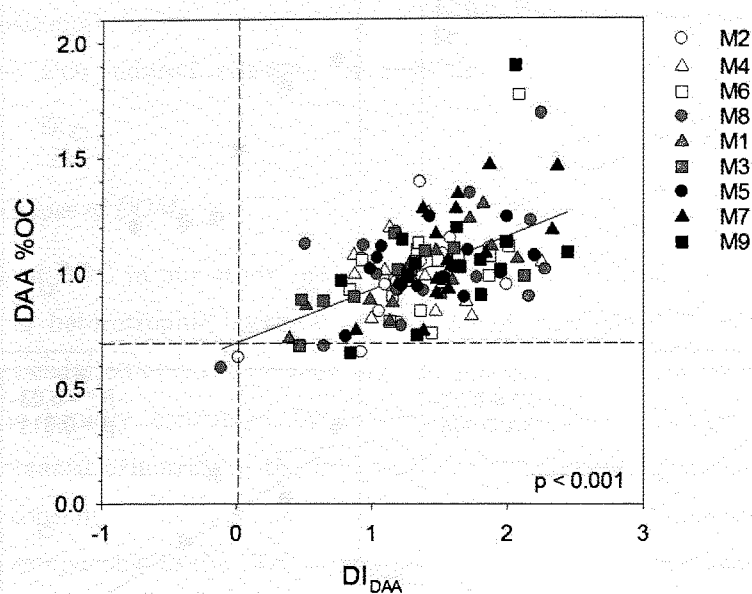


Figure 5 Linear Regression Analysis of the amino-acid based degradation index (DI_{DAA}) and the carbon-normalized yield of dissolved amino acids (DAA %OC).

Effect of seawater pH on DOM dynamics

The mean deviations of DOC, DON, TAA, DAA concentrations, and amino acid composition of the nine mesocosm were tested for dependence on CO_2 -induced changes in seawater pH. No significant effect of pH on DOC, DON and AA concentrations was detected (data not shown). In general, amino acid compositions were similar between mesocosms. Four amino acids could be identified where pH had an effect on their relative concentration (Figure 6). In the total fraction, concentrations of Phe, Thr, and Ileu were significantly higher (0.2 – 0.4 mol%) in lowest pH compared to the highest pH mesocosms. In contrast, the concentration of Gly was 1 mol% lower in the low pH mesocosms compared to the control mesocosms ($p < 0.05$). In the dissolved fraction, Phe and Gly concentrations were not significantly affected by pH. The relative concentrations of Thr and Ileu showed the same trend as in the total fraction with higher concentrations 0.8 mol% and 0.2 mol% ($p < 0.05$) respectively in the lowest pH mesocosm. Overall the amino acid composition was relatively stable over time and between treatments. Any compositional changes of single amino acids were small (≤ 1 mol%).

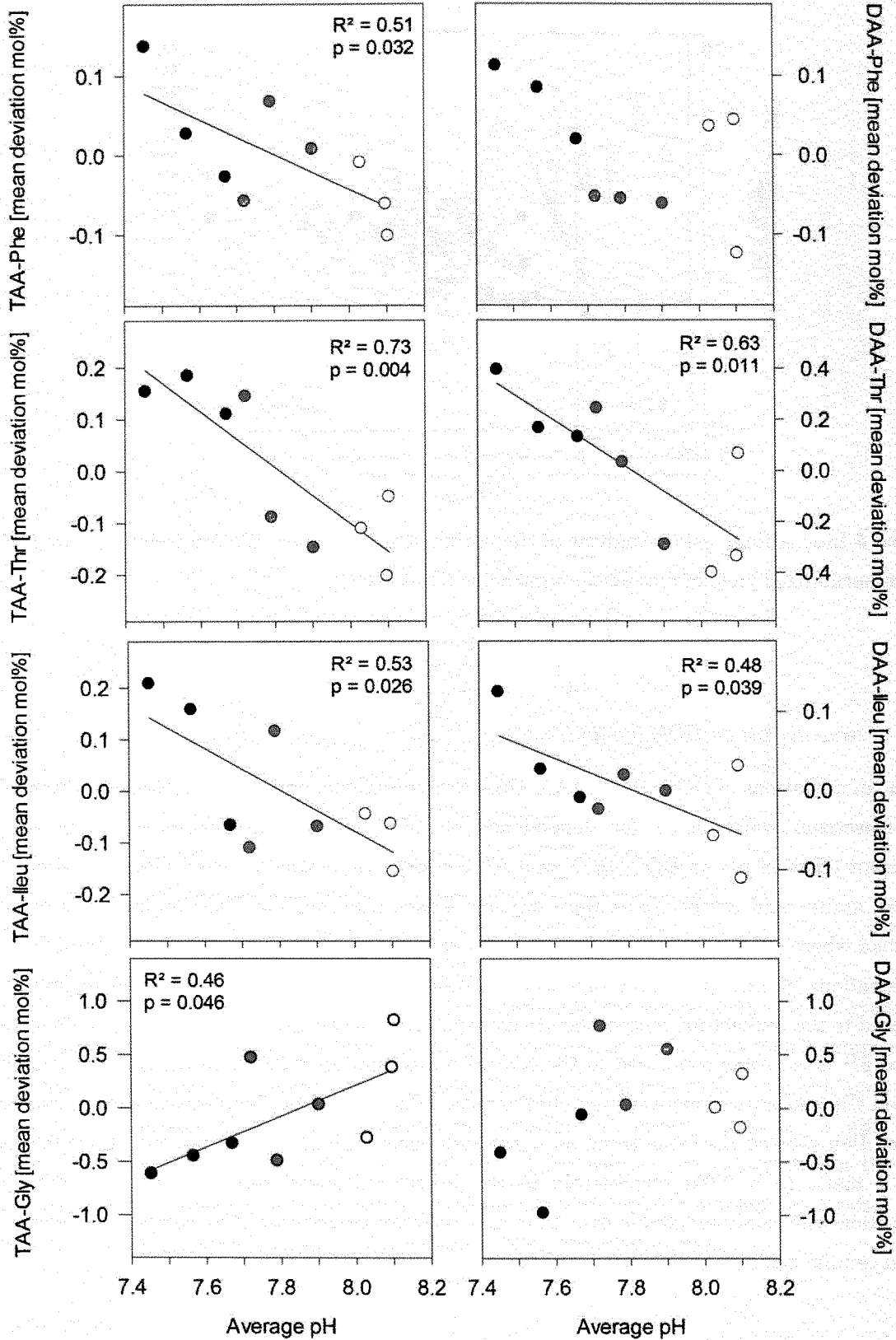


Figure 6 Mean deviation of total and dissolved phenylalanine (Phe), threonine (Thr), isoleucine (Ileu), and glycine (Gly) compared to average pH in the appropriate mesocosms. Linear Regression lines indicate a significant correlation ($p < 0.05$).

4.4.5 Discussion

We studied compositional changes in amino acids as well as DOM dynamics in a natural plankton community under CO₂-enduced changes in seawater pH during a large-scale mesocosm experiment.

Net production and loss of DOM

The accumulation of DOC in our study indicates either the production of refractory DOM or a severe limitation of heterotrophic bacterial growth. DOM is released by algae during lysis of senescent cells or exudation of carbon compounds during nutrient limitation. In general, freshly produced DOM is rapidly consumed by heterotrophic bacteria. During this study, DOC concentrations increased primarily in the late stages of the first and the second phytoplankton bloom while chl *a* concentrations declined. On average, DOC concentrations increased by 1.3 - 1.6 $\mu\text{M C d}^{-1}$ which is comparable to the DOC accumulation during previous mesocosm studies in the same fjord ($\sim 1.3 \mu\text{M C d}^{-1}$, Engel et al. 2004a, $\sim 1 \mu\text{M C d}^{-1}$, Schulz et al. 2008). Some compounds of DOM, such as polysaccharides, may coagulate and form transparent exopolymer particles (TEP) (Alldredge et al. 1993; Verdugo et al. 2004). Peaks in TEP concentration occurred two days after the chl *a* maxima when DOC accumulation rates were high and TEP production was enhanced at high $p\text{CO}_2$ (Endres et al. in prep.). This indicates that the actual amount of DOC release may have been even higher, especially in the high $p\text{CO}_2$ treatments, compared to the determined concentrations.

In contrast to DOC, no significant increase in DON concentrations was observed. Production of DON may have been counterbalanced by loss processes such as microbial degradation or aggregation into gel particles. In fact, high activities of the protein-degrading enzyme leucine aminopeptidase were measured during this experiment (Endres et al. in prep.) indicating nitrogen-limitation of the microbial community. We assume that bacteria used DON as alternative nitrogen source preventing the accumulation of DON. Leucine aminopeptidase activities were significantly increased with $p\text{CO}_2$ and this faster recycling of DON may have favoured bacterial growth under high $p\text{CO}_2$ (Endres et al. in prep.).

Although aminopeptidase activity and TEP production were increased with CO₂, no statistically significant CO₂ effect on absolute DOC and DON concentration could be determined in this study. This is in accordance with previous studies that detected no effect or just slightly higher accumulation of DOC under elevated CO₂ conditions (Engel et al. 2004a; Engel et al. 2012). We suggest that changes in DOC and DON release may be undetectable with daily sampling because heterotrophic degradation by marine bacteria was tightly coupled to autotrophic production of DOM and rapidly reacting to changes in DOM release. This is confirming previous observations of the central equatorial Pacific where biological activities highly fluctuated but DOC concentrations remained stable (Carlson and Ducklow 1995).

Amino acid composition and contribution to organic matter

The contribution of amino acids to organic carbon increased during both phytoplankton blooms in the total but not in the dissolved fraction indicating that amino acids were mainly enriched in the biomass and DAA released by phytoplankton were probably quickly consumed by heterotrophic bacteria. In the surface ocean, DAA account for 6-12 %OC and 1-2 %ON (Benner 2002). Normalized amino acid yields in this study were in the lower end of these ranges (5-7 %OC and ~1 % ON, respectively).

In general, variations in the amino acid pool were low and the overall composition was comparable between mesocosms over time. Gly, AsX and GlX are well known to be one of the most abundant amino acids (Hubberten et al. 1994; Hubberten et al. 1995). In this study, they accounted for over 50% of the amino acids in the total and the dissolved fraction. While there were temporal changes in total TAA and DAA that may be related to phytoplankton bloom development and degradation, there was no difference between $p\text{CO}_2$ treatments. This suggests that the bulk amount of amino acids was not significantly affected by CO_2 or effects were masked by a fast response of the microbial community at elevated $p\text{CO}_2$.

However, we found temporal and treatment related changes in less concentrated amino-acids such as Arg, Thr, Phe and Ileu. Total Arg concentrations significantly followed the chl α development with a four days delay ($p < 0.02$). Despite the changes in total Arg, dissolved Arg concentrations did not change suggesting that either Arg was not released by the cells or it was rapidly degraded by heterotrophic bacteria. To the best of our knowledge, a temporal cross-correlation between chl α and Arg has not yet been described in marine microalgae. Further studies are necessary to confirm this pattern. Compared to other proteinogenic amino acids, Arg has a high nitrogen-content (4 N-atoms per molecule). Arg and Arg-rich proteins are known to be an important store of organic nitrogen in many plants and Arg is a precursor for the synthesis of plant hormones which play an important role in stress tolerance (Funck et al. 2008). In higher plant species, Arg accumulates under nutrient deficiencies (K, P, Fe, Cl, Zn) due to increased activity of the *de novo* arginine biosynthetic pathway (Rabe and Lovatt 1984). We suggest that, in this study, algal cells actively enriched Arg under nutrient limitation to store nitrogen temporarily within the cell.

It was shown that relative concentrations of Arg and GlX decrease rapidly during bacterial degradation of fresh DOM, indicating preferential utilization by heterotrophs, while the proportions of AsX, Ser, Thr, Gly, Ala, and Val increase as degradation proceeded (Amon et al. 2001). In this mesocosm study, the relative proportion of Thr in the dissolved fraction increased over time and with increased $p\text{CO}_2$ concomitant with the bacterial abundances, indicating that Thr accumulated due to higher bacterial degradation activity.

So far, the relationship between accumulation and degradation of single amino acids on one side and autotrophic or heterotrophic activities on the other side remains unknown. Although compositional changes in amino acids were low (≤ 1 mol%), we identified a significant $p\text{CO}_2$ effect

on four amino acids. While Phe, Thr, Ileu were significantly increased with increasing $p\text{CO}_2$, Gly concentrations were lowered. We can speculate that the increase in Phe, Thr, and Ileu may be related to the stimulation of bacterial activities under high $p\text{CO}_2$. Glycine is one of the most prominent amino acids in the ocean (Hubberten et al. 1994; Hubberten et al. 1995) and was the most abundant amino acid in this study. The relative decrease in Gly concentration in the high $p\text{CO}_2$ mesocosms was roughly counterbalanced by increased concentrations of Phe, Thr, and Ileu. Further investigations are needed to elucidate the relationship between biological activities, species composition and amino acid dynamics. To study the effect of increasing $p\text{CO}_2$ on amino acids, a detailed look on the variations of single amino acids may help to indicate changed biological activity, different physiological response of bacteria, and bioavailability of the organic material.

Amino acid composition as indicator of organic matter production and degradation

In earlier studies, Davis et al. (2009) suggested that carbon-normalized AA yields may be used to detect changes during the early stages of degradation while the degradation index (DI_{DAA}) was more suitable to look on intermediate timescales of years (Davis et al. 2009). We compared these two amino-acid based indicators to determine the diagenetic state of DOM. Both indicators correlated significantly ($p < 0.001$) and appear suitable for examining freshly produced DOM during phytoplankton blooms.

Overall, DOM was freshly produced and highly bioavailable throughout the sampling period. When the first bloom declined and before the second phytoplankton bloom was induced by nutrient addition (~day 13-15), bioavailability was decreased probably due to high bacterial activity and low primary production in the post-bloom phase. No CO_2 -related effect on DOM bioavailability was detected. However, we found small but significant changes in amino acid composition between treatments. These changes seemed to be below the sensitivity of current methods to describe the diagenetic status of organic matter. Further studies on DOM dynamics under the aspect of environmental change should consider this. The dynamics of single amino acids may be sensitive enough to detect even small changes in autotrophic production and microbial processing of DOM.

Conclusion

Despite a significant stimulation of gel particle production, enzymatic degradation of organic matter and bacterial growth with CO_2 , only minor changes in the DOM pool were detected during this mesocosm study. We suggest that heterotrophic degradation by marine bacteria was tightly coupled to autotrophic production of DOM and rapidly reacting to changes in the amount or composition of DOM. Nevertheless, small changes in the relative concentrations of less abundant amino acids were detected at elevated CO_2 conditions. These small compositional differences have potential as indicators of changes in production and degradation of organic matter. However,

further studies are needed to examine how lasting these compositional changes may be and if they may influence amino acid composition in the ocean on larger time-scales.

4.4.6 Acknowledgments

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4.5 Manuscript III

Response of *Nodularia spumigena* to $p\text{CO}_2$ - Part 2: Exudation and extracellular enzyme activities

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4.5.1 Abstract

The filamentous and diazotrophic cyanobacterium *Nodularia spumigena* plays a major role in the productivity of the Baltic Sea as it forms extensive blooms regularly. Under phosphorus limiting conditions *Nodularia spumigena* has a high enzyme affinity for dissolved organic phosphorus (DOP) by production and release of alkaline phosphatase. Additionally, they are able to degrade proteinaceous compounds by expressing the extracellular enzyme leucine aminopeptidase. As atmospheric CO₂ concentrations are increasing, we expect marine phytoplankton to experience changes in several environmental parameters including pH, temperature, and nutrient availability. The aim of this study was to investigate the combined effect of CO₂-induced changes in seawater carbonate chemistry and of phosphate deficiency on the exudation of organic matter, and its subsequent recycling by extracellular enzymes in a *Nodularia spumigena* culture. Batch cultures of *Nodularia spumigena* were grown for 15 days under aeration with low (180 µatm), medium (380 µatm), and high (780 µatm) CO₂ concentrations. Obtained pCO₂ levels in the treatments were on median 315, 353, and 548 µatm CO₂, respectively. Extracellular enzyme activities as well as changes in organic and inorganic compound concentrations were monitored. CO₂ treatment-related effects were identified for cyanobacterial growth, which in turn influenced the concentration of mucinous substances and the recycling of organic matter by extracellular enzymes. Biomass production was increased by 56.5% and 90.7% in the medium and high pCO₂ treatment, respectively, compared to the low pCO₂ treatment. In total, significantly more mucinous substances accumulated in the high pCO₂ treatment reaching 363 µg Xeq L⁻¹ compared to 269 µg Xeq L⁻¹ in the low pCO₂ treatment. However, cell-specific rates did not change. After phosphate depletion, the acquisition of P from DOP by alkaline phosphatase was significantly enhanced. Alkaline phosphatase activities were increased by factor 1.64 and 2.25, respectively, in the medium and high compared to the low pCO₂ treatment. We hypothesize from our results that *Nodularia spumigena* can grow faster under elevated pCO₂ by enhancing the recycling of organic matter to acquire nutrients.

4.5.2 Introduction

Cyanobacteria play an important role in the productivity of the Baltic Sea and form regularly extensive blooms (Finni et al., 2001). One of the typical bloom-forming species is the filamentous *Nodularia spumigena* (Sellner, 1997) which has the physiological capacity to fix atmospheric nitrogen. The estimated annual amount of fixed nitrogen in the Baltic Sea by cyanobacteria is 180–430 Gg N and equal to the total nitrogen input from rivers (480 Gg N yr⁻¹) and atmospheric deposition (~200 Gg N yr⁻¹) (Larsson et al., 2001; Schneider et al., 2003). Therefore, *Nodularia* is highly important for ecosystem functions in the Baltic Sea.

Nutrient concentrations, especially nitrogen and phosphorus, are the potential limiting factors for phytoplankton growth in the ocean with phosphorus being suggested as the more important nutrient for long-term productivity (Tyrell, 1999). As inorganic phosphorus is depleted rapidly, a potential alternative for phytoplankton growth is the utilization of dissolved organic phosphorus (DOP) (Björkman and Karl, 1994; Nausch, 1998a; Nausch and Nausch, 2006). Its availability may be an important factor influencing the distribution of diazotrophic cyanobacteria in the Baltic Sea (Niemi, 1979) as phosphate bioavailability controls the ability to fix N₂ (Paytan and McLaughlin, 2007). In the Baltic Sea, the DOP fraction in surface waters ranges between 0.20 to 0.23 µM in the central basin (Nausch et al., 2004) and 0.50 to 0.90 µM in the Gulf of Riga (Pöder et al., 2003) and can constitute as much as 70–100% of total phosphorus (Kononen et al., 1992; Nausch et al., 2004). This DOP pool is estimated to support 20% (range of 12–30%) of the phytoplankton production in the field (Mather et al., 2008). Hence, the ability to use this source efficiently is of great ecological importance for diazotrophs during nutrient limitation (Degerholm et al., 2006; Paytan and McLaughlin, 2007). *Nodularia* and similar species appear well adapted to the stratified and P-limited surface waters, which are predominantly found in these waters of the Baltic Sea in summer (Degerholm et al., 2006).

Gel particles play an important role in aggregation and export of organic and inorganic matter (Logan et al., 1995; Passow et al., 2001; Shipe et al., 2002; Engel et al., 2004b). Those particles consisting mainly of acidic sugars are called transparent exopolymer particles (TEP) and can be visualised under the microscope by Alcian Blue staining. Proteinaceous particles can be stained with Coomassie Blue and are therefore referred to as Coomassie stainable particles (CSP). Both, TEP and CSP, are potential food sources for bacteria but also substrate to grow upon. One origin of these exopolymers in the sea is exudation of carbon compounds by phytoplankton under nutrient limitation (Passow, 2002; Engel et al., 2002a; Engel et al., 2004a). Relatively little is known about exudation and gel particle formation in cyanobacteria communities (Engel et al., 2002b; Engel, 2002). Changes in light intensity were shown to have an increasing effect on exudation of DON and DOC in cultures of *Nodularia spumigena*. Up to 89 % of the fixed nitrogen and 53 % of the fixed carbon were released during the light period (Wannicke et al., 2009). Besides exudation, various species of unicellular and filamentous cyanobacteria produce large amounts of extracellular polymeric substances consisting predominantly of polysaccharides

(Otero and Vincenzini, 2004) which form a mucus layer around the cell. The function of this mucus layer and factors regulating the production, however, are not understood yet (Nausch, 1996; Otero and Vincenzini, 2004).

Microbes consume carbohydrates and proteins to meet their energy and nutrient requirements and to build up biomass. Therefore, they hydrolyse high molecular weight dissolved organic matter (DOM) with extracellular enzymes (Chrost et al., 1989). Highest activity rates and affinity of extracellular enzymes are usually measured towards the end of a phytoplankton bloom (Chrost et al., 1989). While α - and β - glucosidase and aminopeptidase activity were typically found to be associated with the bacterial fraction, alkaline phosphatase activity was associated also with phytoplankton (Chrost et al., 1989). Recent studies showed that cultured, axenic strains of *Nodularia spumigena* also seem to be able to express leucine aminopeptidase to degrade proteins (Stoecker et al., 2005).

The alkaline phosphatase catalyses the hydrolytic break-down of the PO_4^{3-} end from DOM and is expressed in response to phosphate limitation in many bacterial and phytoplankton species (Azam et al., 1983; Chrost and Overbeck, 1987; Beardall et al., 2001; Labry et al., 2005). In the Baltic Sea, alkaline phosphatase activity seems to be directly related to the abundance of heterocystous cyanobacteria (Nausch et al., 2004). Increasing phosphatase activities were determined with decreasing phosphate concentrations during *Nodularia* blooms (Huber and Hamel, 1985a, 1985b). Cultures of *Trichodesmium* IMS101 can grow with DOP as the only source of phosphorus, suggesting that DOP should be included in estimates of P availability in surface waters (Mulholland et al., 2002).

Due to rising atmospheric $p\text{CO}_2$ the ocean pH decreases constantly since the beginning of the anthropocene around 1800 (Zalasiewicz et al., 2008). By the year 2100, a further decrease by 0.3 units from current conditions is expected (Wolf-Gladrow et al., 1999; Feely et al., 2010). The atmospheric CO_2 concentrations increased since glacial periods from 180 μatm to 380 μatm nowadays and, for the future, we expect values around 780 μatm for the year 2100 (Parry et al., 2007; Meehl et al., 2007; Raupach et al., 2007). The effect of ocean acidification on marine microbes and the turnover of organic matter are little explored (Joint et al., 2010). During the past decades, an increase in the frequency (Sellner et al., 2003) and extension (Kahru et al., 1994) of cyanobacteria blooms has been detected and mainly been attributed to global warming and eutrophication (O'Neil et al., 2012). Considering the fact that marine microbes already experience high variations in CO_2 and pH, they might have the flexibility to accommodate pH change and therefore the microbial driven biogeochemical processes will probably not change dramatically (Joint et al., 2010). On the other side, it has been shown that pH has a regulating effect on enzyme activities. Some hydrolytic enzymes have been shown to have their optimum at a pH below present seawater pH. Piontek et al. (2010) showed that a decrease in seawater pH as expected for the near future increases enzymatic hydrolysis rates of polysaccharides and accelerates the bacterial degradation of organic carbon. During a mesocosm study aminopeptidase activity was significantly higher at elevated $p\text{CO}_2$ (Grossart et al., 2006).

A direct coupling between inorganic carbon acquisition and organic carbon exudation was observed during incubation experiments in the central Baltic Sea (Engel, 2002). Future increase in atmospheric CO₂ may however not necessarily lead to a higher exudation rate as the latter seemed to be already at its maximum under present CO₂ concentration in some ecosystems (Engel, 2002). Nevertheless, a mixed mesocosm phytoplankton community treated with high CO₂ showed significantly enhanced TEP production normalized to cell abundance (Engel et al., 2004a). Increasing *p*CO₂ may potentially affect N₂ fixation and increase the release of DOM but also the recycling of nutrients by *Nodularia* and may therefore have consequences for nutrient cycling and the export of organic matter in the Baltic Sea. So far, there are only two studies published on the effects of increasing CO₂ on *Nodularia* spp. (Czerny et al., 2009; Wannicke et al., 2012). Yet observations on changes in extracellular enzyme activities and turnover of organic matter in cyanobacteria blooms due to ocean acidification are still lacking.

This is the second of three companion papers on a major culture experiment with *Nodularia spumigena* (Wannicke et al., 2012; Unger et al., 2013). The aim of this study was to examine the relationship between *p*CO₂ and diazotrophic growth of *Nodularia spumigena* and the related fluxes of carbon, nitrogen, and phosphorus. In particular we wanted to investigate the effect of ocean acidification on the exudation of organic matter and its subsequent recycling by extracellular enzymes in a *Nodularia spumigena* batch experiment. Enzyme activities as well as changes in organic and inorganic compound concentrations were measured over 15 days of incubation at three different *p*CO₂ levels.

4.5.3 Material & Methods

Cultivation and experimental setup

Details of the experimental set-up and culture conditions can be found in Wannicke et al. (2012). Axenic precultures of the cyanobacterium *Nodularia spumigena* were grown in F/2 medium in batch bottles at 15°C with a light cycle of 16:8 (cool, white fluorescent lighting, 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$). The precultures were aerated for three days with premixed gases (Linde gas) of 180, 380, and 780 ppm CO_2 , representing past, present, and future atmospheric $p\text{CO}_2$, respectively. The premixed gas was filtered through seawater to increase humidity of the air to avoid water evaporation from the batch bottles. During the duration of the experiment the batch cultures were aerated for one hour per day. Aeration was not sufficient to yield the target $p\text{CO}_2$ concentrations (Wannicke et al., 2012) and carbonate chemistry may have been altered additionally by cellular carbon uptake. However, the three obtained $p\text{CO}_2$ levels differed from each other and therefore we refer to them as “low”, “medium” and “high” $p\text{CO}_2$ treatments. The low $p\text{CO}_2$ treatment ranged from 249 to 499 $\mu\text{atm CO}_2$ with a median of 315 μatm . The medium $p\text{CO}_2$ treatment reached 287 to 571 μatm with a median of 353 μatm . The high $p\text{CO}_2$ level was 395 to 630 μatm with a median of 548 μatm (Wannicke et al., 2012).

At the beginning of the experiment (day 0), 36 bottles with nutrient depleted (below detection limit), four month aged and sterile-filtered Baltic Sea water were inoculated with *N. spumigena* to a starting concentration of 0.8 $\mu\text{g chl } a \text{ L}^{-1}$. To stimulate growth, 0.35 $\mu\text{M PO}_4^{3-}$ were added at day 0 and on day 3 as phosphate was already depleted in the cultures. Three bottles with sterile seawater remained as a blank to determine background concentrations of inorganic and organic compounds in the seawater. The bottles were aerated with premixed gases of the three $p\text{CO}_2$ levels resulting in 12 replicates of each CO_2 treatment. We followed the build-up and decline of the phytoplankton blooms over a 15-day period, with sampling on days 0, 3, 9 and 15. Three replicate bottles of each CO_2 treatment were harvested at each sampling point. In the following we refer to the average concentration of the three replicates and its standard deviation for all parameters. The experiment was conducted at 23°C and the day to night cycle was adjusted to 16:8 h with a light intensity of 200 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

Carbonate chemistry

The carbonate chemistry was determined by measuring pH and dissolved inorganic carbon (DIC) on each sampling day. The pH was measured using an electrode (Knick Mikroprozessor pH Meter 761 with Type SE 100 glass electrode), repeatedly calibrated with NBS buffer. Values of pH are given relative to total scale. DIC was analysed according to Johnson et al. (1993) directly after sampling using the colorimetric SOMMA system. We used carbon reference material provided by A. Dickson (University of California, San Diego). Analysis precision was $\pm 2 \mu\text{mol kg}^{-1}$. $p\text{CO}_2$ and total alkalinity (TA) were calculated with CO2SYS (Lewis et al., 1998).

Analysis of inorganic and organic compounds

Determination of inorganic nutrients (NH_4^+ , NO_3^- and PO_4^{3-}) was accomplished by filtering 60 mL sample through a combusted GF/F filter and measured colorimetrically in a spectrophotometer U 2000 (Hitachi-Europe GmbH, Krefeld, Germany) according to the method of Grasshoff et al. (1983). Ammonium concentrations remained undetectable in the course of the experiment.

To measure the chlorophyll *a* content (chl *a*), 100 mL water samples were filtered onto Whatman GF/F filters and the filters were stored in liquid nitrogen or at -80°C . After thawing, they were extracted with 96% ethanol for at least 3 h and the chl *a* fluorescence was measured with a TURNER fluorometer (10-AU-005) at an excitation wavelength of 450 nm and an emission of 670 nm (HELCOM, 2005). The chlorophyll concentration was calculated according to the method of Jeffrey and Welschmeyer (1997).

Samples for dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) analysis were filtered through combusted GF/F filters, collected in 20 mL combusted (8 h, 500°C) glass ampoules, acidified with 80 μL of 85% phosphoric acid and stored at $0-2^\circ\text{C}$ for 10 months. DOC and TDN concentrations were determined simultaneously in the filtrate by high temperature catalytic oxidation with a Shimadzu TOC-VCSH analyser equipped with a Shimadzu TNM-1 module. DOC and TDN concentrations were average values of quadruplicate measurements. Values of TDN were corrected for nitrate, and ammonium, and thereafter referred to as DON.

For the determination of total phosphorus (TP) 40 mL unfiltered samples were taken and stored at -20°C up to two months. For measuring dissolved phosphorus (DP) 40 mL samples were filtered through combusted (450°C , 4 h) Whatman GF/F filters. The thawed samples were oxidized with an alkaline peroxodisulfate solution (Grasshoff et al., 1983) in a microwave ($\mu\text{Prep-A}$) to convert organic phosphorus into dissolved inorganic phosphorous (PO_4^{3-}). The PO_4^{3-} concentration was determined photometrically ($\lambda = 885 \text{ nm}$) in a 10 cm cuvette. The detection limit was 0.01 μM . Dissolved organic phosphorus (DOP) was calculated by subtracting PO_4^{3-} from DP. Particulate organic phosphorus (POP) was calculated by subtracting DP from TP.

For analysis of particulate organic carbon (POC) and nitrogen (PON) concentration, 200 mL were filtered onto GF/F filters and stored at -20°C . Concentrations were measured by means of flash combustion in a Carlo Erba EA 1108 at 1020°C and a Thermo Finnigan Delta S mass spectrometer.

Nodularia filament and bacteria cell counts

Fifty mL samples were fixed with acetic Lugol's (KI/I₂) solution (1% v/v final concentration). Within the subsequent four weeks, abundance, cell length and width of *Nodularia* sp. filaments were determined with an inverted Leica microscope at 100x magnification (Utermöhl, 1958). For bacterial cell counts, 4 mL samples were preserved with 100 μl formaldehyde (1% v/v final concentration), shock frozen in liquid nitrogen and stored at -70°C for three months until

measurement. A stock solution of SYBR GREEN (Molecular Probes) was prepared by mixing of 1 μL dye with 49 μL dimethyl sulfoxide (DMSO, Sigma Aldrich, 1:16 diluted). A 3 μL potassium citrate solution, 10 μL of the dye stock solution and 10 μL fluoresbrite microspheres (Polysciences) were added to 300 μL of the thawed sample and incubated for 30 min in the dark. These samples were then analysed using a flow cytometer (Facs Calibur, Becton Dickinson) following the method of Gasol and del Giorgio (2000) at medium flow rate. Calculations were done using the software program "Cell Quest Pro".

Transparent exopolymer particles (TEP) and Coomassie stainable particles (CSP)

Alcian Blue stains the acidic mucopolysaccharide layers surrounding cells (mucus) as well as free aggregates of acidic sugars, so-called transparent exopolymer particles (TEP). As we cannot distinguish between these two with this method, we refer to them as "mucinous substances" (Leppard, 1995). For analysis, 15 mL samples were filtered onto 0.4 μm Nuclepore filters, stained with 1 mL of a calibrated Alcian Blue solution and rinsed with several mL of ultrapure water. All samples were filtered in triplicates. Before use, the staining solution was filtered (0.2 μm) to avoid particles in the dye solution. The filters were stored at -20°C for 2-6 weeks until spectrophotometrical analysis. The amount of Alcian Blue adsorption per sample was determined colorimetrically according to Passow and Alldredge (1995). Each filter was incubated for 3 h with 6 mL of 80% H_2SO_4 in order to dissolve the particles and then the solution was measured at 787 nm with an UV-Vis spectrophotometer (Shimadzu UV-1700 PharmaSpec). The total concentration of mucinous substances is given in xanthan gum equivalent (Xeq), as xanthan gum was used for calibration.

