

Aggregation of Marine Pico-Cyanobacteria

by

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ABSTRACT

Marine pico-cyanobacteria of the genera *Synechococcus* and *Prochlorococcus* carry out nearly two thirds of the primary production in oligotrophic oceans. These cyanobacteria are also considered an important constituent of the biological carbon pump, the photosynthetic fixation of CO₂ to dissolved and particulate organic carbon and subsequent export to the ocean's interior. But single cells of these cyanobacteria are too small to sink, so their carbon export has to be mediated by aggregate formation and/or consumption by zooplankton that produce sinking fecal pellets. In this dissertation, I investigated for the first time the aggregation of these cyanobacteria by studying the marine *Synechococcus* sp. strain WH8102 as a model organism. I first found in culture experiments that *Synechococcus* cells aggregated and that such aggregation of cells was related to the production of transparent exopolymeric particles (TEP), known to provide the main matrix of aggregates of eukaryotic phytoplankton. I also found that despite the lowered growth rates, cells in the nitrogen or phosphorus limited cultures had a higher cell-normalized TEP production and formed a greater total volume of aggregates with higher settling velocities compared to cells in the nutrient replete cultures. I further studied the *Synechococcus* aggregation in roller tanks that allow the simulation of aggregates settling in the water column, and investigated the effects of the clays kaolinite and bentonite that are commonly found in the ocean. In the roller tanks, *Synechococcus* cells formed aggregates with diameters of up to 1.4 mm and sinking velocities of up to 440 m/d, comparable to those of larger eukaryotic phytoplankton such as diatoms. In addition, the clay minerals increased the number but reduced the size of aggregates, and their ballasting effects increased the sinking velocity and the carbon export potential of

the aggregates. Lastly, I investigated the effects of heterotrophic bacteria on the *Synechococcus* aggregation, and found that heterotrophic bacteria generally resulted in the formation of fewer, but larger and faster sinking aggregates, and eventually led to an enhanced aggregation of cells and particles. My study contributes to the understanding of the role of marine pico-cyanobacteria in the ecology and biogeochemistry of oligotrophic oceans.

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DISSERTATION INTRODUCTION

1.1 The Biological Carbon Pump

The biological carbon pump (BCP) describes the photosynthetic fixation of CO₂ to dissolved and particulate organic carbon by phytoplankton and subsequent export to the ocean's interior (Falkowski et al., 2000; Neuer et al., 2002). Through this pump, organic carbon can be transported to depth through particulate organic carbon (POC) flux, via mixing of dissolved organic carbon (DOC), and by active migration by zooplankton (Emerson, 2014). POC sinks gravitationally to the deep sea sediments at different velocities, mediating vertical mass fluxes and driving elemental cycling on a range of time scales (Honjo et al., 2008; Neuer et al., 2014). Sinking particles, some of which can be macroscopic (>0.5 mm in diameter, also referred to as “marine snow”), are mainly aggregates of living organisms, detritus, fecal and inorganic matter (Alldredge et al., 1993). These marine snow particles are formed from smaller particles via two major pathways: (1) grazing, which generates fecal pellets and/or large feeding structures, and (2) aggregation, the physical process by which smaller particles collide and stick together to form larger, rapidly sinking aggregates (Passow et al., 2001; Burd & Jackson, 2009). Regardless of the transporting mechanism or pathway, some portion of the organic carbon fixed by the BCP will eventually get buried in the sediments of the deep sea.

There are three major components of the BCP: new production, carbon export flux and carbon sequestration flux (Passow & Carlson, 2012). In today's oceans, approximately 10-25% of the carbon fixed by phytoplankton sinks below the euphotic

zone (“export flux”, at ca. 100 m depth), with around 10% of this export flux reaching the base of the mesopelagic zone (“sequestration flux”, at ca. 1000 m depth) (Falkowski et al., 2000; Passow & Carlson, 2012). The oceans have sequestered nearly half of the fossil-fuel CO₂ emitted into the atmosphere since pre-industrial times, and the atmospheric CO₂ concentration would be 150-200 ppmv higher without all the phytoplankton in the ocean (Falkowski et al., 2000; Sabine et al. 2004). The BCP in today’s oceans are thus playing an important role in regulating atmospheric CO₂.

1.2 Aggregation of Marine Pico-Cyanobacteria

Phytoplankton cells used to be considered the main constituents of oceanic POC. However, recent studies have focused on the formation of exo-polysaccharide particles as another important source of POC (Engel et al., 2004). These exo-polysaccharide particles are also referred to as “transparent exopolymer particles” (TEP), which can further facilitate the aggregation and subsequent settling of small cells during phytoplankton blooms (Alldredge et al., 1993; Passow et al., 2001). Through this pathway, large and rapidly sinking aggregates can also form from small particles (including single cells) via colliding and sticking together (Burd & Jackson, 2009).

Transparent exopolymer particles (TEP) are described as a class of large, discrete gel-like particles that are formed abiotically from dissolved extracellular polymeric substances (EPS) exuded by microorganisms (Alldredge et al., 1993; Passow, 2000). TEP have been found to provide the main matrix of all marine snow aggregates and serve as a substrate and microhabitat for attached bacteria (Alldredge et al., 1993; Passow &

Allredge, 1994). Owing to their fractal, surface-reactive nature, TEP support the coagulation processes with other suspended particles such as phytoplankton cells, leading to the formation of large, fast sinking aggregates (Engel, 2000; Engel et al., 2004; Burd & Jackson, 2009). TEP are especially important during phytoplankton blooms in which they facilitate the aggregation and subsequent sedimentation of phytoplankton cells that otherwise would be too small to sink (Passow et al., 2001), and thus enhance the carbon export to depth.

The formation of TEP from EPS presents an important, rapid abiotic pathway for the transformation of dissolved organic carbon into particulate form, other than the conventional microbial uptake/growth (Allredge et al., 1993; Engel et al., 2004). In the field, the length of individual TEP can range from 3-5 μm up to several 100 μm (Passow & Allredge, 1994), and their concentration in different oceanic environments ranges from 10 to 900 $\mu\text{g/L}$ xanthan gum equivalent (Passow & Allredge, 1995) depending on season, depth, and plankton community composition (Wurl et al., 2011). During phytoplankton blooms, the potential importance of TEP is actually two-fold since in addition to the phytoplankton cells that are involved in the aggregate formation, TEP themselves can also contribute appreciably to the carbon export (Passow et al., 2001; Engel et al., 2004; Deng et al., 2015).

Cyanobacteria are a widespread group of photoautotrophic microorganisms that play an important role in marine ecosystems, especially in oligotrophic regions. Although two of the most important marine pico-cyanobacteria, *Synechococcus* and

Prochlorococcus often co-occur, they have been adapted to different ecological and biogeochemical conditions. *Synechococcus* is widespread in all marine environments from high latitudes to the tropics (Neuer, 1992; Partensky et al., 1999), with its ability to regulate photochemistry over a wide range of temperatures (Mackey et al., 2013). Generally it is more abundant in nutrient-rich than in oligotrophic waters and its vertical distribution is restricted to the upper euphotic zone (Partensky et al., 1999) due to its high demands for light and nitrogen (to maintain the N-rich phycobilisome, Wyman et al., 1985; Kana et al., 1992). *Prochlorococcus*, despite of its narrower geographical distribution, is much more abundant in warm oligotrophic waters compared to *Synechococcus* (Partensky et al., 1999; Flombaum et al., 2013).

For instance, at the Bermuda Atlantic Time-series Study (BATS) site located in the Sargasso Sea (western North Atlantic Subtropical Gyre), *Synechococcus* represent around one-third of the autotrophic POC biomass (DuRand et al., 2001), but have traditionally not been considered as important contributors to the oceanic carbon export due to their small size, lack of natural ballasting minerals and tightly coupled micrograzer control (Bienfang, 1981; Worden & Binder, 2003). Thus, their aggregation and subsequent settling have not received the same attention as that of other groups of phytoplankton such as diatoms and coccolithophorids.

However, recent studies have hypothesized that cyanobacteria contribute to the oceanic carbon export in proportion to their net primary production, via formation and gravitational sinking of aggregates and/or consumption of those aggregates by

zooplankton (Richardson & Jackson 2007). In fact, *Synechococcus* derived aggregates are estimated to contribute 2-13% of the total POC flux measured by sediment traps at the BATS site (Brew et al., 2009; Lomas & Moran, 2011). Their significant contributions to the total carbon export were also confirmed by DNA-based molecular analyses of sediment trap material at the BATS site (Amacher et al., 2013).

1.3 Objectives of My Study

To explain the observations above, laboratory studies are needed to:

1) Test if *Synechococcus* cells, despite of their small size and lack of natural ballasting minerals, can form sinking aggregates that would be able to sink out of the euphotic zone and reach depths.

2) Investigate the potential role of TEP in such aggregation and the effects of nutrient limitation on the TEP production and aggregate formation of *Synechococcus*.

3) Investigate the effects of various factors, e.g., clay minerals and heterotrophic bacteria, which could potentially alter the aggregate characteristics and the carbon export of aggregation by these pico-cyanobacteria.

I intended to answer these questions in my dissertation in three chapters as follows: in Chapter 1, I investigate the effects of nutrient limitation on cell growth, TEP production and aggregate formation of marine *Synechococcus* in growth experiments; in Chapter 2, I investigate the effects of clay minerals on the aggregation and subsequent settling of marine *Synechococcus* using roller tanks (Shanks & Edmondson, 1989); and in Chapter 3, I further investigate the potential effects of heterotrophic bacteria, together

with clays, on the aggregation of marine *Synechococcus* and *Prochlorococcus* using roller tanks. Considering the facts that 1) pico-cyanobacteria are the dominant contributors to the oceanic new production in oligotrophic regions; 2) their large and fast sinking aggregates and/or consumption of those aggregates by zooplankton are one of the main constituents of the carbon export flux; and 3) these aggregates' characteristics also impact the POC flux attenuation through the water column and eventually the carbon sequestration flux in the deep sea, my study will improve the understanding of the role of marine pico-cyanobacteria like *Synechococcus* and *Prochlorococcus* in the biological carbon pump of oligotrophic oceans.

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CHAPTER 1

Effects of Nutrient Limitation on Cell Growth, TEP Production and Aggregate

Formation of Marine *Synechococcus*

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Abstract. Cyanobacteria are considered to play an important role in the oceanic biological carbon pump, especially in oligotrophic regions. A recent laboratory study using roller tanks showed that marine *Synechococcus* cells, despite of their small size and lack of natural ballasting minerals, can still form aggregates that sink at measureable velocities in seawater. Our objective here is to investigate the mechanism behind such aggregation by studying the potential role of transparent exopolymeric particles (TEP) and the effects of nutrient (nitrogen or phosphorus) limitation on the TEP production and aggregate formation of these pico-cyanobacteria. Our results show that despite of the lowered growth rates, cells in the nutrient limited cultures had larger cell-normalized TEP production, and formed greater total volume of aggregates that resulted in higher settling velocities compared to cells in the nutrient replete cultures. This study contributes to our understanding in the physiology of marine *Synechococcus* as well as their role in the ecology and biogeochemistry in oligotrophic oceans.

Key words. Nutrient limitation, Marine *Synechococcus*, TEP production, Aggregate formation

2.1 Introduction

Many aquatic microorganisms are able to synthesize and secrete large amounts of extracellular polymeric substances (EPS), especially when growth conditions deteriorate. These exopolymer secretions can exist as tight cell capsules or as free loose slimes (Decho, 1990; De Philippis & Vincenzini, 2003) in the colloidal/gel organic carbon pool of the ocean (Verdugo et al., 2004; Thornton et al., 2007). Polysaccharides comprise a main fraction of these exopolymer secretions (Biddanda & Benner, 1997; Pereira et al., 2009) and can sometimes account for more than 50% of total primary production (Baines & Pace, 1991). As a major component of the EPS, acid polysaccharides (APS) have a high molecular mass and a high concentration of covalently bound sulfate and carboxylate groups (Mopper et al., 1995; Thornton et al., 2007). APS are very “sticky”, i.e., tend to bind themselves and other negatively charged units such as bacteria cells and clay particles via cation bridging (Kiørboe & Hansen, 1993; Bhaskar & Bhosle, 2005), and are also more resistant to hydrolysis by bacteria (Aluwihare & Repeta, 1999).

In seawater, APS will form abiotically within hours to days larger colloids and eventually a class of large, discrete, gel-like particles: transparent exopolymer particles (TEP) (Alldredge et al., 1993; Passow, 2000). TEP have been found to provide the main matrix of all marine snow aggregates, and serve as a substrate and microhabitat for attached bacteria (Alldredge et al., 1993; Passow & Alldredge, 1994). Owing to their fractal, surface-reactive nature, TEP support the coagulation processes with other suspended particles such as phytoplankton cells, leading to the formation of large, fast sinking aggregates (Engel, 2000; Engel et al., 2004; Burd & Jackson, 2009). Thus, they

are especially important during phytoplankton blooms in which they facilitate the aggregation and subsequent sedimentation of phytoplankton cells that otherwise would be too small to sink (Passow et al., 2001) and enhance the carbon export to depth.

The formation of TEP from APS presents an important, rapid abiotic pathway for the transformation of dissolved organic carbon into particulate form, other than the conventional microbial uptake/growth (Alldredge et al., 1993; Engel et al., 2004). During phytoplankton blooms, the potential importance of TEP is actually two-fold since in addition to the phytoplankton cells that are involved in the aggregate formation, TEP themselves can also contribute appreciably to the carbon export (Passow et al., 2001; Engel et al., 2004; Deng et al., 2015).

The ultimate source of exo-polysaccharides is microbial secretion, a process regulated by a cell internal imbalance of carbon and nutrient assimilation. Studies of EPS/TEP production have shown that the extracellular release patterns are species-specific and depend on both the physiological state of cells, such as growth phase as well as the environmental conditions, such as nutrient availability (Passow, 2002; Radić et al., 2006). Though EPS/TEP are also produced by actively growing cells, it is widely accepted that their production increases during the late stationary and decline phases of growth (Passow, 2002), when environmental stressors as well as cell senescence, death and lysis become increasingly important processes leading to the accumulation of EPS/TEP (Baldi et al., 1997; Berman-Frank et al., 2007).

Kjørboe et al. (1990) showed that the stickiness of diatom cells increased significantly as cell growth ceased and cells became nutrient limited. The (cell-normalized) TEP production by various phytoplankton groups were observed to increase in the cases of nitrogen, phosphorus, iron and silicon limitation (Corzo et al., 2000; Mari et al., 2005; Berman-Frank et al., 2007). Nutrient limitation can also affect the interaction between heterotrophic bacteria and phytoplankton on TEP production (Gaerdes et al., 2012). Actually cell-normalized TEP production may increase as growth rate decreases, independent of the factors triggering the reduction such as nutrient limitation or self-shading (Passow, 2002). Based on biological stoichiometry, nutrient limitation implies excess carbon fixed in autotrophs. This proportion of carbon that can not be used for cell growth has to be excreted to maintain the autotrophs' elemental homeostasis (Hessen et al., 2004).

The unicellular, coccoid pico-cyanobacteria of the genus *Synechococcus* are important primary producers in oceans from high latitudes to the tropics (Neuer, 1992; Partensky et al., 1999). Deng et al. (2015) showed in a recent laboratory study using roller tanks that marine *Synechococcus* cells, despite of their small size and lack of natural ballasting minerals, can still form aggregates that sink at measureable velocities (>100 m/d) in seawater. Our objective here is to investigate the mechanism behind such aggregation by studying the potential role of TEP and the effects of nutrient (nitrogen or phosphorus) limitation on the TEP production and aggregate formation of these pico-cyanobacteria. Based on theoretical considerations and previous studies' suggestions (Corzo et al., 2000; Marie et al., 2005; Berman-Frank et al., 2007), we formulate the

following three **hypotheses**: 1) TEP play an important role in *Synechococcus* aggregation; 2) when cells become nutrient limited, they have larger cell-normalized TEP production and thus form more aggregates; and 3) these aggregates result in higher settling velocities compared to cells grown in the nutrient replete condition.

2.2 Methods

2.2.1 Experimental setup.

Axenic batch cultures of the marine *Synechococcus sp.* strain WH8102 (CCMP 2370) obtained from the National Center for Marine Algae and Microbiota (NCMA) were grown at 25 °C, and a light intensity of 90-100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in a 14h:10h light-dark cycle, and on rocking platforms to better simulate the natural open water condition. Autoclaved IMR medium (Eppley et al., 1967, Table 1) in artificial seawater (Sigma Seasalt, at 30‰ salinity) was used as the replete growth medium. Reduced nitrogen and phosphorus concentrations that could result in significantly lowered growth rates (Table 2) in the nutrient limiting treatments (Table 1) were determined based on preliminary experiments. Each nutrient treatment was replicated.

We are presenting results of three experiments (EXP. 1, EXP. 2 and EXP. 3), and each experiment was carried out using different batches of cultures (Table 1). EXP. 1 and EXP. 2 had a nitrogen limited treatment, and EXP. 3 had nitrogen and phosphorus limited treatments compared to a nutrient replete treatment. In addition to monitoring the cell growth, aggregate formation and TEP production in each nutrient treatment as in EXP. 2,

we measured the Chl-*a* content and settling velocity in EXP. 3. Settling velocity was also determined in EXP. 1 using different methods (see below).

The liquid medium “f/2_PM” (with bacto-peptone and methylamine-HCL, as suggested by NCMA) was used to regularly test for contamination by heterotrophic bacteria and fungi, and only cultures that had negative results were considered axenic and were used to inoculate experimental treatments. We used 5-10% inoculum into 1L growth medium at the beginning, and started the sampling of 50 mL cultures every two to three days using sterile techniques until their late stationary and decline phases were reached (after ca. 10 days). Settling velocity was determined only at the end of culture experiments.

2.2.2 Cells and aggregates.

Synechococcus cells and aggregates were analyzed after being fixed with glutaraldehyde (1%) and filtered onto polycarbonate filters (black, 0.2 µm pore-size). Using an epifluorescence microscope (EFM, Zeiss Axioscope), cells (and aggregates in EXP. 1) were counted under blue-light excitation to visualize phycoerythrin fluorescence as in Amacher et al. (2009), and the concentration was calculated in number per mL. Aggregates in EXP. 1 were also observed using a scanning electron microscope (SEM, JEOL 6300) to depict aggregate matrices, as well as using a combination of epifluorescence and brightfield microscopy (Logan et al., 1994) to investigate the association between TEP and *Synechococcus* cells.