For microscopic analysis of mucinous substances size and abundance, 5 mL samples were filtered in duplicates onto 0.4 μm Nuclepore filters, stained and incubated with 1 mL of Alcian Blue for 3 sec, and rinsed with distilled water. Samples for the proteinaceous gel particles (CSP) were processed identically except that they were stained and incubated with 1 mL of Coomassie Blue solution for 30 sec. CSP may play an important role as organic N source for heterotrophic organisms. They were barely stained in the mucus surface coating of *Nodularia* so CSP can be considered as discrete particles during this experiment. The filters were stored on cytoclear slides at -20°C until microscopic analysis (Engel, 2009). Slides were analyzed with a light microscope connected to a color video camera with 400 \times magnification. About 2 \times 30-35 frames per slide were taken in a cross section. Particles were counted and sized semi-automatically using the software Image J (Rasband, 1997-2011). CSP size can be compared by calculating the equivalent spherical diameter (ESD) of each particle. Total mucinous substance concentration determined by Xeq was significantly related to total area of mucinous substances determined by microscopic analysis ($n=37$, $R^2=0.73$, $p < 0.001$).

Extracellular enzyme activities

Fluorogenic model substrates are used to quantify potential *in situ* extracellular enzyme activities (Hoppe et al., 2002). The activity of extracellular enzymes (alkaline phosphatase, α - and β -glucosidase, and leucine-aminopeptidase) was determined by using 4- methylumbelliferyl (MUF)-phosphate, 4-MUF- α -glucopyranoside, 4-MUF- β -glucopyranoside, and L-leucine-4-methyl-7-coumarinylamide (MCA), respectively (Hoppe, 1983). The fluorescent substrate analogues were added to subsamples of 180 μ L volume and incubated in duplicates for 3.5 - 4.5 h in the dark at 25°C. Seven different substrate concentrations ranging from 0 to 150 μ M (0, 1, 5, 10, 20, 50, and 150 μ M) were tested. Sample fluorescence was measured in microtiter plates with a fluorometer (FLUOstar OPTIMA, BMG Labtech, excitation 355 nm, emission 460 nm). Calibration was carried out with solutions of MUF and MCA. Detection limit for the fluorescent dye was 25 nM for MUF and 10 nM for MCA. To eliminate background fluorescence effects and to ensure that there is no significant substrate hydrolysis due to abiotic processes control samples for every concentration of substrate added were measured with distilled water or sterilized seawater. In order to consider pH effects on the fluorescence intensity of MUF, standard solutions were adjusted to pH 7.88, 7.99, 8.0, 8.35, and 8.66. V_{\max} is the maximum rate achieved by the system at saturating substrate concentrations. The activity of extracellular enzymes was calculated as the maximum hydrolysis rate (V_{\max}) using the software SigmaPlot 12.0. The Michaelis-Menten constant K_m is the substrate concentration at which the reaction rate is half of V_{\max} .

Data analysis and statistics

Calculations, statistical tests and illustration of the data were performed with the software packages Microsoft Office Excel 2003 and Sigma Plot 12.0 (Systat). Values given are the average of three replicates. To compare different $p\text{CO}_2$ treatments an independent t-test was used. The significance level for all tests was $p < 0.05$. Data were tested for normality using the Shapiro-Wilk test. The relation between the organic parameters, nitrogen fixation rate (actual rates are presented in Wannicke et al. (2012)) and enzyme activities was assessed by the Spearman Rank Order Correlation. For pairs with $R^2 > 0.5$, correlation coefficient $|R| > 0.7$ and p -values below 0.05, there is a strong significant relationship (**) between the two variables. Pairs with correlation coefficient $|R|$ between 0.3 and 0.7 and p -values below 0.05, there is a weak significant relationship (*) between the two variables. The pairs of variables with positive correlation coefficients R tend to increase together. For the pairs with negative correlation coefficients R one variable tends to decrease while the other increases. There was one outlier in the data set (Sample 180-II on day 9), which contained double amount of PO_4^{3-} at the beginning. It was removed to ensure equal starting conditions of the replicates.

4.5.4 Results

Biomass production

Detailed information on general bloom development, nitrogen fixation rates and cell productivity is given in Wannicke et al. (2012) and will only briefly be summarized here. *Nodularia* filament abundance, chl *a* concentration, as well as POC concentrations increased over time in all treatments until day 9, which was supposed to be around the maximum of the bloom. The chl *a* concentration rose from 0.8 $\mu\text{g chl } a \text{ L}^{-1}$ on day 0 up to 4.6 μg in the low and 7.3 $\mu\text{g L}^{-1}$ in the high $p\text{CO}_2$ treatment on day 9 (Fig. 1). We refer to this period as the “growth phase”. From day 9 to day 15, chl *a* concentrations decreased to 1.5 μg at low and 4.4 μg at high $p\text{CO}_2$, respectively. In general, chl *a* concentrations increased with $p\text{CO}_2$. POC concentrations increased by 81.9 $\mu\text{mol C L}^{-1}$ in the low, 128.1 $\mu\text{mol C L}^{-1}$ in the medium and 156.2 $\mu\text{mol C L}^{-1}$ in the high $p\text{CO}_2$ treatment (Fig. 2). Thus, POC increase was significantly elevated by 56.5% and 90.7% at medium ($p = 0.005$) and high ($p = 0.029$) $p\text{CO}_2$ treatments, respectively, compared to the low $p\text{CO}_2$ treatment. Specific growth rates (μ) per day were calculated based on changes in filament abundance, chlorophyll *a* (chl *a*), particulate organic nitrogen (PON) and particulate organic carbon (POC) for the three $p\text{CO}_2$ treatments from day 0 to day 9. Compiled growth rates based on all parameters, presented in Wannicke et al. (2012), were significantly different between the $p\text{CO}_2$ treatments ($p < 0.05$ and $p = 0.001$). Based on POC data, growth rates were $0.0980 \pm 0.0480 \text{ d}^{-1}$ in the low, $0.1500 \pm 0.0070 \text{ d}^{-1}$ in the medium, and $0.2060 \pm 0.0200 \text{ d}^{-1}$ in the high $p\text{CO}_2$ treatments.

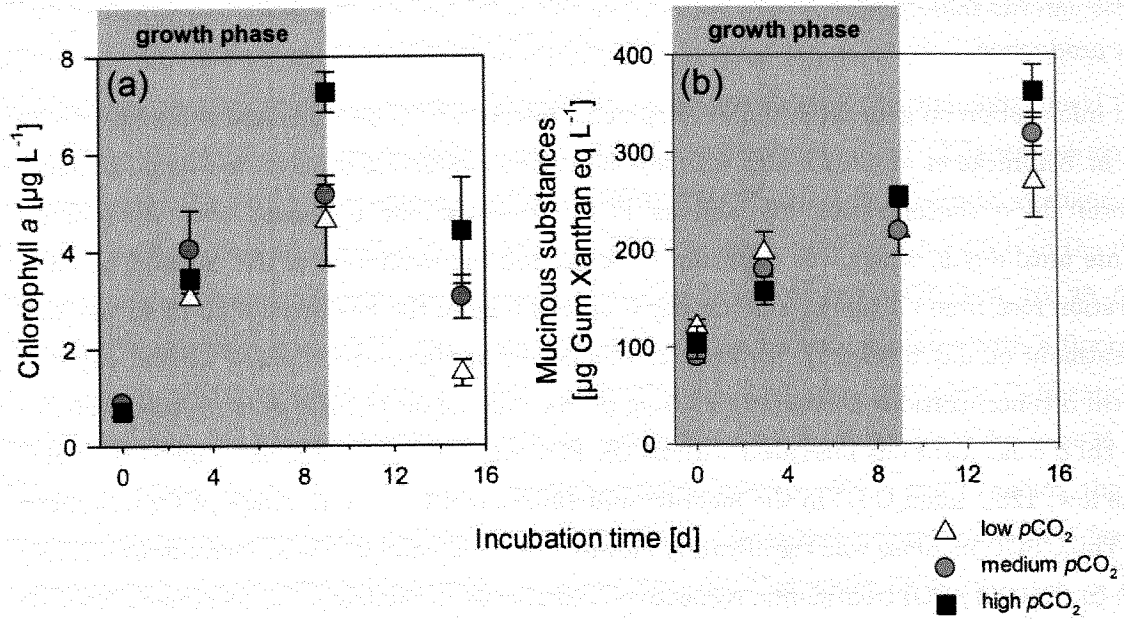


Fig. 1. Chlorophyll *a* concentration (a) and mucinous substance concentration (b) over time at low (triangle), medium (circle) and high (squares) $p\text{CO}_2$. Symbols display the mean of three replicates with standard deviation.

Heterotrophic bacteria cells counts at the start of the experiment were below the blank value (Wannicke et al., 2012). No significant growth of heterotrophic bacteria was observed over time. Bacterial cell numbers on average were $4.5 \pm 2.3 \times 10^5$ cells L^{-1} in the low, $2.4 \pm 2.1 \times 10^5$ cells L^{-1} in the medium and $4.8 \pm 2.8 \times 10^5$ cells L^{-1} in the high $p\text{CO}_2$ treatment (Wannicke et al., 2012, Table 2). Standard deviations were relatively high and cell numbers varied between replicates and over time probably due to methodological constraints but no systematic increase was observed over time. If some bacteria were attached to each other, to *Nodularia* or to gel particles, bacterial abundance determined by flow cytometry might have been underestimated. On the other hand, non-viable bacteria might have been included in the enumeration since also extracellular nucleic acids and dead, DNA containing, cells stain with the dye SYBR green that is used for flow cytometry, as discussed in Wannicke et al. (2012).

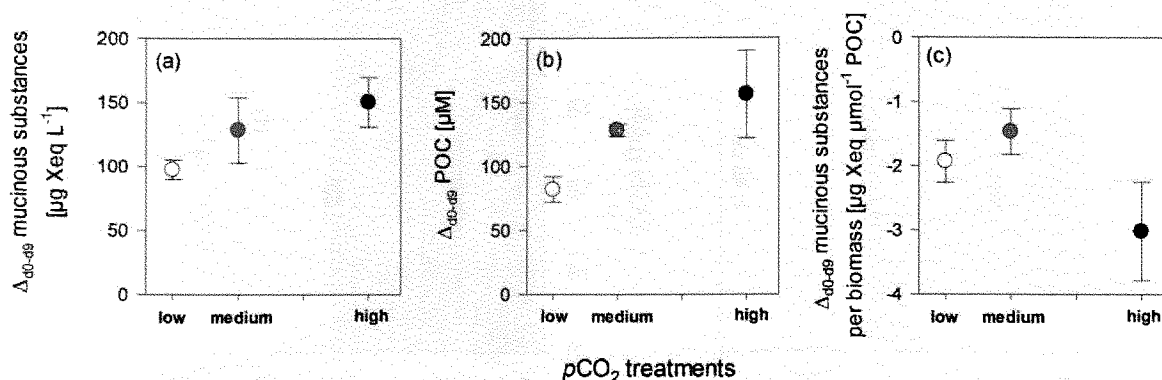


Fig. 2. Comparison of biomass production and accumulation of mucinous substances during the growth phase (day 0 to day 9). Symbols display the mean of three replicates with standard deviation.

Exudation and formation of gel particles

DOC concentrations were $303 \pm 26 \mu\text{mol L}^{-1}$ in the low, $309 \pm 21 \mu\text{mol L}^{-1}$ in the medium, and $313 \pm 36 \mu\text{mol L}^{-1}$ in the high $p\text{CO}_2$ treatment. The increase in DOC during the growth phase (day 0 to day 9) in all treatments ranged between 2 and $59 \mu\text{mol DOC L}^{-1}$. Differences between the single $p\text{CO}_2$ treatments were not significant and differences between replicates were higher than between treatments (Fig. 3). The average DON concentration was slightly but significantly lower in low $p\text{CO}_2$ treatment with $15 \pm 1.0 \mu\text{mol DON L}^{-1}$ compared to the high $p\text{CO}_2$ treatment with $17 \pm 1.2 \mu\text{mol DON L}^{-1}$ ($p = 0.045$). DON decreased during the growth phase in all treatments by up to $4 \mu\text{mol DON L}^{-1}$. Differences in decrease between the single $p\text{CO}_2$ treatments were not statistically significant and differences between replicates were higher than between treatments (Fig. 3). For more details on changes in DOM and C:N:P stoichiometry see Wannicke et al. (2012).

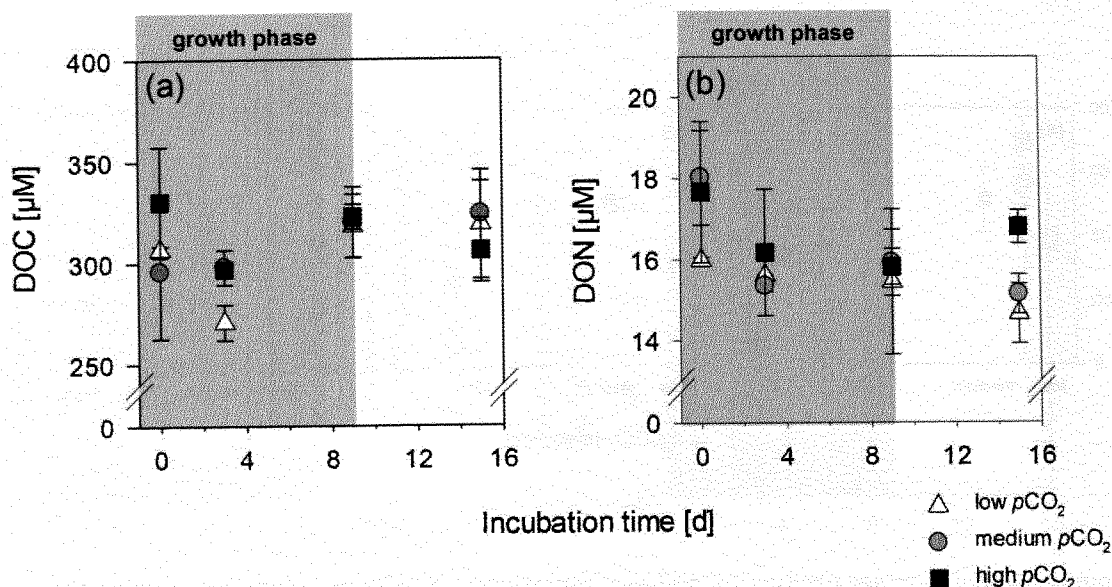


Fig. 3. DON and DOC concentrations over time at low (triangle), medium (circle) and high (squares) $p\text{CO}_2$. Symbols display the mean of three replicates with standard deviation.

Total concentrations of mucinous substances ranged from 90 to 363 $\mu\text{g Xeq L}^{-1}$. The total concentration doubled within the bloom phase from 90 - 120 μg on day 0 up to 218 - 255 $\mu\text{g Xeq L}^{-1}$ on day 9 (Fig. 1). From day 9 to day 15 of the experiment *Nodularia* biomass decreased, while mucinous substance concentration was still increasing. Highest increase was determined in the high $p\text{CO}_2$ treatments reaching a final concentration of 363 $\mu\text{g Xeq L}^{-1}$ compared to 319 μg in the medium and 269 $\mu\text{g Xeq L}^{-1}$ in the low $p\text{CO}_2$ treatment. During the growth phase significantly more mucinous substances accumulated in the high $p\text{CO}_2$ treatment compared to the low $p\text{CO}_2$ treatment ($p = 0.039$; Fig. 2a).

Mucinous substance concentration normalized to biomass (POC) amounted between 3.9, 2.8 and 4.5 $\mu\text{g Xeq } \mu\text{mol}^{-1}$ POC on day 0 in the low, medium and high $p\text{CO}_2$ treatment and decreased over time by 49.8 %, 51.3 % and 66.8 %, respectively, during the growth phase. The high $p\text{CO}_2$ treatments showed the strongest decrease in mucinous substance concentration normalized to biomass (Fig. 2c). However, this trend was not significantly correlated to $p\text{CO}_2$. From day 9 to day 15 mucinous substance concentration per POC recovered to 3.3 μg in the low and the high $p\text{CO}_2$ treatment and 3.5 $\mu\text{g Xeq } \mu\text{mol}^{-1}$ POC in the medium $p\text{CO}_2$ treatment.

For further information on gel particle size and abundance, a microscopic analysis was performed. Most cyanobacteria filaments seemed to be coated by mucus which was stained by Alcian Blue (Fig. 4a) while CSP was mostly observed as “free” particles in the seawater (Fig. 4c, 4d). Additionally, free TEP particles were observed (Fig. 4b). Due to methodological constraints it was not possible to quantify TEP or mucus separately.

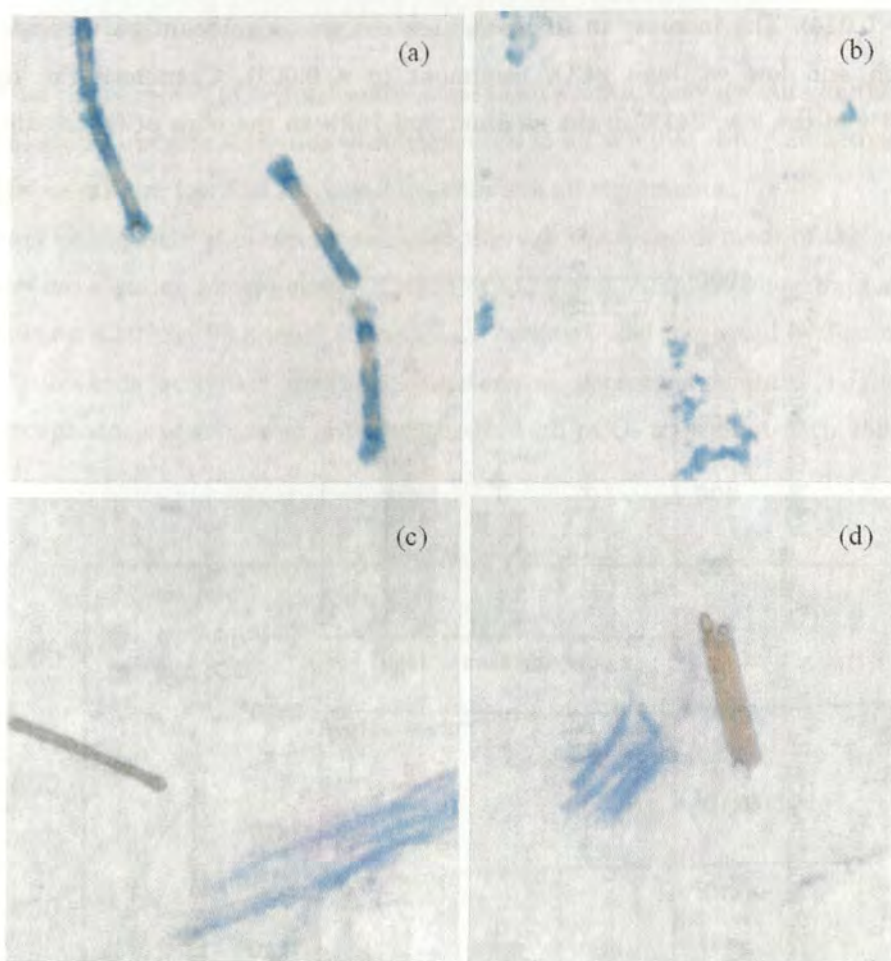


Fig. 4. Microscopic view of *Nodularia spumigena* cultures covered with a mucus coating (a), stained TEP (b) and CSP (c, d)

The microscopic analysis revealed an increase in CSP area during the growth phase (day 0 to day 9) in all treatments (Fig. 5a). Additionally, CSP area differed significantly between low and medium ($p = 0.042$) as well as between low and high ($p = 0.009$) $p\text{CO}_2$ treatments. CSP area increased by $57.0 \text{ mm}^2 \text{ L}^{-1}$ (= 32%) in the low, by $331.4 \text{ mm}^2 \text{ L}^{-1}$ (= 193%) in the medium and by $580.4 \text{ mm}^2 \text{ L}^{-1}$ (= 389%) in the high $p\text{CO}_2$ treatment during the growth phase. CSP abundance clearly increased during the growth phase, i.e. by 355%, 174% and 148% in the low, medium and high $p\text{CO}_2$ treatment, respectively. Variability between replicates was high (standard deviation ranged between 19% and 78%). The average ESD of CSP differed significantly between low and high $p\text{CO}_2$ treatment ($p = 0.032$). During the growth phase, CSP size decreased by $0.84 \pm 0.15 \mu\text{m}$ in the low and $0.22 \pm 0.27 \mu\text{m}$ in the medium $p\text{CO}_2$ treatment, while in the high $p\text{CO}_2$ treatment average ESD of CSP increased by $0.47 \pm 0.45 \mu\text{m}$ (Fig. 5c). The area covered by filaments was calculated from average filament length, width and abundance in each replicate. On day 0, filament area ranged between 131.3 and $171.6 \text{ mm}^2 \text{ L}^{-1}$ and increased up to 615.3 , 833.1 and $1264.4 \text{ mm}^2 \text{ L}^{-1}$ in the low, medium and high $p\text{CO}_2$ treatment (Fig. 5a). The increase in filament area was significantly different between low and high as well as between medium and high $p\text{CO}_2$

treatments ($p = 0.014$). The increase in filament numbers was significantly different between medium vs. high and low vs. high $p\text{CO}_2$ treatment ($p < 0.001$). Cyanobacteria abundance increased by 134% in the low, 241% in the medium and 753% in the high $p\text{CO}_2$ treatment (Fig. 5b).

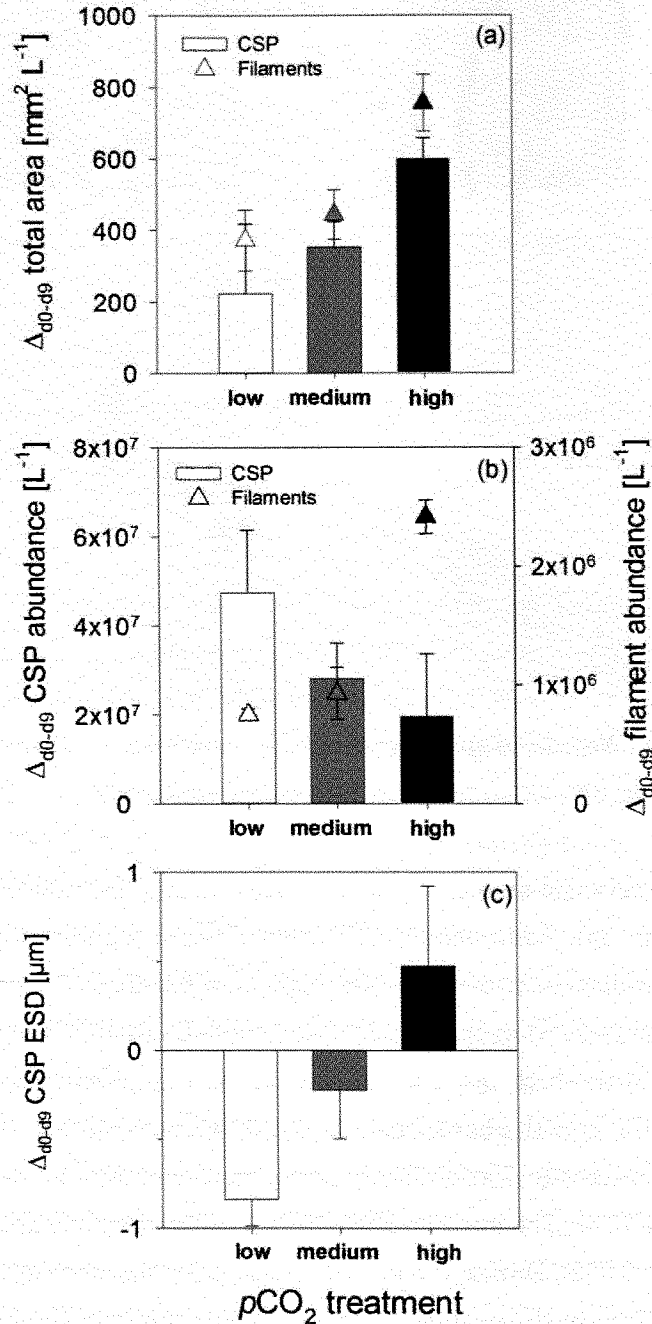


Fig. 5. Variation of the filaments and CSP during the growth phase (day 0 to day 9) in the low (white), medium (grey) and high (black) $p\text{CO}_2$ treatments: (a) total area of the filaments and CSP; (b) filament and CSP abundance; (c) equivalent spherical diameter (ESD) of CSP. Symbols and bars display the mean of three replicates with standard deviation.

Enzyme activities and recycling of organic matter

To determine the turnover of organic matter due to enzymatic cleavage outside the cyanobacterial cells, extracellular enzyme activities were measured in all samples. Enzyme activities at the start of the experiment were low and increased over time in all treatments.

The turnover of the DOP pool can be assessed through the measurement of the activity of the P-specific enzyme alkaline phosphatase (Mather et al., 2008). Alkaline phosphatase activity (APA) ranged between 62.9 and 93.6 $\text{nmol L}^{-1} \text{h}^{-1}$ (V_{max}) on day 0 and increased by factor 10 until day 9 (Fig. 6). Afterwards activities remained constant or decreased slightly. Highest increase in alkaline phosphatase activity was observed in the high $p\text{CO}_2$ treatment with 958.23 $\text{nmol L}^{-1} \text{h}^{-1}$ on day 9.

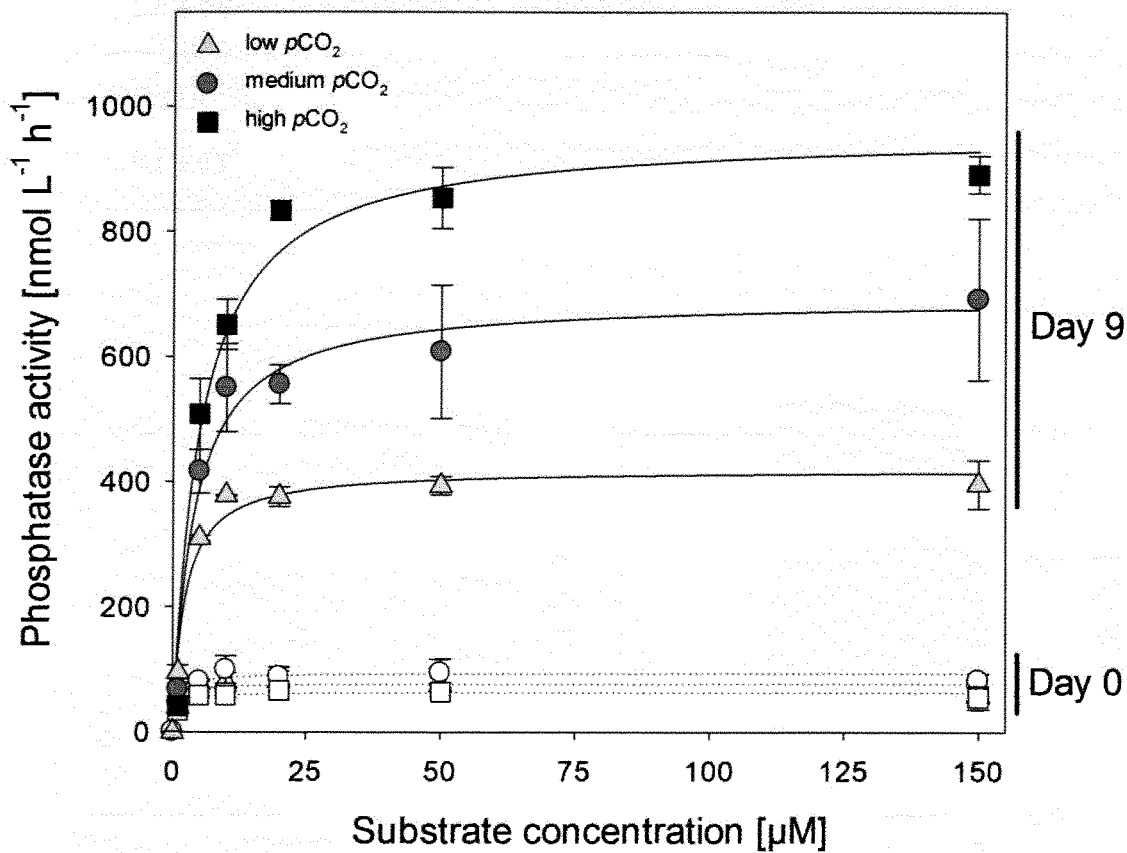


Fig. 6. Total alkaline phosphatase activity at day 0 and day 9 of low (triangle), medium (circle) and high (square) $p\text{CO}_2$ treatments. Symbols display the mean of three replicates with standard deviation.

Table 1. Phosphatase activity characteristics: V_{\max} is the maximum rate achieved by the system at saturating substrate concentrations in nmol per litre and hour; the Michaelis-Menten constant K_m is the substrate concentration in μM at which the reaction rate is half of V_{\max} . $R^2 > 0.97$; Substrate concentrations ranged from 1–150 μM ; number of substrate concentrations used for each regression = 7.

		<i>pCO₂ treatment</i>			
			low	medium	high
Day	V_{\max}	$\frac{\text{nmol}}{\text{L} \times \text{h}}$	76.7 ± 5.8	93.6 ± 4.4	62.9 ± 2.6
0	K_m	μM	0.6 ± 0.4	0.9 ± 0.3	0.7 ± 0.2
Day	V_{\max}	$\frac{\text{nmol}}{\text{L} \times \text{h}}$	419.5 ± 17.0	693.2 ± 36.8	958.2 ± 54.5
9	K_m	μM	2.2 ± 0.5	3.9 ± 1.0	4.9 ± 1.2

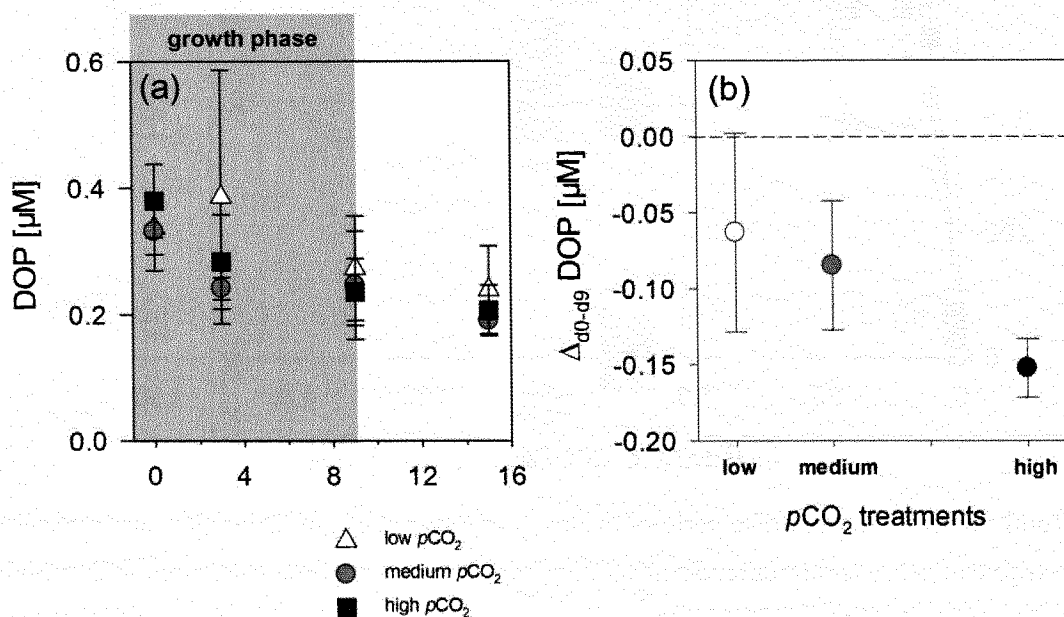


Fig. 7. DOP concentration over time at low (triangle), medium (circle) and high (squares) $p\text{CO}_2$ (a) and changes from day 0 to day 9 (b). Differences between treatments are not significant. Symbols display the mean of three replicates with standard deviation.

K_m values of the alkaline phosphatase ranged between 0.6 and 0.9 μM at the beginning of the experiment and increased up to 2.2 μM in the low, 3.9 μM in the medium and 4.9 μM in the high $p\text{CO}_2$ treatment (Table 1). As the K_m values of the alkaline phosphatase increased with increasing $p\text{CO}_2$, the substrate affinity of the enzyme decreased.

APA showed significantly negative correlation with PO_4^{3-} concentration (Table 2). On the third day of the experiment PO_4^{3-} was depleted, so the cells were likely P limited. Fastest decrease and hence uptake of PO_4^{3-} was observed at high $p\text{CO}_2$. From day 3 onwards, there was a net loss in DOP in all treatments (Fig. 7a). The net consumption of DOP differed between CO_2 treatments with 0.06 μM at low and 0.15 μM at high $p\text{CO}_2$ but differences were not significant due to high variations between replicates (Fig. 7b).