In EXP. 2 and EXP. 3, aggregates were analyzed using a Multisizer 3 particle counter (Beckman Coulter). Samples in duplicates were diluted to a 1-10% particle concentration with Isoton II (Beckman Coulter) solution and aggregates were sized and quantified with a 100 μm aperture tube. The volume concentration of aggregates was calculated in μm^3 per mL within the size (equivalent spherical diameter) range of 5-60 μm .

2.2.3 TEP and Chlorophyll-*a*.

TEP were determined using a dye-binding assay for the spectrophotometric measurement as in Passow & Alldredge (1995). 10 mL of sample in duplicates were filtered at low, constant vacuum (10 cm of Hg) onto polycarbonate filters (0.4 μm pore-size), and TEP on the filters were quickly stained by filtering 0.5 mL of Alcian Blue (8GX, Sigma) solution (0.02%) at pH 2.5 (adjusted with acetic acid). After being stained, filters were rinsed with distilled water to remove excess dye and were kept frozen until transferred into 5 mL of 80% sulfuric acid and soaked for 2-3 hours (gently agitated 2-3 times). Absorption at 787 nm was measured using a spectrophotometer (Shimadzu UV-1601) against distilled water. We choose to present our TEP as absorption instead of in concentration units as gum xanthan equivalent due to the uncertainty in calibration of different batches of the Alcian Blue dye used.

The chlorophyll-*a* (Chl-*a*) concentration was determined by filtering 10 mL of sample in duplicates onto GF/F filters, which were kept frozen until extraction in 5 mL of 90% acetone for about 24 hours in a refrigerator. Fluorescence was measured using a

fluorometer (Turner Designs TD700) as in Welschmeyer (1994), which was calibrated using Chl-*a* standards (Sigma) in $\mu\text{g L}^{-1}$.

2.2.4 Settling velocity.

The settling velocity was determined using the SETCOL method as described in Bienfang (1981) for cells and aggregates collected on the last day of EXP. 3. Uniformly mixed culture medium in each nutrient treatment was settled in 1 L cylinders (33 cm in height) for 0.5 hour, during which biomass progressively accumulated in the bottom region of the settling column. At the end of the settling period, the bottom 100 mL of the cylinders were sampled for analysis by carefully siphoning out all the media above using a peristaltic pump (Masterflex).

The settling velocity was calculated based on the change of biomass in vertical distribution over the settling time and converted to velocities in centimeters per day (Bienfang, 1981). We measured the changes of both Chl-*a* and aggregate concentration in the settling columns. In EXP. 1, aggregates ($>100 \mu\text{m}$) were analyzed using epifluorescence microscopy, while in EXP. 3 measurements were conducted using the Multisizer for aggregate (5-60 μm) volume concentration.

2.3 Results

2.3.1 *Synechococcus* aggregates and TEP.

Using different microscopic observations we could confirm the aggregation of *Synechococcus* cells in our culture flasks. The size of aggregates observed under the

epifluorescence microscope (EFM) ranged from 3-5 μm to over 100 μm (Fig. 1 A). The scanning electron microscope (SEM) image (Fig. 1 B) depicts an aggregate matrix, which supposedly consists mainly of transparent exopolymer particles (TEP, Alldredge et al., 1993), binding groups of cells together. In addition, aggregates as well as most single cells observed under the epifluorescence microscope (Fig. 1 C) appeared to be stained with Alcian Blue dye in brightfield (Fig. 1 D), illustrating the existence of many TEP or its exopolymer precursors (Passow & Alldredge, 1995; Thornton et al., 2007) as cell coatings. This indicates that most TEP produced by *Synechococcus* attach closely to cells and bind them together as the major component of the aggregate matrix, rather than being free in the medium. This close association between TEP and *Synechococcus* cells was also supported by a centrifugation test (data not shown) showing that in the final supernatant, the TEP concentration correlated to the decreasing cell abundance as the centrifugation time/speed increased, despite of the difference in excess density between TEP and cells.

2.3.2 Cell growth and aggregate formation.

In general, *Synechococcus* cells in the nutrient replete cultures had larger abundance and reached the maximum cell abundance and growth rate a few days later compared to in the nutrient limited cultures (Fig. 2 A, C). We calculated the maximum growth rate during the exponential growth phase in each nutrient treatment (see specific sampling days in Table 2). Significant differences in the maximum growth rates were observed between the nutrient replete and limited cultures in both experiments (T-tests,

Table 2), confirming the growth limiting effect of the reduced nutrient concentrations in our experimental treatments (Table 1).

In general, around 90% of the aggregates analyzed using the Multisizer were in the size range of 5-10 μm , and the nutrient limited cultures had larger total aggregate (5-60 μm) volume concentration compared to the replete cultures, especially over the lag and early exponential growth periods (Fig. 2 B, D). We calculated the total aggregate to cell volume ratio (Table 3), assuming the single cell volume to be $1 \mu\text{m}^3$, when cells grew at the maximum rate in each nutrient treatment (Table 2). The nitrogen limited culture in EXP. 2 (4.9 ± 0.6) and phosphorus limited culture in EXP. 3 (0.68 ± 0.01) had significantly higher (T-tests, Table 3) ratios compared to the nutrient replete cultures (0.72 ± 0.09 in EXP. 2 and 0.38 ± 0.04 in EXP. 3). The nitrogen limited culture in EXP. 3 (11 ± 5) also had a higher (but non-significant) ratio compared to the replete culture (0.38 ± 0.04).

2.3.3 TEP production and Chl-*a* content.

In general, the TEP concentration was higher in the nutrient limited cultures compared to in the replete cultures over the lag and early exponential growth periods (Fig. 3 A, B). On the day when cells grew at the maximum rate (Table 2), the cell-normalized TEP production (in absorption) in the nitrogen limited culture in EXP. 2 (9.0 ± 1.5) and phosphorus limited culture in EXP. 3 (5.5 ± 0.05) were significantly larger (T-tests, Table 3) compared to in the nutrient replete cultures (1.5 ± 0.3 in EXP. 2 and 2.8 ± 0.2 in EXP. 3). The nitrogen limited culture in EXP. 3 (50 ± 20) also had a larger (but

non-significant) cell-normalized TEP production compared to the replete culture (2.8 ± 0.2).

In EXP. 3, we also analyzed the changes of Chl-*a* concentration (Fig. 3 C) which were generally positively associated with the cell abundance. On the day when cells grew at the maximum rate (Table 2), the cell-normalized Chl-*a* content in the phosphorus limited culture (1.0 ± 0.08 fg) was significantly smaller (T-tests, Table 3) compared to in the nutrient replete culture (2.6 ± 0.04 fg). The nitrogen limited culture (2.2 ± 0.2 fg) also had a smaller (but non-significant) cell-normalized Chl-*a* content compared to the replete culture (2.6 ± 0.04 fg).

2.3.4 Settling velocity.

In EXP. 3, we calculated settling velocities based on the changes of both Chl-*a* concentration as well as aggregate volume concentration (5-60 μm , analyzed using the Multisizer) in the settling columns in each nutrient treatment (Fig. 4). As we can expect, settling velocities based on the sinking aggregates were much higher than the Chl-*a* based values (Table 4) that represent the bulk biomass including single cells. In addition, settling velocities in the nitrogen limited culture (52 ± 19 cm/d, Chl-*a* based) and phosphorus limited culture (341 ± 13 cm/d, aggregate based) were significantly higher (T-tests, Table 4) compared to in the nutrient replete culture (0.02 ± 1.92 cm/d, Chl-*a* based and 183 ± 14 cm/d, aggregate based). This indicates again the greater proportion of cells involved in the aggregate formation, in addition to the higher total aggregate to cell volume ratio presented earlier in the nutrient limited treatments.

In EXP. 1, we calculated settling velocities using the epifluorescence microscopic analysis of aggregates larger than 100 μm (Table 4). These aggregates ($>100 \mu\text{m}$) were much less abundant (ca. 10-100/mL) compared to the smaller aggregates (5-60 μm) analyzed using the Multisizer (over 10^4 - 10^5 /mL). Settling velocities of these larger and faster sinking aggregates reached up to 67 ± 9 m/d in the nitrogen limited culture, significantly higher (T-tests, Table 4) than in the nutrient replete culture (5.7 ± 1.8 m/d).

2.4 Discussion

2.4.1 *Synechococcus* aggregation and TEP.

The microscopic observations (Fig. 1) support our first hypothesis that TEP play an important role in *Synechococcus* aggregation, by attaching closely to cells and binding them together as the major component of the aggregate matrix. The existence of many Alcian Blue stainable cell coatings in our *Synechococcus* was also observed in some diatoms like *Nitzschia* (Crocker & Passow, 1995; Passow, 2002). In addition to the free TEP, these TEP coatings could result in a high cell stickiness that may also facilitate the aggregation of our *Synechococcus*. Furthermore, the changes of total aggregate (5-60 μm) volume concentration (Fig. 2 B, D) were positively associated with the TEP concentration (Fig. 3 A, B) of cultures in different nutrient treatments over the growth periods. Specifically on the days when cells grew at the maximum rate (Table 2), strong positive correlations between total aggregate volume concentration and the corresponding TEP concentration (Fig. 5) were observed in both EXP. 2 and EXP. 3.

The Passow & Alldredge (1995) method used for our TEP measurements is semi-quantitative since the Alcian Blue dye binds differently to various acid polysaccharides, and the binding capability also varies in different batches of dye. We therefore used the spectrophotometrical absorption to present our TEP without calibration to concentration as gum xanthan equivalents. The absence of calibration between experiments (batches of dye) would not influence the effects of nutrient limitation we found within each single experiment. However, comparisons of absolute values between experiments would be impacted, e.g., the offset in regression lines of total aggregate volume concentration and the corresponding TEP concentration (Fig. 5) observed between EXP. 2 and EXP. 3.

2.4.2 Effects of nutrient limitation.

In general, despite of the lowered growth rates (Table 2), nutrient limited cultures had larger cell-normalized TEP production (Table 3), formed greater total volume of aggregates (Table 3), and resulted in higher settling velocities (Table 4) compared to replete cultures, supporting our second and third hypotheses. In addition, our *Synechococcus* cultures were more sensitive to nitrogen compared to phosphorus limitation (Fig. 2 c). Most marine *Synechococcus* (including our strain) have N:P ratios above the Redfield ratio (Bertilsson et al., 2003; Heldal et al., 2003). These high N:P ratios could result from the high nitrogen demands of *Synechococcus* to maintain their N-rich photosynthetic protein complex, the phycobilisome (Wyman et al., 1985; Kana et al., 1992). In addition, *Synechococcus* are able to better respond to phosphorus scarcity by lowering the thresholds for (K_s) to maintain relative high uptake rates (V_{max} , “Michaelis–Menten kinetics”, Lomas et al., 2014), lowering their maximum growth rates with

reduced allocation to the P-rich ribosomal RNA (“growth rate hypothesis”, Elser et al., 2003) and using non-phosphorus lipids like sulfolipids (Van Mooy et al., 2009, Mouginot et al., 2015). This low phosphorus tolerance of *Synechococcus* should be clearly an advantage in the oligotrophic oceans compared to other eukaryotic phytoplankton.

It can be noted that the total aggregate volume and TEP concentration in the nutrient replete cultures exceeded those in the nitrogen/phosphorus limited cultures (Fig. 2 b, d, Fig. 3 a, b) after the day when cells grew at the maximum rate (Table 2), mainly due to the much larger number of cells (though cell-normalized values were smaller) in the nutrient replete cultures. When cultures reached the late stationary and decline phases, other important factors such as the senescence, death and lysis of cells could have altered the cellular TEP production (Baldi et al., 1997; Passow, 2002; Berman-Frank et al., 2007) in addition to the nutrient availability. Our method for quantifying cells by epifluorescence microscopy would exclude non-fluorescent or dead cells and underestimate the actual cell numbers in the stationary and decline phases. Thus, when calculating cell-normalized values (Table 3), we focused on the day when cells grew at the maximum rate during the exponential growth phase to enable comparisons of fast growing cells between different nutrient treatments (as in Corzo et al., 2000).

Most of the aggregates (>95 %) formed over the growth periods had sizes within the upper limit (60 μm) of the size spectrum determined by our particle counter, which are usually referred to as “tiny aggregates” or “suspended aggregates” (De La Rocha et al., 2008; Deng et al. 2015). These aggregates have average settling velocities of up to 3.4

± 0.1 m/d in our nutrient limited treatments, which may not be enough for aggregates to sink out of the ocean mixed layer. However, by using roller tanks we found that *Synechococcus* can form visible aggregates that had sizes and sinking velocities (ca. 1.4 mm and 440 m/d, Deng et al., 2015) comparable to marine snow in the ocean (Alldredge & Gotschalk, 1988; Peterson et al., 2005). This indicates that the small, “suspended aggregates” formed in our culture experiments can be further incorporated into larger, visible aggregates via colliding and sticking together with other single cells or aggregates that settle at different velocities (Burd & Jackson, 2009) in the ocean.

2.4.3 Implications for the oligotrophic oceans.

Studies have shown that different phytoplankton groups, including many filamentous cyanobacteria such as *Trichodesmium* (Berman-Frank et al., 2007) and *Anabaena* (Bittar & Vieira, 2010) can generate TEP abundantly. Our study adds important information on the TEP production by marine *Synechococcus*, one of the most abundant and widespread unicellular pico-cyanobacteria (Neuer, 1992; Partensky et al., 1999). Together with recent laboratory experiments on the marine *Synechococcus* aggregation using roller tanks (Deng et al., 2015), the mechanism behind such aggregation and *Synechococcus*'s contribution to the carbon export in the oligotrophic oceans, e.g., at the Bermuda Atlantic Time-series Study (BATS) site (Lomas & Moran, 2011; Amacher et al., 2013), can be elucidated. Despite of their small size and lack of natural ballasting minerals, *Synechococcus* can still form sinking aggregates by producing TEP and thus potentially reach depths below the euphotic zone.

An important conclusion from our study is that a reduction of growth rate will increase the cell-normalized TEP production and aggregation of *Synechococcus*, which is consistent with many previous studies on various phytoplankton groups (Table 5). This growth rate reduction can result from 1) the reduced nutrients (or altered ratios) in the starting growth medium as in our case, Corzo et al. (2000), and Mari et al. (2005); 2) the natural aging of cultures with nutrient depletion or self-shading (Kiørboe et al., 1990; Passow, 2002); and 3) other types of environmental stressors such as high irradiance and oxidative state (Berman-Frank et al., 2007). As Passow (2002) pointed out, cell-normalized TEP production may increase as growth rate decreases, independent of the factors triggering the reduction. Cell abundance of *Synechococcus* in the field (e.g., ca. 10^4 cells/mL in the spring mixed layer at the BATS site, DuRand et al., 2001) is much lower compared to in laboratory cultures (ca. 10^7 cells/mL in the replete growth medium). However, our results imply that *Synechococcus* in a nutrient limited environment, representative of the vast oligotrophic ocean gyres, would have a greater aggregation potential as they have a higher cell-normalized TEP production than the nutrient replete cells.

Aggregation will also result in different ecological consequences for phytoplankton themselves. As an example, aggregation increases the functional size of pico-cyanobacteria, making them better avoid microzooplankton grazing but become more susceptible to grazing by larger zooplankton (Pernthaler, 2005). *Synechococcus* are observed abundantly in mesozooplankton guts and fecal pellets (Wilson & Steinberg, 2010). In addition, the enhanced TEP production and aggregation can benefit

phytoplankton in environments that result in sub-optimal growth conditions. For instance, Koblížek et al. (2000) proposed that cell aggregation represents a fast adaptive reaction to excess light by enhancing packaging and self-shading. The enhanced TEP production and aggregation are also associated with the limitation by nutrients, e.g., at the end of phytoplankton blooms. Via the formation and settling of aggregates, cells that are nutrient limited will be able to reach deeper depths with higher nutrient availability.

2.5 References

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2.6 Tables and Figures

Table 1

Nutrient concentrations and parameters measured in each experiment.

Nutrient Treatment	Nutrient Concentration			Parameters
	N (μ M)	P (μ M)	N:P	
EXP. 1	Replete IMR	500	35	14
	N-limitation	5	35	0.14
Aggregate observation, Settling velocity				
EXP. 2	Replete IMR	500	35	14
	N-limitation	5	35	0.14
Cell growth rate, Aggregate volume conc., TEP production				
EXP. 3	Replete IMR	500	35	14
	N-limitation	5	35	0.14
	P-limitation	500	0.35	1400
Cell growth rate, Aggregate volume conc., TEP production, Chl- α content, Settling velocity				

Table 2

Maximum cell abundance, maximum growth rate, and results (P values) of T-tests (df=1, one-tailed) comparing the nutrient replete with limited cultures on growth rate in EXP. 2 and EXP. 3. Significant results ($\alpha=0.05$) are shown in bold.

	Max. Cell Abundance ($\times 10^6$ cells/mL)			Max. Growth Rate (d)		
	Value	Time	Value	Value	Time	P (vs. Replete)
EXP. 2	Replete	51 \pm 9	Day 7	0.59 \pm 0.06	Day 3-5	N/A
	N-limited	9.7 \pm 0.8	Day 7	0.32 \pm 0.06	Day 1-3	0.05
EXP. 3	Replete	95*	Day 11	0.70 \pm 0.01	Day 4-6	N/A
	N-limited	4.0 \pm 0.1	Day 8	0.09 \pm 0.02	Day 1-4	<0.005
	P-limited	45 \pm 2	Day 11	0.38 \pm 0.08	Day 4-6	<0.005

*lack of replication due to loss of one replicate on Day 8

Table 3

Total aggregate (Agg.) to cell volume ratio, cell-normalized TEP production and Chl-a content of cultures in different nutrient treatments (on the day when cells grew at the maximum rate, Table 2). P values give results of T-tests ($df=1$, one-tailed) for comparisons between treatments in EXP. 2 and EXP. 3. Significant results ($\alpha=0.05$) are shown in bold.

	Total Agg. Vol. / Total Cell		TEP (absorption $\times 10^{-19}$) / Cell		Chl-a (fg) / Cell	
	Value	P (vs. Replete)	Value	P (vs. Replete)	Value	P (vs. Replete)
EXP. 2	Replete (Day 5)	0.72 \pm 0.09	N/A	1.5 \pm 0.3	N/A	
	N-limited (Day 3)	4.9 \pm 0.6	0.01	9.0 \pm 1.5	0.02	
EXP. 3	Replete (Day 6)	0.38 \pm 0.04	N/A	2.8 \pm 0.2	N/A	2.6 \pm 0.04
	N-limited (Day 4)	11 \pm 5	0.14	50 \pm 20	0.12	2.2 \pm 0.2
	P-limited (Day 6)	0.68 \pm 0.01	0.01	5.5 \pm 0.05	<0.005	1.0 \pm 0.08

Table 4

Settling velocities calculated based on the changes of Chl-*a* and aggregate (Agg.) concentrations in the settling columns, on the last day in different nutrient treatments. *P* values give results of *T*-tests (*df*=1, one-tailed) for comparisons between treatments in EXP. 1 and EXP. 3. Significant results ($\alpha=0.05$) are shown in bold. MS: Multisizer analysis for aggregate volume concentration, EFM: Epifluorescence microscopic analysis for aggregate number concentration.

Settling	EXP. 1 (Agg. >100 μm)				EXP. 3 (Agg. 5-60 μm)			
Velocity (cm/d)	Chl- <i>a</i>	P (vs. Replete)	Agg. (EFM)	P (vs. Replete)	Chl- <i>a</i>	P (vs. Replete)	Agg. (MS)	P (vs. Replete)
Replete	8.3 ± 0.3	N/A	568 ± 183	N/A	0.02 ± 1.92	N/A	183 ± 14	N/A
N-limited	54 ± 8	0.05	6748 ± 887	0.01	52 ± 19	0.05	234 ± 86	0.30
P-limited					18 ± 11	0.12	341 ± 13	0.007

Table 5

Summary of studies investigating the effects of different limiting factors on the TEP production of various phytoplankton groups. (+) positive, (-) negative and (?) ambiguous effect.

	Limiting Factor	Parameter & Effect	Reference
<i>Thalassiosira pseudonana</i> (diatom)	Culture Aging	Cell Stickiness (+)	Kiorboe et al. 1990
<i>Chaetoceros calcitrans</i> (diatom)	Nitrate	Cell-normalized TEP production (+)	Corzo et al. 2000
<i>Chaetoceros affinis</i> (diatom)	Phosphate and Silicic Acid	Cell-normalized TEP production (?)	Passow 2002
<i>Phaeocystis globosa</i> (prymnesiophyte)	Nitrate to Phosphate Ratio	TEP concentration (+)	Mari et al. 2005
<i>Trichodesmium</i> (cyanobacteria)	Iron and Phosphate	TEP concentration (+)	Berman-Frank et al. 2007
<i>Synechococcus</i> (cyanobacteria)	Nitrate and Phosphate	Cell-normalized TEP production (+)	This Study

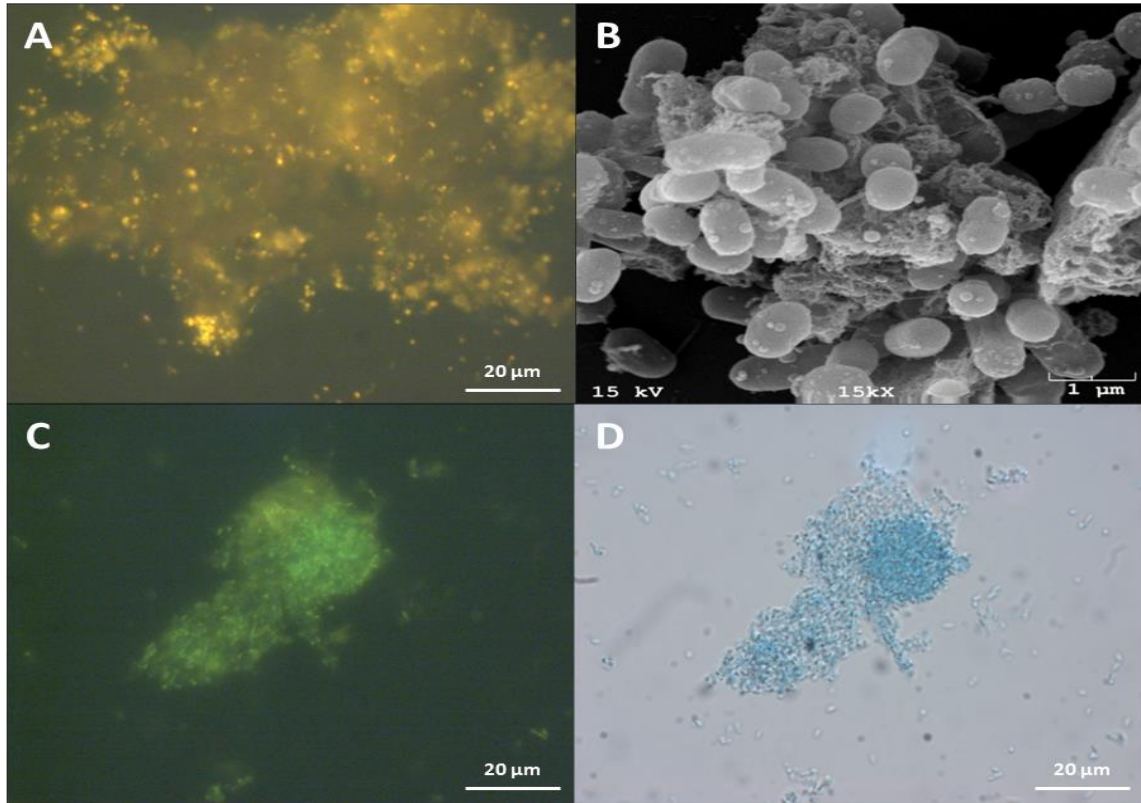


Figure 1. Epifluorescence microscope (EFM) image of a *Synechococcus* aggregate (nutrient replete treatment) under blue-light excitation (A); Scanning electron microscope (SEM) image of cells attached to an aggregate matrix (B); EFM image (C) and its corresponding brightfield image (D) of an Alcian Blue stained aggregate.

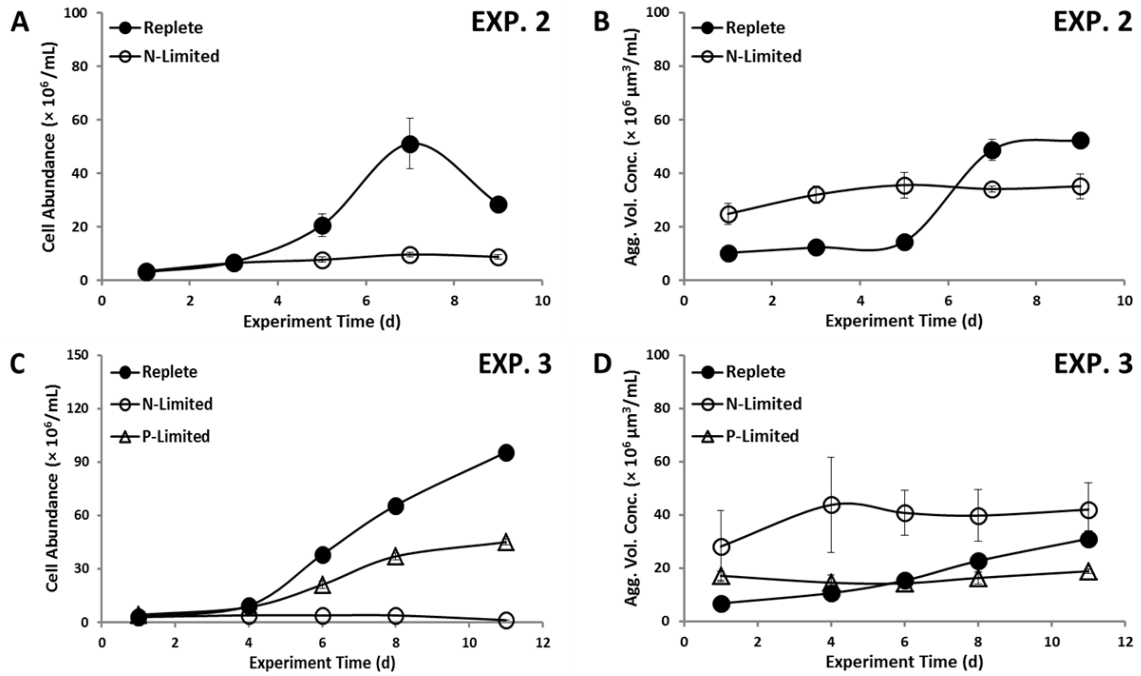


Figure 2. Growth of *Synechococcus* cells (A, C) and changes of aggregate (5-60 μm) volume concentration (B, D) in the nutrient replete (black circles), nitrogen limited (open circles) and phosphorus limited (open triangles) cultures in EXP. 2 and EXP. 3.

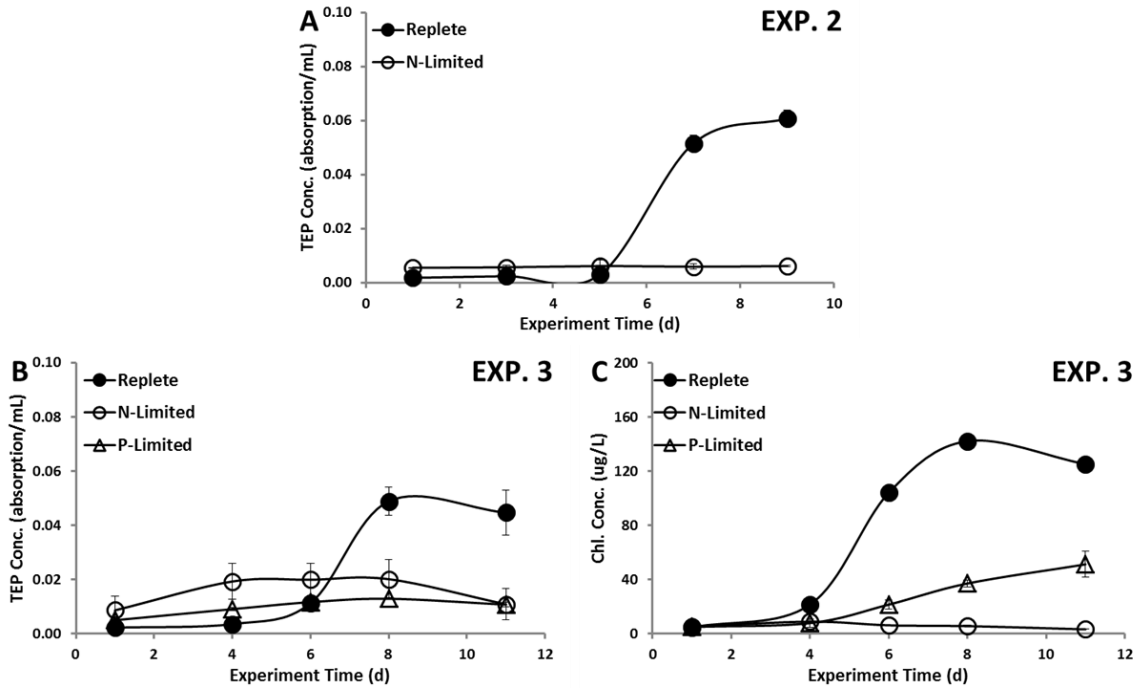


Figure 3. Changes of TEP (A, B) and Chl-a (C) concentration in the nutrient replete (black circles), nitrogen limited (open circles) and phosphorus limited (open triangles) cultures in EXP. 2 and EXP. 3.

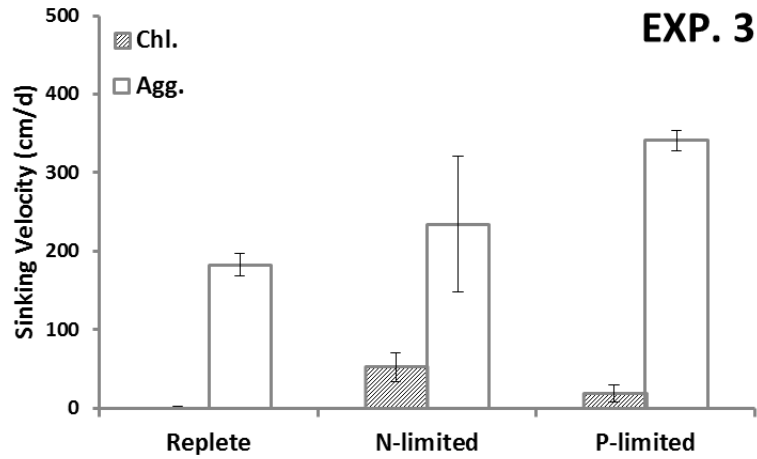


Figure 4. Settling velocities calculated based on the changes of Chl-*a* (shaded bars) and aggregate volume (5-60 μm , open bars) concentrations in the settling columns, on the last day in each nutrient treatment in EXP. 3.

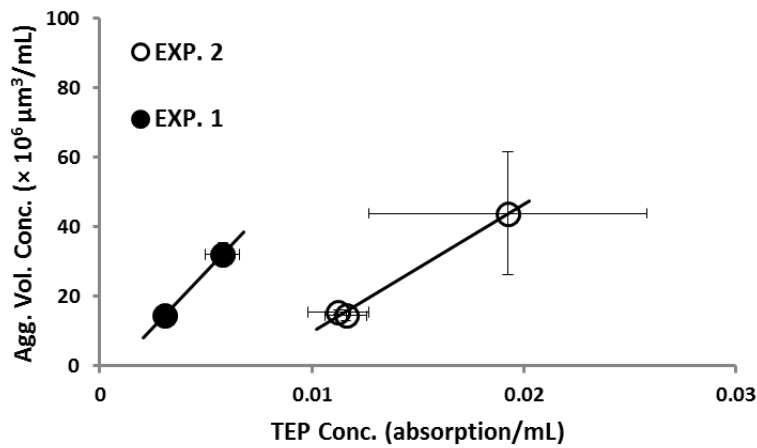


Figure 5. Correlations between total aggregate (5-60 μm) volume concentration and the corresponding TEP concentration of cultures in different nutrient treatments, on the day when cells grew at the maximum rate (Table 2) in EXP. 2 and EXP. 3.

CHAPTER 2

Effects of Clay Minerals on the Aggregation and Subsequent Settling of Marine

Synechococcus

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Abstract. Cyanobacteria are considered to play an important role in the oceanic biological carbon pump, especially in oligotrophic regions. But since single cells are too small to sink, their carbon export has to be mediated by aggregate formation and possible consumption by zooplankton producing sinking fecal pellets. Here we study the aggregation and subsequent settling of the ubiquitous marine pico-cyanobacterium *Synechococcus* as a model organism in roller tanks and investigate the effects of the clays kaolinite and bentonite in a series of concentrations. We found that (1) *Synechococcus* cells formed aggregates as large as 1.4 mm in diameter that sank at velocities of up to 440 and 660 m d⁻¹ with and without ballasting clays, respectively; (2) clay minerals increased the number and reduced the size of aggregates, and their ballasting effects increased the excess density and sinking velocity of aggregates; (3) kaolinite, with its lower cation exchange capacity, resulted in a greater number of smaller and slower sinking aggregates compared to bentonite; and (4) based on our experimental conditions, *Synechococcus* could potentially export carbon 2-3 times of that contained in their cells via aggregation, likely due to the scavenging of transparent exopolymer particles and dissolved organic matter. The presented results have relevance for our understanding of the role of cyanobacteria in the ecology and biogeochemistry in today's ocean and also elucidate mechanisms of carbon export in the early Proterozoic ocean.

Key words. Clay minerals, Marine *Synechococcus*, Aggregate and settling, Roller tanks

3.1 Introduction

The biological carbon pump describes the photosynthetic fixation of CO₂ to dissolved and particulate organic carbon (POC) by phytoplankton and subsequent export to the ocean's interior (Falkowski et al., 2000). POC sinks gravitationally to the deep sea at different velocities, mediating vertical mass fluxes and driving elemental cycling on a range of time scales (Neuer et al., 2014). Sinking particles, some of which can be macroscopic (>0.5 mm in diameter, also referred to as “marine snow”), are mainly aggregates of living organisms, detritus, fecal and inorganic matter (Alldredge et al., 1993).

Phytoplankton cells used to be considered the main constituents of oceanic POC. However, recent studies have focused on the formation of exo-polysaccharide particles as another important source of POC (Engel et al., 2004). These exo-polysaccharide particles are also referred to as “transparent exopolymer particles” (TEP), which can further facilitate the aggregation and subsequent settling of small cells during phytoplankton blooms (Alldredge et al., 1993; Passow et al., 2001). Through this pathway, large and rapidly sinking aggregates can also form from small particles (including single cells) via colliding and sticking together (Burd & Jackson, 2009).

Armstrong et al. (2002) and Klaas & Archer (2002) proposed in their “ballast hypothesis” that oceanic POC is quantitatively associated with suspended minerals (silicates and carbonates). The ballasting mineral particles are thought to increase the density of POC and protect it from microbial degradation, and thus influence the POC

transport through the water column. In a contradictory view, sinking POC scavenges suspended mineral particles until their carrying capacity is reached (Passow, 2004; Passow & De La Rocha, 2006). In either scenario suspended minerals will influence the oceanic POC flux, and their role in the aggregation and subsequent settling of phytoplankton still remains an active area of research.

Suspended minerals have long been known to enhance the flocculation and sedimentation of phytoplankton aggregates in situ (Avnimelech et al., 1982; Søballe & Threlkeld, 1988). Also in laboratory studies, the aggregation of diatoms and their interaction with suspended minerals have been investigated using roller tanks, as in Shanks & Edmondson (1989). Passow & De La Rocha (2006) and De La Rocha et al. (2008) showed in their roller tanks experiments that as the concentration of suspended minerals increased, the POC to dry weight (DW) ratio of diatom aggregates decreased to 2-5% before leveling off; also aggregates decreased in size and porosity and eventually fragmented into a much greater number of smaller and denser ones. The addition of suspended minerals accelerated the formation of large aggregates, while their effects on the sinking velocity of those aggregates were ambiguous and also varied with the type of suspended minerals used (Hamm, 2002; Passow & De La Rocha, 2006). Since size and porosity have opposite effects on aggregate sinking velocity, the relationship between aggregate POC to mineral ratio and sinking velocity is not straightforward.