Table 2. Correlation of alkaline phosphatase activity (APA) with biomass of filamentous cyanobacteria (POC, chl α), PO_4^{3-} , DOP, $p\text{CO}_2$, pH, N_2 fixation, and bacterial cell counts ($n = 36$, except for PO_4^{3-} ($n = 35$) and N_2 fixation ($n=32$); R = correlation coefficient). Correlation level is marked as * for low significance ($0.03 < |R| < 0.7$, $p < 0.05$) and ** for strong significance ($|R| > 0.7$, $p < 0.05$), nc: for no correlation

Parameter	Correlation coefficient R	Correlation
AP activity vs.		
PO_4^{3-}	-0.73	**
DOP	-0.64	*
POC	0.86	**
chl α	0.73	**
$p\text{CO}_2$	-0.55	*
pH	0.55	*
N_2 fixation rate	-0.65	*
Bacterial cell number	0.13	nc

Activities of α - and β -glucosidases remained very low during the whole experiment, but increased slightly toward the end of the experiment (Table 3). On day 0, glucosidase activities remained below the detection limit. Highest activities were measured on day 15, ranging between 15.5 - 18.8 nmol L⁻¹ h⁻¹ for α -glucosidase and 16.1 - 18.7 nmol L⁻¹ h⁻¹ for β -glucosidase. Variation between replicates was higher than between treatments. Glucosidase activities are usually assigned to heterotrophic bacteria which degrade organic carbon compounds. Therefore, bacterial cell-specific activities were calculated for both glucosidases (Table 3). Standard deviations were high due to high variability in bacterial cell counts between the replicates. Cell-specific activities increased over time in all treatments with highest increase in the medium $p\text{CO}_2$ treatment. This was mainly due to a decrease in bacterial abundance over time in this treatment, while in the low and high $p\text{CO}_2$ treatments bacterial cell numbers increased slightly.

Table 3. Glucosidases and leucine aminopeptidase activities at different $p\text{CO}_2$ (V_{max} and standard deviation; n=3) on day 15 (= day of highest activities)

Enzyme			$p\text{CO}_2$ treatment		
			low	medium	high
α -glucosidase	total	$\frac{\text{nmol}}{\text{L} \times \text{h}}$	15.6 ± 2.8	15.5 ± 2.7	18.8 ± 7.9
	per cell	$\frac{\text{amol}}{\text{L} \times \text{h}}$	28.5 ± 19.9	77.6 ± 22.6	46.9 ± 51.2
β -glucosidase	total	$\frac{\text{nmol}}{\text{L} \times \text{h}}$	18.7 ± 2.4	16.1 ± 2.9	18.1 ± 7.1
	per cell	$\frac{\text{amol}}{\text{L} \times \text{h}}$	33.0 ± 21.1	81.4 ± 14.7	42.9 ± 44.7
leucine aminopeptidase	total	$\frac{\text{nmol}}{\text{L} \times \text{h}}$	268.0 ± 133.8	375.6 ± 60.7	244.6 ± 58.2

No leucine aminopeptidase (LAP) activity was detectable on day 0 (< 10 nmol L⁻¹ h⁻¹) but activities increased during the growth phase up to 105.17, 241.21 and 168.04 nmol L⁻¹ h⁻¹ in the low, medium and high $p\text{CO}_2$ treatment (Fig. 8). Highest activities were measured with 268.0, 375.6 and 244.6 nmol L⁻¹ h⁻¹ on day 15 (Table 3). Bacterial cell-specific activities could not be calculated for leucine aminopeptidase as there is no possibility to distinguish between enzymes that are produced by cyanobacteria or heterotrophic bacteria. No significant correlation was found between APA (Table 2) or LAP (Table 4) activity and bacterial cell numbers, but both showed a

significantly positive correlation with cyanobacteria biomass (POC, chl α) and a significantly negative correlation with N_2 fixation rates (Table 4). Correlation between both enzymes and pCO_2 or pH was low.

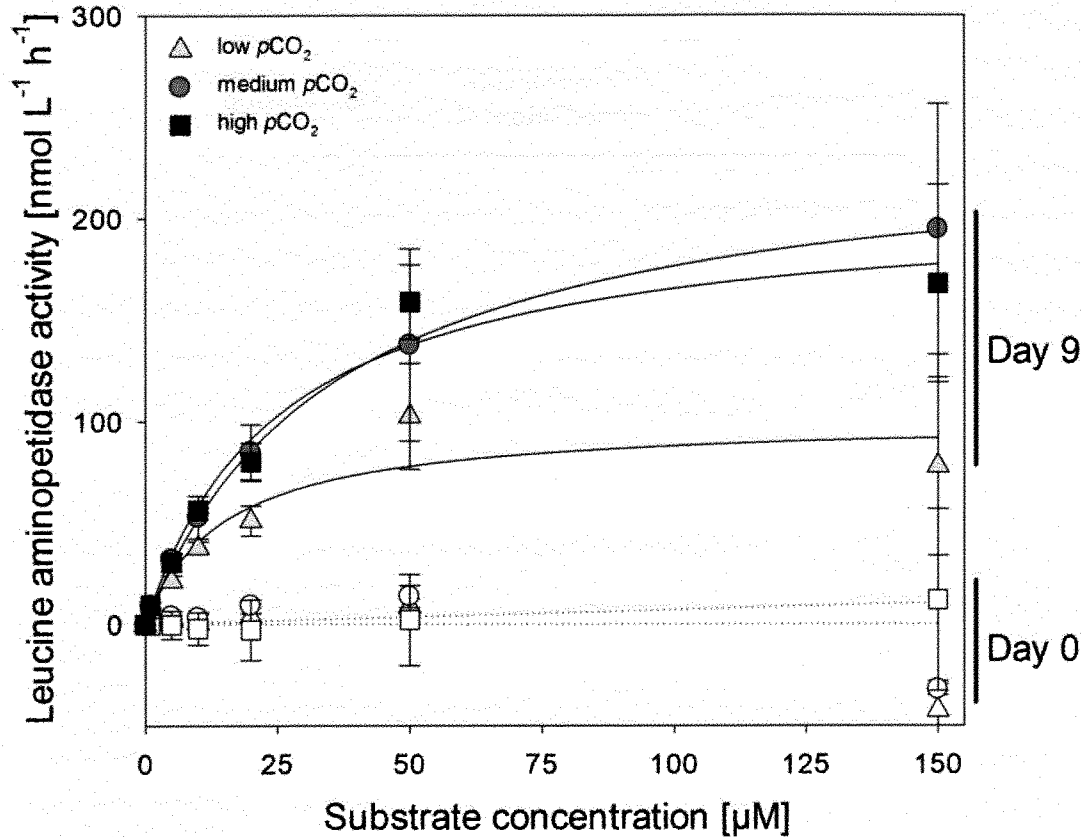


Fig. 8. Aminopeptidase activities on day 0 and day 9 of low (triangle), medium (circle) and high (square) pCO_2 treatments. Symbols display the mean of three replicates with standard deviation.

At the end of the experiment, cyanobacteria cells were filtered out (2.7 μm GF/D filter). Extracellular enzymes in the medium however cannot be removed by this filtration. We determined extracellular enzyme activities of the filtrate and found that 1-2% of the APA and 2-59% of the LAP activity remained. Summarizing our results, we can say that (1) AP was mainly attached to *Nodularia* cell surface, (2) LAP was mainly released to the medium, (3) degradation of DOP was driven by *Nodularia* while heterotrophic degradation of DOP was negligible and (4) we cannot ascribe LAP activity solely to *Nodularia spumigena*. Heterotrophic bacteria might also have contributed to LAP to a certain degree although they did not build up biomass. Heterotrophic bacteria attached to the filaments may be responsible for the slight increase in glucosidase activity towards the end of the experiment and also for some percentage of LAP activity.

Table 4. Correlation of leucine aminopeptidase activity with biomass of filamentous cyanobacteria (POC, chl *a*), dissolved inorganic and organic P, *p*CO₂, pH, N₂ fixation and bacterial cell counts (n = 36, except for NO₃, DON (n = 35) and N₂ fixation (n=32); R = correlation coefficient). Correlation level is marked as * for low significance (0.03 < |R| < 0.7, *p* < 0.05) and ** for strong significance (|R| > 0.7, *p* < 0.05), nc: for no correlation

Parameter	Correlation coefficient R	Correlation
LAP activity vs.		
NO ₃	-0.46	*
DON	-0.34	*
POC	0.71	**
chl <i>a</i>	0.52	*
<i>p</i> CO ₂	-0.63	*
pH	0.64	*
N ₂ fixation rate	-0.73	**
Bacterial cell counts	0.03	nc

4.5.6 Discussion

The aim of this study was to investigate the effect of CO₂-induced changes in seawater carbonate chemistry on the exudation of organic matter and its subsequent recycling by extracellular enzymes in a *Nodularia spumigena* culture. *Nodularia spumigena* growth was induced by adding phosphate on day 0 and day 3. The growth peak occurred simultaneously around day 9 in all treatments. Afterwards cell numbers declined since phosphorus was depleted. Three CO₂ conditions were applied by aerating the cultures with 180 ppm, 380 ppm and 780 ppm CO₂. Obtained *p*CO₂ values were on median 315 µatm in the low, 353 µatm in the medium, and 548 µatm in the high *p*CO₂ treatment (Wannicke et al., 2012). In general, *p*CO₂ had a stimulating effect on *Nodularia spumigena* growth and N₂ fixation (Wannicke et al., 2012). These findings are in accordance with earlier findings that suggested growth and POC production of cyanobacteria to be increased by elevated *p*CO₂. (Hutchins et al., 2007; Barcelos e Ramos et al., 2007; Kranz et al., 2010). Increased cyanobacterial growth in turn was influencing exudation, nutrient uptake and recycling of organic matter by extracellular enzymes in our study.

Exudation and formation of gel particles by Nodularia spumigena

Highest changes in mucinous substance concentration were observed at high *p*CO₂ levels (Fig. 2). Cells of *Nodularia spumigena* are covered by a mucus surface coating (Nausch, 1996; Engel et al., 2002b) that includes acidic mucopolysaccharide and is therefore stained by Alcian Blue. Thus, an increased biomass production in the high *p*CO₂ treatment leads to apparently higher mucinous substance concentrations. Field measurements revealed similar concentrations in the Baltic Sea as found in our study (Engel et al., 2002b). When the cyanobacteria bloom declined, mucinous substance concentrations still increased in our study (Fig. 1). This was pronounced at high *p*CO₂. In accordance with the accumulation of mucinous substances, the increase in POC and chl *a* concentration was significantly higher with increasing *p*CO₂ during the growth phase (Fig. 2). Yet, the ratio of mucinous substances to POC concentration was decreasing during the growth phase and increasing afterwards. This indicates that biomass production was more stimulated than carbon exudation and mucus production during the first nine days of the incubation. As growth slowed down and filament numbers decreased, more carbon was released either by active exudation or cell lysis. Therefore, our conclusion is that cell-specific production of mucinous substances was not effected by CO₂ but due to higher biomass we found more mucinous substances in the high *p*CO₂ treatments.

Due to methodological constraints, we cannot discriminate between acidic polysaccharides contained in TEP and in mucus. While TEP is formed by coagulation and aggregation of exuded DOC, large mucopolysaccharide molecules are released by cells and coagulate in the mucus layer (Nausch, 1996). It has been proposed that the mucus layer has the function to protect the autotrophic cells against degradation by heterotrophic bacteria and help the cells to aggregate

(O'Neil and Roman, 1992). Thus, it would be interesting to investigate in further studies which factors influence the production of mucus and to quantify the amount per cell. For heterotrophic bacteria it is known that maximal extracellular polymeric substance production occurs under nutrient limitation in the presence of an excess carbon source (Sutherland, 1985). In *Nostoc*, a filamentous N₂-fixing freshwater cyanobacterium, extracellular polymeric substances serve as a sink for the excess fixed carbon under unbalanced C:N metabolism (Otero and Vincenzini, 2004). This mucus production happens especially under high $p\text{CO}_2$ conditions, where the potential rate of CO₂ fixation exceeds that of N₂ fixation. In our study, ratios of fixed CO₂ to fixed N₂ were higher than the Redfield ratio with maximum values in the medium $p\text{CO}_2$ treatment (Wannicke et al., 2012) which does not suggest potential higher production of mucus in the high $p\text{CO}_2$ treatments. Hence, we suggest that principally more TEP, and not mucus, is formed at higher $p\text{CO}_2$ after growth decelerated.

In contrast to TEP, CSP are barely stained in the mucus surface coating of *Nodularia* so CSP can be considered as discrete, protein-containing particles during this experiment. CSP area was significantly positive correlated with $p\text{CO}_2$. CSP abundance increased over time in all treatments with highest increase in the low $p\text{CO}_2$ treatment. The average size of the individual CSP decreased at low and medium but increased at high $p\text{CO}_2$ (Fig. 5). This indicates that CSP were possibly degraded in the low and the medium $p\text{CO}_2$ treatment to more but smaller particles while more material was aggregating to bigger particles in the high $p\text{CO}_2$ treatment. This might be explained by increasing stickiness and changing composition of the organic matter and especially by a higher concentration of acidic sugars (Alldredge et al., 1993; Engel et al., 2004a), which could also hint towards the formation of free TEP.

Transferred to the field, an increase in gel particles would change the aggregation of filaments and the export of organic matter towards deeper layers of the ocean. Additionally, it may also effect bacterial growth as mucus is a suitable substrate for marine bacteria. However, the composition changes of organic matter, especially sugars and amino acids, under elevated $p\text{CO}_2$ need to be investigated more in detail to proof this assumption.

Production and subsequent recycling of dissolved organic matter by extracellular enzymes

Some studies propose that N₂ fixation and subsequent release of DON may be a mechanism to cope with excess light energy on a short time scale (Lomas et al., 2000; Wannicke et al., 2009). So far, no report on the effect of $p\text{CO}_2$ on DON release exists, while exudation of DOC was shown to be stimulated with increasing $p\text{CO}_2$ (Borchard and Engel, 2012). In the present study, DOC concentrations slightly increased in all treatments during the growth phase, while DON and DOP concentrations decreased (Fig.3 and Fig. 7). Our results indicate that the algae were releasing DOC to the seawater but we cannot confirm a stimulating effect of elevated $p\text{CO}_2$ on DOC exudation. In contrast, DON concentrations decreased which may be explained by enzymatic degradation to acquire nitrogen. As CSP area was increasing during our experiment and with

$p\text{CO}_2$, the DON loss may also be explained by aggregation processes to CSP. The growth of *Nodularia spumigena* after phosphate depletion on day 3, as well as the continuing POP formation between days 3 to 15 (Unger et al, 2013), indicated the utilization of DOP as organic source for phosphorus. Further aspects of changes in DOM and stoichiometry of particulate organic matter are discussed in Wannicke et al. (2012). Unger et al. (2013) describe in detail changes in the turnover of P and the composition of the P pool in our study.

Different compounds of organic matter may be released by primary producers under elevated $p\text{CO}_2$ but also extracellular enzymes may modulate the composition and properties of organic matter. To determine the turnover rates of organic matter due to enzymatic cleavage, extracellular enzyme activities of four key enzymes in carbon and nutrient cycling (alkaline phosphatase, α - and β -glucosidase, and leucine aminopeptidase) were followed over time in all treatments. Enzyme activities at the start of the experiment were low and increased over time in all treatments.

Leucine aminopeptidase is a major enzyme in microbial degradation of organic matter. Most of its activity was found to be associated with particles (Hoppe et al., 2002). *Nodularia spumigena* is able to express leucine aminopeptidase to degrade proteins (Stoecker et al., 2005) and to acquire amino acids. In terms of magnitude, LAP activities determined during this study are in the range of those previously measured in the Baltic Sea (Nausch, 1998b). Nausch and co-workers determined an average activity of $263.1 \pm 128.4 \text{ nmol L}^{-1} \text{ h}^{-1}$ in autumn in the Pomeranian Bight. In our study, leucine aminopeptidase activity (LAP) positively correlated with cyanobacterial biomass and negatively with N_2 fixation rates (Table 4). N_2 fixation decreased over time in all treatments while LAP activity increased. In the end of the vegetation period, senescent algae exude polymeric substances, such as proteins, which have to be enzymatically cleaved before uptake (Hoppe et al., 2002). This might explain the temporal increase in leucine aminopeptidase activity in our study especially when *Nodularia* stopped growing and filament numbers decreased. We conclude that *Nodularia* might have changed their N acquisition strategy from N_2 fixation towards enzymatic degradation of DOM over time.

Enzyme activities may respond quickly to changes in pH but also in substrate availability and their kinetics are modified to benefit best from environmental conditions. In this context, it is important to distinguish between direct $p\text{CO}_2$ - and pH-effects on microbial activity from effects induced by CO_2 -related changes in autotrophic organic matter production. Piontek and coworkers (2010) suggest that rates of enzymatic hydrolysis by marine glucosidase assemblage are not at their maximum at present-day seawater pH. Our results show an indirect effect of increasing $p\text{CO}_2$ on LAP activities due to increased cyanobacterial growth. Correlation between LAP activity and $p\text{CO}_2$ was low. However, a direct pH effect could, in theory, have been masked by differences in the quantity or nature of enzymes and their substrates, but such effects cannot be detected with the experimental methods used here. This is in good accordance with previous results from a mesocosm study where increasing $p\text{CO}_2$ changed the photosynthetic production of dissolved and particulate organic matter and therefore increased enzyme activities (Grossart et al., 2006).

In our study, initial PO_4^{3-} concentrations were below $0.5 \mu\text{M}$ and APA was negatively correlated to PO_4^{3-} concentration over time (Table 2). It is well documented that under P-limiting conditions *Nodularia spumigena* has a high uptake affinity for DOP by production and release of alkaline phosphatase (Nausch, 1998a; Wu et al., 2012). A direct relationship between intracellular PO_4^{3-} concentration and APA was shown in Huber and Hamel (1985a) and indicated that cellular rather than external phosphorus sources controlled APA. A threshold of APA around $1 \mu\text{M}$ PO_4^{3-} was defined (Nausch, 1998a). In terms of magnitude, APA determined during this study is comparable with data given by Nausch (1998a) for the Baltic Sea ($5\text{--}550 \text{ nM h}^{-1}$). We conclude that *Nodularia* expressed more alkaline phosphatase to acquire phosphorus from organic sources when PO_4^{3-} concentrations declined.

We found increased APA by ~factor 1.64 and 2.25 in the medium and high compared to the low $p\text{CO}_2$ treatment (Fig. 6). Correlation between pH and the increase of APA was low (Table 2). The alkaline phosphatase has its optimum between pH 7.5 and 10 (e.g. Healey and Hendzel, 1979; Münster, 1992) depending on origin and composition of the enzyme assemblage. Other enzymes were shown to have their optimum at a pH below present seawater pH (e.g. Münster, 1992; Grossart et al., 2006; Piontek et al., 2010). Therefore, we assume that pH may also have a direct stimulating effect on APA.

Increased APA in the high $p\text{CO}_2$ treatments may have supported the growth of *Nodularia* by reducing P-limitation. While APA was increasing, DOP concentrations decreased in parallel (Fig. 7). The rapid hydrolysis and recycling of DOP by *Nodularia* may have stimulated POP (biomass) production. On the other side, increased C availability in the high $p\text{CO}_2$ treatments may have stimulated *Nodularia* growth which in turn increased the expression of more alkaline phosphatase and this consecutively supported *Nodularia* growth. This point needs further examination, because it cannot be resolved completely here. Our results are in accordance with another study investigating the effect of elevated $p\text{CO}_2$ on APA during a mesocosm experiment under phosphate depletion (Tanaka et al., 2008). To sum up, we hypothesize that DOP recycling by alkaline phosphatase was stimulated by increasing $p\text{CO}_2$ and likely beneficial for *Nodularia* growth.

The K_m values of APA in our experiment increased over time and tended to be higher at high $p\text{CO}_2$ but differences were not significant (Table 1). Higher K_m values indicate decreasing substrate affinity. Enzymes catalyzing the same reaction may occur in more than one molecular form (isoenzymes), characterized by different half-saturation constants (K_m), temperature, and pH optima (Hoppe, 2003). Substrate specificity of phosphatase is little explored. The increasing K_m values in our study might be explained by the production of different and less-specific isoenzymes of the alkaline phosphatase. This could be a mechanism for *Nodularia* to access more and different compounds of the DOP pool under severe P-limitation. Because total APA and K_m values were likewise increasing, enzyme efficiency ($V_{\text{max}}:K_m$ ratio) did not increase with increasing $p\text{CO}_2$. In terms of magnitude K_m values determined here are in the range of values measured in the Central North Pacific Ocean (Perry and Eppley, 1981), and in the Gulf of Biscay

(Labry et al., 2005) but below values from the Baltic Sea, which ranged between 4 and 37 μM (Grönlund et al., 1996). In conclusion, phosphate affinity of the alkaline phosphatase tended to be lower in the high $p\text{CO}_2$ treatment but enzyme efficiency remained.

To investigate the effect of $p\text{CO}_2$ on production and exudation of organic matter by *Nodularia*, we tried to exclude heterotrophic bacteria to avoid fast degradation of labile components. Although a low number of heterotrophic bacteria were still present in the treatments, we did not detect active growth. In our study, AP and LAP activities could be mostly assigned to cyanobacteria although we cannot completely exclude the contribution of heterotrophic bacteria to LAP activity. This is in accordance with previous studies that found extracellular enzyme activity in association with *Nodularia* sp. (Huber and Hamel, 1985b; Stoecker et al., 2005). Around 37% of APA and 30% of LAP activity were associated with cyanobacteria during a *Nodularia* dominated bloom in the Baltic Sea (Stoecker et al., 2005). However, heterotrophic bacteria may have contributed to organic matter degradation which is reflected in slightly increasing α - and β -glucosidase activities over time. In the field heterotrophic bacteria may compete with cyanobacteria for nutrients and contribute significantly to the recycling of organic matter especially in the decline of algal blooms (Chrost et al., 1989; Jacquet et al., 2002). However, cyanobacteria are efficient in using DOP and therefore successful competitors with heterotrophic bacteria for phosphate (Vahtera et al., 2007; Michelou et al., 2011).

Implications for the Baltic Sea under environmental change

Ocean acidification is only one of several environmental changes caused by rising atmospheric CO_2 concentrations. To provide a realistic picture of climate change for the marine ecosystem, the multiple effects of environmental change need to be addressed (Rost et al., 2008). Combined effects of climate change (e.g., CO_2 and temperature) may compensate or amplify direct effects of increasing CO_2 levels alone. Similar to pH, temperature may affect stability, hydrolyzation rates and substrate affinity of enzymes (Hernandez et al., 2002). Temperature may also enhance diazotrophic growth as suggested by O'Neil et al. (2012) and consequently expand range and timing of diazotrophic cyanobacteria blooms in the future. Higher temperatures will also reduce the mixing of the upper ocean due to increased thermal stratification and suppress the upwelling of nitrate (Doney, 2006), further promoting the growth of diazotrophic organisms such as *Nodularia spumigena*.

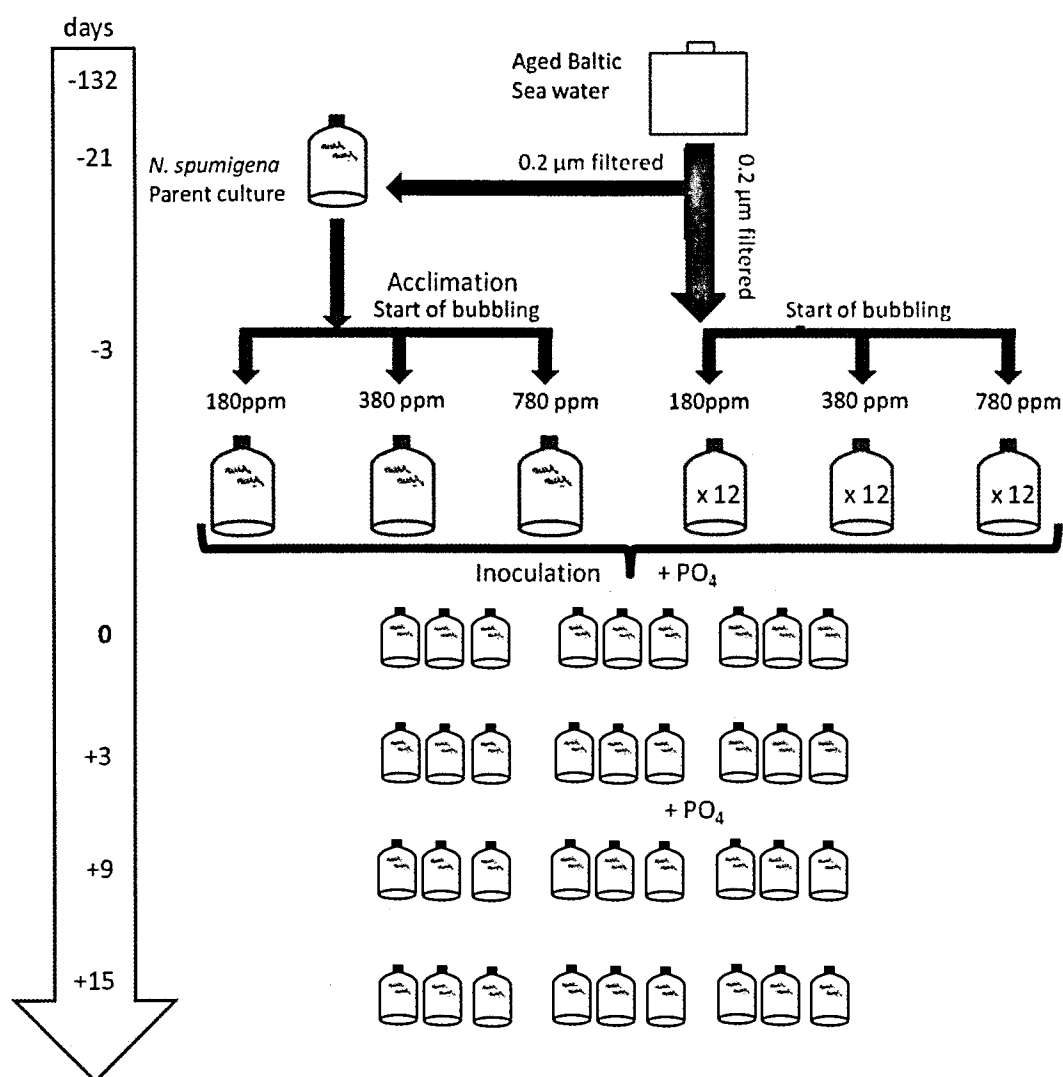
On the other side, the on-going increase in global N loading may potentially increase P limitation in marine ecosystems (Dyhrman et al., 2007). P is an important driver of microbial dynamics in marine systems which lead to an intense competition for P between phototrophic and heterotrophic microorganism and forces them to improve their P-cycling efficiencies. As *Nodularia* is able to utilize DOP more efficiently compared to other phytoplankton in the Baltic Sea (Vahtera et al., 2010) they may benefit from decreasing seawater pH and possibly also from ocean warming. This may promote a succession of cyanobacterial communities towards this

genus. It was recently shown that the cyanobacterium *Aphanizomenon ovalisporum* promotes inorganic phosphate supply by secreting toxins, which induces alkaline phosphatase in other phytoplankton species (Bar-Yosef et al., 2010; Raven, 2010). Some *Nodularia* strains produce the cyanotoxin nodularin during the summer bloom (Sivonen et al., 1989; Sellner et al., 2003), which accumulates in blue mussels (Kankaanpää et al., 2007), flounders (Kankaanpää H. et al., 2005) and seabirds (Sipiä et al., 2004). Mass occurrences of *Nodularia* due to climate change might lead to an overall increase in cyanotoxin concentration. These toxins might spread further in the ocean and accumulate in the invertebrates and vertebrates with unknown consequences for the marine food-web and eventually humans. Furthermore, extended blooms may lead to higher aggregation and export of filaments to deeper waters. Enhanced microbial degradation there could enhance oxygen consumption and consequently expand the already existing oxygen-deficient zone in the deeper waters of the Baltic Sea.

Conclusion

Our results suggest that *Nodularia spumigena* can grow faster under elevated $p\text{CO}_2$ supported by enhanced recycling of organic matter to satisfy nutrient demands. Aggregation of filaments and the export of organic matter towards deeper water layers may change due to the formation of more gel particles by *Nodularia spumigena*. To predict the effect of climate change on cyanobacterial dynamics and organic matter cycling in the sea, we need to broaden the focus by including the combined effects of all projected changes in environmental conditions.

4.5.7 Supplementary material



Appendix A. Schematic overview of the experimental set up and time flow of the single steps taken from preparation (-132 days) to the end of the experiment (+15 days). See Wannicke et al. (2012) for detailed information.

4.5.8 Acknowledgments

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4.6 Manuscript IV

Response of *Nodularia spumigena* to $p\text{CO}_2$ - Part 1: Growth, production and nitrogen cycling

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4.6.1 Abstract

Heterocystous cyanobacteria of the genus *Nodularia* form extensive blooms in the Baltic Sea contributing substantially to the total annual primary production. Moreover, they dispense a large fraction of new nitrogen to the ecosystem, when inorganic nitrogen concentration in summer is low. Thus, it is of great ecological importance to know how *Nodularia* will react to future environmental changes, in particular to increasing carbon dioxide (CO₂) concentrations and what consequences there might arise for cycling of organic matter in the ocean. Here, we determined carbon (C) and dinitrogen (N₂) fixation rates, growth, elemental stoichiometry of particulate organic matter and nitrogen turnover during batch growth of the heterocystous cyanobacterium *Nodularia spumigena* under low (median 315 µatm), mid (median 353 µatm), and high (median 548 µatm) CO₂ concentrations. Our results demonstrate an overall stimulating effect of rising *p*CO₂ on C and N₂ fixation, as well as on cell growth. An increase in *p*CO₂ during incubation days 0 to 9 resulted in an elevation in growth rate by $84 \pm 38\%$ (low vs. high *p*CO₂) and $40 \pm 25\%$ (mid vs. high *p*CO₂), as well as in N₂ fixation by $93 \pm 35\%$ and $38 \pm 1\%$, respectively. C uptake rates showed a high standard deviation for C fixation, because increase at high *p*CO₂ from day 0 to day 3 was accompanied by a decrease in uptake rates at day 9 relative to mid ($60 \pm 60\%$) and low *p*CO₂ ($9 \pm 44\%$).

Additionally, elevation in the carbon as well as nitrogen to phosphorus quota of the particulate biomass formed (POC:POP and PON:POP) was observed at high *p*CO₂. Our findings suggest that rising *p*CO₂ stimulates the growth of heterocystous diazotrophic cyanobacteria, in a similar way as reported for non-heterocystous diazotrophs. Implications for biogeochemical cycling and food web dynamics, as well as ecological and socio-economical aspects in the Baltic Sea are discussed.

4.6.2 Introduction

In summer, the heterocystous diazotrophic cyanobacteria of the genus *Nodularia* form extensive blooms in the open Baltic Sea with more than 200 mg m⁻³ wet weight (Wasmund, 1997), along with cyanobacteria of the genus *Aphanizomenon*. These blooms are usually promoted by low nitrogen-to-phosphorus ratios in the surface waters (e.g. Niemistö et al., 1989; Nausch et al., 2008a; Raateoja et al., 2011), exhibiting an average annually primary production rate of ~21 mol C m⁻² yr⁻¹ in the Baltic Proper (Wasmund et al., 2001b). The capacity of community N₂ fixation in the Baltic Sea is comparable to nitrogen inputs from the land and atmosphere (e.g. Larsson et al., 2001; Wasmund et al., 2001b; Wasmund et al., 2005b). Annual N₂ fixation rates during a moderate bloom in the Baltic Proper were averaged to 101–263 mmol N m⁻² yr⁻¹ (Wasmund et al., 2001a).

A significant fraction of the newly fixed nitrogen can be directly released by cyanobacteria, thereby dispensing 35 to 80% of nitrogen into the surrounding environment (Wannicke et al., 2009; Ploug et al., 2011). Nitrogen fixed by diazotrophs can be transferred to lower food web levels via dissolved organic matter (Ohlendieck et al., 2000) and to higher trophic levels by grazing directly on cyanobacteria or indirectly via the microbial loop (Engström-Ost et al., 2011). The extra load of nitrogen thus increases overall ecosystem productivity and meets 20 to 90% of the nitrogen requirements for community primary production during summer blooms (Sörensson and Sahlsten, 1987; Larsson et al., 2001; Wasmund et al., 2005a). Aggregation and sedimentation of net primary production accounts for ~30 up to 72% of biomass loss from the upper mixed layer which is a considerable proportion of the seasonal sinking flux (Lignell et al., 1993; Heiskanen and Leppänen, 1995). This process is supported by the formation of aggregates including *Nodularia* filaments (Engel et al. 2002).