Lithogenic clay minerals can derive from various sources, including dust deposition, volcanic or hydrothermal venting and continental weathering, etc. The two

clay minerals studied here, kaolinite and bentonite, have been commonly used and compared when investigating clay effects on phytoplankton aggregation (Hamm, 2002; Satterberg et al., 2003; Verspagen et al., 2006). Bentonite is commonly of volcanic origin and kaolinite is an important continental weathering product. These two clay minerals differ in their effective surface area as well as cation exchange capacity (CEC), and both are lower for kaolinite compared to bentonite (Van Olphen, 1977). CEC is considered to affect the “aggregation efficiency” of clay minerals with phytoplankton (Hamm, 2002; Satterberg et al., 2003; Verspagen et al., 2006), and kaolinite has been shown to be much less efficient, i.e., a greater amount of kaolinite particles would be required for the same effect of increased phytoplankton aggregation.

Cyanobacteria are a widespread group of photoautotrophic microorganisms that play an important role in marine ecosystems, especially in oligotrophic regions. The unicellular, coccoid pico-cyanobacteria of the genus *Synechococcus* are important primary producers in oceans from tropical to high latitudes (Neuer, 1992; Partensky et al., 1999). For instance, at the Bermuda Atlantic Time-series Study (BATS) site located in the Sargasso Sea (western North Atlantic Subtropical Gyre), *Synechococcus* represent around one-third of the autotrophic POC biomass (DuRand et al., 2001), but have traditionally not been considered as important contributors to the oceanic carbon export due to their small size, lack of natural ballasting minerals and tightly coupled micrograzer control (Worden & Binder, 2003). Thus, their aggregation and subsequent settling have not received the same attention as that of other groups of phytoplankton such as diatoms and coccolithophorids.

However, recent studies have hypothesized that cyanobacteria contribute to the oceanic carbon export in proportion to their net primary production, via formation and gravitational sinking of aggregates and/or consumption of those aggregates by zooplankton (Richardson & Jackson, 2007). In fact, *Synechococcus* derived aggregates are estimated to contribute 2-13% of the total POC flux measured by sediment traps at the BATS site (Brew et al., 2009; Lomas & Moran, 2011). Their significant contributions to the total carbon export were also confirmed by DNA-based molecular analyses of sediment trap material at the BATS site (Amacher et al., 2013). To explain the latter observations, laboratory studies will need to be conducted to test the mechanism that *Synechococcus* cells, despite of their small size and lack of natural ballasting minerals, can still form aggregates and sink at measureable velocities in the ocean.

Our objective is to study the aggregation and subsequent settling of the ubiquitous marine pico-cyanobacterium *Synechococcus* as a model organism in roller tanks and to investigate the effects of the clays kaolinite and bentonite in a series of concentrations. We will address the following questions: 1) whether aggregation is a mechanism to explain how marine *Synechococcus* reach depths far below the euphotic zone; 2) whether aggregate characteristics of *Synechococcus* cells are different from those of larger, eukaryotic cells and can be affected differently by clay minerals; and 3) whether their carbon export potential will be enhanced in the presence of ballasting clays. This study is aimed to improve our understanding of the role of cyanobacteria in the ecology and

biogeochemistry in today's ocean as well as to elucidate mechanisms of carbon export in the early Proterozoic ocean.

3.2 Methods

3.2.1 *Synechococcus* culturing.

Axenic batch cultures of the marine *Synechococcus* sp. strain WH8102 (CCMP 2370) from the National Center for Marine Algae and Microbiota (NCMA) were grown in flasks on rocking platforms. The cultures were kept in a reach-in chamber (Conviron), at 25 °C, and a light intensity of 90-100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in a 14h:10h light-dark cycle for a growth period of 9 days, when late exponential growth phase was reached. Autoclaved IMR medium (Eppley et al., 1967, 500 μM nitrogen and 35 μM phosphorus) in artificial seawater (Sigma Seasalt, at 30‰ salinity) was used as the growth and experimental medium throughout the experiments.

We used the liquid medium “f/2-PM” (with bacto-peptone and methylamine-HCL) as suggested by NCMA to regularly check for contamination by heterotrophic bacteria and fungi, and only cultures confirmed to be axenic were used for experiments.

3.2.2 Aggregate formation.

Synechococcus cultures were diluted with autoclaved medium to ca. 10^6 cells mL^{-1} and incubated in transparent, cylindrical (1.25 L) roller tanks (Shanks & Edmondson, 1989). In addition to a control treatment, the clays kaolinite and bentonite (Sigma, analytical), dissolved and autoclaved in seawater in a series of concentrations (0.5, 5 and

50 mg L⁻¹), were added to the cell suspensions. In each clay experiment, eight duplicating roller tanks in total were rotated on a rolling platform at around 3.5 rotations per minute (rpm), at room temperature (ca. 25 °C) and in darkness for one week.

The size distributions of the clay particles in the kaolinite and bentonite stock solutions (50 mg L⁻¹) were analyzed using a Multisizer 3 particle counter (Beckman Coulter). Both clay particles were smaller than 30 µm in equivalent spherical diameter (ESD), and their size distributions were similar with the median diameter of 2.90 µm (kaolinite) and 2.87 µm (bentonite), respectively (Fig. 6). It should be noted that the asymmetry of the peaks and the lower limits of the size distributions reflected the detection limit of the particle counter but not the absence of particles smaller than 2 µm.

3.2.3 Aggregate count, size and sinking velocity.

When the roller tanks were taken down, the number of visible aggregates (>0.1 mm) formed in each roller tank was counted, and their sizes (diameters of five aggregates) were measured using a dissecting microscope (Zeiss Stereoscope) with a calibrated ocular micrometer. Visible aggregates were then gently transferred with a wide-bore syringe pipette into a 1 L settling cylinder (released at 1 cm under the air-water interface) to determine their sinking velocities. The settling cylinder was filled with artificial seawater at the same salinity, and allowed to stabilize at room temperature before measurements. The sinking velocities of 10 aggregates from each roller tank were measured with a stopwatch through a vertical distance of 32.6 cm in the settling cylinder and converted to velocities in meters per day.

3.2.4 Aggregate excess density.

The aggregate excess density ($\Delta\rho$) was determined using the Navier-Stokes drag equation (1) as in Iversen & Ploug (2010):

$$\Delta\rho = \frac{C_D \rho_w v^2}{\frac{4}{3} g D} \quad (1)$$

C_D is the dimensionless drag force defined in Eq. 2 for a Reynolds number (Re , defined as in Eq. 3) >1 , ρ_w is the density of seawater (1.0196 g cm^{-3} , at $25 \text{ }^\circ\text{C}$ and 30‰ salinity), v is the measured sinking velocity in cm s^{-1} , g is the gravitational acceleration of 981 cm s^{-2} , and D is the measured diameter in cm.

$$C_D = \left(\frac{24}{Re} \right) + \left(\frac{6}{1 + Re^{0.5}} \right) + 0.4 \quad (2)$$

$$Re = \frac{v D \rho_w}{\mu_w} \quad (3)$$

μ_w is the dynamic viscosity of seawater ($0.9565 \times 10^{-2} \text{ g cm}^{-1} \text{ s}^{-1}$, at $25 \text{ }^\circ\text{C}$ and 30‰ salinity).

3.2.5 Aggregate dry weight and particulate organic carbon content.

The aggregate dry weight (DW) was determined by filtering 10-40 aggregates from each roller tank onto pre-weighed and pre-combusted GF/F filters in four replicates. Aggregates were broken up and washed with Milli-Q water to remove salt in pore-water before filtered, and dried in a desiccator at $55 \text{ }^\circ\text{C}$ for 2-3 days before weighted again on a balance (Mettler Toledo XS105). The aggregate particulate organic carbon (POC) content was further determined using two of the same GF/F filters. Samples were packed into tin-foils and analyzed in a CHN Elemental Analyzer (Perkin Elmer 2400).

3.2.6 Suspended aggregates and single cells.

Suspended aggregates in the medium were characterized and quantified before and after incubation using a Multisizer 3 particle counter (Beckman Coulter). Samples in duplicates were diluted to a 1-10% particle concentration with Isoton II (Beckman Coulter) solution and aggregates were analyzed with a 100 μm aperture tube. The volume concentration of aggregates was calculated in μm^3 per mL within the equivalent spherical diameter (ESD) range of 5-60 μm (we did not observe any aggregate larger than 60 μm in the background medium).

Single cells in the medium were analyzed before and after incubation using an epi-fluorescence microscope (Zeiss Axioscope). Samples fixed with glutaraldehyde (1%) were filtered onto polycarbonate filters (black, 0.2 μm pore-size). *Synechococcus* cells were counted under blue excitation to visualize phycoerythrin fluorescence as in Amacher et al. (2009), and the concentration was calculated in cells per mL.

3.2.7 Chlorophyll-*a*.

Chlorophyll-*a* (Chl-*a*) content in single cells and suspended aggregates in the medium was analyzed before and after incubation. It was determined by filtering 10 mL sample in duplicates onto GF/F filters, which were kept frozen until extraction in 5 mL 90% acetone at 4 °C for 24 hours. The fluorescence was measured using a fluorometer (Turner Designs TD700) as in Welschmeyer (1994) and the concentration was calibrated in $\mu\text{g L}^{-1}$.

3.3 Results

3.3.1 Aggregate formation.

In both experiments aggregate formation started within the first 24 hours of incubation, and the formation rate was generally accelerated with the increasing concentration of clay treatments. After the week-long incubation, the medium in roller tanks had become much clearer and lighter colored, indicating that single cells and clay particles had been scavenged and incorporated into the visible aggregates that had formed over time.

Synechococcus cells in the control treatment formed aggregates as large as 1.4 mm in diameter (Fig. 7). In the clay treatments, the number of aggregates formed was greater and their sizes were smaller with the increase of clay concentration in general (Fig. 7). In the ANOVA tests (Table 6, followed by the Tukey multiple comparisons of means, at 95% confidence level in all statistical tests), aggregates had significantly larger sizes in the control compared to the clay treatments in the bentonite experiment (Fig. 7). We also compared the effect of two clay types using paired T-tests (Table 6), and observed that more aggregates (non-significant) formed in the kaolinite treatment that had significantly smaller sizes compared to those formed in the bentonite treatment (Fig. 7).

3.3.2 Aggregate sinking velocity and excess density.

Synechococcus aggregates in the control treatment sank at velocities of up to 440 m d⁻¹ (Fig. 8). In the clay treatments, both aggregate sinking velocity and excess density

generally increased along with the clay concentration (Fig. 8), despite of their smaller sizes. Aggregates had significantly higher sinking velocities and excess densities in the highest, 50 mg L⁻¹ clay treatment compared to the control and lower concentration clay treatments in both clay experiments (ANOVA tests in Table 6, Fig. 8). When comparing the effect of two clay types using paired T-tests (Table 6), aggregates formed in the kaolinite treatment had significantly slower sinking velocities but higher (non-significant) excess densities compared to those formed in the bentonite treatment (Fig. 8).

3.3.3 Aggregate particulate organic carbon to dry weight ratio.

The aggregate particulate organic carbon (POC) to dry weight (DW) ratio generally decreased with the increasing concentration of clay treatments, saturating at 3-5% (Fig. 9). Aggregates had significantly lower POC to DW ratios in the 5 mg L⁻¹ and 50 mg L⁻¹ clay treatments compared to the control in the bentonite experiment (ANOVA tests in Table 6, Fig. 9). When comparing the effect of two clay types using paired T-tests (Table 6), no significant difference was observed in the aggregate POC to DW ratio between the two clay treatments.

3.3.4 Suspended aggregate and single cell concentration.

In the medium after incubation, the volume concentration of suspended aggregates (5-60 µm) increased while the single cell concentration decreased with the increasing concentration of clay treatments in general (Fig. 10). Single cell concentration was significantly higher in the control compared to the clay treatments in the kaolinite experiment (ANOVA tests in Table 6, Fig. 10). When comparing the effect of two clay

types using paired T-tests (Table 6), no significant difference was observed in the suspended aggregate and single cell concentration between the two clay treatments.

3.3.5 Chl-*a* concentration.

In the medium after incubation, the final Chl-*a* concentration generally decreased with the increasing concentration of clay treatments (Fig. 11), though not significantly different in the ANOVA tests (Table 6). When comparing the effect of two clay types using paired T-tests (Table 6), the Chl-*a* concentration in the kaolinite treatment was significantly lower compared to that in the bentonite treatment (Fig. 11).

3.4 Discussion

3.4.1 Aggregate formation of marine *Synechococcus*.

We have experimentally investigated, to our knowledge for the first time, the aggregate formation and characteristics of the marine pico-cyanobacterium *Synechococcus* (sp. strain WH8102). Under conditions simulated in our roller tanks, *Synechococcus* cells in the control treatment formed aggregates as large as 1.4 mm in diameter (Fig. 7), more than 1000 times the size of a single cell (ca. 1 μm). In addition, their aggregates sank at velocities of up to 440 m d^{-1} without any ballasting clays (Fig. 8), comparable to diatom aggregates studied in roller tanks (Hamm, 2002; Iversen & Ploug, 2010, Table 7), as well as to marine snow in the ocean (Alldredge & Gotschalk, 1988; Peterson et al., 2005). These results on the formation and gravitational sinking of aggregates furthermore point to a mechanism why marine *Synechococcus* have been

found at depths below the euphotic zone (Lomas & Moran, 2011) as well as in shallow particle traps (Amacher et al., 2013) at the BATS site.

3.4.2 Effects of clay addition on aggregation.

Clay minerals have a high affinity to other negatively charged units such as bacteria or polysaccharide particles for coagulation via cation bridging (Verspagen et al., 2006; De La Rocha et al., 2008). As the clay concentration increased in our clay treatments, clay minerals increased the number and reduced the size of aggregates (Fig. 7). These results are consistent with studies of diatom aggregation with different concentrations of suspended minerals (Hamm, 2002; Passow & De La Rocha, 2006; De La Rocha et al., 2008, Table 7). In addition, aggregates showed a steady increase in sinking velocity over the whole range of clay concentrations in both clay experiments (Fig. 8). This contradicts the observation in Hamm (2002) who found an ambiguous relationship between mineral concentration and diatom aggregate sinking velocity, and indicates that the ballasting effect of clay minerals exceeded the negative effect of smaller sizes in *Synechococcus* aggregation. It has to be shown in further studies if our results for these similarities and differences compared to larger, eukaryotic aggregation also apply to other pico-phytoplankton groups.

Compared to the “fluffy” aggregates formed in the control treatment, aggregates had both higher compactness (indicated by the decrease of aggregate size and porosity, Fig. 7) and higher solid hydrated density (indicated by the decrease of aggregate POC to DW ratio, Fig. 9) as the clay concentration increased. The combination of these two

effects resulted in the higher “excess density” of aggregates (as derived from aggregate size and sinking velocity, Eq. 1). Aggregate POC to DW ratio decreased and saturated at 3-5% in the 5 mg L⁻¹ or 50 mg L⁻¹ clay treatments (Fig. 9), similar to the values observed in the studies of diatom aggregates (Passow & De La Rocha, 2006; De La Rocha et al., 2008, Table 7) and sinking particles collected in the deep sea (Armstrong et al., 2002).

Also in the medium after incubation and aggregation, there were fewer single cells and more suspended aggregates (5-60 µm) as the clay concentration increased (Fig. 10), consistent with what De La Rocha et al. (2008) described as “tiny aggregates”. This indicates that the clay particles may have enhanced the scavenging of single cells and their incorporation into suspended aggregates, and further into larger, visible aggregates. Passow et al. (2014) showed that the clay particles could act as more efficient coagulators compared to TEP in driving aggregate formation and determining aggregate characteristics.

Studies investigating the “aggregation efficiency” of different clay minerals with phytoplankton (Hamm, 2002; Satterberg et al., 2003; Verspagen et al., 2006) found that kaolinite was much less efficient, which was explained by its lower cation exchange capacity (CEC, 30-150 meq kg⁻¹) compared to bentonite (700-1000 meq kg⁻¹, Van Olphen, 1977). In our study, kaolinite resulted in significantly smaller (Fig. 7) and slower sinking (Fig. 8, Table 6, consistent with Hamm, 2002) aggregates compared to bentonite. However, the final Chl-*a* concentration in the kaolinite treatment was significantly lower (Fig. 11, Table 6, consistent with Verspagen et al., 2006) compared to bentonite,

indicating that in total there was a greater amount of *Synechococcus* cells and kaolinite particles incorporated into aggregates. This is confirmed by the higher total organic and inorganic material (see below) in all visible aggregates formed in the kaolinite treatment (Fig. 12), and can be explained by the greater surface to volume ratio of the smaller aggregates, and thus enhanced aggregation in the kaolinite treatment.

It has to be noted that there were some discrepancies in most results of the control treatments between the two clay experiments. This could be due to a difference in the initial cell concentration (0.58×10^6 cells mL⁻¹ in the kaolinite and 0.62×10^6 cells mL⁻¹ in the bentonite experiment) as well as possible physiological state of the cultures before incubation, i.e., one could have been more advanced and closer to stationary phase. However, this does not affect the general principles and conclusions on the effects of clay minerals on aggregate formation and characteristics found in our study. For instance, though the aggregate size in the control treatment of the kaolinite experiment was much smaller than that in the bentonite experiment, it will still illustrate a recognizable decreasing trend of size in the bentonite experiment after replacing the data of bentonite control with that of kaolinite control (Fig. 7). In addition, some of the variability observed between replicating roller tanks limited our ability to get significant differences in the effect of increasing clay concentrations.

3.4.3 Biomass aggregation fraction and carbon export potential.

Compared to the visible (>0.1 mm), fast sinking aggregates (up to 660 m d⁻¹), single cells of *Synechococcus* are too small to sink and their tiny aggregates (5-60 µm)

have sinking rates of only a few meters per day (unpublished data). We refer to the percentage of the initial biomass being incorporated into visible aggregates (instead of remaining in single cells and suspended aggregates) at the end of aggregation as the “biomass aggregation fraction”. A larger biomass aggregation fraction would increase the POC contained in aggregates to potentially sink out of the euphotic zone; we call this then the “carbon export potential”.

The decrease in the final Chl-*a* concentration (contained in single cells and suspended aggregates in the medium) as the clay concentration increased (Fig. 11) indicates that the clay minerals enhanced the biomass aggregation fraction, up to 95% in the highest, 50 mg L⁻¹ clay treatment (Fig. 13). We carried out a control experiment using a *Synechococcus* culture grown in a flask without rotation that showed that the Chl-*a* concentration did not decrease after a week-long incubation even when kept in darkness (data not shown). Thus, the biomass aggregation fraction calculated above is likely not overestimated due to natural cell senescence and death over the incubation period.

We calculated the ratio of total aggregate POC (POC content in all visible aggregates formed in each roller tank) to that contained in the initial cells to estimate the carbon export potential (Fig. 13). The per cell POC content obtained from our *Synechococcus* cultures that were harvested from the exponential growth phase with replete nutrients was found to be 875 ± 64 fg C cell⁻¹, around three times the cellular carbon content found in oligotrophic ocean regions like the BATS site (ca. 300 fg C cell⁻¹, Lomas & Moran, 2011). Thus, the total POC content in initial cells in each roller tank

was around 0.66 mg. Given the range of total aggregate POC from the CHN analysis (1.3-1.8 mg), *Synechococcus* could potentially export carbon almost 2 and 3 times (average for the bentonite and kaolinite treatment, respectively) of that contained in their initial cells via aggregation (Fig. 13). This could be explained by other carbon sources including the incorporation of suspended aggregates and transparent exopolymer particles (TEP, Alldredge et al., 1993; Passow & Alldredge, 1995), as well as the transformation of dissolved organic matter (DOM) in the initial medium into particulate forms (De La Rocha et al., 2008). DOM analysis carried out on the medium of our *Synechococcus* cultures that were harvested from the exponential growth phase showed that, in one roller tank, TEP and total DOM can each contain around 0.1 mg and 1 mg of carbon, which is 15% and 1.5 times of the total POC content in initial cells.

We also calculated the total particulate organic matter (POM, by doubling POC as in Neuer et al., 2004) and particulate inorganic matter (PIM, as DW minus POM) contained in all visible aggregates formed in each roller tank. Both the total aggregate POM and PIM showed a general increase in the clay treatments (Fig. 12), which again indicates that the clay minerals, especially kaolinite, enhanced the carbon export potential via aggregation (Fig. 13). It should also be noted that the total aggregate PIM often exceeded the initial amount of clay particles added, especially in the control and low concentration clay treatments (Fig. 12). Though marine *Synechococcus* are found to accumulate silicon in natural seawater (Baines et al., 2012), this effect should hardly contribute any PIM as we are using a defined medium without added silica. The excess total aggregate PIM could then either reflect the association of cations with the organic

exopolymers (De La Rocha et al., 2008) or indicate that ions in aggregate pore-water were not removed thoroughly in the washing step (see Methods). This effect would be expected to contribute more in aggregates with higher porosity, and would lead to the possible underestimation of the aggregate POC to DW ratios formed in the control and low concentration clay treatments (Fig. 9).

3.4.4 Implications of *Synechococcus* aggregation for the oceanic environment.

Our study thus shows that via aggregation *Synechococcus* could potentially export carbon 2-3 times of that contained in their single cells. In the oceanic environment, the organic carbon pool of extracellular polymeric substances (EPS, its major component acid polysaccharides are considered as the dissolved precursors of TEP, Passow, 2000; Thornton et al., 2007) can be much greater compared to the organic carbon pool associated with planktonic organisms and can provide a large carbon source to be incorporated into aggregates during phytoplankton blooms (Verdugo et al., 2004). In addition, we show that clay minerals have positive effects on both the formation and gravitational sinking of *Synechococcus* aggregates. Though the clay concentrations used in our experiments were higher than the general oceanic particle concentrations ranging from 0.5 to 250 $\mu\text{g L}^{-1}$ (Passow & De La Rocha, 2006, and references within), it has to be taken into consideration that suspended minerals will keep being incorporated into aggregates as they sink through the water columns. Most importantly, clay concentrations used in our experiments are in the range of those widely used in other aggregation studies with larger, eukaryotic phytoplankton, ensuring that our results can be compared with those obtained in these earlier studies (Table 7).

In oligotrophic ocean regions like the BATS site, *Synechococcus* cell concentration can reach up to 5.6×10^4 cells mL⁻¹ in the spring mixed layer, with an average carbon concentration of $5.9 \mu\text{g C L}^{-1}$ in the upper 200 m depth (Durand et al., 2001). Assuming that via aggregation *Synechococcus* could potentially export carbon twice of that contained in their single cells (Fig. 13), with a growth rate sufficient to maintain the average carbon concentration ($5.9 \mu\text{g C L}^{-1}$) in the upper 200 m depth and an average aggregate sinking velocity of 500 m d^{-1} , *Synechococcus* derived aggregates could contribute to a POC flux of $6 \text{ g C m}^{-2} \text{ d}^{-1}$ out of the upper layer. This is certainly a maximum value under optimized environmental conditions, and greatly exceeds that measured in the field (ca. $0.5 \text{ mg C m}^{-2} \text{ d}^{-1}$ at 200 m depth, Lomas & Moran, 2011). Also, when investigating field samples collected at different depths at the BATS site in July 2012, we could hardly observe any intact *Synechococcus* aggregates using epi-fluorescence microscopy (data not shown).

The much smaller POC flux measured and the lack of aggregates observed in the field compared to the aggregate formation observed in our laboratory study could be explained by lower cell densities, differences in clay concentrations, types and sizes, as well as various other processes. Zooplankton grazing and fragmentation, bacterial degradation, viral lysis, and water turbulence etc. could all undoubtedly alter both the formation and gravitational sinking of *Synechococcus* derived aggregates. As an example, pico-cyanobacteria like *Synechococcus* can avoid micro-zooplankton grazing by

aggregation (Pernthaler, 2005; Jezberová & Komárková, 2007). At the same time, their aggregates will become more available to grazing by larger zooplankton. And mobile heterotrophic bacteria and nano-flagellates guided by chemotaxis, i.e., elevated DOC concentrations on and around aggregates (Pernthaler, 2005), will also contribute to aggregate degradation in the field.

Nevertheless, our results are important as they illustrate the potential of *Synechococcus* aggregation in carbon export. These findings have strong implications when considering preferred oceanic scenarios in the future or past. For instance, oligotrophic ocean regions are hypothesized to increase in areal extent with future warming and increasing stratification (Polovina et al., 2008), and *Synechococcus* may become an increasingly dominant contributor to the phytoplankton community (Lomas et al., 2010). In addition, pico-cyanobacteria could have played a critical role in an early ocean dominated by prokaryotes. Their aggregation and subsequent settling could have been the only mechanism of carbon export before the evolution of eukaryotic grazers and subsequent production of sinking fecal pellets, which was critical for the formation of an oxygenated environment during the late Archean and early Proterozoic (ca. 2.8-2.2 Ga) period. Though back then primary productivity may have been limited by low nutrient availability (Anbar & Knoll, 2002; Bjerrum & Canfield, 2002), the absence of eukaryotic grazer control and the great amount of lithogenic clay particles derived from hydrothermal venting (bentonite) and continental weathering (kaolinite) (Tosca et al., 2010) could have resulted in a greater carbon export flux by these pico-cyanobacteria.

3.5 References

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3.6 Tables and Figures

Table 6

Results (P values on various experimental parameters) of ANOVA ($df_1=3$, $df_2=4$) testing the effect of increasing concentrations of kaolinite or bentonite, as well as paired T-tests ($df=7$, two-tailed) comparing the effect of the two clay types. Significant results ($\alpha=0.05$) are shown in bold.

Parameter	ANOVA test	ANOVA test	Paired T-test
	Kaolinite	Bentonite	Kaolinite vs. Bentonite
Aggregate Count	0.258*	0.261	0.245
Aggregate Size	0.057	0.014	0.034
Agg. Sinking Velocity	<0.005	0.033	<0.005
Agg. Excess Density	0.005	0.007	0.094
Agg. POC to DW Ratio	0.112*	0.031	0.071
Suspended Agg.	0.108	0.133	0.282
Single Cell	<0.005	0.104*	0.087
Chl- <i>a</i>	0.674*	0.108*	0.029

**Non-parametric, Kruskal-Wallis rank sum test was used instead of ANOVA test due to unequal variances of duplicating roller tanks.*

Table 7

Summary of results from the studies investigating aggregation of various phytoplankton groups with different concentrations and types of suspended minerals in comparison to findings of this study. (+) positive, (-) negative, and (?) ambiguous relationship; CEC = cation exchange capacity; POC = particulate organic carbon, DW = dry weight.

Phytoplankton group	Suspended mineral			Parameters				Reference
	Type	Conc. (mg L ⁻¹)	Aggregate size (mm)	Sinking velocity (m d ⁻¹)	POC to DW ratio (%) (at saturation)	Aggregation efficiency		
Diatom: <i>Thalassiosira punctigera</i> and <i>Skellonema costatum</i>	Kaolinite, illite and Smectite	Control, 5-100 mg L ⁻¹	0.4-4.2 mm, Clay Conc. (-)	230-720 m d ⁻¹ , Clay Conc. (?)			Hamm 2002	
Diatom: <i>Thalassiosira weissflogii</i> and Copepod detritus	Illite and Calcium carbonate	0.01-50 mg L ⁻¹	1.0-3.6 mm, Mineral Conc. (-)	Clay CEC (+)	1.5-3%, Mineral Conc. (-)		Passow and De La Rocha 2006	
Diatom: <i>Skellonema costatum</i> and <i>Chaetoceros decipiens</i>	Biogenic Silica and Calcium carbonate	Control, 0.1-40 mg L ⁻¹	1.1-2.1 mm, Mineral Conc. (-)		2-5%, Mineral Conc. (-)		De La Rocha et al. 2008	
Cyanobacteria: <i>Microcystis</i>	Kaolinite and Bentonite	250-750 mg L ⁻¹		Positively buoyant to sedimentation		Clay CEC (+)	Verspaigen et al. 2006	
Diatom: <i>Skellonema costatum</i> and Coccolithophorid: <i>Emiliania huxleyi</i>	None	N/A	1.7-2.5 mm	110-250 m d ⁻¹			Iverson and Ploug 2010	
Cyanobacteria: <i>Synechococcus</i>	Kaolinite and Bentonite	Control, 0.5-50 mg L ⁻¹	0.24-1.4 mm, Clay Conc. (-)	360-780 m d ⁻¹ , Clay Conc. (+)	3-5%, Clay Conc. (-)	Clay CEC (+)	This study	

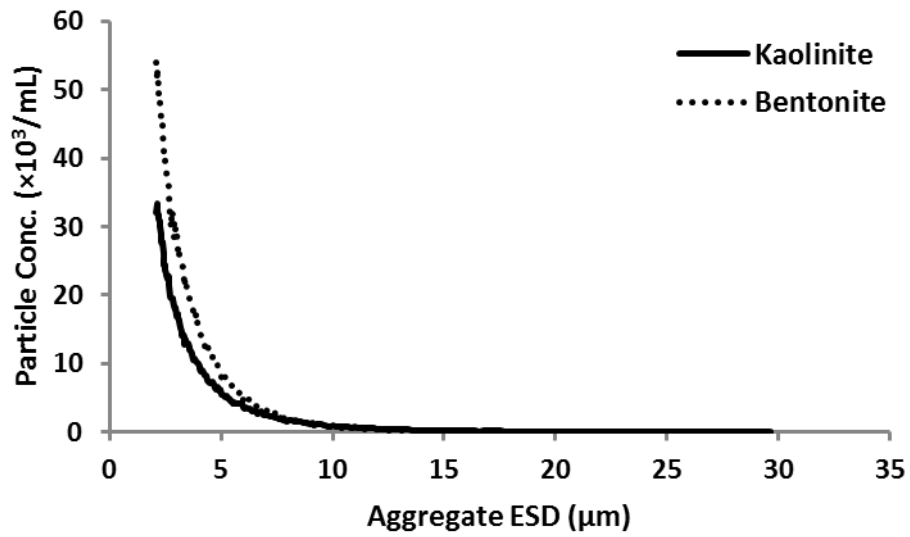


Figure 6. Size distributions (Equivalent Spherical Diameter, ESD) of clay particles in the kaolinite (solid line) and bentonite (dashed line) solutions (each 50 mg L⁻¹) using the Multisizer 3 particle counter.

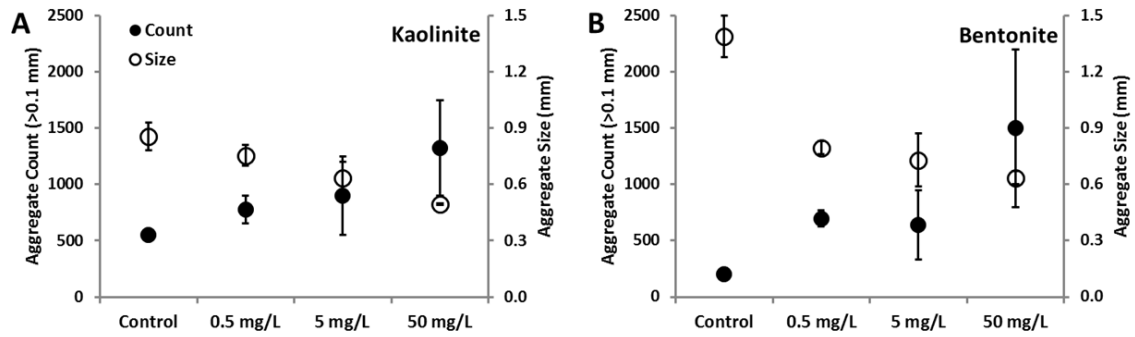


Figure 7. Number of aggregates (>0.1 mm, black circles) and their sizes (open circles) at the end of the aggregation experiments with added kaolinite (A) and bentonite (B). Error bars indicate Standard Error of the Mean (SEM).

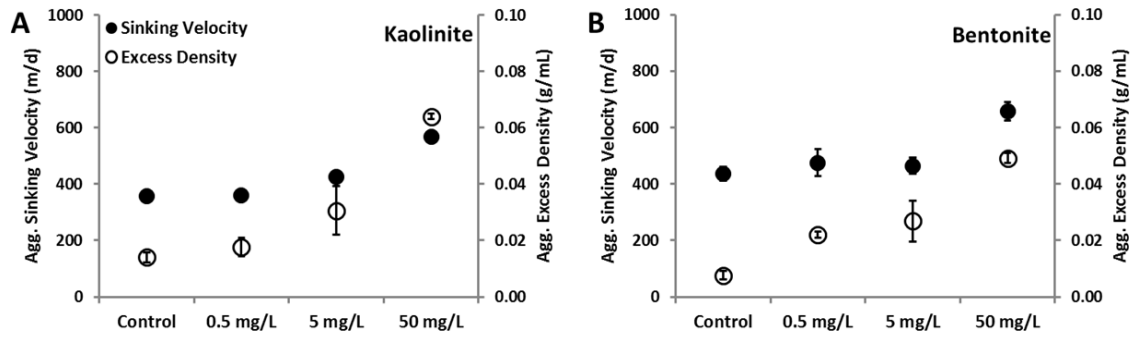


Figure 8. Aggregate sinking velocity (black circles) and excess density (open circles) at the end of the aggregation experiments with added kaolinite (A) and bentonite (B). Error bars indicate Standard Error of the Mean (SEM).

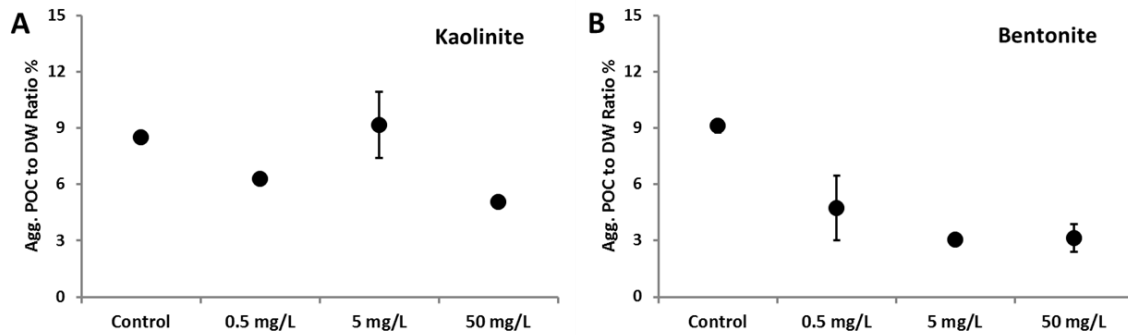


Figure 9. Aggregate particulate organic carbon (POC) to dry weight (DW) ratio at the end of the aggregation experiments with added kaolinite (A) and bentonite (B). Error bars indicate Standard Error of the Mean (SEM).

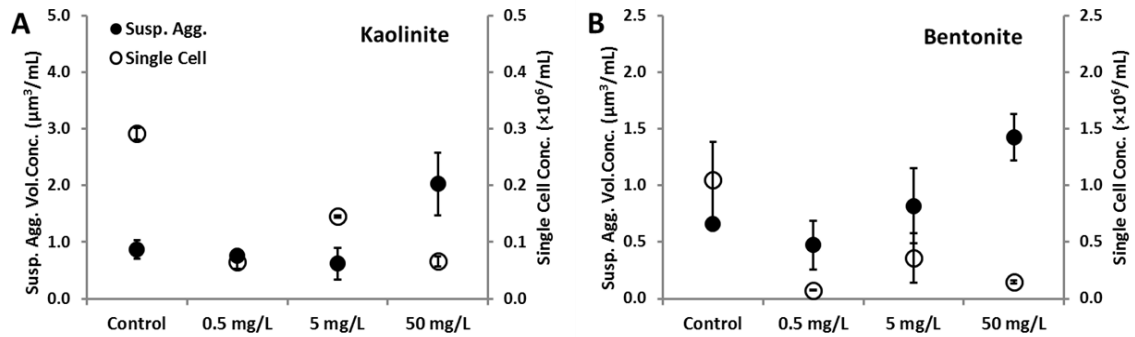


Figure 10. Volume concentration of suspended aggregates (5-60 μm , black circles) and single cell concentration (open circles) in the medium at the end of the aggregation experiments with added kaolinite (A) and bentonite (B). Error bars indicate Standard Error of the Mean (SEM). Note the differences in the axis scales between (A) and (B).