To date, it is not well understood and barely investigated, how future changes in climate caused by anthropogenic elevation of atmospheric CO₂ concentration will affect *Nodularia* performance and their potential to alter biogeochemical fluxes. At present day an atmospheric partial pressure of CO₂ (*p*CO₂) of 380 ppm prevails being elevated by 27% compared to the pre-industrial times of 280 ppm. This accounts for the highest levels since approximately half a million years (e.g. Lüthi et al., 2008). With atmospheric CO₂ dissolving in seawater, it is expected that current *p*CO₂ in the oceans will nearly double to 780 µatm by 2100, and lower the ocean's pH by about 0.35 units (IPCC, 2007), assuming that emissions will carry on at the present rate. This has severe implications for marine phytoplankton, as they appear to directly respond to increasing *p*CO₂ by altering their physiology (e.g. Riebesell et al., 2007), relative abundance (e.g. Tortell et al., 2002), and biogeography (e.g. Boyd and Doney, 2002). Additionally, unicellular marine cyanobacteria such as *Synechococcus* and *Prochlorococcus* can show species-specific responses to increasing *p*CO₂ (e.g. Fu et al., 2007). Several studies demonstrate that elevated *p*CO₂ supports C and N₂ fixation, as well as growth rates in the non-heterocyst diazotroph *Trichodesmium* (Hutchins et al., 2007; Levitan et al., 2007; Barcelos e Ramos et al., 2007). It has been hypothesized that these

trends are facilitated by changes in activity of the carbon concentrating mechanism (CCM) and modified protein activity (Levitan et al., 2010a; and 2010b; Kranz et al., 2011) of the enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (RUBISCO) resulting in a decrease of energy and nutrient demand of the cell at high $p\text{CO}_2$. The enzyme RUBISCO has a naturally low affinity to CO_2 . Subsequently, energy saved can be relocated to other metabolic processes such as N_2 fixation. But experimental data so far are not able to prove this hypothesis on a gene expression level and no publication is available to verify this notion for heterocystous cyanobacteria.

In general, there is little knowledge on the response of heterocystous cyanobacteria to $p\text{CO}_2$. Czerny et al. (2009) directly addressed the effects of different $p\text{CO}_2$ conditions on growth and C fixation of the genus *Nodularia* and observed an overall detrimental effect of rising $p\text{CO}_2$ on the cells associated with a decrease in growth and production. They suggested that this pattern could be typical for heterocystous cyanobacteria compared to non-heterocystous cyanobacteria of the genus *Trichodesmium* and potentially relate to physiological and structural dissimilarities of both cyanobacteria groups. Even less information is available on the coupling of fluxes of carbon, nitrogen and phosphorus in relation to $p\text{CO}_2$ mediated by heterocystous cyanobacteria.

The purpose of this study was to examine the relationship between $p\text{CO}_2$ and diazotrophic growth of *Nodularia spumigena* and the related fluxes of carbon, nitrogen and phosphorus. Cultures of Baltic Sea *Nodularia spumigena* isolates were grown in a batch mode at three different $p\text{CO}_2$ levels (low, medium and high), which were supposed to simulate glacial (180 μatm), present day values (380 μatm) and values projected for the year 2100 (780 μatm). Here, we present data on growth and production parameters, as well as N_2 fixation and nitrogen turnover in response to increasing $p\text{CO}_2$. Carbon cycling and extracellular enzyme activities, as well as phosphorous cycling and utilization of dissolved organic phosphorous (DOP) will be presented in two companion publications (Endres et al., 2012, Unger et al., in prep).

4.6.3 Material & Methods

Culture condition and design of the batch culture experiment

The experimental set-up we applied can be divided into three parts: firstly, the preparation of aged seawater, secondly, culturing of the parent culture and its acclimation to the $p\text{CO}_2$ treatments and thirdly, inoculation with pre-acclimated cultures, amendment with DIP and the actual experimental run. The three individual steps taken are described in details below and illustrated in figure 1.

The first part began with sampling of 1000 litre of seawater in the open Baltic Sea (54.22749°N, 12.1748°E, salinity of 9.1 psu), four months before the start of the experiment. Plankton growth was allowed for three months resulting in aged sea water with low concentration in dissolved inorganic nutrients. After this growth phase, sterilization was achieved by UV light treatment and 0.2 μm filtration under a clean bench. Concentrations of inorganic nutrients in this seawater (DIN and DIP) were below the detection limit.

The second part included the growth of three litre parent culture of the heterocystic cyanobacterium *N. spumigena*, which was isolated by L. Stal and coworkers (NIOO) from the Baltic Sea and maintained since 2000 at the Leibniz Institute for Baltic Sea Research in batch cultures in F/2 medium free of any combined inorganic N compounds. Three weeks prior to the start of the experiment, an axenic parent culture was transferred to sterile filtered aged Baltic Sea water amended with 0.3 $\mu\text{mol l}^{-1}$ PO_4 every week to assure an exponential growth phase. The parent culture was cultured at 15°C in a walk-in incubation chamber under 16:8 hours light:dark cycle (cool, white fluorescent lighting, 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). One week before the start of the acclimation period, the parent culture was still in a stationary growth phase and removed from the walk-in incubation chamber to a climate controlled room. There, temperature was increased to 23°C (representing typical summer temperatures at the Baltic Sea water surface) and light supply raised to 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (light: dark cycle of 16:8 hours, cool, white fluorescent lighting.). The chosen light intensity was rather low compared to light intensities experienced by cyanobacteria in summer time in the Baltic Sea (ca. 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). Higher light intensities (>200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) were not achievable by using fluorescent light and would have led to an unwanted large increase in experimental temperature. Cultures were routinely mixed by manually shaking to prevent adhesion of cyanobacteria to the walls of the culture vessels.

Acclimation of the *N. spumigena* parent culture to the different $p\text{CO}_2$ level started three days before the beginning of the experiment. The parent culture was separated into three pre-cultures, one litre for each CO_2 treatment, and acclimated to the target $p\text{CO}_2$ by aeration with premixed gases (Linde gas). Three levels of $p\text{CO}_2$ were used, i.e. 180 μatm representing glacial conditions, 380 μatm representing present day and 780 μatm representing year 2100 conditions (Boer et al., 2000). During the course of the experiment (including the acclimation period; 18

days), aeration took place in the early afternoon (2 pm) for one hour per day in order to avoid continuously high turbulence that could potentially harm the integrity of the cyanobacteria filaments.

Aeration, however, was not sufficient to yield equilibration with the pre-mixed gases, i.e. the calculated $p\text{CO}_2$ (from CT and pH) values in the pre-cultures were 402 μatm , 422 μatm and 548 μatm for the glacial, present-day and future $p\text{CO}_2$ treatments, respectively. For this reason, we re-defined the three $p\text{CO}_2$ levels based on the true $p\text{CO}_2$ determined during the experiment. These are the low $p\text{CO}_2$ treatment (median 315 μatm), medium $p\text{CO}_2$ treatments (median 398 μatm) and high (median 548 μatm) $p\text{CO}_2$ treatment.

The third part started with the inoculation of each experimental bottle. After three days of pre-acclimation, chlorophyll a concentrations were determined in the three pre-cultures, yielding 28 $\mu\text{g chl a l}^{-1}$ in the low $p\text{CO}_2$ treatment, 27 $\mu\text{g chl a l}^{-1}$ in the mid and 38 $\mu\text{g chl a l}^{-1}$ in the high $p\text{CO}_2$ scenario. In order to inoculate the same quantity, 0.8 $\mu\text{g Chl a l}^{-1}$, we added 296 ml to replicate bottles of the low $p\text{CO}_2$ treatment, 308 ml to the mid $p\text{CO}_2$ treatment and 221 ml to replicate bottles of the high $p\text{CO}_2$ treatment. Heterotrophic bacteria cells counts at the start of the experiment were below the blank value of 1000 cells l^{-1} . Overall, bacterial biomass during the course of the experiments (18 d, 15 d+ 3 days acclimation) never exceeded 1% of cyanobacterial biomass. After inoculation with *N. spumigena* each of the 36 bottles was amended with phosphate to 0.35 $\mu\text{mol l}^{-1}$ at time 0 and at day 3.

Four sampling time points were chosen for the three $p\text{CO}_2$ treatments (time 0, +3, +9 and +15 days, Fig. 1) with three replicate bottles harvested at each time point. Each bottle contained 10 litres of aged and sterile filtered seawater that had been aerated with premixed gases for three days in parallel with the pre-cultures. One replicate bottle of the low $p\text{CO}_2$ treatment at day 9 was omitted in the data compilation, due to inaccurate inoculation with DIP. Samples were taken between 8 and 9 am, before daily aeration.

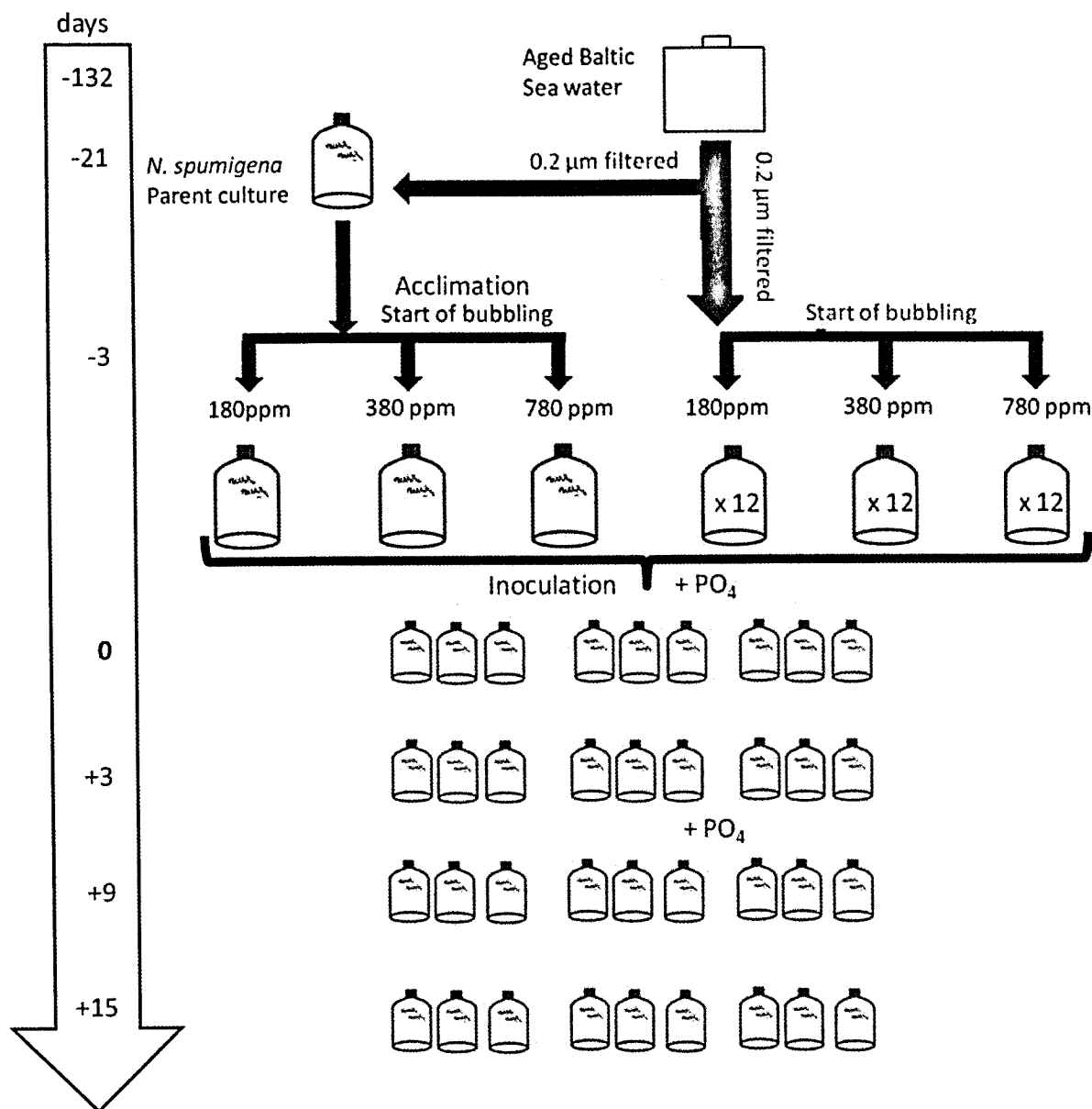


Fig. 1. Schematic overview of the experimental set up and time flow of the single steps taken from preparation (-132 days) to the end of the experiment (+15 days). See text for detailed information.

Carbonate chemistry

pH was measured with an electrode (Knick Mikroprozessor pH Meter 761 with Typ SE 100 glass electrode), calibrated directly before measurement with standard NBS buffer. Values of pH are given relative to the total scale. Total carbon (CT) was analysed directly after sampling using the colorimetric SOMMA system according to Johnson et al. (1993). The system was calibrated with carbon reference material provided by A. Dickson (University of California, San Diego) and yielded a precision of about $\pm 2 \mu\text{mol kg}^{-1}$. Total alkalinity and $p\text{CO}_2$ were calculated using CO2SYS (Lewis et al., 1998) parallel to CT, pH, salinity and temperature.

Nutrient and chlorophyll a analysis

Dissolved inorganic nutrients (NH_4^+ , NO_3^- and PO_4^{3-}) were determined colorimetrically from 60 ml filtered subsamples (combusted GF/F) using a spectrophotometer U 2000 (Hitachi-Europe GmbH, Krefeld, Germany) according to Grasshoff et al. (1983). The detection limits were $0.02 \mu\text{mol l}^{-1}$ for DIP, $0.05 \mu\text{mol l}^{-1}$ for ammonium and $0.05 \mu\text{mol l}^{-1}$ for NO_3^- . A subsample of 100 ml was filtered onto Whatman GF/F filters for chlorophyll *a* analysis, immediately after sampling. Filters were stored in liquid nitrogen or at -80°C and were extracted with 96% ethanol for at least 3 hrs. Chlorophyll *a* fluorescence was measured with a TURNER fluorometer (10-AU-005) at an excitation wavelength of 450 nm and an emission of 670 nm (HELCOM, 2005). Chlorophyll *a* concentrations were calculated according to the method of Jeffrey and Welschmeyer (1997).

Nodularia filament and bacteria cell counts

Subsamples of 50 ml were taken for phytoplankton analysis (preserved with acetic Lugol's (KI/I₂) solution to 1% fixation) and counted using an inverted microscope (Leica) (Utermöhl, 1958) at 100x magnification. Cell length and diameter were measured using a micrometer eyepiece and converted to biovolume assuming the geometrical approximation of a cylinder.

Bacteria were counted using a flow cytometer (Facs Calibur, Becton Dickinson). Four ml samples were preserved with 100 μl formaldehyde (1% v/v final concentration), shock frozen in liquid nitrogen and stored at -70°C until measurement. A stock solution of SYBR GREEN (Molecular Probes) was prepared by dilution of 1 μl dye with 49 μl DMSO. Three μl potassium citrate solution, 10 μl of the dye stock solution and 10 μl fluoresbrite microspheres (Polysciences) were added to 300 μl of the thawed sample and incubated for 30 min in darkness. Cell counting was done at a medium flow rate and calculations were performed using the software program "Cell Quest Pro". Mean abundance of heterotrophic bacteria was $4.45 \pm 2.28 \cdot 10^5$ cells l^{-1} (low $p\text{CO}_2$), $2.38 \pm 2.09 \cdot 10^5$ cells l^{-1} (mid $p\text{CO}_2$) and $4.80 \pm 2.82 \cdot 10^5$ cells l^{-1} (high $p\text{CO}_2$).

Dissolved organic matter (DON, DOC, DOP)

For analysis of dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) subsamples were filtered through pre-combusted GF/F filters, collected in 20 ml pre-combusted (8 hrs, 500°C) glass ampoules, acidified with 80 μl of 85% phosphoric acid and stored at $2-5^\circ\text{C}$ in a refrigerator. TDN and DOC concentrations were determined simultaneously by high temperature catalytic oxidation with a Shimadzu TOC-VCSH analyser. In the auto sampler, 18 ml of sample volume plus 9 ml of ultrapure (Type 1) water (in pre-combusted vials) were acidified with 50 μl HCl (1 M) and sparged with oxygen (150 ml min^{-1}) for 6 min to remove all inorganic C. 100 μl sample volume was injected directly on the catalyst (heated to 720°C). Detection of the generated CO_2 was performed with an infrared detector. Final DOC concentrations were average values of quadruplicate measurements. If the coefficient of variation exceeded 0.1%, up to 4 additional analyses were performed and outliers were eliminated. Total N was quantified by a

chemiluminescence detector (gas flow oxygen: 0.6 l min⁻¹). After every 8th sample, one standard for quality control and one blank was measured. Values of TDN were corrected for nitrate, and ammonium, and thereafter referred to as DON.

Subsamples (40 ml) for the determination of total (TP) and dissolved phosphorus (DP) were stored at -20°C until processing either unfiltered (for TP) or filtered through pre-combusted (450°C, 4 h) Whatman GF/F filters (for DP). Thawed samples were oxidized with an alkaline peroxodisulfate solution (Grasshoff et al., 1983) in a microwave (MarsXpress, CEM) to convert organic phosphorus into DIP. The subsequent DIP determination was done using a 10 cm-cuvette reducing the detection limit to 0.01 µmol l⁻¹. Dissolved organic phosphorus (DOP) was calculated as the difference between dissolved phosphorus (DP) and dissolved inorganic phosphorous (DIP), detected as described above.

Particulate organic matter analysis (PON, POC, POP)

Stable N and C isotope ratios (⁶¹⁵N-PON, ⁶¹³C-POC), as well as PON and POC concentration were measured by means of flash combustion in a Carlo Erba EA 1108 at 1020°C and a Thermo Finnigan Delta S mass-spectrometer. Filters containing particle samples were trimmed, sectioned and then loaded into tin capsules and pelletised for isotopic analysis. Particulate organic phosphorus (POP) was calculated as the difference between total and dissolved phosphorus.

Isotopic analysis and rates measurements (primary production, N₂ fixation)

The stable N and C isotope ratios measured for each sample were corrected for values obtained from standards with defined N and C isotopic compositions (International Atomic Energy Agency IAEA: IAEA-N1, IAEA-N2, NBS 22 and IAEA-CH-6) by means of mass balance. Values are reported relative to atmospheric N₂ (⁶¹⁵N) and VPDB (⁶¹³C- Vienna Peedee belemnite). The analytical precision for both stable isotope ratios was ± 0.2‰. Calibration material for C and N analysis was acetanilide (Merck). N₂ fixation activity was measured using the ¹⁵N-N₂ assay, C fixation using the ¹³C-NaHCO₃ assay. Tracer incubations were terminated by gentle vacuum filtration (<200 mbar) through pre-combusted GF/F filters. These filters were dried at 60°C and stored for isotopic analysis. Rates were calculated using the approach of Montoya et al. (1996). Incubation time for rate measurements was 6 hrs, guaranteeing a sufficient dissolution of the ¹⁵N gas in the incubation bottle (method consideration (Mohr et al., 2010)).

Statistical analysis

Statistical analyses were done using the software SPSS 9.0 and Sigma Plot 10. The effect of the pCO₂ treatment on biological and chemical variables was tested by analysis of variance of data (ANOVA, t-test). Dependencies of growth and production parameters from other environmental parameters were tested using Pearson's correlation "stepwise" multiple regression analysis. Prior to ANOVA and correlation analysis, data were tested for normality and homogeneity of variances

using Wilk-Shapiro and Levene's tests. Linear regression analysis was applied to calculate growth rates from changes in natural logarithm transformed filament/cell numbers, PON, POC, as well as chlorophyll a values.

4.6.4 Results

Carbonate chemistry

Throughout the study, the $p\text{CO}_2$ treatments were different with respect to pH and total carbon (CT), as well as calculated total alkalinity (AT) and $p\text{CO}_2$ (Table 1). The $p\text{CO}_2$ treatments differed significantly in pH and CT between mid and high $p\text{CO}_2$ treatment, as well as between the low and high $p\text{CO}_2$ treatment ($p \leq 0.001$, $n = 12$, Supplemental Table 2). Ranges of calculated values for the single treatments were for the low $p\text{CO}_2$ treatment 248.5 μatm - 498.6 μatm with a median of 315.7 μatm (mean value 340 ± 80 μatm), for the mid $p\text{CO}_2$ treatment 286.5 μatm - 571.1 μatm with a median of 353.3 μatm (mean value 398 ± 104 μatm) and for the high $p\text{CO}_2$ treatment 395.2 μatm - 630.4 μatm with a median of 548.8 μatm (mean value 508 ± 89 μatm). The large deviation in $p\text{CO}_2$ within the treatments occurred partly because CO_2 was consumed during photosynthesis. The calculated $p\text{CO}_2$ was significantly different between all three $p\text{CO}_2$ set-ups ($p \leq 0.001$, $n = 12$, Supplemental Table 2).

Inorganic nutrients

There were no significant differences in concentrations of inorganic nutrients between $p\text{CO}_2$ treatments (Supplemental Table 2). Dissolved inorganic phosphate was depleted in all treatments after three days of incubation (Table 2). DIP amended on day 3 was again below the detection limit at day 9. Throughout the experiment, mean concentration of dissolved inorganic nitrogen ($\text{DIN} = \text{NO}_3^- + \text{NO}_2^-$) was 0.26 ± 0.1 $\mu\text{mol l}^{-1}$ in the low $p\text{CO}_2$ treatment, 0.13 ± 0.1 $\mu\text{mol l}^{-1}$ in the mid $p\text{CO}_2$ treatment and 0.1 ± 0.1 $\mu\text{mol l}^{-1}$ in the high $p\text{CO}_2$ treatment (Table 2), whereas ammonium was not detectable. Due to the uptake of nutrients during cell growth, an inverse relationship was observed between DIP and abundance, chlorophyll a, PON and POP ($R^2 = -0.567$, -0.686 and -0.599 , 0.359 , respectively, $p \leq 0.05$ and $p \leq 0.01$, $n = 12$, Supplemental Table 1).

Dissolved organic matter (DOM)

DOM concentration and stoichiometry did not differ significantly between the treatments. DOC concentrations were 303 ± 26 $\mu\text{mol l}^{-1}$ in the low $p\text{CO}_2$ treatment, 309 ± 21 $\mu\text{mol l}^{-1}$ in the mid and 313 ± 36 $\mu\text{mol l}^{-1}$ in the high $p\text{CO}_2$ treatment. During the first 3 days of the experiment, DOC concentration decreased in the low and high $p\text{CO}_2$ treatment by 35 and 33 $\mu\text{mol l}^{-1}$, respectively, while it increased in the mid $p\text{CO}_2$ treatment by 2 $\mu\text{mol l}^{-1}$. From thereon, until the end of the experiment at day 15, concentrations of DOC increased by 24 $\mu\text{mol l}^{-1}$ in the low $p\text{CO}_2$ treatment, by 13 $\mu\text{mol l}^{-1}$ in the mid $p\text{CO}_2$ treatment and by 5 $\mu\text{mol l}^{-1}$ in the high $p\text{CO}_2$ treatment,

respectively. DON concentrations were $15 \pm 1.0 \mu\text{mol l}^{-1}$ in the low, $16 \pm 1.3 \mu\text{mol l}^{-1}$ in the mid and $17 \pm 1.2 \mu\text{mol l}^{-1}$ in the high $p\text{CO}_2$ treatment. They were reduced in the low compared to high $p\text{CO}_2$ level indicating a higher accumulation of DON at high $p\text{CO}_2$. From day 3 onwards, DON concentration decreased by $0.3 \mu\text{mol l}^{-1}$, $0.11 \mu\text{mol l}^{-1}$ and $0.9 \mu\text{mol l}^{-1}$, respectively. Nevertheless, it has to be kept in mind that calculated differences in concentration were of the same magnitude as standard deviation of the single measurements and have to be considered carefully. DON showed a significantly negative correlation with PON, POP and pH ($R^2 = -0.351$, -0.574 and -0.619 , $p < 0.05$ and $p \leq 0.01$, $n = 12$, Supplemental Table 1) and positive ones with C fixation, PO_4^{3-} , $p\text{CO}_2$ and CT ($R^2 = 0.557$, 0.599 and 0.622 , $p \leq 0.01$, $n = 12$, Supplemental Table 1). DOP concentrations were $0.3 \pm 0.1 \mu\text{mol l}^{-1}$ in the low, $0.27 \pm 0.1 \mu\text{mol l}^{-1}$ in the mid and $0.27 \pm 0.08 \mu\text{mol l}^{-1}$ in the high $p\text{CO}_2$ treatment. Mean values for DOC:DON ratios were 20 ± 3 (low $p\text{CO}_2$), 19 ± 2 (mid $p\text{CO}_2$) and 19 ± 2 (high $p\text{CO}_2$). Mean DOC:DOP ratios were 1094 ± 383 (low $p\text{CO}_2$), 1249 ± 421 (mid $p\text{CO}_2$) and 1243 ± 378 (high $p\text{CO}_2$). Ratios of DON:DOP were 57 ± 14 (low $p\text{CO}_2$), 64 ± 17 (mid $p\text{CO}_2$) and 66 ± 19 (high $p\text{CO}_2$).

Table 1: Carbonate system variables for the four sampling time points. pH and total carbon (C_T) were measured, total alkalinity (A_T) and pCO_2 in seawater were calculated from pH and C_T using CO2SYS (Lewis and Allison, 1998). Values are means and standard deviations of three replicates (except one replicate bottle of the 180 ppm treatment at day 9). Samples were taken between 8 and 9 am, after daily aeration.

Date	pCO_2 Target [μatm]	Incubation Time [d]	pH Total scale	C_T [$\mu mol\ kg^{-1}$]	A_T [$\mu mol\ kg^{-1}$]	pCO_2 [μatm]
29.3.2010	180	0	8.02 ± 0.02	1651.9 ± 8.9	1724.2 ± 6.4	472.9 ± 29.9
	380	0	7.95 ± 0.01	1656.8 ± 3.6	1713.4 ± 2.8	561.0 ± 8.7
	780	0	7.93 ± 0.03	1676.7 ± 2.7	1730.3 ± 2.6	590.7 ± 35.3
1.4.2010	180	3	8.16 ± 0.03	1607.4 ± 10.2	1709.7 ± 3.2	330.1 ± 26.3
	380	3	8.09 ± 0.03	1617.5 ± 11.4	1703.5 ± 3.5	388.8 ± 36.0
	780	3	7.94 ± 0.02	1667.9 ± 1.9	1720.5 ± 5.1	576.8 ± 23.3
7.4.2010	180	9	8.22 ± 0.04	1570.4 ± 14.6	1690.8 ± 5.0	276.7 ± 27.1
	380	9	8.18 ± 0.03	1581.1 ± 2.8	1690.9 ± 5.9	305.3 ± 20.3
	780	9	8.07 ± 0.02	1620.7 ± 5.3	1702.3 ± 3.8	412.2 ± 16.9
13.4.2010	180	15	8.20 ± 0.04	1568.0 ± 11.4	1683.3 ± 22.6	291.8 ± 29.3
	380	15	8.14 ± 0.01	1595.9 ± 4.9	1696.7 ± 2.0	339.6 ± 12.5
	780	15	8.03 ± 0.06	1626.9 ± 21.1	1702.2 ± 6.7	452.9 ± 82.2

Table 2: Abiotic and biotic variables for the four sampling time points. Values are means and standard deviations of three replicates (except one replicate bottle of the 180 ppm treatment at day 9).

Date	$p\text{CO}_2$ treatment	PO_4^{3-} [μM]	DIN [$\mu\text{mol L}^{-1}$]	Chlorophyll <i>a</i> [$\mu\text{g L}^{-1}$]	Bacterial abundance [10^5 L^{-1}]	DOC [$\mu\text{mol L}^{-1}$]	DON [$\mu\text{mol L}^{-1}$]	DOP [$\mu\text{mol L}^{-1}$]
29.3.2010	low	0.29 ± 0.02	0.22 ± 0.31	0.74 ± 0.08	4.31 ± 2.54	306.5 ± 18.6	16.02 ± 0.07	0.34 ± 0.04
	medium	0.32 ± 0.08	0.28 ± 0.16	0.87 ± 0.09	5.61 ± 2.22	295.6 ± 23.9	18.02 ± 1.17	0.33 ± 0.06
	high	0.34 ± 0.02	0.22 ± 0.02	0.71 ± 0.04	3.14 ± 0.22	330.1 ± 76.3	17.67 ± 1.74	0.38 ± 0.06
1.4.2010	low	0.05 ± 0.02	0.41 ± 0.35	3.04 ± 0.12	2.68 ± 0.82	270.6 ± 8.7	15.61 ± 0.01	0.27 ± 0.02
	medium	0.03 ± 0.01	0.24 ± 0.03	4.04 ± 0.79	4.95 ± 1.68	297.8 ± 8.5	15.35 ± 0.14	0.24 ± 0.02
	high	0.04 ± 0	0.22 ± 0.02	3.46 ± 0.07	4.33 ± 1.65	296.8 ± 1.6	16.18 ± 1.56	0.28 ± 0.07
7.4.2010	low	0.01 ± 0.01	0.28 ± 0.27	3.38 ± 2.27	5.14 ± 1.56	308.7 ± 19.6	15.44 ± 1.79	0.27 ± 0.08
	medium	0.02 ± 0.02	0.20 ± 0.12	5.15 ± 0.23	5.41 ± 2.39	319.9 ± 17.2	15.91 ± 0.82	0.25 ± 0.09
	high	0.01 ± 0	0.14 ± 0.15	7.27 ± 0.42	5.34 ± 3.85	322.6 ± 6.1	15.81 ± 0.44	0.23 ± 0.04
13.4.2010	low	0.03 ± 0.01	0.20 ± 0.04	1.49 ± 0.28	6.62 ± 3.22	318.7 ± 26.8	14.65 ± 0.74	0.24 ± 0.07
	medium	0.02 ± 0.01	0.21 ± 0.15	3.05 ± 0.45	2.20 ± 0.33	323.7 ± 16.4	15.13 ± 0.49	0.19 ± 0.02
	high	0.02 ± 0.01	0.10 ± 0	4.41 ± 1.09	6.10 ± 5.24	306.0 ± 15.8	16.79 ± 0.41	0.21 ± 0.04

Responses of *Nodularia*

Nodularia abundance

There was a steady increase in the abundance of *Nodularia* filaments in all $p\text{CO}_2$ treatments until day 9 (Fig. 2). Afterwards, abundance remained in a stationary phase. *Nodularia* abundance correlated significantly positive with chlorophyll a, POC, PON and POP ($R^2 = 0.74, 0.83, 0.88$ and 0.88 , $p < 0.01$, $n = 12$).

Mean filament length in the high $p\text{CO}_2$ treatment increased from $74 \pm 28 \mu\text{m}$ (day 0) to $88 \pm 38 \mu\text{m}$ (day 9), but this trend was not statistically significant. Filament length in the low $p\text{CO}_2$ treatment and mid $p\text{CO}_2$ treatments increased from $67 \pm 24 \mu\text{m}$ to $117 \pm 53 \mu\text{m}$, and from $71 \pm 29 \mu\text{m}$ to $100 \pm 50 \mu\text{m}$, respectively. Differences in filament length between treatments were not statistically significant, but filaments were slightly shorter at the highest $p\text{CO}_2$ by $\sim 23\%$. On day 3, there was a significant higher number of heterocysts per filament in the high $p\text{CO}_2$ treatment compared to the mid $p\text{CO}_2$ ($p = 0.005$, $n = 50$, data not shown) and to the low $p\text{CO}_2$ treatment ($p = 0.0001$, $n = 50$, data not shown). From thereon, heterocyst number per filament decreased significantly in all treatments, while differences between the treatments were no longer statistically significant.

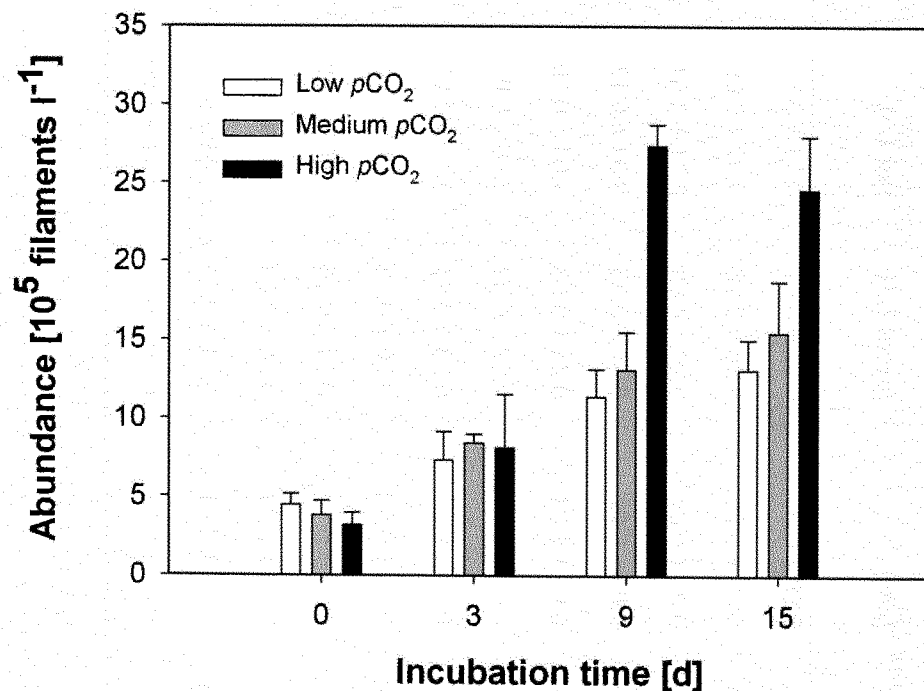


Fig. 2. Changes in abundance with time for the three $p\text{CO}_2$ treatments labelled with the partial pressure of premixed gas with which the cultures were aerated (low $p\text{CO}_2$ white bars; medium $p\text{CO}_2$, grey bars; high $p\text{CO}_2$, black bars). Bars represent mean values of three replicates with respective standard deviation.