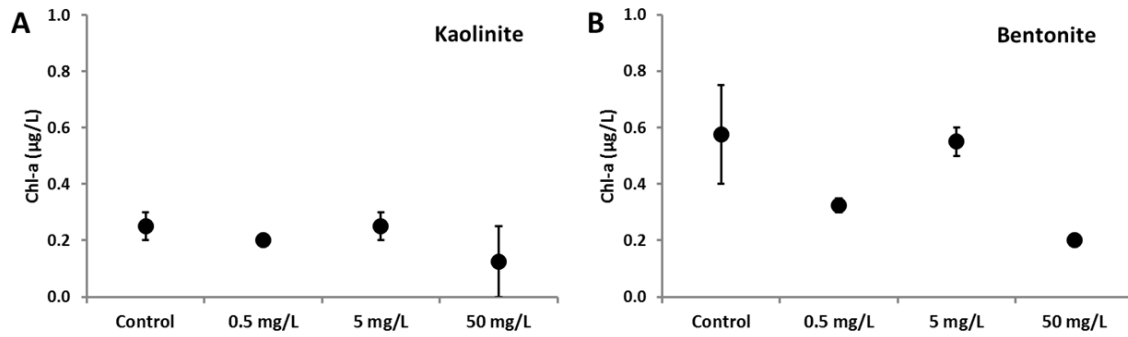


Figure 11. Final Chl-a concentration in the medium at the end of the aggregation experiments with added kaolinite (A) and bentonite (B). Error bars indicate Standard Error of the Mean (SEM).

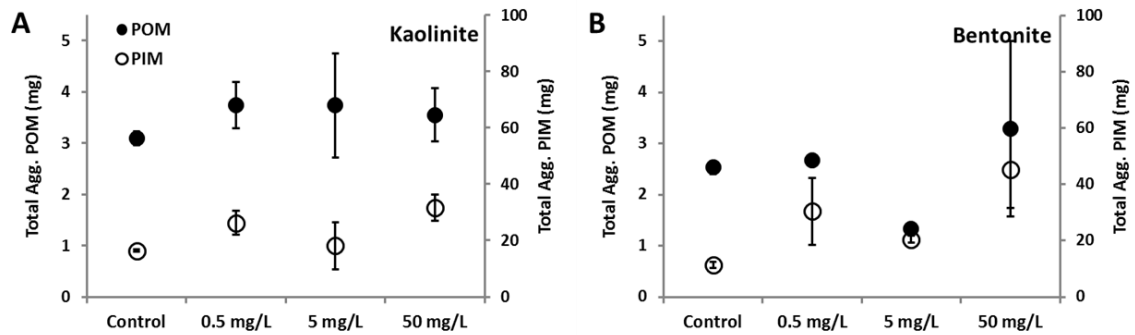


Figure 12. Total aggregate particulate organic matter (POM, black circles) and particulate inorganic matter (PIM, open circles) at the end of the aggregation experiments with added kaolinite (A) and bentonite (B). Error bars indicate Standard Error of the Mean (SEM).

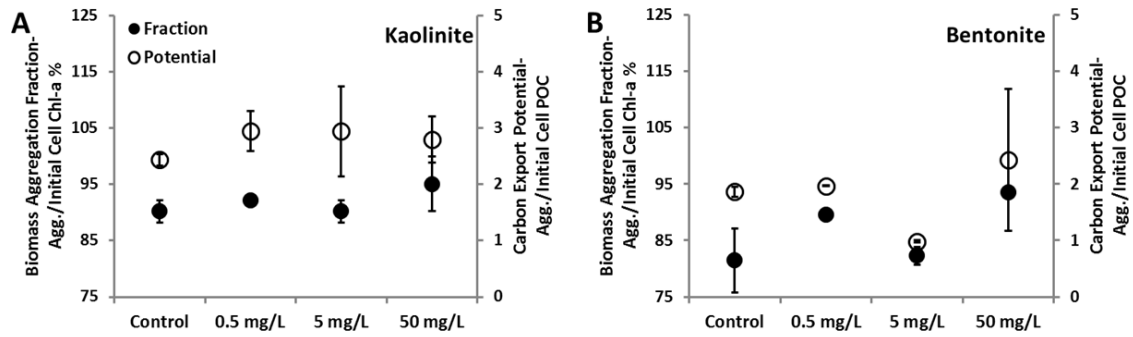


Figure 13. Biomass aggregation fraction (ratio of aggregate to initial cell Chl-*a*, black circles) and carbon export potential (ratio of aggregate to initial cell POC, open circles) in the aggregation experiments with added kaolinite (A) and bentonite (B). Error bars indicate Standard Error of the Mean (SEM).

CHAPTER 3

Effects of Heterotrophic Bacteria and Clay Minerals on the Aggregation of Marine

Synechococcus and *Prochlorococcus*

Abstract. Marine pico-cyanobacteria of the genera *Synechococcus* and *Prochlorococcus* are dominant primary producers and are considered to play an important role in the biological carbon pump of oligotrophic oceans. Recent laboratory studies have found that marine *Synechococcus* cells, despite of their small size and lack of natural ballasting minerals, can form sinking aggregates by producing transparent exopolymer particles (TEP). My objective here is to further study the aggregation of these pico-cyanobacteria by investigating the effects of heterotrophic bacteria, together with the clay kaolinite, using roller tanks. I show that *Prochlorococcus* is able to form sinking aggregates comparable to *Synechococcus* in the presence of heterotrophic bacteria. Secondly, I found that the presence of heterotrophic bacteria generally resulted in the formation of fewer, larger and faster sinking aggregates and eventually led to an enhanced aggregation of cells and particles, supposedly due to the enhanced production of TEP. Thirdly, I propose that clay particles and TEP likely act in different ways in driving aggregation and determine aggregate characteristics, though both of them can eventually help the incorporation of cells and particles into aggregates. My study contributes to the understanding of the role of cyanobacteria in the ecology and biogeochemistry of oligotrophic oceans.

4.1 Introduction

Phytoplankton cells used to be considered the main constituents of oceanic particulate organic carbon (POC). However, recent studies have focused on the formation of exo-polysaccharide particles as another important source of POC (Engel et al., 2004). These exo-polysaccharide particles are also referred to as “transparent exopolymer particles” (TEP), which can further facilitate the aggregation and subsequent settling of small cells during phytoplankton blooms (Alldredge et al., 1993; Passow et al., 2001). Through this pathway, large and rapidly sinking aggregates can also form from small particles (including single cells) via colliding and sticking together (Burd & Jackson, 2009).

Many phytoplankton groups (diatoms, coccolithophores, cyanobacteria etc.) are able to generate TEP abundantly, especially when their growth rates decrease, e.g., due to nutrient limitation (Corzo et al., 2000; Passow, 2002; Deng et al., in revision). Though phytoplankton appears to be the most significant source of TEP, heterotrophic bacteria can possibly enhance the TEP production by phytoplankton (Passow et al., 2001; Passow, 2002). TEP have been found to serve as a substrate and microhabitat for attached bacteria in addition to providing the main matrix of marine snow aggregates (Alldredge et al., 1993; Passow & Alldredge, 1994). From the other perspective, bacteria can also influence the accumulation and composition of TEP or their exopolysaccharide precursors (Grossart et al., 2006a; Gärdes et al., 2012) and thus the stickiness of phytoplankton cells using enzymes and extracellular products (Smith et al., 1995; Alderkamp et al., 2007).

Since TEP play an important role in phytoplankton aggregation both in the field and in laboratory experiments (Passow et al., 1994; Passow et al., 2001; Deng et al., in revision), I would expect that the quantity and characteristics of phytoplankton aggregates can also be altered by heterotrophic bacteria. Such aggregate-bacteria interaction has been found in many diatoms (Grossart et al., 2006b; Gärdes et al., 2012) and Gärdes et al. (2010) showed that certain diatom-associated bacteria are even required for aggregation. Marine bacteria and phytoplankton are thought to closely interact in the ‘phycosphere’, which is the micro-zone surrounding phytoplankton cells/aggregates (Bell & Mitchell, 1972). The initial attachment of heterotrophic bacteria to phytoplankton aggregates can be characterized by encounter models (Kiørboe et al., 2003). As the degradation processes, mobile bacteria can find phytoplankton aggregates guided by chemotaxis, i.e., to elevated DOC concentrations on and around aggregates (Blackburn et al., 1998).

Marine pico-cyanobacteria are important primary producers and make significant contribution to the carbon export in oligotrophic oceans like the Sargasso Sea in the western North Atlantic Subtropical Gyre (DuRand et al., 2001; Lomas & Moran, 2011; Amacher et al., 2013). Although two of the most important marine pico-cyanobacteria, *Synechococcus* and *Prochlorococcus*, often co-occur, they have been adapted to different ecological and biogeochemical conditions. *Synechococcus* is widespread in all marine environments from high latitudes to the tropics (Neuer, 1992; Partensky et al., 1999), with its ability to regulate photochemistry over a wide range of temperatures (Mackey et

al., 2013). Generally it is more abundant in nutrient-rich than in oligotrophic waters and its vertical distribution is restricted to the upper euphotic zone (Partensky et al., 1999) due to its high demands for light and nitrogen (to maintain the N-rich phycobilisome, Wyman et al., 1985; Kana et al., 1992). *Prochlorococcus*, despite of its narrower geographical distribution, is much more abundant in warm oligotrophic waters compared to *Synechococcus* (Partensky et al., 1999; Flombaum et al., 2013). At the Bermuda Atlantic Time-series Study (BATS) site, *Synechococcus* usually peaks during the winter/spring bloom period while *Prochlorococcus* reaches peak abundance during the summer/fall months (DuRand et al., 2001; De Martini et al., in review). Also by analyzing the particle trap materials, *Synechococcus* was found to contribute to the POC flux mirroring their seasonal distribution, while *Prochlorococcus* contributed much less to the POC flux despite of their abundances in the water column and seasonal distribution (De Martini et al., in prep.). To explain the latter observations, laboratory studies are needed to test the potential differences in the aggregation of marine *Synechococcus* vs. *Prochlorococcus*.

Recent laboratory studies have found that marine *Synechococcus* cells, despite of their small size and lack of natural ballasting minerals, can still form sinking aggregates by producing TEP, and such TEP production and aggregate formation can be enhanced by nutrient limitation and clay addition (Deng et al., 2015; Deng et al., in revision). These former experiments were conducted using axenic *Synechococcus*. My objective here is to further investigate the effects of heterotrophic bacteria, together with the clay kaolinite, on the aggregation of these pico-cyanobacteria using roller tanks. In this study, I will test

the aggregation of xenic *Synechococcus* and xenic *Prochlorococcus*, and also compare them with the aggregation of axenic *Synechococcus* (from Deng et al., 2015). I was not able to grow axenic *Prochlorococcus* in the culture experiments and thus could not test its aggregation in comparison with xenic *Prochlorococcus*. I will address the following questions: 1) what effects do heterotrophic bacteria have on the aggregation of *Synechococcus*; 2) do effects of clay minerals on *Synechococcus* aggregation differ in the presence of heterotrophic bacteria; and 3) does *Prochlorococcus* form sinking aggregates in the presence of heterotrophic bacteria.

4.2 Methods

4.2.1 Culturing of *Synechococcus* and *Prochlorococcus*.

Xenic batch cultures of the marine *Synechococcus* sp. strain CCMP 837 and *Prochlorococcus marinus* strain CCMP 1986 from the National Center for Marine Algae and Microbiota (NCMA) were grown in 1 L flasks on rocking platforms. The cultures were kept in a reach-in chamber (Convion), at 25 °C, and a light intensity of 90-100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in a 14h:10h light-dark cycle for a growth period of 9 days, when late exponential growth phase was reached. Autoclaved IMR medium (Eppley et al., 1967) and Pro 99 medium (Moore et al., 2007) in artificial seawater (Sigma Seasalt) were used as the growth and experimental medium for *Synechococcus* and *Prochlorococcus*, respectively.

4.2.2 Aggregate formation.

Synechococcus or *Prochlorococcus* cultures were diluted with autoclaved medium to ca. 10^6 cells mL^{-1} and then incubated in transparent, cylindrical (1.25 L) roller tanks (Shanks & Edmondson, 1989). In addition to a control treatment, the clay kaolinite (Sigma, analytical), dissolved and autoclaved in seawater in a series of concentrations (0.5, 5 and 50 mg L^{-1}), was added to the cell suspensions. In each culture experiment, four treatments each with two replicate tanks were rotated on a rolling platform at around 3.5 rotations per minute (rpm), at room temperature (ca. $25 \text{ }^\circ\text{C}$) and in darkness for one week.

4.2.3 Aggregate count, size and sinking velocity.

When the roller tanks were taken down, the number of visible aggregates (>0.1 mm) formed in each roller tank was counted, and their sizes (diameters of three to five aggregates) were measured using a dissecting microscope (Zeiss Stereoscope) with a calibrated ocular micrometer. Visible aggregates were then gently transferred with a wide-bore syringe pipette into a 1 L settling cylinder (released at 1 cm under the air-water interface) to determine their sinking velocities. The settling cylinder was filled with artificial seawater at 30‰ salinity, and allowed to stabilize at room temperature before measurements. The sinking velocities of nine aggregates from each roller tank were measured with a stopwatch through a vertical distance of 32.6 cm in the settling cylinder and converted to velocities in meters per day.

4.2.4 Aggregate excess density.

The aggregate excess density ($\Delta\rho$) was determined using the Navier-Stokes drag equation (1) as in Iversen & Ploug (2010):

$$\Delta\rho = \frac{C_D \rho_w v^2}{\frac{4}{3} g D} \quad (1)$$

C_D is the dimensionless drag force defined in Eq. 2 for a Reynolds number (Re , defined as in Eq. 3) >1 , ρ_w is the density of seawater (1.0196 g cm^{-3} , at $25 \text{ }^\circ\text{C}$ and 30‰ salinity), v is the measured sinking velocity in cm s^{-1} , g is the gravitational acceleration of 981 cm s^{-2} , and D is the measured diameter in cm.

$$C_D = \left(\frac{24}{Re} \right) + \left(\frac{6}{1 + Re^{0.5}} \right) + 0.4 \quad (2)$$

$$Re = \frac{v D \rho_w}{\mu_w} \quad (3)$$

μ_w is the dynamic viscosity of seawater ($0.9565 \times 10^{-2} \text{ g cm}^{-1} \text{ s}^{-1}$, at $25 \text{ }^\circ\text{C}$ and 30‰ salinity).

4.2.5 Suspended aggregates and single cells.

Suspended aggregates in the medium were analyzed before and after incubation using a Multisizer 3 particle counter (Beckman Coulter). Samples in duplicates were diluted to a 1-10% particle concentration with Isoton II (Beckman Coulter) solution and aggregates were sized and quantified with a $100 \text{ }\mu\text{m}$ aperture tube. The volume concentration of aggregates was calculated in μm^3 per mL within the size (equivalent spherical diameter) range of 5-60 μm .

Single cells in the medium were analyzed before and after incubation using an epi-fluorescence microscope (Zeiss Axioscope). Samples were fixed with glutaraldehyde

(1%) and stained with DAPI (4,6-diamidino-2-phenylindole, 0.03M) as in Amacher et al. (2009), and then filtered onto polycarbonate filters (black, 0.2 μm pore-size). DAPI is a fluorescent stain that binds to DNA under UV excitation. *Synechococcus* cells were counted under blue excitation to visualize phycoerythrin fluorescence; *Prochlorococcus* and heterotrophic bacteria cells were counted under UV excitation, and were distinguished based on their shapes (coccoid for *Prochlorococcus* vs. rod shaped for heterotrophic bacteria). The cell concentrations were all calculated in number per mL.

4.3 Results

4.3.1 Aggregate formation.

In both experiments, the *Synechococcus* and *Prochlorococcus* cells formed visible aggregates and the clay kaolinite generally increased the number and decreased the sizes of these aggregates (Fig. 14). These results are consistent with the general conclusions from earlier aggregation experiments with axenic *Synechococcus* (CCMP 2370, Deng et al., 2015). However, this time I observed several tanks in both culture experiments (see specific treatments in Fig. 14) that formed just one or two large cell flocculates (Fig. 15), which had never been found in my earlier aggregation experiments with axenic *Synechococcus* (Deng et al., 2015). The cell flocculates did not have a well-defined shape but existed as a string or film (up to ca. 10 cm in diameter) that could fold/unfold while rotating in the tanks (Fig. 15).

Roller tanks that formed only one or two cell flocculates were not analyzed and I then used the non-parametric, Kruskal-Wallis rank sum tests (Table 8) instead of

ANOVA to test for statistical significance (at 95% confidence level in all statistical tests). Despite of the general increase in aggregate number and decrease in aggregate size as the clay concentration increased in both culture experiments (Fig. 14), no significant difference was observed (Table 8) when comparing aggregates between different clay treatments.

I also compared aggregate between xenic *Synechococcus* and *Prochlorococcus*, as well as between xenic *Synechococcus* and axenic *Synechococcus* (from Deng et al., 2015) using paired T-tests (Table 8). Xenic *Synechococcus* formed significantly larger aggregates compared to xenic *Prochlorococcus* and significantly fewer, larger aggregates compared to axenic *Synechococcus* in the control and low (0.5 mg/L, 5 mg/L) clay treatments (Fig. 14, Table 8).

4.3.2 Aggregate sinking velocity and excess density.

The highest aggregate sinking velocities were observed in the 0.5 mg/L clay treatments in both experiments, up to over 1000 m/d, while the calculated aggregate excess density generally increased with increasing clay concentration (Fig. 16). I did not observe significant difference in the Kruskal-Wallis rank sum tests (Table 8) in aggregate sinking velocity or excess density between different clay treatments. When comparing xenic *Synechococcus*, xenic *Prochlorococcus*, and axenic *Synechococcus* (from Deng et al., 2015) using paired T-tests (Table 8), xenic *Synechococcus* formed aggregates that had significantly higher sinking velocities compared to axenic *Synechococcus* in the control and low (0.5 mg/L, 5 mg/L) clay treatments (Fig. 16). No significant difference in

aggregate excess density was observed (Table 8) despite that xenic *Prochlorococcus* generally formed aggregates with higher excess densities compared to xenic *Synechococcus* (Fig. 16).

4.3.3 Suspended aggregate and single cell concentration.

Suspended aggregate (5-60 μm , volume concentration) and single cell concentrations in the background medium (BGM) were measured before and after incubation, and I calculated their fraction left in the final BGM compared to in the initial BGM. In general, the fraction of suspended aggregates left increased while the fraction of single cells left (both cyanobacteria and heterotrophic bacteria) decreased as the clay concentration increased (Fig. 17). Significant differences were observed in the ANOVA tests (Table 9) in *Synechococcus* cells (xenic *Synechococcus* experiment) and bacteria cells (xenic *Prochlorococcus* experiment) between different clay treatments. When comparing xenic *Synechococcus*, xenic *Prochlorococcus*, and axenic *Synechococcus* (from Deng et al., 2015) using paired T-tests (Table 9), xenic *Synechococcus* had a significantly smaller fraction of suspended aggregates and single cells left in the BGM compared to xenic *Prochlorococcus* (Fig. 17). Xenic *Synechococcus* also had generally (but non-significantly, Table 9) a larger fraction of single cells left in the BGM compared to axenic *Synechococcus* (Fig. 17).

4.4 Discussion

4.4.1 Effects of clay addition on aggregate characteristics.

In both culture experiments, the clay kaolinite generally increased the number and decreased the sizes of aggregates formed in the roller tanks (Fig. 14), which is consistent with the general conclusions from earlier aggregation experiments with axenic *Synechococcus* (CCMP 2370, Deng et al., 2015) as well as similar studies of diatom aggregation (Hamm, 2002; Passow & De La Rocha, 2006; De La Rocha et al., 2008). In these experiments, there were multiple factors influencing the aggregation, i.e., increasing clay concentrations, different cyanobacteria groups and the presence or absence of heterotrophic bacteria. The latter two ultimately influence the amount of TEP available during aggregation. Passow et al. (2014) showed that clay particles could act as more efficient coagulators compared to TEP in driving aggregate formation and determining aggregate characteristics. In my control and low (0.5 mg/L, 5 mg/L) clay treatments, most aggregate characteristics were different between xenic *Synechococcus*, xenic *Prochlorococcus*, and axenic *Synechococcus* (from Deng et al., 2015). However, many of these aggregate characteristics became indistinguishable in the highest (50 mg/L) clay treatment. This can be seen from the paired T-tests (Table 8) that I often observed significant differences only in the control and low (0.5 mg/L, 5 mg/L) clay treatments, but not in all four treatments when comparing the aggregate characteristics of xenic *Synechococcus*, xenic *Prochlorococcus*, and axenic *Synechococcus*. Thus, my results indicate that the clay particles in high concentrations tend to strongly “define” the aggregation under the specific experimental conditions. For instance, at the highest (50 mg/L) clay concentration, xenic *Synechococcus*, xenic *Prochlorococcus*, and axenic

Synechococcus all formed a few thousands aggregates in each tank that were about 0.44 mm in diameter and sank at velocities of about 600 m/d (Fig. 14, 16).

4.4.2 Effects of heterotrophic bacteria on aggregate characteristics.

While I have shown in the preceding paragraph that clay particles in high concentrations tend to strongly “define” the aggregation, heterotrophic bacteria could result in “undefined” cell flocculates. Such cell flocculates were only observed in the control and lowest (0.5 mg/L) clay treatments (Fig. 14), in which the defining effect of clay particles may not have been strong enough to lead to the formation of the many spherical aggregates that I would normally observe. Interestingly also, though such cell flocculates had never been found in my earlier aggregation experiments with axenic *Synechococcus* (Deng et al., 2015) grown in nutrient replete media, I had observed such cell flocculates with axenic *Synechococcus* grown in nitrogen limited media (data not presented). This indicates again that the effects of heterotrophic bacteria on aggregation may ultimately be related to the amount of TEP available. An enhanced production of TEP, resulting from either heterotrophic bacteria (Passow et al., 2001; Passow, 2002) or nutrient limitation (Deng et al., in revision), could possibly lead to the incorporation of cells and particles all into just one single, large flocculation without it breaking into many smaller aggregates. This can be explained by the fact that TEP could act as glue, which would scavenge cells and particles until their carrying capacity is reached (Passow et al., 2001; Passow, 2004).

In addition to the formation of only one or two cell flocculates in several tanks (Fig. 15), heterotrophic bacteria also altered the characteristics of the many spherical aggregates that I would normally observe in the other tanks. My results show that xenic *Synechococcus* formed fewer but larger aggregates compared to xenic *Prochlorococcus* and axenic *Synechococcus* (Fig. 14), and the larger sizes of xenic *Synechococcus* aggregates also resulted in their higher sinking velocities compared to those formed by axenic *Synechococcus* (Fig. 16). I did not observe significant difference in sinking velocity between xenic *Synechococcus* and *Prochlorococcus*, since xenic *Prochlorococcus* generally formed aggregates with higher excess densities (despite of their smaller sizes) compared to xenic *Synechococcus* (Fig. 16). To explain the differences in aggregate number and sizes, I propose that there was a larger amount of TEP available in the aggregation of xenic *Synechococcus* compared to xenic *Prochlorococcus* and axenic *Synechococcus*, since *Synechococcus* is known to be able to generate TEP abundantly (Deng et al., in revision; it is so far unknown for *Prochlorococcus*) and heterotrophic bacteria are thought to enhance its TEP production (Passow et al., 2001; Passow, 2002). The presumably enhanced production of TEP, acting as glue, could have helped the incorporation of cells and particles and resulted in the formation of fewer, larger and faster sinking (if the excess density was close) aggregates of xenic *Synechococcus* compared to those formed by xenic *Prochlorococcus* and axenic *Synechococcus*.

Another important observation is that the highest aggregate sinking velocities were observed in the 0.5 mg/L clay treatments in both xenic *Synechococcus* and

Prochlorococcus experiments (Fig. 16). This contradicts what was found by Deng et al. (2015) that aggregates of axenic *Synechococcus* had a steady increase in sinking velocity as the clay concentration increased. This indicates that in the aggregation of xenic *Synechococcus* and *Prochlorococcus*, the positive effect of larger aggregate sizes on sinking velocity exceeded the negative effect of reduced ballasting clays in the low clay treatments, supposedly due to the enhanced production of TEP. On the other hand, the calculated aggregate excess density generally increased with increasing clay concentration (Fig. 16), which is consistent with the earlier aggregation experiments with axenic *Synechococcus* (Deng et al., 2015). The increase of aggregate excess density with increasing clay concentration was related to the lower aggregate porosity and POC to dry weight ratio in the study by Deng et al. (2015), and should be observed regardless of the changes in aggregate sinking velocity (see the Navier-Stokes drag equation (1) used for the calculation of aggregate excess density).

4.4.3 Effects of clay vs. TEP on driving aggregation and its implications.

In the background medium (BGM) after incubation and aggregation, there were fewer single cells and more suspended aggregates (5-60 μm) as the clay concentration increased (Fig. 17), which validates the assumption that the clay particles may have enhanced the scavenging of single cells and their incorporation into suspended aggregates, and further into larger, visible aggregates (De La Rocha et al., 2008; Deng et al., 2015). The aggregate incorporation of single cells included both cyanobacteria and heterotrophic bacteria (Fig. 17). I observed a decrease in the ratio of cyanobacteria to heterotrophic bacteria (in the BGM, Fig. 18) as the clay concentration increased in the

xenic *Synechococcus* but not in the xenic *Prochlorococcus* experiment. Thus, it is hard to conclude whether the clay particles in high concentrations would favor cyanobacteria over heterotrophic bacteria in the aggregate incorporation of single cells.

To be able to compare the “aggregation fraction” (Deng et al., 2015) between xenic *Synechococcus*, xenic *Prochlorococcus*, and axenic *Synechococcus* (from Deng et al., 2015), I calculated the ratios of suspended aggregate (5-60 μm) and single cell (cyanobacteria and heterotrophic bacteria) concentrations in the BGM at the end of the aggregation experiments compared to in the initial BGM. There are two possible scenarios: on one hand, 1) the larger sizes of xenic *Synechococcus* aggregates could have incorporated more cells and particles compared to xenic *Prochlorococcus*; while on the other hand, 2) a larger number of smaller aggregates (with greater surface to volume ratios) of xenic *Prochlorococcus* could have enhanced the overall aggregation fraction compared to xenic *Synechococcus*. My results supported the first scenario, showing that the aggregation of xenic *Synechococcus*, with a smaller number of larger aggregates (Fig. 14), had a smaller fraction of suspended aggregates and single cells left in the BGM and thus had a larger aggregation fraction compared to xenic *Prochlorococcus* (Fig. 17).

Though both clay particles and TEP are thought to be efficient coagulators and help the incorporation of cells and particles into aggregates, they seem to act in different ways in driving aggregation. Here I propose that the aggregation of cyanobacteria with clay addition consists of two stages. In stage one, TEP act as the main coagulator/glue and scavenge cells and particles until their carrying capacity is reached (Passow et al.,

2001; Passow, 2004). When comparing the aggregation of different cyanobacteria that may ultimately be related to the difference in the amount of TEP available as in my study, a smaller number of larger aggregates of xenic *Synechococcus* would lead to a larger aggregation fraction compared to xenic *Prochlorococcus*. In the second stage, clay particles take the main role in reshaping (decreasing aggregate size and porosity) and possibly fragmenting aggregates (Passow & De La Rocha, 2006; De La Rocha et al., 2008) that would have greater surface to volume ratios. When comparing the aggregation with different clay additions as in Deng et al. (2015), a larger number of smaller aggregates in the kaolinite treatment would lead to a larger aggregation fraction compared to in the bentonite treatment.

These results imply that though heterotrophic bacteria can reduce the potential carbon export by degrading the organic matter of cyanobacteria aggregates, they may also enhance the TEP production and thus the aggregation of cells and particles. Also, *Prochlorococcus* was found to form sinking aggregates comparable to those of *Synechococcus*. Thus, the higher contribution by *Synechococcus* compared to *Prochlorococcus* to the POC flux measured in the particle traps at BATS (De Martini et al., in prep.) is more likely due to the difference in their grazer utilization (Christaki et al., 1999; De Martini et al., in review) and digestion (Gorsky et al., 1999). Besides these implications, further studies are in need to 1) validate the presumably enhanced production of TEP in the presence of heterotrophic bacteria by testing the TEP production of axenic vs. xenic *Synechococcus* and axenic vs. xenic *Prochlorococcus* in culture experiments; and 2) show the overall effect of heterotrophic bacteria on the

carbon export potential of cyanobacteria aggregation, considering another important process during aggregation: the degradation of organic matter by heterotrophic bacteria (Ploug & Grossart, 2000; Iversen & Ploug, 2010).

4.5 Conclusion

In conclusion, I show for the first time to my knowledge, that *Prochlorococcus* is able to form sinking aggregates comparable to *Synechococcus* in the presence of heterotrophic bacteria. Secondly, I found that the presence of heterotrophic bacteria generally resulted in the formation of fewer, larger and faster sinking aggregates and eventually led to an enhanced aggregation of cells and particles, supposedly due to the enhanced production of TEP. Thirdly, I propose that clay particles and TEP likely act in different ways in driving aggregation and determine aggregate characteristics, though both of them can eventually help the incorporation of cells and particles into aggregates. My results provide important knowledge about the effects of heterotrophic bacteria, in addition to nutrient availability and clay minerals (Deng et al., 2015; Deng et al., in revision), on the aggregation of these important marine pico-cyanobacteria.

4.6 References

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4.7 Tables and Figures

Table 8

Results (P values on sinking aggregates) of Kruskal-Wallis testing the effect of increasing kaolinite concentrations on aggregation, as well as paired T-tests (df=3, two-tailed) comparing the aggregation of different cyanobacteria in the control and low (0.5 mg/L, 5 mg/L) clay treatments (T-test 1) and in all four treatments (T-test 2). Significant results ($\alpha=0.05$) are shown in bold.

Parameter	Kruskal-Wallis		Paired T-test 1		Paired T-test 2	
	X-Pro.	X-Syn.	X-Syn. vs X-Pro.	X-Syn. vs A-Syn.	X-Syn. vs X-Pro.	X-Syn. vs A-Syn.
Count	0.17	0.15	0.13	0.25	0.29	0.02
Size	0.19	0.13	0.07	0.09	0.03	0.04
Sinking Velocity	0.23	0.13	0.86	0.06	0.90	0.03
Excess Density	0.19	0.17	0.06	0.48	0.12	0.54

Table 9

Results (P values on background medium, BGM) of ANOVA ($df_1=3, df_2=4$) testing the effect of increasing kaolinite concentrations on aggregation, as well as paired T-tests ($df=3$, two-tailed) comparing the aggregation of different cyanobacteria in the control and low (0.5 mg/L, 5 mg/L) clay treatments (T-test 1) and in all four treatments (T-test 2). Significant results ($\alpha=0.05$) are shown in bold.

Parameter	ANOVA	ANOVA	Paired T-test 1	Paired T-test 1	Paired T-test 1	Paired T-test 2	Paired T-test 2
	X-Pro.	X-Syn.	X-Syn. vs X-Pro.	X-Syn. vs A-Syn.	X-Syn. vs X-Pro.	X-Syn. vs X-Pro.	X-Syn. vs A-Syn.
Suspended Agg.	0.33	0.34	0.03	0.13	0.11	0.18	
Cyanobacteria Cell	0.10*	0.004	0.04	0.10	0.14	0.06	
Het. Bacteria Cell	0.01	0.24	0.03	N/A	0.06	N/A	

**Non-parametric, Kruskal-Wallis rank sum test was used instead of ANOVA test due to unequal variances of duplicating roller tanks.*

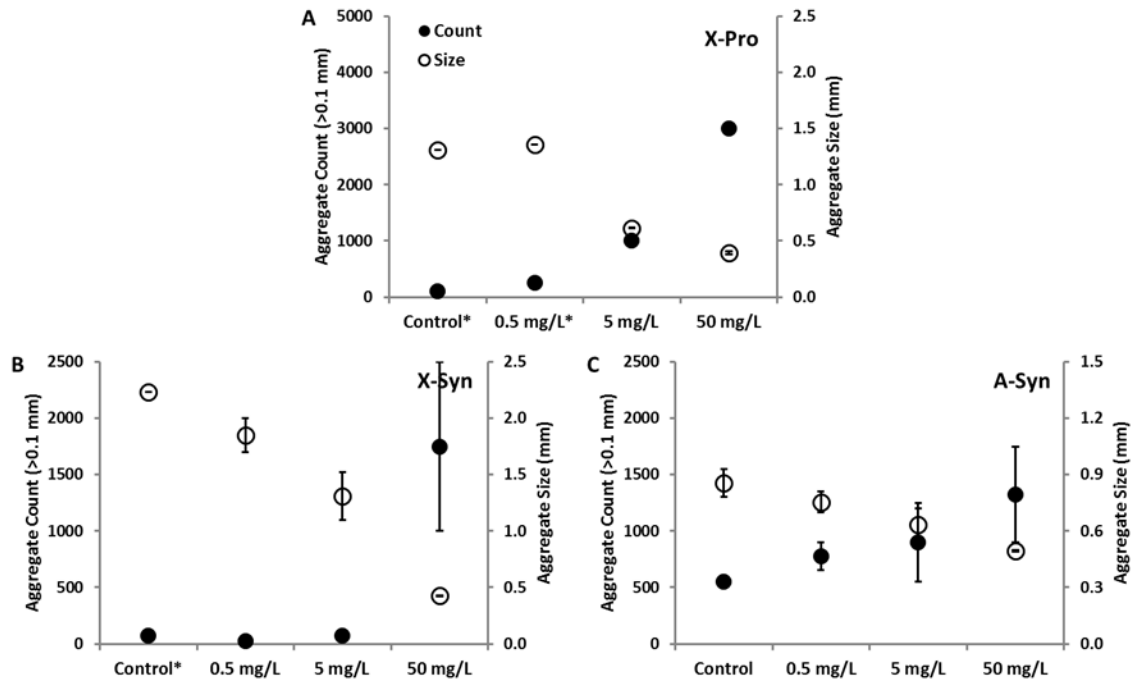


Figure 14. Number of aggregates (>0.1 mm, black circles) and their sizes (open circles) at the end of the aggregation experiments of A) Xenic *Prochlorococcus* (X-Pro), B) Xenic *Synechococcus* (X-Syn) and C) Axenic *Synechococcus* (A-Syn, from Deng et al. 2015), with added kaolinite in a series of concentrations. Asterisks indicate treatments without replicate tanks (one tank had formed the cell flocculates and was removed). Error bars indicate Standard Error of the Mean (SEM). Note the differences in the axis scales.

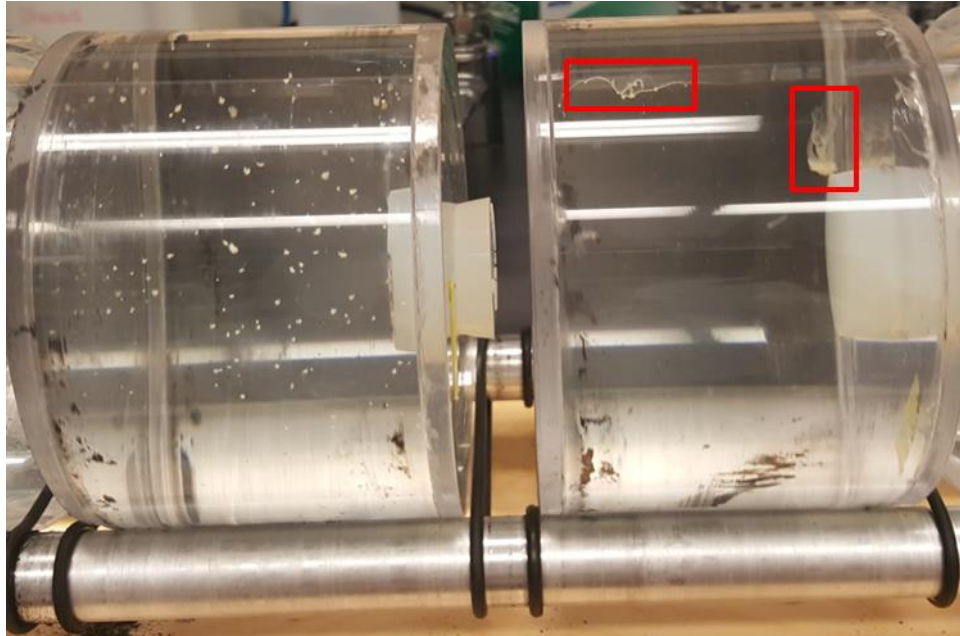


Figure 15. Comparison of the formation of the many spherical aggregates of xenic *Prochlorococcus* in the left tank and the two cell flocculates (red squares) in the right tank at the end of the aggregation experiment in the lowest (0.5 mg/L) clay treatment.