Chlorophyll a

Chlorophyll *a* increased over time in all $p\text{CO}_2$ treatments (Table 2). Highest mean chlorophyll *a* values occurred in the high $p\text{CO}_2$ treatment ($3.96 \pm 0.42 \mu\text{g l}^{-1}$), medium values at the mid $p\text{CO}_2$ treatment ($3.28 \pm 0.72 \mu\text{g l}^{-1}$), and lowest values at low $p\text{CO}_2$ levels ($2.28 \pm 0.79 \mu\text{g l}^{-1}$). Nevertheless, only differences between the high $p\text{CO}_2$ treatment and the low $p\text{CO}_2$ treatment were statistically significant according to the t-test ($p = 0.009$, $n = 12$).

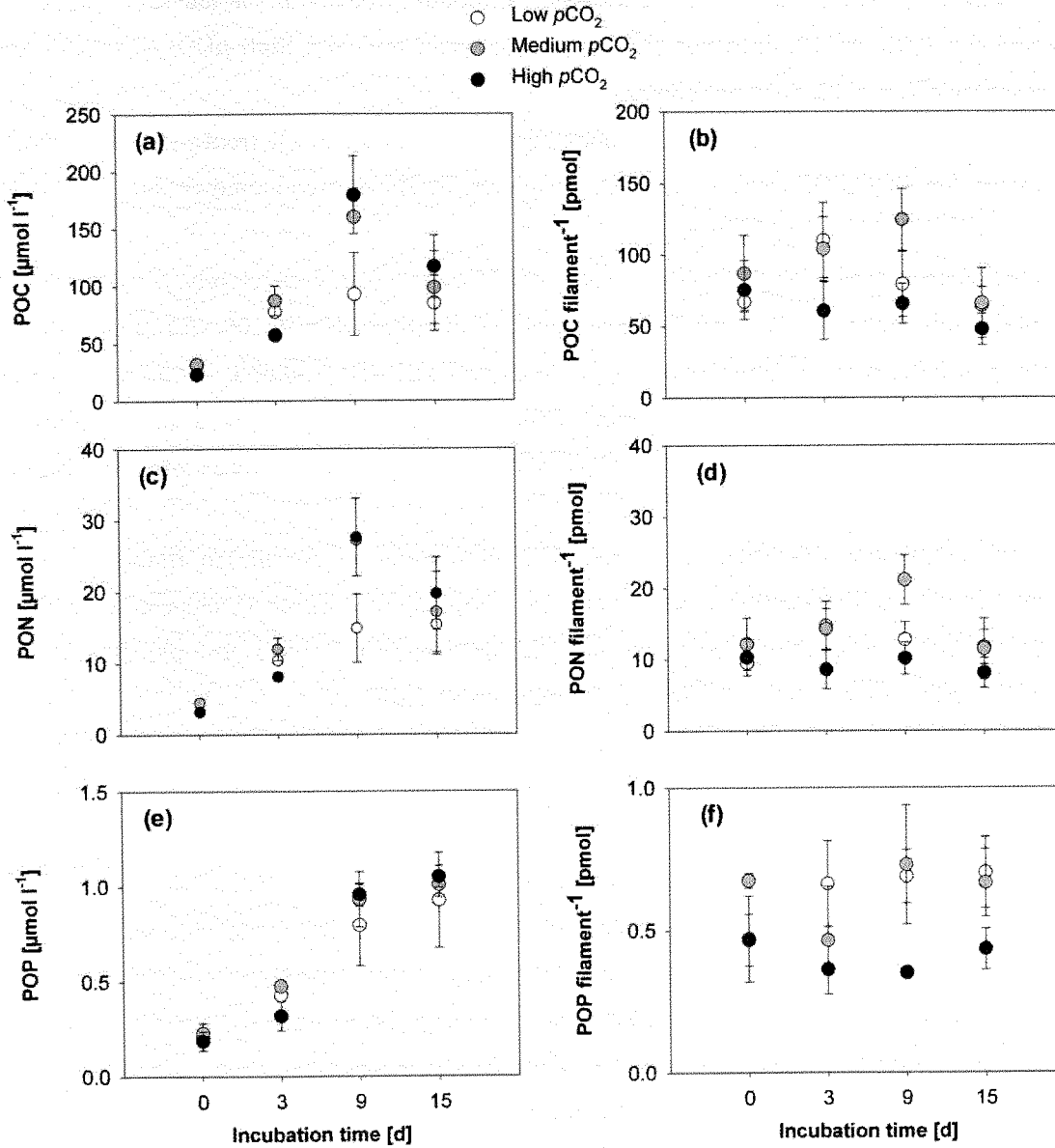


Fig. 3. Time depended variation in particulate organic carbon (POC), particulate organic nitrogen (PON), particulate organic phosphorous (POP) per volume (a,c,e) and per filament (b,d,f) for the three $p\text{CO}_2$ treatments (low $p\text{CO}_2$, white circles; medium $p\text{CO}_2$, grey circles; high $p\text{CO}_2$, black circles). Values are means and standard deviations of three replicates.

Concentration and stoichiometry of particulate organic matter (POM)

Concentrations of POC, PON and POP increased in all $p\text{CO}_2$ treatments, but most pronounced at the high $p\text{CO}_2$ level (Fig. 3). POC and PON concentration differed significantly between $p\text{CO}_2$ treatments with highest concentrations being observed at high $p\text{CO}_2$ (Table 2). Normalized to filament abundance, however, POC, PON, and POP were lowest in the high $p\text{CO}_2$ treatment (Fig. 3). Thereby, differences in POC content per filament were statistically significant for the mid vs. the high $p\text{CO}_2$ level ($p = 0.05$, $n = 12$, data not shown), but not for the other combinations of $p\text{CO}_2$ levels. PON and POP per filament differed significantly between the low and high $p\text{CO}_2$ ($p = 0.05$ and $p = 0.01$, $n = 12$, data not shown) and between mid and high $p\text{CO}_2$ ($p = 0.05$ and $p = 0.01$, respectively, $n = 12$, data not shown).

Box-plots of POM elemental composition demonstrate an elevation in all treatments relative to Redfield ratios for POC:POP and PON:POP, but near Redfield stoichiometry for POC:PON (Fig. 4).

Elemental ratios decreased with $p\text{CO}_2$ ($R^2 = -0.552$, -0.653 and -0.634 , respectively, $p \leq 0.01$, $n = 2$, Supplemental Table 1), as well as with biomass specific C fixation ($R^2 = -0.708$, -0.732 and -0.711 , respectively, $p \leq 0.01$, $n = 12$, Supplemental Table 1) and DIP ($R^2 = -0.633$, -0.653 and -0.634 , respectively, $p < 0.01$, $n = 12$, Supplemental Table 1). This might imply a more balanced incorporation of C, N, and P at higher $p\text{CO}_2$, if we assume incorporation of nutrients according to Redfield ratios.

POC:PON, POC:POP and PON:POP increased with abundance of *Nodularia* filaments ($R^2 = 0.751$, 0.795 and 0.789 , respectively, $p \leq 0.01$, $n = 12$, Supplemental Table 1), chlorophyll a ($R^2 = 0.823$, 0.883 and 0.829 , respectively, $p < 0.01$, $n = 12$, Supplemental Table 1) and pH ($R^2 = 0.529$, 0.556 and 0.529 , respectively, $p \leq 0.01$, $n = 12$, Supplemental Table 1).

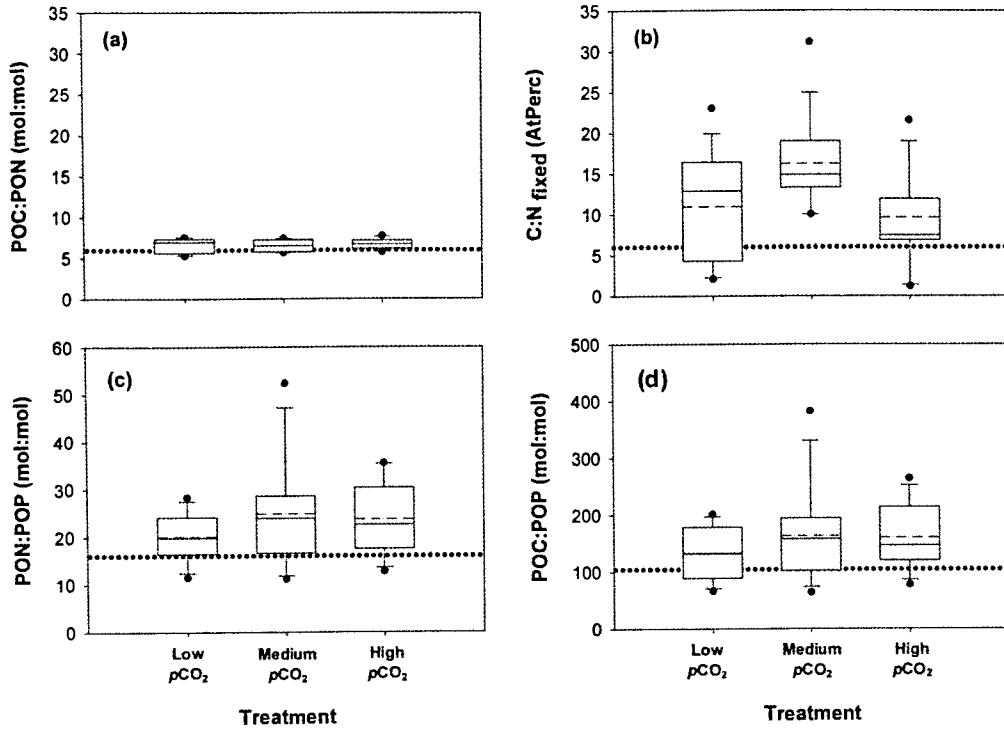


Fig. 4. Box plot ($n = 12$) of particulate organic matter stoichiometry and stoichiometry of carbon to nitrogen fixed for the three $p\text{CO}_2$ treatments (low $p\text{CO}_2$, medium $p\text{CO}_2$, high $p\text{CO}_2$). a) Molar ratio of particulate organic carbon to particulate organic nitrogen (POC:PON), b) atom percent ratio of carbon and nitrogen fixed, c) molar ratio of particulate organic carbon to particulate organic phosphorous (POC:POP), d) molar ratio of particulate organic nitrogen to particulate organic phosphorous (PON:POP). Dotted lines represent Redfield stoichiometry. Dashed dotted line represents mean values.

Growth rates

Growth rates calculated for the exponential growth phase (day 0-9) from changes in POC and PON were lower than growth rates derived from abundance and chlorophyll a in the low $p\text{CO}_2$ treatment, while they were equal in the mid and high $p\text{CO}_2$ treatment (Fig. 5). Compiled growth rates based on all parameters were significantly different between the $p\text{CO}_2$ treatments ($p < 0.05$ and $p = 0.001$, $n = 12$, Supplemental Table 2) with the highest growth rate at high $p\text{CO}_2$ ($0.212 \pm 0.018 \text{ d}^{-1}$).

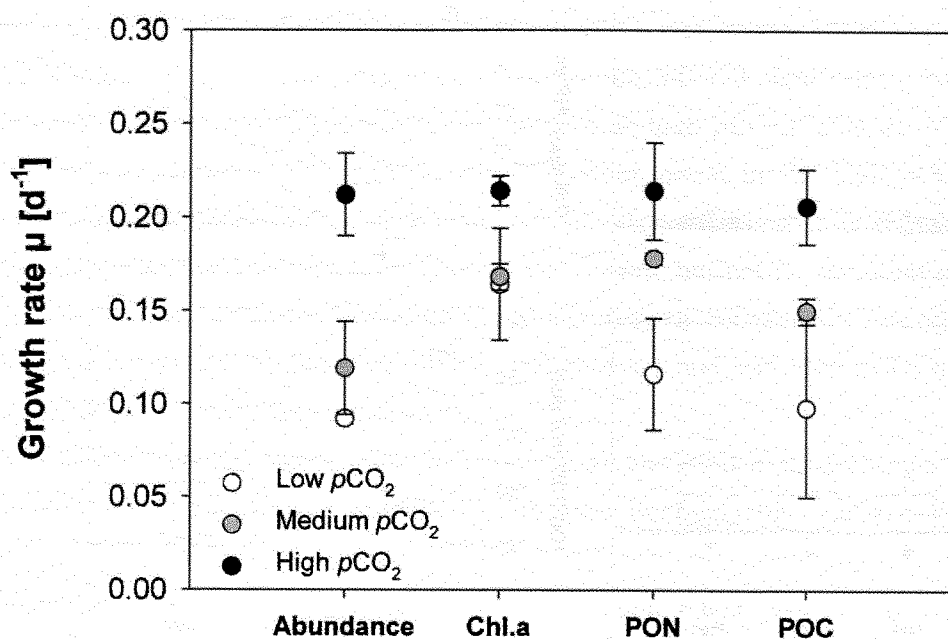


Fig. 5. Calculated growth rates (μ) per day based on changes in abundance, chlorophyll a (Chl a), particulate organic nitrogen (PON) and particulate organic carbon (POC) for the three $p\text{CO}_2$ treatments (low $p\text{CO}_2$, open circles; medium $p\text{CO}_2$, grey circles; high $p\text{CO}_2$, black circles). Symbols represent means of 12 values with standard deviations.

C and N₂ fixation

Biomass specific C and N₂ fixation rates decreased with incubation time in all $p\text{CO}_2$ treatments (Fig. 6). Mean values of C fixation averaged over the sampling period of 15 days were 21 ± 15 nmol C $\mu\text{mol POC}^{-1} \text{h}^{-1}$ at low $p\text{CO}_2$, 30 ± 19 nmol C $\mu\text{mol POC}^{-1} \text{h}^{-1}$ at mid $p\text{CO}_2$ and 35 ± 31 nmol C $\mu\text{mol POC}^{-1} \text{h}^{-1}$ at high $p\text{CO}_2$. Mean values of N₂ fixation, excluding day 3 were 0.32 ± 0.18 nmol N $\mu\text{mol POC}^{-1} \text{h}^{-1}$ at low $p\text{CO}_2$, 0.36 ± 0.21 nmol N $\mu\text{mol POC}^{-1} \text{h}^{-1}$ at mid $p\text{CO}_2$ and 0.48 ± 0.20 nmol N $\mu\text{mol POC}^{-1} \text{h}^{-1}$ at high $p\text{CO}_2$. Mean values of N₂ fixation of day 3 were 2.56 ± 0.44 nmol N $\mu\text{mol POC}^{-1} \text{h}^{-1}$ at low $p\text{CO}_2$, 3.89 ± 0.12 nmol N $\mu\text{mol POC}^{-1} \text{h}^{-1}$ at mid $p\text{CO}_2$ and 5.36 ± 0.32 nmol N $\mu\text{mol POC}^{-1} \text{h}^{-1}$ at high $p\text{CO}_2$. C uptake rates showed high standard deviations within treatments and in between sampling days. Nevertheless, C fixation in the high $p\text{CO}_2$ treatment was elevated compared to the other two treatments by 97% (high vs. low) and 44 % (high vs. mid) at day 0 and day 3, but this effect diminished afterwards.

Statistically significant differences between $p\text{CO}_2$ treatments according to ANOVA and Tukey's post hoc test were observed for biomass specific C and N₂ fixation rates ($p \leq 0.05$ and $p \leq 0.001$, Supplemental Table 2). C and N₂ fixation increased significantly with $p\text{CO}_2$ ($R^2 = 0.747$ and 0.362 , $p \leq 0.01$ and $p \leq 0.05$, $n = 12$, Supplemental Table 1). Ratios of $C_{\text{fixed}}: N_{\text{fixed}}$ were higher than the Redfield ratio and yielded maximum values at mid $p\text{CO}_2$ (C:N = 16:1) and lowest values at high $p\text{CO}_2$ (C:N = 9.6) (Fig. 4).

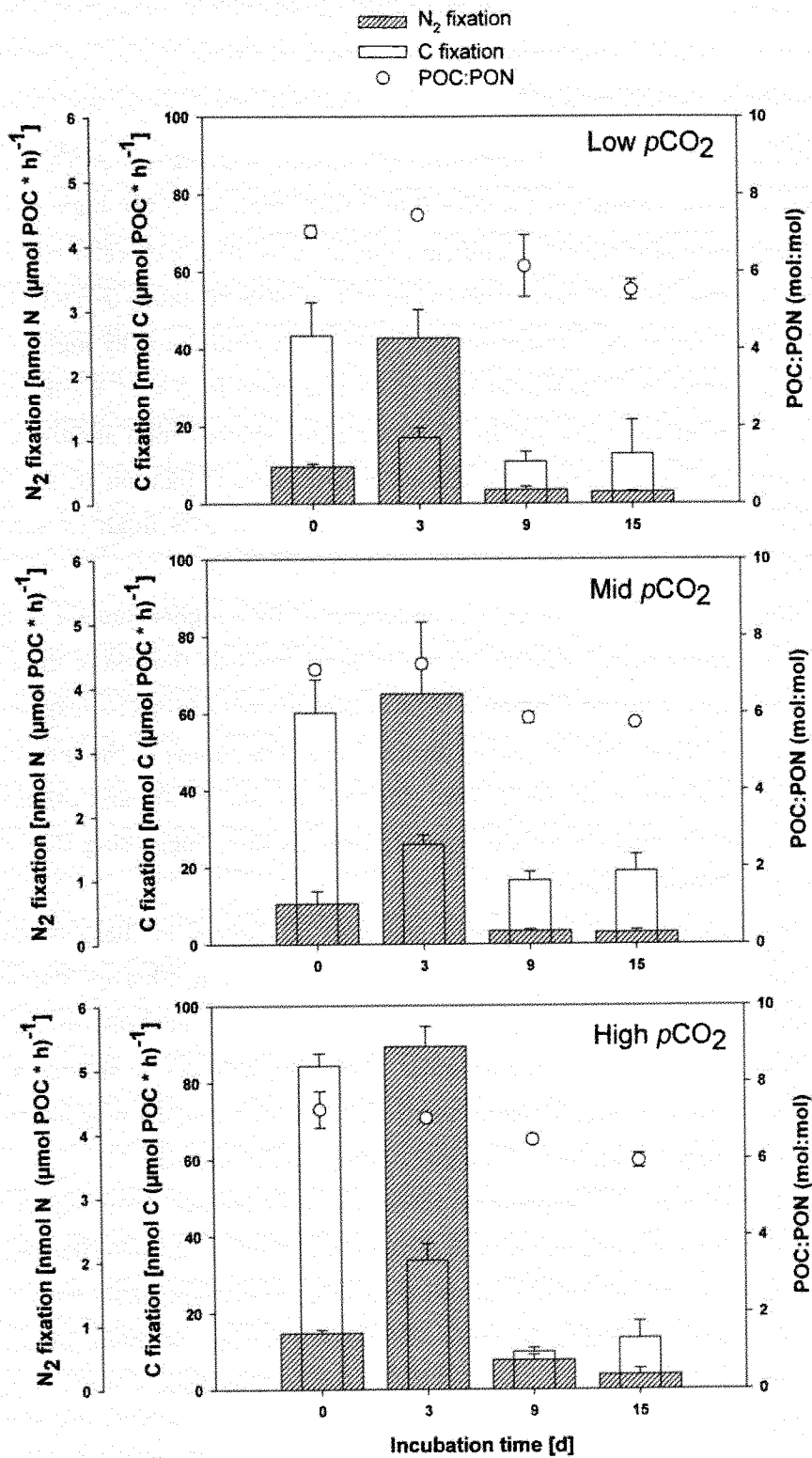


Fig. 6. Changes in biomass specific C (non filled bar charts), N_2 fixation (filled bar charts) and corresponding molar POC:PON ratios (scatter plots) versus incubation time for the three $p\text{CO}_2$ treatments low $p\text{CO}_2$, medium $p\text{CO}_2$, high $p\text{CO}_2$. Bars and scatter represent mean values of three replicates with respective standard deviation.

4.6.5 Discussion

Growth and production under different pCO₂ conditions

In this study we assessed the response of *Nodularia spumigena* to changes in pCO₂. Growth rates in terms of biomass increase calculated for days 0 to 9 were highest in the high pCO₂ scenario (508 ± 89 µatm). They were statistically significant elevated by 40 ± 25% relative to mid pCO₂ (398 ± 104 µatm) and by even 84 ± 38 % relative to low pCO₂ (340 ± 80 µatm) ($p = 0.001$, $n = 12$). In the same time period (0-9 days), N₂ fixation seemed to be more stimulated by high pCO₂ than C uptake (Fig.6). Biomass specific C fixation at high pCO₂ increased by 9 ± 44 % compared to mid pCO₂ and by 60 ± 60% relative to low pCO₂. C uptake rates showed high standard deviations within treatments and in between sampling days. Nevertheless, C fixation in the high pCO₂ treatment was elevated compared to the other two treatments by 97% (high vs. low) and 44% (high vs. mid) at day 0 and day 3, but this effect diminished afterwards.

Elevation in N₂ fixation at the highest pCO₂ was accompanied by a higher number of heterocysts per filament. This went along with a shortening of filaments towards the end of the experiment (not statistically significant), because filaments tend to become more instable, fragile and break more easily. Nevertheless, heterocyst frequency declined over the course of the incubation in all treatments. Heterocyst frequency in *Nodularia* (Lindahl et al., 1980) and *Aphanizomenon* (Riddolls, 1985) has been shown to correlate with N₂ fixation rate, suggesting that it could be used as an indicator for N₂ fixation capacity.

If this tendency in morphology is repeatedly observed in future studies, it might have implications for grazing on filamentous cyanobacteria by zooplankton. Shorter filaments might not provide sufficient morphological grazing resistance, apart from chemical resistance. Moreover, Chan et al. (2004) demonstrated that in the presence of grazers, heterocysts showed a decline in N₂ fixation rates by 40%. By reducing filament length, zooplankton grazing may act to pre-empt cyanobacteria blooms by suppressing N₂ fixation and cyanobacterial growth.

The stimulative effect of high pCO₂ disappeared during the time course of our experiment from day 9 onwards, along with a complete exhaustion of the inorganic P pool. Nevertheless, the DOP pool was exhausted to a greater proportion in the high pCO₂ treatment along with a higher P concentration per filament indicating a more efficient P usage at high pCO₂. Further investigation of the different DOP components and P turnover will be discussed in two companion manuscripts (Endres et al., 2012; Unger et al., in prep.).

In our study growth rates increased with increasing pCO₂, despite DIP limitation, indicating a stimulating effect of DIC availability. This suggests a co-limitation by C and P in our experimental set-up at low and mid pCO₂ conditions, which might be applied to the Baltic Sea in summer, as well. A deficiency in DIP seems to be partly counter-balanced by excess C, which is

opposing the concept of Liebig's law of only one limiting nutrient, which has already been noted by e.g. Arrigo (2005) and Hutchins et al. (2007).

The first and only study available so far reporting the response of *Nodularia* growth and primary production to changing $p\text{CO}_2$ conditions was published by Czerny et al. (2009), who hypothesized a detrimental effect of high $p\text{CO}_2$ on *Nodularia* growth. Both studies investigating *Nodularia* performance, Czerny et al. (2009) and ours, used culture conditions that favored the formation of single filaments without visible formation of larger aggregates. Czerny et al. continuously rotated their incubation bottles using a plankton wheel, representing a closed DIC manipulation set-up. We used slight agitation by manually rotating the bottles once a day and aeration at a low flow-rate, representing an open DIC manipulation. The different methods used might partly explain the observed opposing trends. Czerny et al. (2009) adjusted the pH by acid/base manipulation, which changes total alkalinity (TA) at constant dissolved inorganic carbon (DIC). Concentrations of DIC, HCO_3^- , and CO_3^{2-} in their study might have been lower than their actual target values, because seawater pH controls the relative proportion of the carbonate species and induces a lower percentage increase in HCO_3^- compared to a reduction of pH achieved by e.g. aeration or by co-adding carbonate ions along with acid (e.g. NaHCO_3 , e.g. Gattuso and Lavigne, 2009). This fact might have dampened a possible positive $p\text{CO}_2$ effect.

Additionally, it has been shown that light intensity strongly influences the magnitude of stimulation of growth and production by $p\text{CO}_2$ (e.g. Kranz et al., 2010), with significantly elevated rates at high $p\text{CO}_2$ and light conditions. Light intensity in our experiment was higher by a factor of 2.4 compared to those given by Czerny et al., (2009) (200 vs. 85 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, respectively). Experimental temperature differed by a factor of 1.3 with higher incubation temperatures in our study. Furthermore, DIP concentrations were non-limiting in the Czerny et al., (2009) study with 5 $\mu\text{mol l}^{-1}$, while reaching limiting conditions in our study after 3 days with DIP values near the detection limit. Czerny and co-workers hypothesized that the negative effect of high $p\text{CO}_2$ on N_2 fixation and growth occurred, because translocation of amino acids from heterocysts to vegetative cyanobacteria cells was restrained by a reduction in extracellular pH. However, intracellular amino acid translocation is not necessarily directly dependent on the external pH, because ionic exchange between adjacent cells takes place through the microplasmodesmata. These are intercellular channels linking cytoplasm of cells where intracellular pH is kept constant (Mullineaux, 2008; Flores and Herrero, 2010). Therefore, it is unlikely that amino acids will pass the outer and inner layers of the heterocyst envelope, but they will diffuse within a continuous periplasm and are re-imported into the cytoplasm of vegetative cells (Flores et al., 2006). Furthermore, Nicolaisen et al. (2009) showed that the outer membrane in heterocystous cyanobacteria is an efficient permeability barrier for glutamate and retains this metabolite within the filament. Nevertheless, a lower extracellular pH might hypothetically explain the reduction in N_2 fixation in the Czerny study by restraining the transport of nitrogenous metabolites, but it cannot explain the pronounced decrease in growth rate detected in parallel to a relative small reduction in N_2 fixation rate.

Supporting evidence towards a stimulation of growth and production at high $p\text{CO}_2$ has been shown previously for non-heterocystous cyanobacteria of the genus *Trichodesmium* both by adjustment of $p\text{CO}_2$ by aeration with pre-mixed gases (Hutchins et al., 2007; Kranz et al., 2009) or acid/base accomplished with DIC addition (Barcelos e Ramos et al., 2007). In these experiments, growth rates increased from present day to future $p\text{CO}_2$ levels by 34 to 38% and decreased by 30 to 50% comparing the glacial vs. the present day $p\text{CO}_2$ levels (Levitan et al., 2007; Barcelos e Ramos et al., 2007). Furthermore, Hutchins et al. (2007) detected no growth in *Trichodesmium* cultures at $p\text{CO}_2$ conditions below 150 ppm.

Several researchers observed an elevation of N_2 fixation rates by approximately 35 to 40% (with a maximum of even 400%) over the respective $p\text{CO}_2$ range (Barcelos e Ramos et al., 2007; Levitan et al., 2007; Hutchins et al., 2007; Kranz et al., 2009). So far, all experiments were done with laboratory cultures, while field measurements are still scarce. Until today, there is only one publication, Hutchins et al. (2009), which reports a stimulation of cyanobacterial N_2 fixation rates by $p\text{CO}_2$ during three experimental runs (6, 21 and 41%) in a field population of the Gulf of Mexico.

The underlying molecular and cell-physiological mechanisms of the beneficial effect of a high $p\text{CO}_2$ environment, however, are still speculative. Levitan et al. (2010a; 2010b) and Kranz et al. (2009; 2010) assume energy savings achieved by down-regulating carbon concentration mechanisms (CCM). The acquisition of carbon in cyanobacteria involves the use of CCM to compensate for a low $p\text{CO}_2$ in aqueous environments, which are typically lower than the half saturation constant of RUBISCO, the major enzyme involved in C fixation. These CCMs often include bicarbonate transporter that allow access to the larger DIC reservoir (Tortell and Morel, 2002). *Trichodesmium*, as well as *Nodularia* both belong to the β -group of cyanobacteria, classified based on the structural differences in RUBISCO (Badger et al., 2002). Both cyanobacteria share CCM components and possess a surplus of one DIC and CO_2 uptake system compared to α -cyanobacteria (BCT1, NADH-I3). The operation of CCMs is energetically expensive and, because cell membranes are freely permeable for CO_2 , additional metabolic costs are incurred in limiting the efflux of CO_2 from the cell. It has been proposed that CCM regulation might occur by changing the gene expression level, but studies by Levitan et al. (2010a; 2010b) and Kranz et al. (2009; 2010) do not support this hypothesis in long-term studies. On the other hand, the discrepancy between CCM gene expression, CCM activity and stimulation of growth and production at high $p\text{CO}_2$, may be due to a modulation of the CCM activity at the translational and post-translational level or alteration of the transporter activity (Levitan et al., 2010b; Kranz et al., 2009, 2010). Within this line of arguments, Kranz et al. (2009) demonstrate an increase in activity of a special CCM transporter component at high $p\text{CO}_2$, the NDH-I4 transporter, a low affinity transporter avoiding efflux of CO_2 from the cytosol by converting CO_2 to HCO_3^- . This elevated activity might lead to enhanced ATP production yielding in an energetic surplus available to fuel N_2 fixation. Regardless of the underlying molecular and cell physiological processes, C and N_2 fixation mechanisms compete for photo-generated reductants and any

reduction in energy demand of the C fixation apparatus can be allocated to other metabolic processes including N₂ fixation and would explain the effect of CO₂ availability on potential C, as well as N₂ fixation. A high plasticity of CCM regulation in *Trichodesmium* under different pCO₂, but also under variable light and temperature conditions and, moreover, in relation to the current N supply (Giordano et al., 2005) has emerged. This may modulate N and corresponding C demands to keep the respective C:N ratio at a constant level.

Overall, our results suggest a stimulating effect of high pCO₂ on *Nodularia*, which may be broadly applicable to non-heterocystous and heterocystous diazotrophic cyanobacteria of group β , unless no other growth factor becomes limiting.

Nitrogen and carbon turnover and elemental stoichiometry under different pCO₂ conditions

Total nitrogen (TN) within our experimental system increased over 9 days by ~10 $\mu\text{mol l}^{-1}$ at low pCO₂, by ~20 $\mu\text{mol l}^{-1}$ at mid pCO₂ and by ~25 $\mu\text{mol l}^{-1}$ at high pCO₂. On a daily basis, N₂ fixation provided sufficient N to explain the buildup of PON and POC. Nevertheless, a discrepancy occurred, because N₂ fixation per day was higher by 0.3 to 1.2 $\mu\text{mol l}^{-1} \text{d}^{-1}$ than the buildup of PON and thus leading to a surplus of N_{fixed} compared to the buildup of PON. Still one has to keep in mind, that N₂ fixation was measured for a period of 6 hours at the sampling day, while accumulation of PON was determined for a longer period of 3 and 6 days, which might introduce errors and makes it inadequate for comparison.

Differences in DON between the mid and low pCO₂ scenarios, as well as mid and high pCO₂ levels were not statistically significant according to ANOVA, but overall a statistically significant positive correlation of DON concentration and pCO₂ was found. This presumes a tendency to elevated exudation of DON at high pCO₂, but caution has to be taken because of the different sampling intervals (3, 6 and 6 days). Moreover, the differences between replicate DOM were in the same concentration range as differences in concentration from one sampling day to the next. Thus, a significant accumulation of DIN due to exudation was not detectable. Again, our sampling interval and method chosen (sampling after 3, 9 and 15 days, exudation calculated from differences in concentration) made it difficult to directly determine exudation, which might indicate a faster cycling of N compounds than could be detected by our experimental approach. There were a constant although low number of heterotrophic bacteria in our incubation bottle, but our measurements revealed that active growth of bacteria did not occur. Therefore, this bacterial contamination might have resulted from a background of non-viable, but SYBR green stainable bacteria since also extracellular nucleic acids and dead, DNA containing, cells will be stained by the dye. In conclusion, uptake of DON and DIN by heterotrophic bacteria should have been negligible. Apart of such potential methodological constrains, some studies have shown that N₂ fixation and subsequent release of DON are possible mechanisms to dissipate excess light

energy on a short term scale (Lomas et al., 2000; Wannicke et al., 2009), while no previous report on the effect of $p\text{CO}_2$ on DON release exists.

There are several studies showing indirectly via TEP and exudate formation (Engel et al., 2002,) or directly (Kim et al., 2011) that DOC production is sensitive to changes in $p\text{CO}_2$. This lack of significant tendencies presumably results from a rapid response of the microbial food web superimposing short term trends of autotrophic processes which might have been significant. On the other hand, Borchard and Engel (2012) recently demonstrated a stimulating effect of greenhouse conditions (high $p\text{CO}_2$ and high temperature) on exudation processes in a laboratory study using *Emiliania huxleyi*. Ratios of newly fixed C:N were above the Redfield ratio and significantly higher than molar C:N ratios of cyanobacteria growing at low and mid $p\text{CO}_2$ ($p = 0.03$ and <0.001 , respectively, $n = 12$, data not shown), whereas ratios of newly fixed C:N and molar C:N did not change at high $p\text{CO}_2$ ($p = 0.08$, $n = 12$, data not shown).

At high $p\text{CO}_2$, a higher N_2 fixation rate along with a higher C fixation rate and a more balanced growth in terms of POC to PON to POP suggests synchronic ammonium incorporation into the carbon skeletons (2-oxoglutarat) through the GS-GOGAT (glutamine synthetase-glutamine oxoglutarate aminotransferase) cycle synthesizing glutamate.

POC:POP, as well as PON:POP in this study were elevated relative to the Redfield ratios in all treatments and deviated significantly between low vs. mid and low vs. high $p\text{CO}_2$ treatments. The positive correlation between POC:POP and PON:POP ratios and biomass (chlorophyll a and abundance) presumes a higher C accumulation relative to N and P and of N relative to P.

In terms of trend and magnitude, our measured elemental ratios are comparable with those given by Hutchins et al. (2007) and Barcelos e Ramos et al. (2007) indicating constant C:N ratios, but an increase in N:P and C:P ratios at high $p\text{CO}_2$. This opposes the trend observed by Levitan et al. (2007), Czerny et al. (2009) and Kranz et al. (2009), who found an increase in C and N quota as well as the ratio at elevated $p\text{CO}_2$. In general, to date there is no consensus on whether phytoplankton elemental ratios are likely to be altered in a systematic manner in a future acidified ocean. Elemental ratios of most of eukaryotic phytoplankton investigated so far either remained near Redfield values, or were increased in some species (Hutchins et al., 2009; Liu et al., 2010 and references therein). Similarly, natural populations display no clear trend in POM stoichiometry either with increased C:N ratios in some studies (Riebesell et al., 2007; Engel et al., 2005) and a decrease in N:P in others (Tortell et al., 2002; Bellerby et al., 2008).

Biogeochemical and ecological implications

Seasonally, cyanobacteria in the Baltic Sea exhibit $p\text{CO}_2$ fluctuations with minimum values close to or below glacial $p\text{CO}_2$ values ($<180 \mu\text{atm}$). In the Gulf of Finland, $p\text{CO}_2$ drops from winter time until May from atmospheric equilibrium values of $\sim 350 \mu\text{atm}$ to $\sim 150 \mu\text{atm}$ due to warming of water and increased sequestration by photosynthetic activity (Schneider et al., 2006). This

corresponds to a decline in $p\text{CO}_2$ of 60%. In July, $p\text{CO}_2$ concentrations rise slightly up to ~ 200 μatm and level off again to a minimum of 100 μatm with the onset of the cyanobacteria bloom.

Thus, the natural cyanobacteria community of the Baltic Sea seems to be periodically exposed to glacial like $p\text{CO}_2$ conditions. If we apply rate measurements obtained in our study at low $p\text{CO}_2$, growth rates, N_2 and C fixation at mid $p\text{CO}_2$ would be lower by up to $34 \pm 29\%$, $30 \pm 29\%$ and $44 \pm 8\%$, respectively.

Nevertheless, this C limitation is balanced periodically by upwelling and turbulent mixing of CO_2 and nutrient rich intermediate winter water (Gidhagen, 1987) with $p\text{CO}_2$ up to 800 μatm (Schneider et al., 2006; Beldowski et al., 2010; Schneider, 2011).

Our results suggest that, as long as growth and production of cyanobacteria in the Baltic Sea are not limited by other factors, e.g. nutrients and light, maximum growth rates of *Nodularia* could potentially rise by $84 \pm 38\%$ due to the predicted increase in $p\text{CO}_2$ throughout the next 100 years. N_2 fixation by *Nodularia* might be elevated by $67 \pm 50\%$ in the next 100 years, if we extrapolate rates we determined in our study (Levitan et al., 2007, Barcelos e Ramos et al., 2007). Current estimates of N_2 fixation by cyanobacteria are about 136 $\text{mmol N m}^{-2} \text{yr}^{-1}$ for the Baltic Proper (Wasmund et al., 2001a, 2005b). If we assume, that our experimental results can also be extrapolated to the field this rate could rise to 227 ± 68 $\text{mmol N m}^{-2} \text{yr}^{-1}$, caused by the expected increases in $p\text{CO}_2$ alone. Nevertheless, this projected increase remains within the natural variability of rate measurements. Subsequently, $p\text{CO}_2$ induced increase in N_2 fixation would elevate the amount of bioavailable nitrogen by the release of dissolved nitrogenous compounds (DIN and DON) corresponding to a release rate of 113 ± 34 $\text{mmol N m}^{-2} \text{yr}^{-1}$ or 182 ± 54 $\text{mmol N m}^{-2} \text{yr}^{-1}$, if we assume that 50% or 80% of total nitrogen fixed by cyanobacteria is exuded (Glibert and Bronk, 1994; Ohlendieck, 2000). However, to date it is not known whether DON exudation will be affected by an increase in $p\text{CO}_2$ itself. In comparison, atmospheric N input to the Baltic Sea accounts for ~ 80 $\text{mmol N m}^{-2} \text{yr}^{-1}$ (Larsson et al., 2001, Rolff et al., 2008), while 45% of the total N input derives from N_2 fixation. Riverine N load is higher and adds up to $76 \cdot 10^3$ $\text{mmol N m}^{-2} \text{yr}^{-1}$ (HELCOM, 2005).

Since diazotrophic cyanobacteria can exploit elemental N_2 , as well as organic N sources, they do not solely rely on dissolved inorganic nitrogen sources like nitrate and ammonia. Moreover, they still can exploit inorganic phosphorous, as well as organic phosphorous, although dissolved inorganic nitrogen is already limiting. As a result, they drive the ecosystem towards P instead of N limitation. Our results suggest that this phenomenon will be amplified in the future ocean, when rate and extend of mass occurrences of diazotrophs develop, in particular when temperature increases at the same time. Cyanobacteria mass developments not only impact N and P cycling in the phototrophic zone, but also reduce oxygen concentrations in the deeper water layers and on the sediment when their biomass settles out. This will increase oxygen consumption and hence expand hypoxia in the Baltic Sea, which are known to release large quantities of inorganic P (Mort et al., 2010).

In addition to this, we have detected an increase in C:P and N:P ratios at high $p\text{CO}_2$. Extrapolating our results to a potential Baltic Sea in 2100 suggests that the nutritional value of organic matter produced in the euphotic zone will decrease in the future ocean. This could impact the efficiency of bacterial degradation on the one hand, and zooplankton production on the other hand, affecting the remineralization potential in deep water layers. Overall, the environmental significance of diazotrophic blooms in the Baltic Sea goes far beyond the detrimental effects of changes in stoichiometry and quantity of degradable biomass to the point of recreational issues. Eutrophication might play a substantial role in the expansion of cyanobacterial blooms (e.g. O'Neil et al., 2012). The future N input into the Baltic Sea, caused by a $p\text{CO}_2$ induced stimulation of cyanobacteria, might counteract the nitrogen load reductions aimed to mitigate eutrophication (e.g. Vahtera et al., 2007; Voss et al., 2011) and in the worst case impair the socio-economic value of the Baltic Sea.

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Response of *Nodularia spumigena* to $p\text{CO}_2$ – Part 3: Turnover of phosphorus compounds

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4.7.1 Abstract

Diazotrophic cyanobacteria often form extensive summer blooms in the Baltic Sea driving their environment into phosphate limitation. One of the main species is the heterocystous cyanobacterium *Nodularia spumigena*. *N. spumigena* exhibits accelerated uptake of phosphate through the release of the exoenzyme alkaline phosphatase that also serves as an indicator of the hydrolysis of dissolved organic phosphorus (DOP). The present study investigated the utilization of DOP and its compounds (e.g., ATP) by *N. spumigena* during growth under varying CO₂ concentrations, in order to estimate potential consequences of ocean acidification on the cell's supply with phosphorus. Cell growth, phosphorus pool fractions, and four DOP-compounds (ATP, DNA, RNA, and phospholipids) were determined in three set-ups with different CO₂ concentrations (341, 399, and 508 μ atm) during a 15-day batch experiment. The results showed rapid depletion of dissolved inorganic phosphorus (DIP) in all p CO₂ treatments while DOP utilization increased with elevated p CO₂, in parallel with the growth stimulation of *N. spumigena*. During the growth phase, DOP uptake was enhanced by a factor of 1.32 at 399 μ atm and of 2.25 at 508 μ atm compared to the lowest p CO₂ concentration. Among the measured DOP compounds, none was found to accumulate preferentially during the incubation or in response to a specific p CO₂ treatment. However, at the beginning 61.9 \pm 4.3% of the DOP were not characterized but comprised the most highly utilized fraction. This is demonstrated by the decrement of this fraction to 27.4 \pm 9.9% of total DOP during the growth phase, especially in response to the medium and high p CO₂ treatment. Our results indicate a stimulated growth of diazotrophic cyanobacteria at increasing CO₂ concentrations that is accompanied by increasing utilization of DOP as an alternative P source.

4.7.2 Introduction

Cyanobacteria bloom events frequently occur in the Baltic Sea in summer (Kahru et al., 1994) and they are dominated by the filamentous diazotrophic cyanobacteria *Nodularia spumigena* and *Aphanizomenon* sp. (Sivonen et al., 1989; Finni, 2001; Vahtera et al., 2005). Calm conditions, a salinity of 7–8, temperatures > 16°C, and a N:P ratio < 8 promote the formation of extensive *Nodularia* blooms in the sea surface layer (Wasmund, 1997). Degerholm et al. (2006) suggested that *Nodularia* sp. is better adapted than *Aphanizomenon* sp. with respect to phosphorus (P) starvation and has a higher affinity for dissolved organic phosphorus (DOP) because of its lower substrate half-saturation constants (K_M) and the higher $V_{max}:K_M$ ratio of the enzyme alkaline phosphatase (AP). These findings were confirmed by Vahtera et al. (2007), who reported that under bloom conditions *Nodularia* is superior to *Aphanizomenon* in its ability to compete for phosphorus at low concentrations, more efficient in acquiring phosphate from organic sources, and better able to grow on intracellular phosphorus stores.

As a constituent of compounds mediating cellular energy transformation and metabolic processes, P is an essential macronutrient for all living organisms (Karl, 2000; Benitez-Nelson, 2000; Nausch and Nausch, 2011). The major forms of P in aquatic ecosystems are dissolved inorganic phosphorus (DIP) and dissolved organic phosphorus (DOP) (Orchard et al., 2010). Among the different forms of DIP, orthophosphate (PO_4^{3-}) is metabolically preferred by phytoplankton and bacteria, based on their direct uptake of this compound through the cell membrane (Løvdal et al., 2007). As P is the limiting nutrient besides nitrogen (N), its availability strongly influences primary production (Smith, 1984; Howarth, 1988; Ruttenberg and Dyhrman, 2005; Elser et al., 2007). After DIP depletion, phytoplankton are able to utilize DOP, as indicated by the increased activity of AP, responsible for hydrolyzing DOP (Ruttenberg and Dyhrman, 2005; Paytan and McLaughlin, 2007).

DOP, together with dissolved organic carbon and nitrogen (DOC and DON, respectively), comprise the dissolved organic matter (DOM) pool (Karl and Björkman, 2002). Measurable DOP components include deoxyribonucleic acid (DNA), ribonucleic acid (RNA) (e.g., Karl and Bailiff, 1989), adenosine-5'-triphosphate (ATP) (e.g., Björkman and Karl, 2001), and phospholipids (PL) (e.g., Suzumura and Ingall, 2001, 2004). DNA, in addition to its fundamental role in heredity for all self-replicating organisms (Karl and Bailiff, 1989), gained further attention when DeFlaun et al. (1987) examined the contribution of its dissolved form to general DOM dynamics. RNA is involved in protein synthesis, which is required for growth (Dortch et al., 1983). Both RNA and DNA are indicators of actively growing, metabolizing cells (Karl and Bailiff, 1989). As reported by Karl and Bailiff (1989), the dissolved DNA and RNA concentrations in marine coastal/estuarine and offshore regions range from 0.56 to 21 $\mu\text{g l}^{-1}$ and from 4.03 to 31.9 $\mu\text{g l}^{-1}$, respectively. ATP, one of the most P-rich organic molecules, mediates energy transfer in all living organisms. Dissolved ATP occurs in seawater in significant concentrations of 0.1–0.6 $\mu\text{g l}^{-1}$ (Azam and Hodson, 1977). Radiolabeled ATP is used to measure the hydrolysis of organic phosphorus

compounds and the uptake of released P (Bentzen and Taylor, 1991; Casey et al., 2009). Phospholipids are ubiquitous in nature, serving as the structural and functional components of biological membranes (Suzumura and Ingall, 2001). They are classified according to their hydrophilic and hydrophobic portions, with dissolved forms providing a reservoir of organic P. So far, only a few studies have examined the distribution and abundance of phospholipids in marine environments, such that our understanding of their function in this respect remains limited (Suzumura, 2005). Parrish (1987) reported a wide range (4–88 $\mu\text{g l}^{-1}$) of dissolved phospholipid concentrations in coastal waters. In Pacific surface waters, concentrations of hydrophobic phospholipid-P between 6 and 16 nmol l^{-1} were measured, thus constituting between 2 and 6% of the DOP pool (Suzumura and Ingall, 2004).

The rising atmospheric CO_2 concentrations in the world's oceans have lowered pH and altered the carbonate chemistry of seawater faster than in the previous thousands of years (Siegenthaler et al., 2005; Hönlisch et al., 2009). These changes are commonly referred to as ocean acidification (Doney et al., 2009). Since preindustrial times until today, the atmospheric CO_2 increased from 280 to 395 ppm (www.esrl.noaa.gov/gmd/ccgg/trends/). By the end of this century, the CO_2 concentration is expected to reach 800 ppm, assuming that anthropogenically induced CO_2 emissions continue to rise at the present rate (IPCC, business-as-usual emission scenario, 2007). At the same time, the average pH of ocean surface waters has fallen by approximately 0.1 units and is expected to decrease a further 0.3–0.4 pH units by 2100 (Orr et al., 2005). At present, the ecological implications of ocean acidification are largely unknown and are therefore the subject of numerous ongoing investigations.

So far, there has been little research directed toward improving our understanding of the effects of elevated $p\text{CO}_2$ on the marine P cycle as it has long been assumed that the P cycle was not directly affected by rising ocean $p\text{CO}_2$. However, indirect responses to the expected changes in C and N cycles are likely and, importantly, may serve as a relatively conservative indicator thereof (Hutchins et al., 2009). Published studies have preferentially concentrated on the cellular P quotas of different cyanobacterial and diatom species. For example, Burkhardt et al. (1999) analyzed the effect of low pH on the C:P ratios of six diatom and one dinoflagellate species, based on the premise that the increasing atmospheric $p\text{CO}_2$ does not affect global ocean Redfield ratios. Hutchins et al. (2007) and Fu et al. (2007) reported no effects of a similar $p\text{CO}_2$ increase on the cellular P quotas of the cyanobacteria *Trichodesmium erythraeum*, *Synechococcus*, and *Prochlorococcus* whereas Czerny et al. (2009) noted a slightly increasing trend in cellular P quotas with elevated $p\text{CO}_2$ in their study of *Nodularia spumigena*. In a Norwegian fjord mesocosm experiment, AP activity (APA) was measured as a means to examine ^{33}P uptake rates and potential DOP utilization under three different $p\text{CO}_2$ concentrations (Tanaka et al., 2008); however, no statistically significant effects of $p\text{CO}_2$ on P biogeochemistry were determined.

This study is part of two other investigations based on the same experimental set-up. Wannicke et al. (2012) and Endres et al. (2013) focused on N-cycling, and exudation and extracellular enzyme activities, respectively. Here we investigated dissolved P pools, in particular their

variation during the growth of the diazotrophic cyanobacterium *Nodularia spumigena* under conditions of $p\text{CO}_2$ elevation, and their contribution to *Nodularia* nutrition. To gain insight into the dynamics of DOP and P metabolism in general, we focused on the changes of DOP and its composition as well as on P transformation processes.

4.7.3 Materials and methods

Experimental setup and conditions

A 15-day batch culture experiment was conducted with the diazotrophic cyanobacterium *Nodularia spumigena* in April 2010. In preparation for the experiment, water from the Baltic Sea (54.22749°N, 12.1748°E) was collected and aged for 4 months to allow the removal of inorganic nutrients by phytoplankton and bacteria. Afterwards the seawater was filtered through 0.2 μm cellulose acetate (CA) filters to remove particulate material and then UV-sterilized for 5 days. Three weeks prior to the start of the experiment, parent cultures of *Nodularia spumigena* were grown in sterile Baltic Sea water in a walk-in cooling chamber (15°C) under controlled light conditions (16:8 h light:dark cycle, 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$). One week before the acclimation phase was started, the parent cultures were allowed to adapt to the experimental temperature of 23°C and to the doubled light supply of 200 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. In the meantime, the sterilized seawater was filtered again through 0.2 μm CA filters under a clean bench into 10 l Nalgene bottles (39 bottles in total). Over a period of 3 days, *Nodularia* maintained in this water was allowed to acclimate to three different CO_2 concentrations by aeration with premixed gases (Linde Gas) of 180 $p\text{CO}_2$ (ppm), 380 $p\text{CO}_2$ (ppm), and 780 $p\text{CO}_2$ (ppm), representing pre-industrial, present, and future $p\text{CO}_2$ conditions, respectively. The acclimated cyanobacteria were then inoculated into thirteen 10 l bottles per treatment. These batch cultures were aerated with the respective CO_2 gases, continuously for the first day and then once a day for 1 h (at 02:00 p.m.). The cultures were routinely mixed by gently shaking the bottles, avoiding aggregate formation and strong turbulence. During sampling, while the $p\text{CO}_2$ -levels were clearly different from one another, the determined $p\text{CO}_2$ -levels deviated from the target values (Fig. 1c). Thus, in the following we refer to them as low ($341 \pm 81 \mu\text{atm}$), medium ($399 \pm 104 \mu\text{atm}$), and high ($508 \pm 90 \mu\text{atm}$) $p\text{CO}_2$ treatments. One bottle per treatment was used to obtain background information regarding nutrient status. In the remaining 36 bottles, the starting concentration of chlorophyll *a* (Chl *a*) of 0.8 $\mu\text{g l}^{-1}$ was adjusted and DIP was added to a final concentration of 0.35 $\mu\text{mol l}^{-1}$. After day 3, an additional 0.35 $\mu\text{mol DIP l}^{-1}$ was added because the phosphate in the medium was nearly depleted. The first set of samples was taken immediately after starting the experiment and then at days 3, 9, and 15 of the incubation. The sampling time for all three conditions was between 8 and 9 am. On each sampling day, three bottles per $p\text{CO}_2$ treatment were harvested. The experimental design is described in detail in Wannicke et al. (2012).

In parallel, the same experimental design and sampling mode were applied to investigate the transformation of DIP, using [^{33}P]PO₄. The difference was that the cultures were maintained in 500-ml-bottles (Schott). At the beginning of the experiment, 50 pM [^{33}P]PO₄ (6.6MBq l⁻¹) (Hartmann Analytics, specific activity 110TBq mmol⁻¹) were added to each bottle, with the radioactivity in the dissolved and the particulate fractions then measured at each sampling point. In addition, biomass parameters such as Chl *a*, particulate organic carbon (POC), and particulate

organic phosphorus (POP) were measured to compare the growth of *Nodularia* in these bottles and in the larger ones.

Carbonate chemistry

The carbonate system was characterized by measuring pH and total dissolved inorganic carbon (C_T) on every sampling day. The pH was determined with an electrode (Knick Mikroprozessor pH Meter 761 with Typ SE 100 glass electrode) and calibrated with standard NBS buffers directly before each measurement. The pH values are expressed relative to the total scale. C_T was analyzed with the colorimetric SOMMA system according to Johnson et al. (1993) and calibrated with carbon reference material provided by A. Dickson (University of California, San Diego). The reported precision of this method is $\pm 2 \mu\text{mol kg}^{-1}$. Total alkalinity (A_T) and $p\text{CO}_2$ were calculated with the program CO2SYS (Lewis et al., 1998). C_T , pH, salinity, temperature, total phosphate, and total silicate were set as parameters for the calculations.

Biomass and cell counts

The chlorophyll *a* (Chl *a*) concentration was determined by filtering 100-ml samples onto Whatman glass-fiber filters (GF/F), applying a vacuum of 200 mbar. The filters were stored in liquid nitrogen or at -80°C until they were extracted with 96% ethanol for at least 3 h. Chl *a* fluorescence was measured with a TURNER fluorometer (10-AU-005) at an excitation wavelength of 450 nm and an emission wavelength of 670 nm (HELCOM, 2005). Calculation of the Chl *a* concentrations was based on the method of Jeffrey and Welschmeyer (1997).

The abundance of *Nodularia spumigena* was determined by preserving 50-ml samples with acetic Lugol's (KI/I₂) solution (1% final concentration). The samples were counted at 100 \times magnification using an inverted Leica microscope (Utermöhl, 1958).

Bacteria were analyzed using a flow cytometer (Facs Calibur, Becton Dickinson) according to Gasol and del Giorgio (2000). Samples of 4 ml were preserved with 100 μl of formaldehyde (1% v/v final concentration), shock frozen in liquid nitrogen, and stored at -70°C until further processing. For analysis a stock solution of SYBR GREEN (Molecular Probes) was prepared by mixing 1 μl of dye with 49 μl of dimethyl sulfoxide (DMSO, Sigma Aldrich). The samples were thawed and 300 μl were then mixed with 3 μl potassium citrate solution, 10 μl of the dye stock solution, and 10 μl fluoresbrite microspheres (Polysciences), followed by incubation in the dark for 30 min. The cells were counted at a medium flow rate. The values of interest were calculated using the software program "Cell Quest Pro".

Inorganic nutrient analyses

Water samples (60 ml) of the batch cultures were filtered through combusted (450°C , 4 h) Whatman GF/F filters and stored at -20°C before the inorganic nutrient concentrations (DIP, nitrate/nitrite, silicate, and ammonium) were determined using the autoanalyzer system

'Evolution III' (Rohde and Nehring, 1979) and standard colorimetric methods (Grasshoff et al., 1983) except for ammonium which was determined manually according to Grasshoff et al. (1983). The detection limit was $0.02 \mu\text{mol l}^{-1}$ for DIP, $0.05 \mu\text{mol l}^{-1}$ for nitrate/nitrite, and $0.1 \mu\text{mol l}^{-1}$ for silicate. Ammonium concentrations were below the detection limit of $0.05 \mu\text{mol l}^{-1}$ throughout the experiment. To estimate the development of the cultures immediately after sampling, DIP concentrations were determined manually according to Murphy and Riley (1962). For data analysis, DIP values from both measurements were pooled.

Organic matter analyses

To determine total and dissolved phosphorus (TP and DP, respectively), 40-ml samples were stored frozen at -20°C either unfiltered or after filtration through $0.2 \mu\text{m}$ CA filters. The thawed samples were then oxidized with an alkaline peroxodisulfate solution (Grasshoff et al., 1983) in a microwave (MWS $\mu\text{Prep-A}$) to convert organic phosphorus into DIP. The procedure lasted 4 h in total including warming, incubating 1 h at 170°C , and cooling. Further DIP analysis was done as described above but using a 10 cm-cuvette, which reduced the detection limit to $0.01 \mu\text{mol l}^{-1}$. DOP was calculated as the difference between DP and DIP. POP was calculated as the difference between TP and DP and is referred to as *Nodularia-P* hereafter.

Both DOC and total dissolved nitrogen (TDN) were analyzed by collecting subsamples in combusted 20-ml glass ampoules (8 h, 500°C), pre-filtered through combusted GF/F filters, followed by acidification with $80 \mu\text{l}$ of 85% phosphoric acid, and stored at $0-2^{\circ}\text{C}$ until further processing. DOC and TDN concentrations were determined simultaneously in the filtrate by high-temperature catalytic oxidation with a Shimadzu TOC-VCSH analyzer equipped with a Shimadzu TNM-1 module. DOC and TDN concentrations were measured as quadruplicates and then averaged. The TDN values were corrected for nitrate/nitrite and ammonium, and subsequently defined as DON.

Particulate organic carbon (POC) and nitrogen (PON) were analyzed by filtering 200-ml samples onto GF/F filters which were subsequently stored frozen at -20°C . Concentrations were measured by means of flash combustion in a Carlo Erba EA 1108 at 1020°C and a Thermo Finnigan Delta S mass-spectrometer.

Dissolved ATP (dATP) was determined according to Björkman and Karl (2001) but modified for Baltic Sea conditions. Samples of 200 ml each were pre-filtered through combusted (4 h, 450°C) Whatman GF/F filters followed by filtration through 0.2 µm CA filters. A Mg(OH)₂ precipitate including the co-precipitated nucleotides was obtained by the addition of 1 M NaOH (0.5% v/v). The precipitate settled overnight and was then centrifuged for 20 min at 1000 x g. The supernatant was aspirated and the precipitate was transferred into 50-ml Falcon tubes, centrifuged again (1.5 h, 1680 x g) to obtain the final pellet, and then resuspended with 5 M HCl, added dropwise. A final pH of 7.2 was reached by the addition of TRIS buffer (pH 7.4, 20 mM, Sigma-Aldrich, T7693). The final volume was recorded and standard concentrations were prepared as for the samples, thus yielding a blank with aged Baltic Sea water and six ATP concentrations (adenosine 5'-triphosphate disodium salt hydrate, Sigma-Aldrich, A2383) ranging from 1 to 20 nmol l⁻¹.

ATP concentrations were measured by the firefly bioluminescence assay using a Sirius Luminometer (Berthold Detection Systems). The 30-µl samples were each treated with 240 µl of firefly lantern extract mixture prepared according to Björkman and Karl (2001). The detection limit was 2.5 pmol ml⁻¹ of the concentrated sample with a precision of <5% at 40 pmol l⁻¹ dATP in the original water sample.

The fluorescence slope of the standard concentrations was used to calculate the dATP concentrations, with correction for the final sample volume. The P-content was calculated based on the fact that 1 mol ATP is equivalent to 3 mol P and is hereafter referred to as dATP-P.

Dissolved phospholipids

The phosphate concentration of dissolved phospholipids (dPL-P) was analyzed according to Suzumura and Ingall (2001, 2004), adopting the method to Baltic Sea conditions. For the extraction of dPL-P, 400-ml aliquots of GF/F (combusted, 4 h, 450°C) and 0.2 µm CA filtered batch samples were stored frozen at -20°C until further processing. The samples were thawed in a water bath at 30°C and then extracted twice with 100 ml of chloroform (Merck 1.07024.2500). The chloroform phase was collected and concentrated to 5 ml in a rotary evaporator (Heidolph Hei-VAP Advantage). The concentrate was then transferred into microwave tubes (suitable for MWS µPrep-A) to completely evaporate the chloroform in a 60°C water bath overnight. Twenty ml of Milli-Q water was added, after which processing in a microwave was the same as described for the analysis of TP and DP. Six standard concentrations ranging from 0 to 125 µg l⁻¹ were prepared by adding the appropriate amounts of a 5 mg PG (L-α-phosphatidyl-DL-glycerol sodium salt, Sigma Aldrich, P8318) ml⁻¹ stock solution to aged seawater. A reagent blank of chloroform was also measured. Based on the slope, the dPL-P concentration was determined. The detection limit was 0.8 nmol l⁻¹.

Dissolved DNA and RNA

Dissolved DNA and RNA were determined according to Karl and Bailiff (1989). For each sample a volume of 200 ml was filtered through combusted GF/F (4 h, 450°C) and 0.2 µm CA filters. The same volume of ethylenediaminetetracetic acid (EDTA, 0.1 M, pH 9.3, Merck, 1.08454.1000) and 4 ml of cetyltrimethylammonium bromide (CTAB, Sigma-Aldrich, H5882) were added. The samples were gently mixed and stored frozen at -20°C for at least 24 h. After defrosting the samples, the precipitate that had formed was collected onto combusted (450°C, 4 h) GF/F filters (25 mm, Whatman), placed into annealed vials, and stored frozen at -80°C until further analysis. Dissolved DNA and RNA (dDNA and dRNA) were detected according to Karl and Bailiff (1989) using the fluorescence-spectrophotometer F2000 (HITACHI) to determine dDNA and the dual-beam-UV/VIS-spectrophotometer U3010 (HITACHI) to determine dRNA. Coupled standards (DNA + RNA) with concentrations between 1–10 µg DNA l⁻¹ (Sigma Aldrich, D3779) and 20–120 µg RNA l⁻¹ (Sigma Aldrich, R1753) were prepared in aged seawater as described above. A reagent blank served as reference and aged seawater as the background control. Dissolved DNA and RNA concentrations were translated into P concentrations by multiplication by a factor of 2.06 nmol P for 1 µg dDNA and 2.55 nmol P for 1 µg dRNA, detected by DP determination in the microwave. Hereafter, these amounts are referred to as dDNA-P and dRNA-P. The detection limit was 10–20 ng for DNA and 250–500 ng for RNA.

The concentrations of the measured DOP fractions (dATP, dPL-P, dDNA-P and dRNA-P) were totaled and the amount subtracted from the total DOP concentration. The difference is defined as the uncharacterized DOP.

[³³P]PO₄ uptake and transformation

Total [³³P]-activity was measured in 1-ml volumes of each of the nine sub-samplings by liquid scintillation counting (Tri-Carb 2800TR, Perkin Elmer).

[³³P]PO₄ incorporated in *Nodularia* filaments was determined by filtering a 5-ml sub-sample onto 0.2 µm polycarbonate (PC) filters pre-soaked with a 20 mM cold PO₄ solution. The filters were rinsed with 5 x 1 ml of particle-free aged seawater. Dissolved inorganic and organic [³³P]-phosphorus were distinguished according to the method described by Ammerman (1993) for the uptake of dissolved ATP. To detect the total dissolved activity, 1 ml of the filtrate (filtrate 1) was transferred into scintillation vials for counting. Activated charcoal (20 mg) and 1 ml 0.03 N H₂SO₄ were then added to the remaining 4-ml filtrate; the mixture was shaken for 15 min and then filtered through 0.45 µm filters. One ml of this filtrate (filtrate 2) was counted again. Filtrate 1 contained inorganic and organic bound [³³P]PO₄; in filtrate 2, organic phosphorus was removed on activated charcoal, leaving only inorganic [³³P]PO₄. Organic, bound [³³P]PO₄ was calculated as the difference between filtrates 1 and 2. The procedure was repeated two times.

In preliminary tests with sterile Milli-Q and aged seawater, the dilution of [³³P] by the addition of 1 ml 0.03 N H₂SO₄ and non-specific binding to charcoal were checked, indicating that 25% of the

variation can be explained by these effects. Therefore, the values of filtrate 2 were corrected by this amount.

Data and statistical analyses

The data and illustrations shown represent the average values of the three parallel incubations, except one outlier (sample: low-II at day 9) in which double the amount of PO₄ was inadvertently added. Statistical significance was tested by an unpaired t-test, with a significance level of $p < 0.05$. Prior to the t-test, the data were tested for normality by the Shapiro-Wilk test. If the normality test failed, a Mann-Whitney rank sum test was used. Correlation analyses were performed using Spearman's rank test, assuming a significant correlation as a correlation coefficient $|R| > 0.6$, and $p < 0.001$. The operations were performed with 'Sigma Plot 11' (Systat Software Inc.).