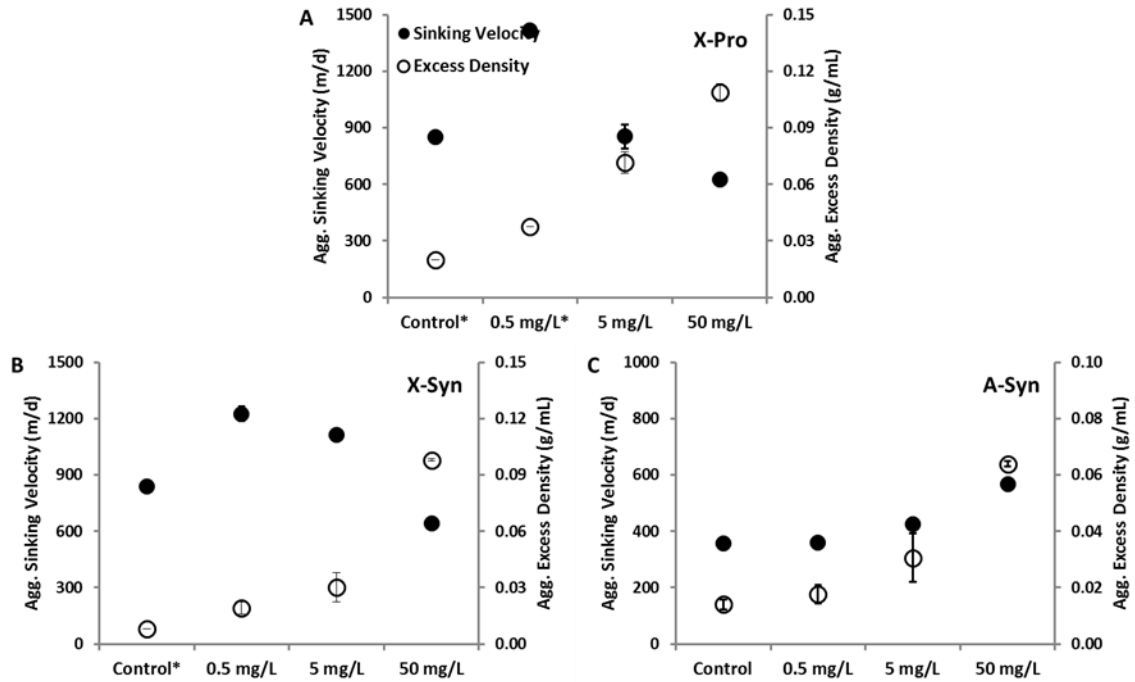


Figure 16. Aggregate sinking velocity (black circles) and excess density (open circles) at the end of the aggregation experiments of A) Xenic *Prochlorococcus* (X-Pro), B) Xenic *Synechococcus* (X-Syn) and C) Axenic *Synechococcus* (A-Syn, from Deng et al. 2015), with added kaolinite in a series of concentrations. Asterisks indicate treatments without replicate tanks (one tank had formed the cell flocculates and was removed). Error bars indicate Standard Error of the Mean (SEM). Note the differences in the axis scales.

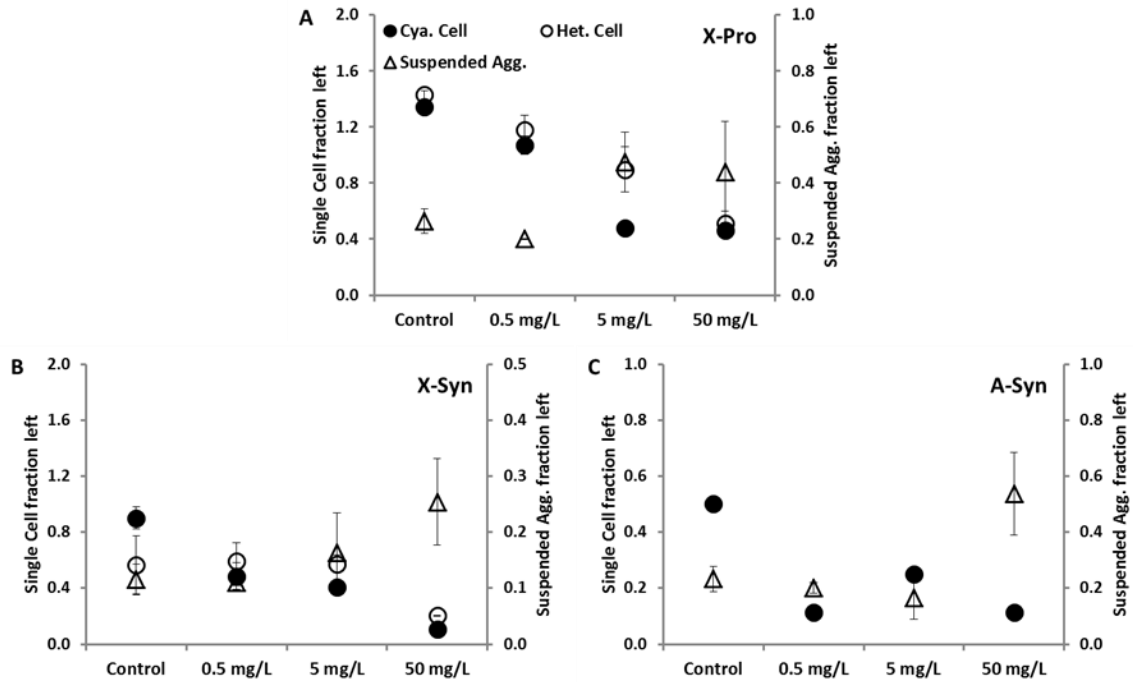


Figure 17. Fraction of single cyanobacteria cells (black circles), heterotrophic bacteria cells (open circles) and suspended aggregates (5-60 μm , open triangles) left in the BGM at the end of the aggregation experiments of A) Xenic *Prochlorococcus* (X-Pro), B) Xenic *Synechococcus* (X-Syn) and C) Axenic *Synechococcus* (A-Syn, from Deng et al. 2015), with added kaolinite in a series of concentrations. Error bars indicate Standard Error of the Mean (SEM). Note the differences in the axis scales.

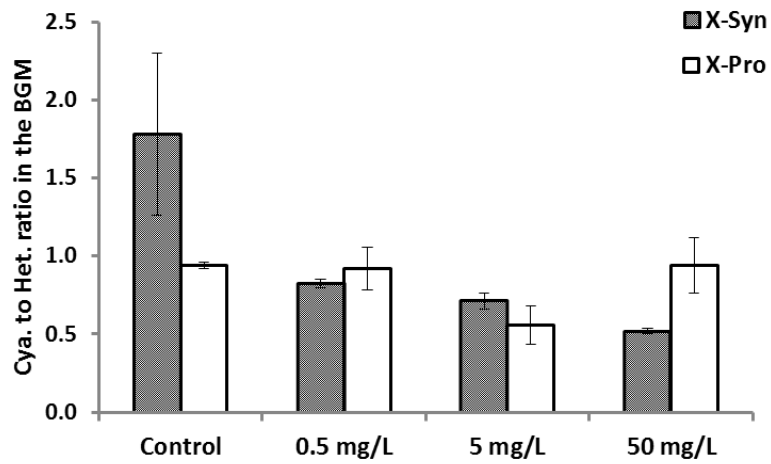


Figure 18. Ratio of cyanobacteria to heterotrophic bacteria in the BGM at the end of the aggregation experiments of *Xenic Synechococcus* (X-Syn, shaded columns) and *Xenic Prochlorococcus* (X-Pro, open columns), with added kaolinite in a series of concentrations. Error bars indicate Standard Error of the Mean (SEM).

CONCLUDING REMARKS

5.1 Contributions of My Study to the Field of Science

By opening the door to the aggregation of marine pico-cyanobacteria, my study contributes to the understanding of the role of these cyanobacteria in the ecology and biogeochemistry of oligotrophic oceans. I have shown that marine pico-cyanobacteria, *Synechococcus* and *Prochlorococcus*, can aggregate and sink as well and as fast as eukaryotic phytoplankton; and there are many factors that can possibly enhance the aggregation of these cyanobacteria, such as TEP and clay minerals. Thus, the fact that intact cyanobacteria aggregates have rarely been observed in the field is more likely due to the lack of proper environments for their aggregation. My research has shown that if we would be able to build a perfect world for the aggregation of these cyanobacteria in oligotrophic ocean regions, as exemplified by the BATS site in the Sargasso Sea, *Synechococcus* derived aggregates could contribute to a POC flux of $6 \text{ g C m}^{-2} \text{ d}^{-1}$ out of the euphotic zone (see 3.4.4).

Again, my estimated contribution of $6 \text{ g C m}^{-2} \text{ d}^{-1}$ is certainly a maximum value under optimized environmental conditions, without considering the various processes in the ocean that can possibly result in the loss of sinking aggregates. For instance, *Synechococcus* are observed abundantly in mesozooplankton guts and fecal pellets (Wilson & Steinberg, 2010; Stukel et al., 2013), providing evidence for the mesozooplankton grazing on *Synechococcus* cells and aggregates. Mobile heterotrophic bacteria and nano-flagellates guided by chemotaxis, i.e., elevated DOC concentrations on

and around aggregates (Blackburn et al., 1998) can also degrade the organic matter of aggregates, at carbon-specific respiration rates of ca. 0.1-0.2 d⁻¹ (Ploug & Grossart, 2000; Iversen & Ploug, 2010). This means that over half of the organic carbon content could be respired after three days settling in the water column. In addition, virus infection is another important control factor, since many species of the genus *Synechococcus* are known as cyanophage hosts (Suttle & Chan, 1994; Mühling et al., 2005). Viruses may be responsible for lysing up to 5-14% of total *Synechococcus* cells on a daily basis in the open ocean (Suttle & Chan, 1994), which could lead to an even larger and faster turnover of the organic carbon content compared to the bacterial degradation.

Nevertheless, my results are important as they illustrate the potential of cyanobacteria aggregation in carbon export. Compared to the estimated contribution of *Synechococcus* to the POC flux in the field (ca. 0.5 mg C m⁻² d⁻¹ at 200 m depth, Lomas & Moran, 2011), we can see how much larger contribution these marine pico-cyanobacteria could make if environmental conditions would change to favor cyanobacteria aggregation. Actually the biggest issue for cyanobacteria aggregation, on top of the various processes leading to the loss of sinking aggregates that I discussed above, is the fact that *Synechococcus* and *Prochlorococcus* are found to be under tightly coupled micrograzer control in oligotrophic oceans like the BATS site (Worden & Binder, 2003). In this scenario, the indirect export, i.e., the grazing of pico-cyanobacteria by micro-protists and the subsequent consumption by mesozooplankton and sinking in fecal pellets, would be the dominant pathway for the often underestimated carbon export by these cyanobacteria (Stukel et al., 2013; De Martini et al., in review). Thus, if

cyanobacteria like *Synechococcus* and *Prochlorococcus* would be able to escape the micrograzer control and form those large and fast sinking aggregates that I found in culture experiments in the first place, they would be able to make a much larger contribution to the direct carbon export than what is found in today's oligotrophic oceans.

5.2 Implications of My Study for Our Knowledge of Earth's Past and Future Oceans

My findings have strong implications for our knowledge of cyanobacteria aggregation and their carbon export potential when considering preferred oceanic scenarios in different time periods of Earth's history. For instance, cyanobacteria or their ancestors could have played a critical role in primary production in an early ocean dominated by prokaryotes (Canfield, 2005; Lyons et al., 2014). Their aggregation and subsequent settling could have been the only mechanism of carbon export before the evolution of eukaryotic grazers and subsequent production of sinking fecal pellets (Logan et al., 1995). The burial of a great amount of carbon in the deep sea sediments via oxygenic cyanobacteria aggregation could have been critical in the early Proterozoic (ca. 2.5-2.2 Ga) period in order for the great oxygenation event to get started (Canfield, 2005; Lyons et al., 2014).

The oceanic primary productivity and carbon cycles in the past could have been very different from today (Lyons & Reinhard, 2009; Ridgwell, 2011). Though primary productivity back in the early Proterozoic (ca. 2.5-2.2 Ga) period might have been limited by the low availability of nutrients and trace metals (Anbar & Knoll, 2002; Bjerrum & Canfield, 2002) as well as high seawater salinity (Knauth, 1998), cyanobacteria are

known for their ability to well respond to these limitations and restrictions. For instance, cyanobacteria are found to better respond to phosphorus scarcity (Van Mooy et al., 2009; Lomas et al., 2014; Mouginit et al., 2015) compared to other eukaryotic phytoplankton, and they show trace metal preferences (Saito et al., 2003) and high salinity tolerance (Knauth, 1998) consistent with a sulfidic and saline early ocean environment (Anbar & Knoll, 2002; Lyons & Reinhard, 2009).

In addition, back then the high atmospheric CO₂ concentration (Rye et al., 1995) could have led to a greater export production at higher C:N ratios (Riebesell et al., 2007; Passow & Carlson, 2012). The large amount of ballasting clay particles (Armstrong et al., 2002) derived from hydrothermal venting (such as bentonite) and early continental weathering (such as kaolinite, Anbar et al., 2007; Tosca et al., 2010) would have also enhanced the sinking of cyanobacteria aggregates as shown in my clay experiments (Deng et al., 2015). Most importantly, the absence of eukaryotic grazer control (Worden & Binder, 2003) could have resulted in a greater carbon export by these marine picocyanobacteria. Chemical weathering is also considered to be critical in controlling the atmospheric O₂ and CO₂ via oxidation and carbonation processes, respectively (Bernier & Maasch, 1996). At the same time, weathering can provide important nutrients, trace metals as well as ballasting clay particles to enhance the biological carbon pump (Barley et al., 2005; Tosca et al., 2010). Specifically, as one of the common products from weathering, kaolinite was found to better enhance the aggregation fraction compared to bentonite that often derives from altered volcanic ash (Deng et al., 2015). Thus, the shift in sources of the ballasting clays from hydrothermal venting (bentonite) to early

continental weathering (kaolinite) might have increased the fraction of export production by the cyanobacteria. In turn, the O₂ production via oxygenic photosynthesis can support the oxidative weathering of pyrites (sulfidic minerals, Berner & Maasch, 1996; Anbar et al., 2007). Thus, there might have been a positive feedback between the oxidative weathering and the biological carbon pump for the accumulation of atmospheric O₂ in the early Proterozoic (ca. 2.5-2.2 Ga) period. However, if we look at a longer time scale, the atmospheric CO₂ sequestration via the biological carbon pump would result in a lower global temperature and a reduced rate of the oxidative weathering (Berner & Maasch, 1996; Barley et al., 2005). Thus, there might also have been a negative feedback to stabilize between the oxidative weathering and the biological carbon pump over long periods of time.

Another case to consider is the oceanic scenario of the near future. Oligotrophic ocean regions are hypothesized to increase in areal extent with future warming and increased stratification (Polovina et al., 2008), and cyanobacteria like *Synechococcus* and *Prochlorococcus* may become increasingly dominant in the phytoplankton community (Lomas et al., 2010; Flombaum et al., 2013). With rising atmospheric CO₂, Riebesell et al. (2007) and Taucher et al. (2015) showed that phytoplankton could potentially play a more important role in the carbon uptake by increasing the production and altering the stoichiometry (i.e., increasing the C:N ratio) of their exported organic matter. In fact, communities dominated by cyanobacteria are usually found to have higher carbon to nutrient ratios compared to those dominated by eukaryotes such as diatoms (Martiny et al., 2013; Teng et al., 2014). An important pathway for the excess carbon fixed by

phytoplankton is exudation as EPS in order to maintain the elemental homeostasis in cell biomass (Hessen et al., 2004). These exuded exo-polysaccharides can further lead to the greater TEP production and aggregate formation (Passow 2000; Passow 2002; Deng et al., in revision), and thus a more efficient biological carbon pump by facilitating the sedimentation of phytoplankton cells that otherwise would be too small to sink to depth (Passow et al., 2001; Deng et al., 2015).

There have been debates on the response of phytoplankton aggregation and carbon export to the future warming and ocean stratification and acidification (Bopp et al., 2001; Passow & Carlson, 2012; Passow et al., 2014). The total biological productivity at the surface may decrease with less nutrient supply from depth (Polovina et al., 2008), and the extent of the POC flux being remineralized at depth may increase in warmer waters (Helmke et al., 2010; Lomas et al., 2010; Marsay et al., 2015). However, it has to be noted that the reduced upwelling currents due to increased stratification can also help prevent the DIC or recalcitrant DOC after remineralization from being brought back to the surface, which may result in an increased carbon sequestration flux in the deep sea (Dutreuil et al., 2009; Jiao et al., 2010). Another important factor that can impact the POC flux attenuation with future warming is the increase in the supply of clay minerals to the ocean from enhanced weathering and dust deposition (Mahowald et al., 1999; Passow & Carlson, 2012). This would increase the ballasting of the sinking POC flux (Armstrong et al., 2002; Neuer et al., 2004), resulting in higher sinking velocities and less remineralization/attenuation (Iversen & Ploug, 2010; Passow et al., 2014; Deng et al., 2015) and thus an increased sequestration flux in the deep sea. The atmospheric CO₂

sequestration via the biological carbon pump can serve to buffer the changes due to anthropogenic CO₂ emissions (Falkowski et al., 2000; Sabine et al., 2004). Ongoing studies and geo-engineering projects have been trying actively to increase the carbon export potential of the biological carbon pump, e.g., by using nutrient fertilization and artificial vertical mixing (Lampitt et al., 2008; Dutreuil et al., 2009). My study highlights the increasing importance of marine pico-cyanobacteria in primary productivity and carbon export via their aggregation and sinking, which has to be taken into consideration in future studies.

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