4.7.5 Results

Carbonate system

Average pH values for the low, medium and high $p\text{CO}_2$ treatments were 8.15 ± 0.08 , 8.09 ± 0.09 , and 7.99 ± 0.07 , respectively. The high $p\text{CO}_2$ treatment differed significantly from the low ($p < 0.001$, $n = 12$) and medium $p\text{CO}_2$ treatments ($p = 0.009$, $n = 12$). The means of the corresponding C_T values were 1598 ± 35 , 1613 ± 30 , and $1648 \pm 27 \mu\text{mol kg}^{-1}$, respectively. The differences in C_T between the low and high as well as the medium and high $p\text{CO}_2$ treatments were significant ($p < 0.001$ and $p = 0.006$, $n = 12$). The calculated values for $p\text{CO}_2$ and A_T (total alkalinity) resulted in an average of $341 \pm 81 \mu\text{atm } p\text{CO}_2$ and $1701 \pm 19 \mu\text{mol kg}^{-1} A_T$ in the low $p\text{CO}_2$ treatment, $399 \pm 104 \mu\text{atm } p\text{CO}_2$ and $1701 \pm 9 \mu\text{mol kg}^{-1} A_T$ in the medium $p\text{CO}_2$ treatment, and $508 \pm 90 \mu\text{atm } p\text{CO}_2$ and $1714 \pm 13 \mu\text{mol kg}^{-1} A_T$ in the high $p\text{CO}_2$ treatment. The significance of the differences in $p\text{CO}_2$ between the low and high as well as the medium and high $p\text{CO}_2$ treatments ($p < 0.001$ and $p = 0.009$, $n = 12$) was verified by a t-test (Fig. 1).

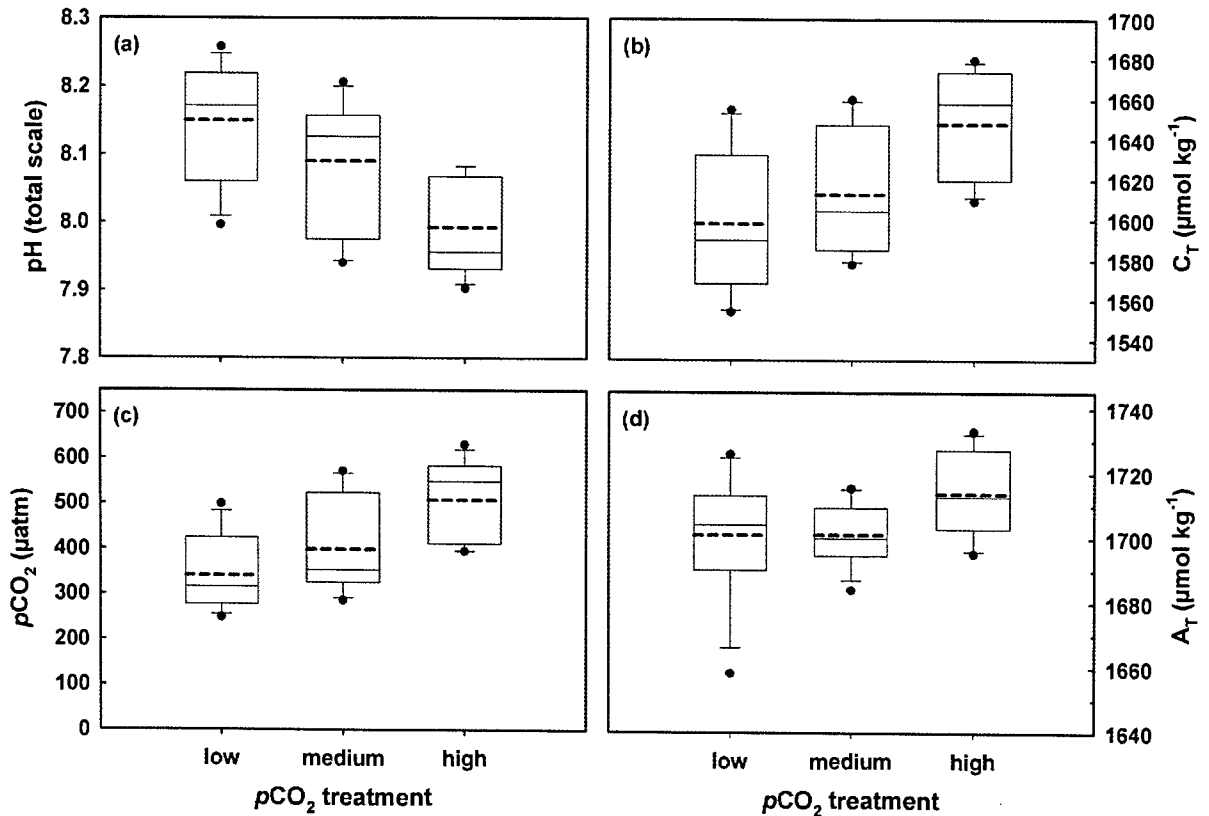


Fig. 1: Box plot ($n = 12$) of the carbonate system for three CO_2 treatments (low, medium, high). Range of the measured values: (a) pH, (b) C_T , and of the calculated values: (c) $p\text{CO}_2$, (d) A_T . The box plots show the range from the start to the end of the experiment (showing each outlier). Solid lines represent the median. Dashed lines represent the mean value.

Nodularia growth and bacterial occurrence

A detailed description of *Nodularia* abundance, filament length, and number of heterocysts in response to changing $p\text{CO}_2$ is given in Wannicke et al. (2012). Briefly summarized, the abundance of *Nodularia spumigena* increased by a factor of 2.5, 3.4, and 8.5 in the low, medium, and high $p\text{CO}_2$ treatment, respectively, until day 9. Afterwards, cyanobacterial growth under low and medium $p\text{CO}_2$ proceeded at a lower rate. At high $p\text{CO}_2$, the abundance declined slightly. A similar trend was observed for chlorophyll a (Chl a). Additionally, the Chl a concentration increased by a factor of 6.1 at low $p\text{CO}_2$, 5.9 at medium $p\text{CO}_2$, and 10.2 at high $p\text{CO}_2$ until day 9 and then dropped, regardless of the CO_2 concentration. Accordingly, the period between day 0 and day 9 was considered to be the growth phase (Fig. 2). During the total time of the experiment, *Nodularia* abundance positively correlated with Chl a , *Nodularia*-P, POC, and PON ($|R| = 0.741$, 0.86, 0.841, and 0.888, $p < 0.001$, $n = 36$).

A comparison of the growth in the small and the large bottles showed no differences. Significant correlations between both experimental set-ups were thus determined ($p < 0.001$, $n = 36$) for Chl a ($|R| = 0.76$), POC ($|R| = 0.817$) and *Nodularia*-P ($|R| = 0.798$).

Heterotrophic bacterial abundance in the low, medium, and high treatments was $4.69 \cdot 10^5 \pm 1.64$, $4.54 \cdot 10^5 \pm 1.59$, and $4.73 \cdot 10^5 \pm 1.28$ cells l^{-1} , respectively. There was no significant increase in abundance of heterotrophic bacteria. Moreover, heterotrophic bacterial abundance did not correlate with any other parameters. Thus, the influence of heterotrophic bacteria could be neglected.

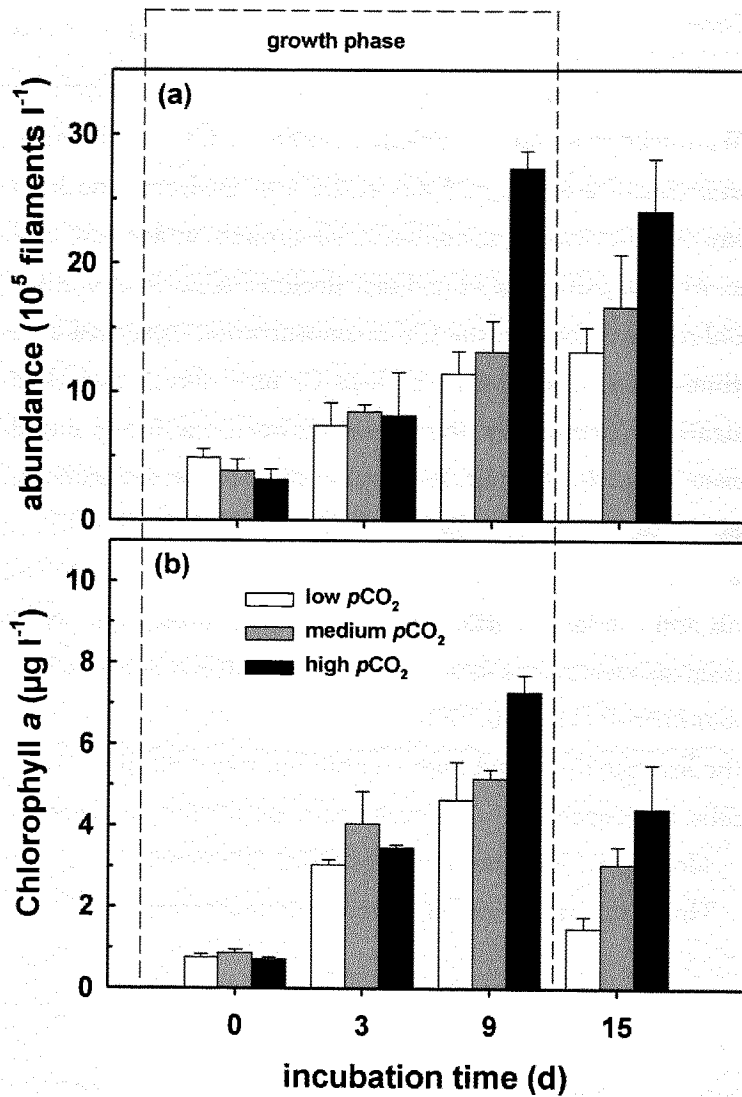


Fig. 2: Development of *Nodularia spumigena* over the sampling time and for the different $p\text{CO}_2$ treatments (low = white bars, medium = grey bars, high = black bars), abundance of *Nodularia spumigena* (a), chlorophyll a distribution (b) (mean values and the respective standard deviation of 3 replicates).

Table 1: The phosphorus pool fractions at the four sampling times. Values are means and standard deviations of three replicates except for DIP where one replicate bottle was excluded (low-II at day 9).

time	$p\text{CO}_2$ treatment	TP [$\mu\text{mol l}^{-1}$]	DP [$\mu\text{mol l}^{-1}$]	<i>Nodularia</i> -P [$\mu\text{mol l}^{-1}$]	DIP [$\mu\text{mol l}^{-1}$]	DOP [$\mu\text{mol l}^{-1}$]
day 0	low	0.83 ± 0.03	0.63 ± 0.04	0.20 ± 0.04	0.29 ± 0.02	0.34 ± 0.04
	medium	0.91 ± 0.08	0.65 ± 0.02	0.26 ± 0.06	0.32 ± 0.08	0.33 ± 0.06
	high	0.87 ± 0.08	0.72 ± 0.04	0.15 ± 0.07	0.34 ± 0.02	0.38 ± 0.06
day 3	low	0.71 ± 0.05	0.31 ± 0.02	0.43 ± 0.03	0.05 ± 0.02	0.39 ± 0.20
	medium	0.77 ± 0.05	0.27 ± 0.01	0.39 ± 0.15	0.03 ± 0.01	0.24 ± 0.02
	high	0.68 ± 0.03	0.32 ± 0.08	0.36 ± 0.09	0.04 ± 0.00	0.28 ± 0.07
day 9	low	1.21 ± 0.04	0.31 ± 0.12	0.80 ± 0.21	0.01 ± 0.01	0.27 ± 0.08
	medium	1.20 ± 0.06	0.27 ± 0.10	0.93 ± 0.15	0.02 ± 0.02	0.25 ± 0.09
	high	1.20 ± 0.02	0.25 ± 0.05	0.96 ± 0.06	0.01 ± 0.00	0.23 ± 0.05
day 15	low	1.20 ± 0.21	0.27 ± 0.06	0.93 ± 0.25	0.03 ± 0.01	0.24 ± 0.07
	medium	1.22 ± 0.06	0.21 ± 0.03	1.01 ± 0.06	0.02 ± 0.01	0.19 ± 0.02
	high	1.27 ± 0.07	0.22 ± 0.05	1.05 ± 0.06	0.02 ± 0.01	0.21 ± 0.04

Phosphorus pool

The initial TP concentration (day 0) was 0.83 ± 0.3 , 0.91 ± 0.08 , and $0.87 \pm 0.08 \mu\text{mol l}^{-1}$ in the low, medium, and high $p\text{CO}_2$ treatment, respectively. An increase of around $0.36 \mu\text{mol l}^{-1}$ at day 9 was due to the additional PO_4 supply after sampling at day 3 (Table 1). The initial concentrations of the DP pool under low, medium, and high $p\text{CO}_2$ were, respectively, 0.63 ± 0.04 , 0.65 ± 0.02 , and $0.72 \pm 0.04 \mu\text{mol l}^{-1}$. During the 15-day incubation, the DP concentration declined by 0.35, 0.44, and $0.50 \mu\text{mol l}^{-1}$. Both the initial amount of DIP and the additional amount of DIP after sampling at day 3 were taken up completely by *Nodularia*. From day 3 onwards, DIP concentrations were below the detection limit (Table 1). Differences in DIP uptake between the respective $p\text{CO}_2$ treatments were not significant. DIP correlated negatively with *Nodularia*-P, POC, PON, Chl *a*, and *Nodularia* abundance ($|R| = -0.843, -0.839, -0.854, -0.822, \text{ and } -0.834, p < 0.001, n = 35$). *Nodularia*-P increased in all treatments over the course of the experiment. The increment during the growth phase accounted for 0.59, 0.70, and $0.77 \mu\text{mol Nodularia-P l}^{-1}$ for the low, medium and high treatments. The amounts obtained in response to medium and high $p\text{CO}_2$ were 1.19- and 1.30-fold higher than those measured under low $p\text{CO}_2$ (Fig. 3a), but differences were not significant. At day 15, there was a slight increase in the *Nodularia*-P concentration in all $p\text{CO}_2$ treatments. *Nodularia*-P correlated negatively with DOP ($|R| = -0.844, p < 0.001, n = 36$) and positively with APA ($|R| = 0.824, p < 0.001, n = 36$).

During the first 3 days, DOP concentrations increased slightly in the low and medium $p\text{CO}_2$ treatments by about 0.05 and $0.02 \mu\text{mol l}^{-1}$, respectively, suggesting that DOP was produced by *Nodularia* at the beginning of the experiment. Under high $p\text{CO}_2$, the DOP concentration decreased already from day 0 until day 3 by $0.10 \mu\text{mol l}^{-1}$. From this point on, the DOP concentration continued to decrease in the high $p\text{CO}_2$ treatment and also in the two other $p\text{CO}_2$ treatments. During the growth phase, DOP utilization varied as a function of $p\text{CO}_2$, with concentrations declined by 0.14, 0.09, and $0.06 \mu\text{mol l}^{-1}$ at high, medium, and low $p\text{CO}_2$, respectively (Fig. 4a). However, DOP uptake differed significantly only between the high and low $p\text{CO}_2$ treatments ($p = 0.04, n = 12$). The decrement in DIP plus DOP by 0.64, 0.69, and $0.77 \mu\text{mol l}^{-1}$ under low, medium, and high $p\text{CO}_2$, respectively, was reflected in the increase of *Nodularia*-P by nearly the same amount (Fig. 3a). The parallel decline of DIP and DOP indicated the utilization of both pools, as confirmed by the positive correlation between both ($|R| = 0.675; p < 0.001, n = 35$).

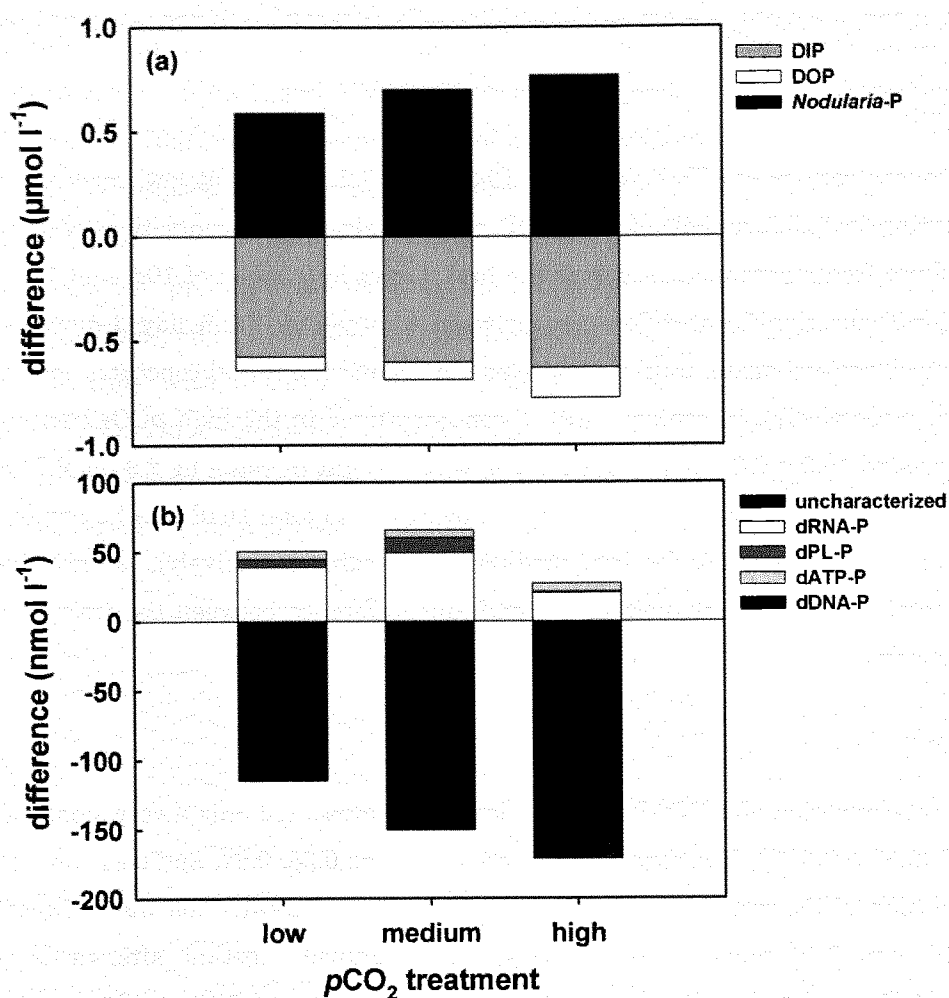


Fig. 3: Changes of the P pool (a) and the DOP pool (b) between day 9 and day 0 of the 15-day incubation experiment for the three different $p\text{CO}_2$ treatments.

DOP components

Dissolved ATP

On sampling days 0 and day 3, dATP-P concentrations remained constant, at $2.5 \pm 0.4 \text{ nmol l}^{-1}$, in all treatments and accounted for $0.7 \pm 0.2\%$ of total DOP. On day 9 dATP-P concentrations escalated by 5.61, 5.23, and 5.60 nmol l^{-1} at low, medium, and high $p\text{CO}_2$, thus comprising 3.1, 3.4, and 3.3%, respectively, of the total amount of DOP. However, at day 15 the dATP-P concentration was reduced by 3.2 (low $p\text{CO}_2$), 2.8 (medium $p\text{CO}_2$), and 3.0 (high $p\text{CO}_2$) nmol l^{-1} (Fig. 4e), without significant differences between treatments. The dATP-P concentration

correlated positively with *Nodularia*-P, POC, PON, and *Nodularia* abundance ($|R| = 0.673, 0.768, 0.816, 0.727, p < 0.001, n = 36$) and negatively with DIP ($|R| = -0.736, p < 0.001, n = 35$).

Dissolved phospholipids

The initial concentrations of dPL-P were 6.7, 10.5, and 15.2 nmol l⁻¹ at low, medium, and high pCO₂, accounting for 2, 3.2, and 4% of total DOP, respectively. The concentrations of dPL-P in the low and medium treatments increased over the first 3 days by a mean of 10.5 and 17.8 nmol l⁻¹, respectively, without significant differences between treatments. From day 3 onwards, dPL-P concentrations decreased again, until they leveled out around the initial concentrations (10.1 and 14.5 nmol l⁻¹, respectively). In contrast, dPL-P concentrations in the high pCO₂ treatment were constant at around 15.9 ± 0.6 nmol l⁻¹ until day 9, with a slight increase by 2.9 nmol l⁻¹ at day 15 (Fig. 4d). By this time, the proportion contributed by dPL-P to total DOP was higher in all three treatments (4.2, 7.6, and 9.3%, for low, medium, and high, respectively), mainly due to the decrease of total DOP. However, neither a significant difference between the treatments nor a correlation to any other parameter was noted.

Dissolved DNA

Overall, the concentration of ddDNA-P was very low and represented only a very small proportion of total DOP (0.07 ± 0.01%). The concentration on day 0 was 0.28, 0.27, and 0.31 nmol l⁻¹ at low, medium, and high pCO₂, respectively. At day 9, the ddDNA-P concentration was reduced by half, to 0.14, 0.14, and 0.16 nmol l⁻¹, respectively, and remained constant afterwards (Fig. 4f). Dissolved DNA-P correlated positively with DOP ($|R| = 0.727, p < 0.001, n = 36$) and negatively with POP, POC, and PON ($|R| = -0.836, -0.637, -0.688, p < 0.001, n = 36$).

Dissolved RNA

Starting concentrations of dRNA at low, medium, and high pCO₂ were 108, 106, and 145 nmol l⁻¹, constituting 32, 32, and 38% of total DOP, respectively. In the low and medium pCO₂ treatments, the concentration increased until day 3, by 46 and 55 nmol l⁻¹, respectively, and then fluctuated around 150.2 ± 3.4 nmol l⁻¹ (low) and 150.5 ± 14.3 nmol l⁻¹ (medium). In contrast, the dRNA concentration of the high pCO₂ treatment first slightly decreased, by 14.5 nmol l⁻¹, before increasing by 35 nmol l⁻¹ at day 9, remaining constant thereafter (Fig. 4c). Thus, the dRNA-P concentrations increased over the course of the 15-day experiment, with the largest increment at low pCO₂ (by 41.7 nmol l⁻¹). At medium and high pCO₂ the increases were minor (28.1 and 12.8 nmol l⁻¹) and differences between both were not significant. Due to the decrease of DOP, the proportions of total DOP contributed by dRNA-P increased with time, finally reaching 63% (low), 71% (medium), and 77% (high) of total DOP. Therefore, dRNA-P accounted for a major fraction of DOP.

Uncharacterized DOP

At the beginning of the experiment, uncharacterized DOP accounted for the majority of the total DOP, amounting to 65.1, 63.7, and 57.0% at low, medium, and high $p\text{CO}_2$, respectively. During the growth phase of *Nodularia*, concentrations of uncharacterized DOP declined by 114.7, 150.3, and 171.2 nmol l^{-1} , respectively. This implied a decrease, albeit not significant, by a factor of 1.31 at medium $p\text{CO}_2$ and 1.49 at high $p\text{CO}_2$ compared to the low $p\text{CO}_2$ treatment. Until day 15, the decrement proceeded, progressively reducing the proportions of uncharacterized DOP to 30.8% (low), 18.6% (medium), and 11.7% (high) of total DOP (Fig. 4b). The uncharacterized DOP fraction correlated positively with dDNA-P ($|R| = 0.738$, $p < 0.001$, $n = 36$) and negatively with *Nodularia*-P, *Nodularia* abundance, and APA ($|R| = -0.82$, -0.682 , -0.681 ; $p < 0.001$, $n = 36$). This correlation analysis supports the finding that uncharacterized DOP was the largest fraction of DOP and thus served as the main source of *Nodularia*-P after DIP depletion.

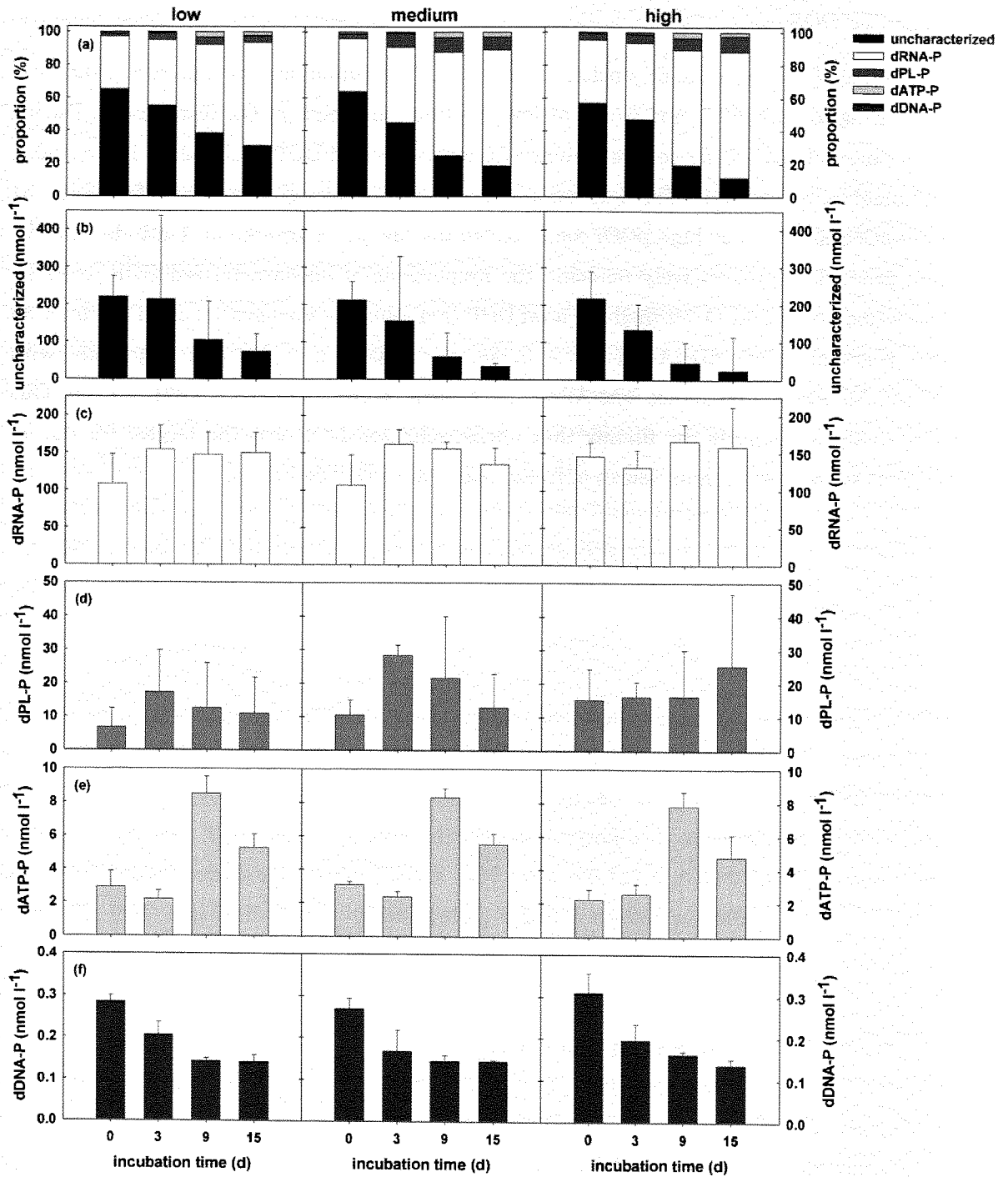


Fig. 4: Composition of DOP as %-proportion (a), and absolute values for the uncharacterized fraction, black (b), dRNA-P, white (c), dPL-P, mid-grey (d), dATP-P, light-grey (e), and dDNA-P, dark-grey (f) over incubation time and for the three $p\text{CO}_2$ treatments (low, medium, and high).

[³³P]PO₄ uptake and transformation

The distribution of [³³P] in the three fractions, *Nodularia*-P, DOP, and DIP, was similar in all CO₂ treatments during the incubation, despite a few deviations. [³³P]PO₄ was incorporated into biomass during the growth phase (Fig. 5), with 15.9–26.3% of the added [³³P]PO₄ occurring in *Nodularia* in all treatments after 3.5 h. Thereafter, [³³P] fixation seemed to be faster in the medium and high pCO₂ treatments, based on a mean at day 3 of 74% and 58%, respectively, detected in the biomass compared to 28% in the low pCO₂ treatment. At day 9, nearly the whole [³³P]PO₄ (94.7-97.8%) was fixed into biomass independent from the pCO₂ treatment. The stagnation or decline of the cyanobacteria population at day 15 (Fig. 2) was accompanied by a strong decrease in cellular P in the medium pCO₂ treatment and a weak decrease in the high pCO₂ treatment. In the low treatment, the proportion of [³³P]PO₄ in *Nodularia* remained as high as at the previous sampling time. Thus, P-turnover was faster under medium and high than under low pCO₂ conditions. [³³P] was released from *Nodularia* predominantly as DIP, with only a small proportion occurring as DOP (Fig. 5a, c, d). The 81% decrease in biomass detected in the medium treatment was combined with a release of 78% as DIP and 3% as DOP. The phosphorus decrease of 6% in the high treatment consisted of 5% as DIP and to 1% as DOP. The transformation of [³³P]PO₄ into DOP by *Nodularia* was generally low (1.4–7.7%), with most already released after 3.5 h. The calculation of DIP transformed into DOP based on the DIP additions indicated that the DIP conversion involved nanomolar concentration ranges (Table 5), in agreement with the decline of DOP (Fig. 3).

Table 5: Quantity of DIP (nmol l⁻¹) transformed into DOP by *N. spumigena* calculated from ³³P-experiments and DIP concentrations at the start and the additional supply at day 3.

time	pCO ₂ treatment		
	low	medium	high
3 days	8.4 ± 1.7	2.4 ± 1.5	4.1 ± 3.9
9 days	21.4 ± 12	13.9 ± 0.7	10.2 ± 2.4
15 days	7.8 ± 4.5	35.5 ± 8.8	18.5 ± 8.8

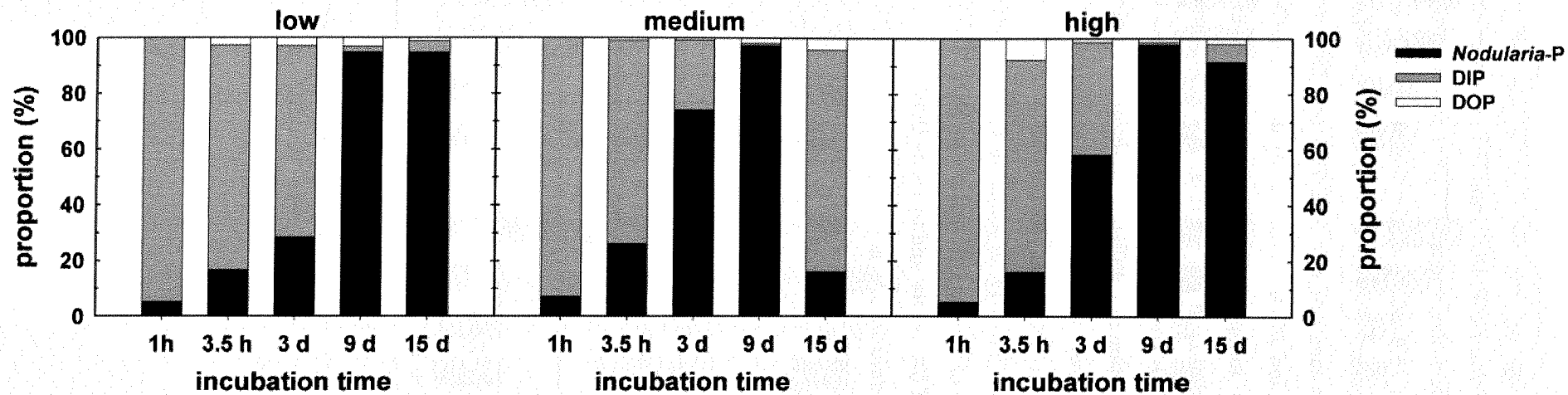


Fig. 5: Proportion of [³³P]PO₄ in the three fractions: Nodularia-P, DIP and DOP over the course of the experiment for the low, medium, and high pCO₂ treatment.

4.7.6 Discussion

This joint study investigated *Nodularia spumigena* growth as well as carbon, nitrogen, and phosphorus transformation under different $p\text{CO}_2$ conditions. Part I focused on growth, production and nitrogen cycling (Wannicke et al., 2012). Part II dealt with exudation and extracellular enzyme activities (Endres et al., 2013). Here (part III), we discuss the turnover of the dissolved P pool, including DOP composition, to illustrate the P-based nutrition of *Nodularia* and its P transformation processes.

CO₂ effects on phosphorus nutrition of Nodularia spumigena

During the first 9 days of the experiment, *Nodularia* growth was significantly enhanced with increasing $p\text{CO}_2$, as evidenced by the increases in Chl *a*, POC, PON, and filament abundances. Carbon and nitrogen fixation rates were stimulated as well (Wannicke et al., 2012). The response of *N. spumigena* to $p\text{CO}_2$ elevation was similar to that reported for the oceanic filamentous cyanobacterium *Trichodesmium* (Barcelos e Ramos et al., 2007; Hutchins et al., 2007; Levitan et al., 2007). When DIP reached the detection limit, *Nodularia* obtained P from DOP. While the DOP concentration decreased in all treatments, the decline was strongest at medium and especially at high $p\text{CO}_2$. Dissolved phosphorus (DIP and DOP) uptake was reflected in the P content of *Nodularia* (Fig. 3a, 5). In the [³³P]-experiments, during the growth phase of *Nodularia* nearly all of the DIP (95–98%) was fixed in biomass. The reason might be different for the higher amount of [³³P]PO₄ retention in the low and high treatments compared to the medium treatment, such as differences in the growth rate. In the low $p\text{CO}_2$ treatment, growth and [³³P]PO₄ incorporation were slower than in the high $p\text{CO}_2$ treatment such that senescence, in which P is released, might not have been reached within the time limits of the experiment. In the high treatment, the greater P demand of *Nodularia* could have caused the persistence of P in the cells. While this hypothesis has to be supported by further experiments, a similar increase in the P demand of *Nodularia* will be likely under the conditions predicted for the Baltic Sea. Even the slight elevation in $p\text{CO}_2$ in our experiment, from 341 to 391 μatm , was shown to have a stimulating effect. Accordingly, small variations in the present $p\text{CO}_2$ (spatially and temporally) can be expected to influence *Nodularia* growth, nitrogen fixation, and P demand. Currently, the $p\text{CO}_2$ in the central Baltic Sea ranges between 120 and 250 μatm during the summer season. Thus, elevation of atmospheric $p\text{CO}_2$ might result in a lower $p\text{CO}_2$ level in seawater as assumed in our experiments.

DOP as phosphorus source

The ability of phytoplankton to utilize DOP as an alternative P source was frequently demonstrated in earlier studies (e.g., Currie and Kalf, 1984; Cotner and Wetzel, 1992; Dyhrman et al., 2006). In fact, it is generally accepted that DOP is the main P source when DIP is

exhausted (Nausch and Nausch, 2004). Accordingly, a high alkaline phosphatase activity (APA) is indicative of DOP utilization (Cembella et al., 1984; Nausch, 1998; Hoppe, 2003) and is often used as an indicator of P stress, especially in association with cyanobacteria (Paasche and Erga, 1988; Wu et al., 2012).

In this joint experiment, DIP depletion and the decrease in DOP occurred in parallel with enhanced APA, as previously reported by Endres et al. (2013). We not only confirmed the negative correlation between APA and DIP and DOP ($|R| = -0.852$, $n = 35$, and -0.635 , $n = 36$, $p < 0.001$) but also found similar degrees of change in APA and DOP (by 2.4- and 1.5-fold) in the high and medium $p\text{CO}_2$ treatments, respectively, versus the low $p\text{CO}_2$ treatment. This is an additional indication for the enhanced P-demand of *Nodularia* with increasing $p\text{CO}_2$.

AP preferentially hydrolyzes phosphomonoester bonds, cleaving orthophosphate from the organic moiety and making it available for cellular assimilation (Sebastián et al., 2004). ATP, DNA, RNA and PL, as the most P-rich organic compounds, are cycled differently due to their varying reactivity (Kolowitz et al., 2001). ATP, a phosphoanhydride, is hydrolyzable by AP (Hernández et al., 1996; Hansen and Heath, 2005). Phosphate is cleaved from DNA and RNA by AP at the 5' terminal end (Hino, 1989) rather than from within the DNA or RNA strands. Phospholipids react with AP only if they contain a phosphate monoester group at the C_3 position of glycerol (Blank and Snyder, 1970). In contrast to these DOP compounds, phosphomonoesters (e.g., sugar phosphates) are easily cleaved by AP and thereby are able to significantly contribute to the bioavailable DOP pool and, therefore, to P-nutrition. Labry et al. (2005) reported that in the Gironde plume phosphomonoester concentrations make up between 11 and 65% of the total DOP pool. In our study, an uncharacterized DOP proportion accounted for 20.4 to 61.9% of total DOP, and may be attributed to phosphomonoesters that were not determined during this study. The importance of this pool of uncharacterized DOP derives from the fact that it accounted for the bulk of P-nutrition in *Nodularia*, a finding supported by the negative correlation with *Nodularia*-P and APA ($|R| = -0.82$ and -0.681 , $p < 0.001$, $n = 36$) as well as the positive correlation with DOP ($|R| = 0.932$, $p < 0.001$, $n = 36$).

Composition of DOP in the presence of Nodularia spumigena

In the laboratory experiment described herein, the DOP concentration and the composition of aged Baltic Sea water changed during the development of *N. spumigena*. Our methods allowed direct determination of dissolved ATP (Björkman and Karl, 2001), dissolved phospholipids (Suzumura and Ingall, 2001, 2004), and dissolved DNA and RNA (Karl and Bailiff, 1989) concentrations. The sum of these components amounted to 38.1–79.6% of the total DOP during the 15-day incubation.

Dissolved ATP concentrations in the batch culture experiments of this study ranged from 399 to 1563 ng l^{-1} and constituted 0.6–3.4% of the DOP pool and thus were higher than those reported by Azam and Hodson (1977) in surface waters at the coast of Southern California by a factor of 1.8 to

24.0. Nawrocki and Karl (1989) reported values similar to ours, i.e., between 23 and 1278 ng l⁻¹, in the upper 100 m of five stations in the Bransfield Strait whereas the concentrations measured by Björkman and Karl (2001) in the subtropical North Pacific gyre were lower (Table 2).

As noted by Suzumura (2005), information on the distribution and abundance of lipid P in marine environments is still lacking. The studies carried out thus far have been limited to the distribution and abundance of dissolved lipids, e.g. phospholipids, in the North Atlantic Ocean, Tokyo Bay, Corpus Christi Bay, and Pacific Ocean (Parrish, 1987; Suzumura and Ingall, 2001, 2004). Ours is the first such study of the Baltic Sea. Moreover, the determined concentrations of lipid P as well as the percentage of total organic P are in agreement with the values reported for the dissolved fraction in pelagic seawater by Suzumura and Ingall (2004) (Table 3).

Table 2: Observed dATP values based on literature data from different oceanic regions.

Sample location	dATP (ng l ⁻¹)	Reference
<i>field observations</i>		
coast, Southern California		
(SIO pier to Point Loma)	65-218	Azam and Hodson, 1977
Saanich Inlet, British Columbia	466	Azam and Hodson, 1977
Gulf stream, Florida		
(range of several stations; 5 m)	22-306	Hodson et al., 1981
Bransfield Strait, Antarctica		
(Jan 87; range of 5 stations, 0-100 m)	23-1278	Nawrocki and Karl, 1989
Subtropical North Pacific gyre		
(range of several stations, 5-125 m)	14.9-41.3	Björkman and Karl, 2001
<i>Laboratory observations</i>		
aged Baltic Sea water (batch		
experiment)	356-1594	present study

Table 3: Lipid P concentrations and percentages of total organic P as shown by Suzumura (2005).

Sample	Sample Description	Concentration	% of total organic P	Reference
Seawater (coastal)	Particulate (>0.7 μm)	90-750 nM P	5.6-11.6	Miyata and Hattori, 1986
Seawater (coastal)	Dissolved (<0.7 μm)	0.7-6.0 nM P	0.1-0.9	Suzumura and Ingall, 2001
	Particulate (>0.7 μm)	31-294 nM P	3.0-13.5	Suzumura and Ingall, 2001
Seawater (pelagic)	Dissolved (<0.7 μm)	4.0-17.9 nM P	1.7-17.6	Suzumura and Ingall, 2004
	Particulate (>0.7 μm)	0.05-1.72 nM P	0.8-34.4	Suzumura and Ingall, 2004
Seawater (aged, batch experiment)	Dissolved (<0.2 μm)	6.7-28.3 nM P	1.7-12.4	present study

Dissolved DNA concentrations were very low throughout the experiment, accounting for a very small proportion of DOP (~0.08%). In fact, they were 8800 times lower than the measured dDNA concentrations from other marine and freshwater areas and therefore can be considered as negligible. The Northern Adriatic Sea is the only site with similarly low dDNA concentrations (Table 4). As suggested by Paul et al. (1990), actively growing phytoplankton might produce small to undetectable amounts of dDNA, with production occurring only in senescent phytoplankton cells. Another plausible explanation is that of Løvdal et al. (2007), who measured accelerated turnover times of dDNA under conditions of P starvation of about 1.5 h instead of 15.6 h under balanced conditions. This and the observed uptake of dDNA-P by *Nodularia* in our studies lead to the assumption that dDNA-P is rapidly used within hours, as it could not be detected with our sampling strategy.

Dissolved RNA has hardly been investigated in aquatic environments. Karl and Bailiff (1989) measured concentrations ranging from 4.03 to 51.1 $\mu\text{g l}^{-1}$ at several stations near Hawaii and the open Pacific Ocean in March 1988. These concentrations are in the lower range of those determined in our study (26–83 $\mu\text{g l}^{-1}$) (Table 4).

Table 4: dDNA and dRNA values for marine and freshwater stations adapt from Karl and Bailiff (1989).

Sample location	dDNA ($\mu\text{g l}^{-1}$)	dRNA	Reference
<i>Marine: coastal/estuarine</i>			
Bombay Harbor, India (range of four stations)	13.4-80.6	—*	Pillai and Ganguly (1972)
Northern Adriatic Sea (range of two stations; 0-30 m)	0.05-0.8†	—	Breter et al. (1977)
Bayboro Harbor, Florida (Mar 86)	18.32(\pm 1.78)	—	DeFlaun et al. (1986)
Bransfield Strait, Antarctica (Dec 86; range of 69 stations)	6-15	—	Bailiff and Karl (1987)
Kaneohe Bay, Hawaii (Mar 88; range of four stations)	2.66-3.15	20.6-31.9	Karl and Bailiff (1989)
Mamala Bay, Hawaii (Mar 88)	1.02(\pm 0.08)	6.67(\pm 2.67)	Karl and Bailiff (1989)
Kahana Bay, Hawaii (Mar 88; water column)	4.70	51.1	Karl and Bailiff (1989)
Northern Baltic Sea, Sweden (June 06, range of four stations, 1 m)	1.3-2.6	—*	Riemann et al. (2009)
<i>Marine: offshore</i>			
N. Pacific Ocean (33°HN, 139°W; 0-400 m)	0.56-1.39	4.03-13.9	Karl and Bailiff (1989)
<i>Freshwater</i>			
Quarry Pond, Hawaii	3.54(\pm 0.03)	23.0(\pm 0.16)	Karl and Bailiff (1989)
Krauss Pond, Hawaii	88	871	Karl and Bailiff (1989)
<i>Laboratory: batch experiments</i>			
aged Baltic Sea water, Germany (batch experiment)	0.01-0.04	26-83	present study

† Assumes DNA is 10% thymine, by weight

* No data available

CO₂ effects on DOP components

Our results demonstrate that the various compounds within the DOP pool developed differentially over time and with $p\text{CO}_2$. The dynamic of dATP-P seems to be not or only marginally influenced by $p\text{CO}_2$. Dissolved ATP-P did not differ significantly between $p\text{CO}_2$ treatments at day 3 and day 9, despite. From day 9 to day 15, the decline in dATP-P (by a mean of $3.04 \pm 0.22 \text{ nmol l}^{-1}$; Fig. 4e) followed the trend of the entire DOP pool. At this stage, *Nodularia* cells were in stationary phase, exhibiting the first signs of decay at day 15. Therefore, rather than the consumption of dATP-P and DOP, a release of the latter would be expected and suggests the continued need of living *Nodularia* cells for P after the more readily available DOP compounds have been consumed. We have no evidence that dATP is used by heterotrophic bacteria, given that their abundances remained at the same low levels as at the start of the experiment.

Under low and medium $p\text{CO}_2$ conditions, dPL-P was released by *Nodularia* from day 0 to day 3, during the period of DIP uptake. Afterwards, dPL-P was utilized by the cyanobacterial cells probably because of the strengthened P demand. Thus, for these two treatments approximately the same concentrations were detected at the start and end of the experiment. In comparison, under high $p\text{CO}_2$ conditions a temporary dPL-P elevation was observed at day 15 and not before. We sampled in intervals of several days and therefore, it is possible that short time elevations of dPL-P were unascertainable before day 15. In contrast to dATP-P, dPL-P was released under high $p\text{CO}_2$, when *Nodularia* growth reached stationary phase or the cells became senescent (Fig. 4d).

The starting concentration of dDNA-P in all treatments was $0.29 \pm 0.02 \text{ nmol l}^{-1}$ and leveled out at $0.14 \pm 0.04 \text{ nmol l}^{-1}$ at the end of the 15-day experiment. The uptake of dDNA-P, even though in the nanomolar range, seemed to be due to the P-rich nature of DNA (Sterner and Elser, 2002) and the strengthened P demand. Throughout the experiment, dDNA-P values were generally low and did not differ significantly between the three $p\text{CO}_2$ treatments (Fig. 4f). The turnover of dDNA-P and other DOP compounds is very short, occurring within hours, so that shorter sampling intervals would have been necessary to estimate the variations. In addition, as mentioned above, Paul et al. (1990) reported low dDNA production during the phytoplankton growth phase. The authors assumed that phytoplankton DNA synthesis primarily occurred at night or that dDNA was released by senescent, dying, or grazed phytoplankton cells. If the synthesis and release of dDNA occur only at night, our sampling time, between 8 and 9 am, would have been unable to detect these changes in dDNA.

In our study, dRNA-P was relatively constant in all $p\text{CO}_2$ treatments and was one of the main contributors to total DOP. However, regardless of the $p\text{CO}_2$ the dRNA-P concentrations were lower at the beginning of the experiment than in the following sampling days. From day 0 to day 9, dRNA-P production was highest in the medium treatment, followed by the low and high treatments (49.3 , 39.1 , and 20.4 nmol l^{-1} , respectively; Fig. 3b). Over the course of the experiment dRNA-P production was highest in the low treatment whereas release was lowest in the high

treatment (41.7 and 12.8 nmol l⁻¹ dRNA-P, respectively), suggesting that with an elevated $p\text{CO}_2$ the release of dRNA-P is reduced due to the strengthened P demand (Fig. 4c).

Overall, DOP is produced as long as either DIP is available or a certain intracellular P-pool exists. We found only a few trends but no significant effects of variable $p\text{CO}_2$ conditions on single metabolic components. Only the total DOP concentration differed significantly as a function of $p\text{CO}_2$ from day 0 to day 9, due to changes in the amounts of compounds other than those specifically detected herein.

Conclusion

Our results indicate that accelerated P turnover can be expected during the cyanobacterial growth period under the $p\text{CO}_2$ conditions predicted for the future Baltic Sea. This implies the faster utilization of DIP as well as DOP. We propose that the stimulating effect on P utilization by the filamentous cyanobacterium *Nodularia spumigena* is indirect, as it is mediated by elevated carbon fixation and is dependent on cyanobacterial growth, which induces a stronger P demand.

There is no trend towards the greater use of dissolved ATP-P, PL-P, RNA-P, and DNA-P under high $p\text{CO}_2$ conditions, but it remains to be confirmed in further investigations. These should include higher sampling resolution to capture the change from release to uptake. Components other than those measured in this study were taken up more intensively and dominated the decrease in the total DOP pool.

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4.7.8 References

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5 Synthesis

The rise in anthropogenic CO₂ emissions correspondingly increases dissolved CO₂ concentrations in the ocean, consequently lowering seawater pH, a process referred to as ‘ocean acidification’. This thesis addresses the questions of (1) how ocean acidification can influence microbial growth and degradation of organic matter and (2) how these biotic changes may be reflected in the organic matter pool. The first part of the thesis deals with the response of a natural plankton community (Manuscripts I and II) to CO₂ enrichment in a Norwegian fjord, while the second part focuses specifically on the diazotrophic cyanobacterium *Nodularia spumigena*, which plays an important role in nitrogen and phosphorus cycling in the Baltic Sea (Manuscripts III-V).

5.1 CO₂ increases the formation of exopolymer substances

The extracellular release of organic compounds by autotrophs increases under excess availability of CO₂ during nutrient limitation (Obnersterer and Herndl 1995; Engel 2002; Engel et al. 2004a; Otero and Vincenzini 2004). These organic molecules, which are thought to be mostly carbon-rich, are partly consumed by heterotrophic bacteria but also act as precursors for the formation of marine gels through coagulation and aggregation of individual molecules to large particles. Gel particles may adsorb high amounts of nutrients and provide a surface for bacteria to attach and grow (Long and Azam 1996; Azam and Malfatti 2007). Consequently, gel particles are hotspots for microbial degradation (Alldredge et al. 1993; Verdugo et al. 2004) and play an important role in formation of aggregates and export of organic and inorganic matter (Logan et al. 1995; Passow et al. 2001; Engel et al. 2004b). Depending on their composition, gel particles can be identified as polysaccharide-rich transparent exopolymer particles (TEP) or proteinaceous Coomassie stainable particles (CSP). Some autotrophic cells, such as filamentous cyanobacterial cells, are coated by a polysaccharide-rich mucus layer. The function of this mucus and factors regulating the production, however, are not yet understood (Nausch 1996; Otero and Vincenzini 2004).

During this thesis, I investigated the formation and accumulation of exopolymer substances (mucus, TEP, CSP) from dissolved precursors produced under elevated CO₂ concentrations. Cell-specific and biomass-corrected production of gel particles in natural plankton communities and the production of mucinous substances (cell-attached mucus and free TEP) in *Nodularia spumigena* cultures were unaffected by CO₂ concentration. However, due to generally higher biomass production the total amount of gel particles may increase under high CO₂ concentrations in the ocean (Manuscripts I and III).

The accumulation of exopolymer substances under elevated CO₂ concentrations may shift the organic matter pool towards larger particle size reinforcing the importance of gel particles in organic matter fluxes and increasing carbon sequestration to the deep ocean. Moreover, exopolymer substances may support marine bacterial growth as surfaces to attach to and as a carbon- or nutrient-rich food source (Manuscript I). Increased microbial degradation activities on gel particles may counteract the export of organic matter to the deep sea preserving more organic matter in the surface ocean. Both scenarios are likely to happen but not necessarily at the same time and place.

5.2 Decreased seawater pH accelerates enzymatic hydrolysis of organic matter

The effect of decreased seawater pH on the activity of four key enzymes in carbon and nutrient cycling were studied during this thesis. Alkaline phosphatase is a key enzyme in natural environments, since it allows phosphorus regeneration of dissolved inorganic orthophosphate from organic molecules. Leucine aminopeptidase is a very widely spread enzyme in marine waters which is involved in the decay of proteins providing organic nitrogen to microbes (Chróst and Siuda 2002). Results of the present studies show that the hydrolysis rates of both alkaline phosphatase and leucine aminopeptidase are accelerated by decreasing seawater pH (Manuscripts I and III) confirming previous studies that also reported stimulation of hydrolytic enzymes such as glucosidases and leucine aminopeptidase (Grossart et al. 2006; Cunha et al. 2010; Piontek et al. 2010; Piontek et al. 2012). As hydrolytic extracellular enzymes catalyze the first step in the break-down of organic matter, the microbial turnover and the recycling of organic nutrients from organic matter in the surface ocean may be more rapid due to ocean acidification supporting bacterial nutrition. Furthermore, extracellular enzymes may dissolve mucus from the surface of algal cells thereby affecting stickiness. This would change aggregation and sinking out of cells during phytoplankton blooms (Azam 1998). Rapid hydrolysis rates on aggregates may effectively solubilize sinking particles to dissolved organic matter (Smith 1992; Grossart & Simon 1998; Ploug and Grossart 2000) decreasing carbon fluxes to the deep ocean.

The exact mechanism of extracellular enzyme stimulation by pH is still unclear. An increased amount of TEP under decreased seawater pH could mean a greater reactive surface area for dissolved enzymes to attach to. Attached enzymes have longer hydrolytic lifetimes than dissolved enzymes (Ziervogel et al. 2007) and therefore may elevate total enzyme activities over time. Extracellular enzymes are directly exposed to seawater pH and direct effects of pH changes on extracellular enzyme activities were shown previously (Muenster 1991; Piontek et al. 2010). Decreasing seawater pH presumably modifies the biochemical structure of the catalytic site of the enzyme improving its hydrolysis rates. Different types of an enzyme (isoenzymes) may vary in their response to pH changes. Extended knowledge on enzyme synthesis, enzyme diversity, and

the regulation of enzyme activities would help to calculate future changes in organic matter hydrolysis in the ocean.

5.3 CO₂ increases bacterial biomass in the surface ocean

Previous studies implied minor effects of ocean acidification on bacterial abundance (Rochelle-Newall 2004; Grossart 2006; Allgaier 2008). During the mesocosm study of this thesis, heterotrophic bacterial growth in a natural plankton community was significantly stimulated at elevated CO₂ concentrations resulting in higher bacterial abundances (Manuscript I). Reasons for the enhanced bacterial success (as discussed in Manuscript I) are increased accumulation of gel particles as substrate to attach to and faster nutrient availability due to enhanced enzymatic hydrolysis rates. Hence, ocean acidification may support the production of heterotrophic bacterial biomass in the surface ocean which may accelerate degradation processes of organic matter and impact on biogeochemical cycling. Heterotrophic respiration in the ocean is the main sink for O₂ and contributes ~50 to >90% of total CO₂ production in the ocean (Sherr and Sherr 1996; Rivkin and Legendre 2001). Enhanced heterotrophic oxygen consumption may consequently reduce O₂ concentrations and further increase inorganic carbon concentrations. At the same time, faster heterotrophic degradation of organic matter in the surface ocean may reduce the fraction of organic matter which is exported and lower the efficiency of particle export and carbon sequestration fluxes to the ocean depth (Passow and Carlson 2012). Future studies should address the question to which degree bacterial populations in the surface ocean may increase respiration of organic matter which is produced by phytoplankton under ocean acidification. A decrease in the exported organic matter fraction may strongly lower the efficiency of carbon sequestration in the ocean and weaken the oceans mitigating effect in atmospheric CO₂ concentrations (Passow and Carlson 2012).

5.4 Diazotrophic cyanobacteria are favored under increased CO₂ concentrations

The increase in oceanic CO₂ concentrations due to anthropogenic CO₂ emissions is expected to stimulate photosynthetic rates of marine phytoplankton (Beardall and Raven 2004; Riebesell et al. 2007) and cyanobacteria (Levitan et al. 2007; Barcelos e Ramos et al. 2007; Hutchins et al. 2009) resulting in higher biomass production. This also holds for the Baltic Sea cyanobacterium *Nodularia spumigena*, which largely increases carbon fixation and growth rates as long as phosphorus is not limiting, leading to an increment in biomass under future CO₂ conditions (Manuscript IV). To sustain higher nitrogen fixation during inorganic phosphorus limitation, cyanobacteria need alternative phosphate sources. *Nodularia spumigena* meet their phosphorus

demand utilizing dissolved organic phosphorous (DOP) (Manuscript V). The enzymatic hydrolysis rates of DOP via the extracellular enzyme alkaline phosphatase accelerated under decreased pH facilitating phosphorus acquisition from organic matter (Manuscripts III).

Rising temperatures due to anthropogenic CO₂ emissions are expected to increase inorganic nutrient limitation due to stronger thermal stratification in the ocean which would lower the exchange of nutrients between deep and surface ocean (Doney 2006). Cyanobacteria may become more competitive under these conditions because of their improved ability to acquire nutrients. In future, blooms of diazotrophic cyanobacteria such as *Nodularia spumigena* may intensify or expand in nitrogen-limited regions or periods. This would provide additional bioavailable nitrogen through the release of dissolved nitrogen compounds (Wannicke et al. 2009) and enhance the turnover of organic phosphorous within these regions supporting primary production (Passow and Carlson 2012).

5.5 Ocean acidification facilitates the acquisition of nutrients from organic matter

Inorganic nutrient concentrations limit autotrophic and heterotrophic growth (Smith 1984; Cotner and Wetzel 1992; Kirchman 1994), thus microbes are forced to utilize alternative nutrient sources such as atmospheric nitrogen, dissolved organic nitrogen and dissolved organic phosphorous (Manuscripts I, III, IV and V). During both experimental studies of this thesis, enhanced enzymatic hydrolysis of organic matter under decreased pH facilitated the acquisition of nutrients from organic matter (Manuscripts I and III). Leucine aminopeptidase and alkaline phosphatase activities, both important enzymes in recycling of nutrients, were significantly accelerated. DON and DOP were preferentially consumed during autotrophic blooms while DOC accumulated (Manuscripts II, IV, and V).

Hence, C:N and C:P ratios of DOM may increase resulting in the accumulation of carbon-rich material and reducing the nutritional value of organic matter in the euphotic zone of the future ocean. Together with the input of more nitrogen due to increased N₂ fixation, this may change nutrient cycling and elemental stoichiometry of DOM in the future ocean. Previous studies on cyanobacteria found increased C:N ratios (Levitan et al. 2007; Czerny et al. 2009) as well as increased N:P and C:P ratios at high CO₂ concentrations (Hutchins et al. 2007; Barcelos e Ramos et al. 2007). However so far, there is no agreement on whether elemental ratios of organic matter are likely to be altered in a systematic way in the future acidified ocean. Furthermore, carbon-rich DOM may become less bioavailable due to microbial transformation (microbial carbon pump) and accumulate over time, increasing the refractory DOM pool (Jiao et al. 2010). The accumulation of carbon-rich DOM and increased productivity due to N₂ fixation in the surface ocean may increase carbon sequestration in the deep sea.

5.6 Autotrophic-heterotrophic coupling in the acidifying ocean

Organic matter derived from autotrophs is an important food source for heterotrophic bacteria, resulting in a tight coupling between autotrophic and bacterial production and abundance (Cole 1982; Cotner and Biddanda 2002). The stimulating effect of ocean acidification on autotrophs may thus be of mutual benefit to heterotrophic bacteria and vice versa: As described above, increasing CO₂ concentrations may induce higher autotrophic growth and biomass (Manuscripts IV) as well as the accumulation of organic matter, especially of exopolymers (Manuscripts I and III), providing a higher amount of organic substrate to heterotrophic organisms. In a previous mesocosm study, phytoplankton growth and community composition strongly depended on CO₂ concentration and very likely affected bacterial production and enzyme activities (Grossart et al. 2006). On the other hand, faster enzymatic hydrolysis of organic compounds due to decreased seawater pH may increase the amount of utilizable monomers in the water supporting both auto- and heterotrophic growth.

As bacterial biomass increases (Manuscripts I and IV) competition for inorganic and organic nutrients between auto- and heterotrophic microbes will be reinforced. Heterotrophic bacteria are generally more successful in acquiring inorganic nutrients (Currie and Kalff 1984; Joint et al. 2002). Additionally, they are able to utilize diverse sources of organic nutrient via enzymatic hydrolysis during nutrient limitation. This could potentially reduce autotrophic biomass production and activity in the future ocean (Thingstad et al. 2008).

5.7 Consequences for DOM composition

The response of auto- and heterotrophic processes to ocean acidification will likely influence the composition of DOM resulting in changed bioavailability or chemical properties of DOM. However, microbial degradation rapidly reacts to changes in the amount or composition of autotrophic produced DOM (Manuscripts II and V) making it difficult to detect CO₂-related changes in the DOM pool. Only 10-20% of the DOM pool can be chemically characterized while the remaining fraction is unknown (Benner 2002). These chemically uncharacterized compounds of marine DOM may be the most utilized ones. During the *Nodularia spumigena* study, the consumption of mostly uncharacterized phosphorus components resulted in a decrease in the DOP pool (Manuscript V). Possible compositional changes were therefore not detectable due to methodological constraints. This highlights the need of new or developing current methods to characterize the chemical composition of bulk DOM in detail.

During the mesocosm study, small compositional changes in amino acids were detected (Manuscript II) which may be related to stimulated bacterial degradation under elevated CO₂

concentrations. Amino acids make up just a minor, nevertheless important, part of marine DOM as they are a central food source for heterotrophic bacteria. Dissolved free amino acids support about 4 – 41% of the bacterial nitrogen demand in the open ocean (Keil and Kirchman 1991, 1999). Bulk DOM concentrations and the diagenetic status of DOM seemed unaffected by CO₂ conditions during this study.

In general, CO₂-induced changes in DOM composition, e.g. amino acid composition, may be hard to identify in short-term experiments but may accumulate and change the bioavailability of DOM in the long-term. Some compounds may accumulate over several years and centuries while others like proteinogenic amino acids become rare and maybe even limiting for microbial growth. Continuously higher production of refractory DOM may lower the average bioavailability of DOM to heterotrophic degradation. Hence, further studies should take longer time scales into account to investigate lasting effects of changes in microbial activities on the DOM pool. However, the bioavailability of organic matter depends also on microbial community composition (Carlson et al. 2004). So far it is unknown how microbial communities may change in the future and how this may affect cycling and composition of DOM.

5.8 Conclusion und research perspectives

From the results of this thesis and considering recent results on ocean acidification research, there are clear signs that marine microbes and organic matter cycling may be directly and indirectly affected by ocean acidification. I infer that ocean acidification will stimulate bacterial growth and increase the microbial turnover of organic matter strengthening the role of the microbial loop in the surface ocean.

Ocean acidification is only one aspect of environmental change besides ocean warming, stratification, eutrophication, and decreasing oxygen concentrations of the ocean (Boyd 2011) due to a rise in global temperature (greenhouse effect). As combined effects may compensate or amplify direct effects of increasing CO₂ levels alone, multiple factors need to be addressed when studying microbial degradation of organic matter. Following global temperature, ocean temperature is predicted to rise by up to 3.1°C until the year 2100 depending on oceanic region and the amount of future anthropogenic CO₂ emissions (Gruber 2011). Temperature has a direct effect on metabolic activities and growth rate of marine bacteria. Bacteria that are better adapted to higher temperatures may thrive, or existing bacterial species may disappear when temperatures go beyond their tolerance range. Similar to pH, ocean warming was found to increase enzyme activities, bacterial production and respiration rates (Piontek et al. 2009; Cunha et al. 2010; Sarmiento et al. 2010). It might as well as enhance polysaccharide release and TEP formation rates (Borchard and Engel 2012). Temperature may also induce higher diazotroph growth (O'Neil et al. 2012). I therefore propose that ocean acidification and warming will act in

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concert to promote or even amplify microbial processes and to reinforce the already dominant role of microbes in the marine biogeochemistry.

The question whether the ocean will mitigate or reinforce the increase of anthropogenic CO₂ in the atmosphere is still unresolved. The results presented here indicate that the potential accumulation of carbon-rich or refractory DOM due to higher microbial activity could increase carbon sequestration in the ocean. However, accelerated degradation of organic matter in the surface ocean may decrease the efficiency of the biological carbon pump as less carbon reaches deeper water layers. Whether the ocean remains a net sink for atmospheric CO₂ may depend on several factors, but the stimulation of the microbial turnover of organic matter is a key aspect when addressing this question.

To date, studies on bacterial response to ocean acidification were not conducted long enough to allow adaptation and evolution of the bacterial community. Short-term perturbation studies on bacterial community composition indicated that the community composition may change under ocean acidification (Allgaier et al. 2008; Krause et al. 2012; Sperling et al. 2013). In the long-term, marine bacteria may adapt genetically to environmental change making it difficult to predict future effects of ocean acidification on community composition and also on organic matter cycling. The genetic, physiological and biogeochemical characterization of adapted bacterial communities, e.g. from natural CO₂ vents, may help to understand more precisely the role of marine bacteria in biogeochemical cycles in the future.

In addition, genomic studies of major bacterial species may clarify the range of catabolic capability of marine bacteria. The genome of the marine carbohydrate-degrading bacteria *Saccharophagus degradans* (Weiner et al. 2008) and *Pseudomonas atlantica* (Copeland et al. 2006) revealed a large variety of polysaccharide-degrading enzyme systems. Deep sea populations of bacteria were found to be better adapted to hydrolyze refractory polysaccharides (Vezi et al. 2005; Lauro and Bartlett 2008) and to use a higher diversity of enzymes compared to bacteria in the surface ocean (DeLong 2006). Further genomic studies may help to learn more about the metabolic potential of marine bacteria and their ability to cope with projected future environmental changes.

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6 Eidesstattliche Erklärung

Hiermit bestätige ich, dass die vorliegende Arbeit mit dem Titel:

Impact of ocean acidification on microbial degradation of organic matter

von mir selbstständig verfasst worden ist und keine weiteren Quellen und Hilfsmittel als die angegebenen verwendet wurden. Die vorliegende Arbeit ist unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden und wurde nicht im Rahmen eines Prüfungsverfahrens an anderer Stelle vorgelegt. Veröffentlichte oder zur Veröffentlichung eingereichte Manuskripte wurden kenntlich gemacht. Ich erkläre mich einverstanden, dass diese Arbeit an die Bibliothek des GEOMAR und die Universitätsbibliothek der CAU weitergeleitet wird.

Kiel, Januar 2013


Sonja Endres